1/31/2022

Flow Cytometric Analysis Of COVID-19 Immune Response

Submitted by – Arinjoy Roy

(Registration No. A01-1112-116-015-2019)

(Examination Roll No. 2022151197)

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College, Rahara

Guided by – Dr. Soumyadip Paul

Assistant Professor, Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College, Rahara

Flow Cytometric Analysis Of COVID-19 Immune Response

Introduction

The recent coronavirus pandemic COVID-2019 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus belongs to the *Sarbecovirus* subgenus (genus *Betacoronavirus*, family *Coronaviridae*). This virus has unique clinical characteristics and is highly contagious with unclear pathological mechanisms. The virus can cause a life-threatening respiratory illness in humans, especially to the people in their late 50s and above, with or without co-morbidities such as diabetes, kidney diseases, heart diseases, etc. Early symptoms of SARS-CoV-2 infection are very similar to that of mild-to-moderate flu, which makes it extremely difficult to classify and shortlisting infected individuals. In addition, inefficient contact tracing of infected individuals is becoming increasingly hard.

Until a successful treatment strategy is appropriately identified, the key to managing this pandemic is greatly dependent on quick and faster detection of infected individuals, followed by isolation of patients from the healthy population. Presently, the detection of the COVID-19 is done by quantitative real-time PCR (qRT-PCR) using unique set of PCR primers.Immunological detection of SARS-CoV-2 antibodies in the blood of infected humans is another method being used for development of rapid detection kits using lateral flow immunochemistry. Reverse transcriptase – LAMP (loop-mediated isothermal amplification) is another potential detection system for SARS-CoV-2, which uses isothermal amplification of the viral nucleic acid using specially designed oligo-nucleotide primers.

Here, an approach for diagnosis of SARS-CoV-2 by screening of test samples (swabs) using flow cytometry. In this process of indirect immunofluorescence where the virus particles are first bound to primary antibodies followed by the complex being labeled by fluorescent secondary antibodies for detection in a flow cell.

1. What Is Flow Cytometry?

Flow cytometry is a laser-based cell biology technique that is used to analyze, count and sort cells of interest from a mixed population. Fluorescence-activated cell sorting (FACS) is a method of choice for analysis and purification of isolated single cells (viz., bacteria, algae, plant and animal cells). FACS can detect and discriminate cells as well as suspended particles by its properties of light scattering and fluorescence (excitation/emission mode). The fluorescence of cells may be obtained using specific fluorochrome reagents, or by using antibodies tagged with a fluorochrome targeted against a cell surface antigen and/or internal constituents in permeabilized cells. Flow cytometry has been used for monitoring cells expressing fluorescent proteins (e.g., GFP)[1] and undergoing DNA replication and cell cycle as well as apoptosis. The tool has also been used for immunophenotyping. FACS has been successfully used for generating qualitative and quantitative data in a broad range of biomedical, clinical and therapeutic research, thereby widening its applications from research to clinical studies.. For researchers investigating COVID-19, flow cytometry can also support studies related to:

Viral entry and infection mechanisms :

- Characterizing SARS-CoV-2
- Cell-virus interactions
- Impact on surface markers; ACE2 receptor expression
- Antiviral development; vaccine strain production

Viral function and its impact on the immune system :

- Phenotyping studies; reduction in absolute CD4 and CD8 T cell counts and population percentages
- T cell exhaustion, senescence, and differentiation
- Cellular inflammation; monitoring monocyte populations

The immune response :

- Response to stimulation with viral proteins/peptides
- Cytokine secretion
- Impact of drugs and treatments

Repurposing drugs for antiviral development :

- Drug screening in specific cell types
- Modulation of viral activity and infectivity
- Identification of compounds
- Toxicity of antiviral compounds

2. Working Principle Of Flow Cytometer :

- A beam of light (usually laser light) of a single wavelength is directed onto a hydro-dynamically focused stream of fluid.
- A number of detectors are aimed at the point where stream passes through the light beam, one in line with the light beam, one in line with the light beam (Forward scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescent detectors).
- Each suspended particle passing through the beam scatters the light in some way and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at as lower frequency than the light source.
- This combination of scattered and fluorescent light is picked up by the detectors, and by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak) it is then possible to extrapolate various types of information about the physical and chemical structure of each individual particle.
- FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness).
- Some flow cytometers on the market have eliminated the need for fluorescence and use only light scatter for measurement.
- Other flow cytometers form images of each cell's fluorescence, scattered light and transmitted light.

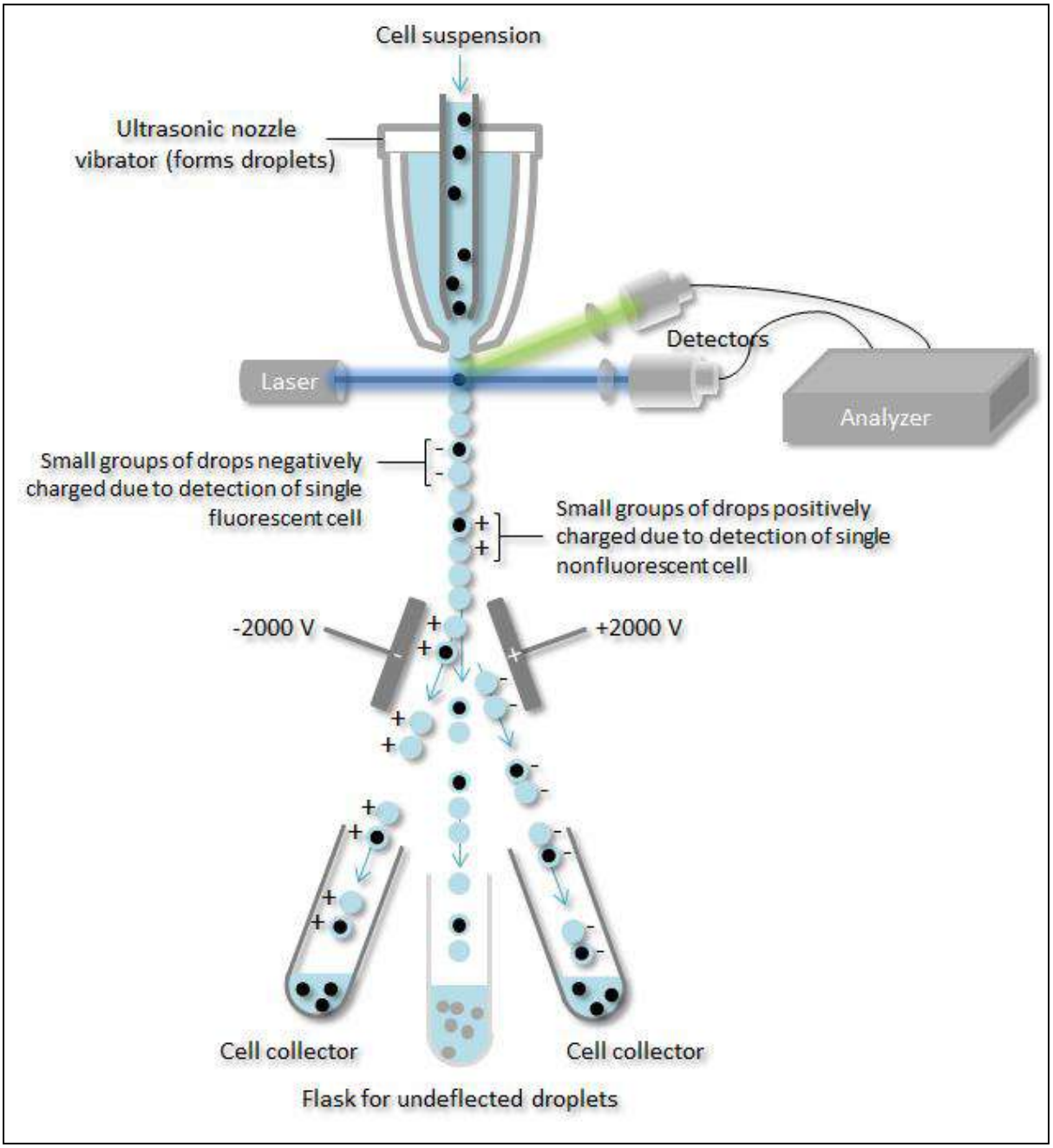


Figure 1: Principle of Flow Cytometer

3. Flow Cytometers:

Modern flow cytometers are able to analyze several thousand particles every second. in "real time", and can actively separate and isolate particles having specified properties. A flow cytometer is similar to a microscope, except that instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters. To analyze solid tissues single-cell suspension must first be prepared.

- A flow cytometer has 5 main components:
- A flow cell - liquid stream (sheath fluid) carries and aligns the cells so that they pass single file through the light beam for sensing.

- A light source - commonly used are lamps (mercury, xenon); high power water-cooled lasers (argon, krypton, dye laser); low power air-cooled Lasers (argon (488nm), red-HeNe (633nm), green-HeNe, HeCd (UV)); diode lasers (blue, green, red, violet).
- A detector and Analogue to Digital Conversion (ADC) system - generating FSC and SSC as well as fluorescence signals.
- An amplification system - linear or logarithmic.

Modern instruments usually have multiple lasers and fluorescence detectors (the current record for a commercial instrument is 4 lasers and 18 fluorescence detectors) Increasing the number of lasers and detectors allows for multiple antibody labeling, and can more precisely identify a target population by their phenotype. Certain instruments can even take digital images of individual cells, allowing for the analysis of fluorescent signal location within or on the surface of cells.

The data generated by flow-cytometers can be plotted in a single dimension, to produce a histogram, or in two dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates".

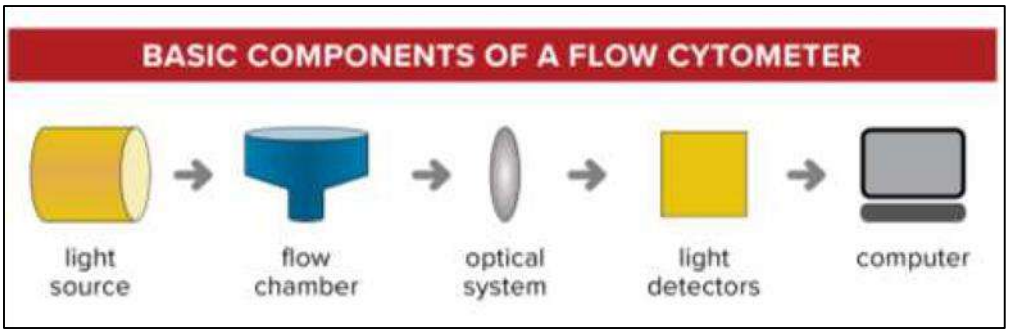


Figure 2 : Basic Components Of A Flow Cytometer

4. Measurable parameter :

Some of the measurable parameters with flow cytometry are;

- Volume of and morphogical complexity of cells.
- Cell pigments such as chlorophyll or phycoerythrin.
- DNA (cell cycle analysis, cell kinetics, proliferation etc.)
- RNA
- Chromosome analysis and sorting (library construction, chromosome paint) Protein expression and localization.
- Transgenic products in-vivo, particularly the green fluorescent protein or related fluorescent proteins.
- Cell surface antigens (cluster of differentiation (CD) markers).
- Intracellular antigens (Various cytokines , secondary mediators etc).
- Nuclear antigens.
- Enzymatic activity.

- pH, intracellular ionized calcium, magnesium, membrane potential.
- Membrane fluidity.
- Apoptosis.
- Cell viability.
- Monitoring elctropermeabilization of cells.
- Oxidative burst.
- Characterizing multidrug resistance (MDR) in cancer cells.
- Glutathione
- Various combinations (DNA/surface antigens, etc).

5. Applications Of Flow Cytometry To COVID-19 :

Until recently, analysis of viruses and viral particles by flow cytometry was limited due to the range of detection (300–500 nm) and the low signal-to-noise ratio of traditional flow cytometers in the lower size range. However, advances in flow cytometry, such as the development of camera-based systems, now allow for the analysis of small particles. Amnis CellStream and ImageStream flow cytometers can both be used to study viruses and viral particles due to the exceptional sensitivity of time delayed integration (TDI) and CCD camera technology, which is unique to Amnis systems. These flow cytometers also offer a low signal-to-noise ratio, further increasing their ability to resolve particles as small as 50 nm with unparalleled sensitivity, using highly customizable systems.

5.1. Currently Used COVID-19 Tests

Currently, there are two types of tests widely used to detect SARS-CoV-2. The first is molecular testing, such as reverse transcription-polymerase chain reaction (RT-PCR tests) which can detect the genetic material of the virus, and antigen tests that look for known proteins associated with the virus. Both of these tests can be used to diagnose a current infection with SARS-CoV-2.

The second class of diagnostic tests are antibody tests. These tests detect the presence of antibodies in the blood that are generated in response to infection with a virus. However, antibodies take time to build up in the body and can linger after the infection has cleared. Therefore, these tests can only be used to determine whether someone has been infected with the virus, they are not diagnostic.

Experts have expressed the importance of accurate and accessible COVID-19 testing in mediating transmission of the virus. This is particularly important while we wait for the vaccine to be rolled out, and until we identify a successful treatment strategy to protect those at-risk.

While PCR tests have long been considered the gold standard, the process is lengthy and the throughput of the system is low. Additionally, the accuracy of antibody-based detection is not high enough to be immune to false or nonspecific results. Due to the

limitations of current tests, there is a need for an accurate, high-throughput and rapid method of testing.

5.2. Using Flow Cytometry to Detect COVID-19

New research has suggested an alternative flow cytometry-based method of testing for the SARS-CoV-2 virus. Flow cytometry is a method that is used across different fields of science to analyze single cells or particles. The technology exposes each particle to lasers and measures the resultant visible light scatter as well as one or more fluorescence parameters.

Data collected on light scatter can indicate the size of the cell, as well as the granularity and internal complexity of a cell. The samples are stained with fluorescent dyes and fluorescently conjugated antibodies which allow scientists to detect the presence of certain viruses or antibodies to viruses.

The team in Mumbai has developed flow cytometry technology to establish a new method of SARS-CoV-2 virus testing. They believe their method will enhance the testing scale of COVID-19 cases. The SARS-CoV-2 is constructed of a 29–30 kb chain of positive single-stranded RNA. Its size ranges from around 70 to 90 nm.

Previous studies have demonstrated how the dengue virion (40–60 nm) can be detected using a combination of fluorescently labeled antibodies and magnetic nanoparticles (MNPs). A team in Mumbai describes how they have adapted this approach, labeling the viral particle surface with antigen-specific primary antibodies and following with a secondary antibody conjugated to a fluorescent dye to identify the presence of the SARS-CoV-2 virus.

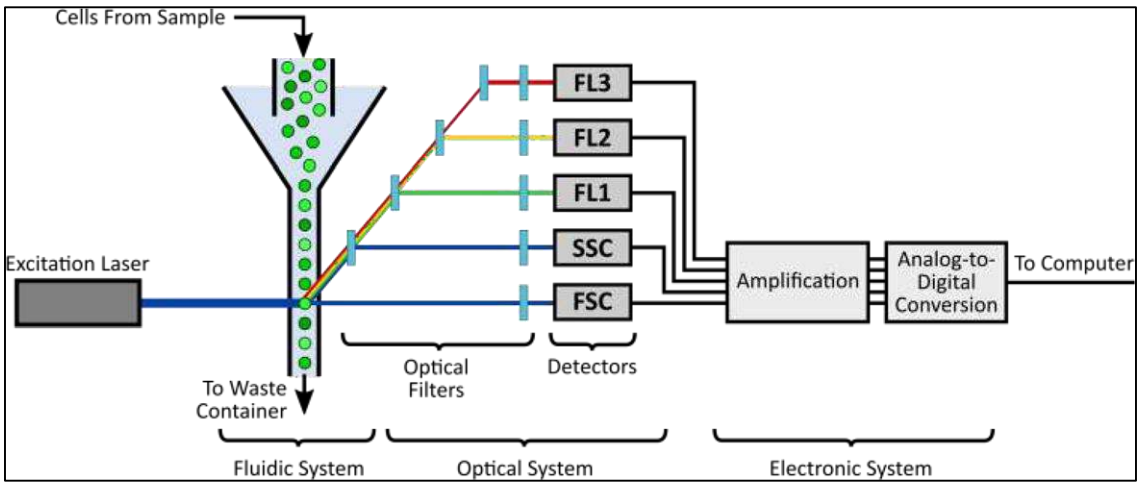


Figure 3 : Process Of Flow Cytometry

The new flow cytometry-based method will likely be vital to the future of COVID-19 testing. The new study demonstrates how the visions of SARS-CoV-2 can effectively be tagged with specific fluorescent-labeled antibodies, which can be detected using advanced flow virometry in a high-throughput manner. The newly established test could process

around 2,000 tests daily using a single set of equipment, offering a reliable, rapid, and accurate method of testing.

The technology has applications in a number of fields like;

- Molecular biology - In the field of molecular biology it is especially useful when used with fluroscence tagged antibodies.
- Marine biology - In marine biology the auto-fluroscent properties of photosynthetic plankton can be exploited by flow cytometry in order to characterize abundance and community structure.
- Protein engineering – It is used in conjugation with yeast display and bacterial display to identify cell surface-displayed protein variants with desired properties.
- Pathology.
- Immunology.
- Plant biology.

Future Direction Of Flow Cytometry

flow cytometry has a place in the analysis of virus-cell interactions. Four major areas on which flow cytometry will likelyhave an impact are (1) Flow cytometry will be used for rapid detection ofvirus-infected cells and other organisms in body fluids ifmonoclonal or polyclonal antibodies that specifically react withcell surface or intracellular viral antigens are available. Examples are cells infected with viruses, such as HTLV-1 and -2, hepatitis B virus, HCV[22], human herpesvirus 6, varicella-zoster virus, and measles virus, inblood and microbes, such as *P. carinii* and *Mycobacteriumtuberculosis*, in pulmonary lavage fluids. Using flow cytometryfor direct detection and possible quantitation of these microbes will save time and expense in the clinical laboratory. (2) Flow cytometry will be used for rapid detection of virusesafter amplification in cell culture. For many viruses that are notcell associated in body fluids, or when there are too few cellsfor a statistically accurate determination by flow cytometry, it ispossible to amplify the number of cells expressing the viralantigens by overnight incubation in cell culture and then topermeabilize, stain, and analyze the virus-infected cells by flowcytometry. Again, this will save considerable time in the clinicallaboratory, particularly when those viruses that take severaldays to produce cytopathic effects in tissue culture are beingstudied. (3) Flow cytometricanalysis of virus-infected cells is an excellent way to monitorthe effects of antiviral or anti-infective agents in vitro and invivo. Further application of this technology will allow screeningof numerous compounds for their antiviral and anti-infectiveactivities. Flow cytometry will continue to be useful in monitoring patients on antiviral chemotherapy to determine the invivo effects of therapeutic agents on their infection. (4) With the development of fluorescent in situ hybridization in suspension, it will be possible to use nucleic acidhybridization methods to detect viruses, bacteria, and othermicroorganisms in cells and quantitate them by flow cytometry. Since flow cytometric analysis of cellsstained for viral antigens will detect only cells that are activelyexpressing viral proteins, the

combination of in situ hybridization and flow cytometry will allow detection of latently infected cells. In addition, the combination of flow cytometry and PCR will provide the sensitivity required to detect and quantitate viruses in individual cells. Of course, flow cytometry will continue to be used to monitor lymphocytes in healthy and diseased people, detect DNA ploidy in tumor cells, and serve as an excellent tool for basic research. Increased use and familiarization with this powerful technology will give the clinician, the laboratorian, and the researcher the ability to move from qualitative science to a more quantitative aspect of their work. In addition, the ability of flow cytometry to measure multiple parameters simultaneously will allow those who are interested in studying the interactions of different cellular and viral components within the cell to perform these experiments. The multiparameter flow cytometric analysis of double- and triple-stained cells will give investigators the opportunity to identify which cells are infected as well as to quantitate the number of virus infected cells. For example, assuming that the CD4+ T lymphocyte is the primary target for HIV, peripheral blood cells could be stained with one or more fluorescently labeled cell surface reagents, and the cells could be permeabilized and treated with one or more fluorescently labeled reagents directed against internal viral antigens. These types of experiments should enable one to identify the peripheral blood cells that are infected with HIV or any virus of interest.

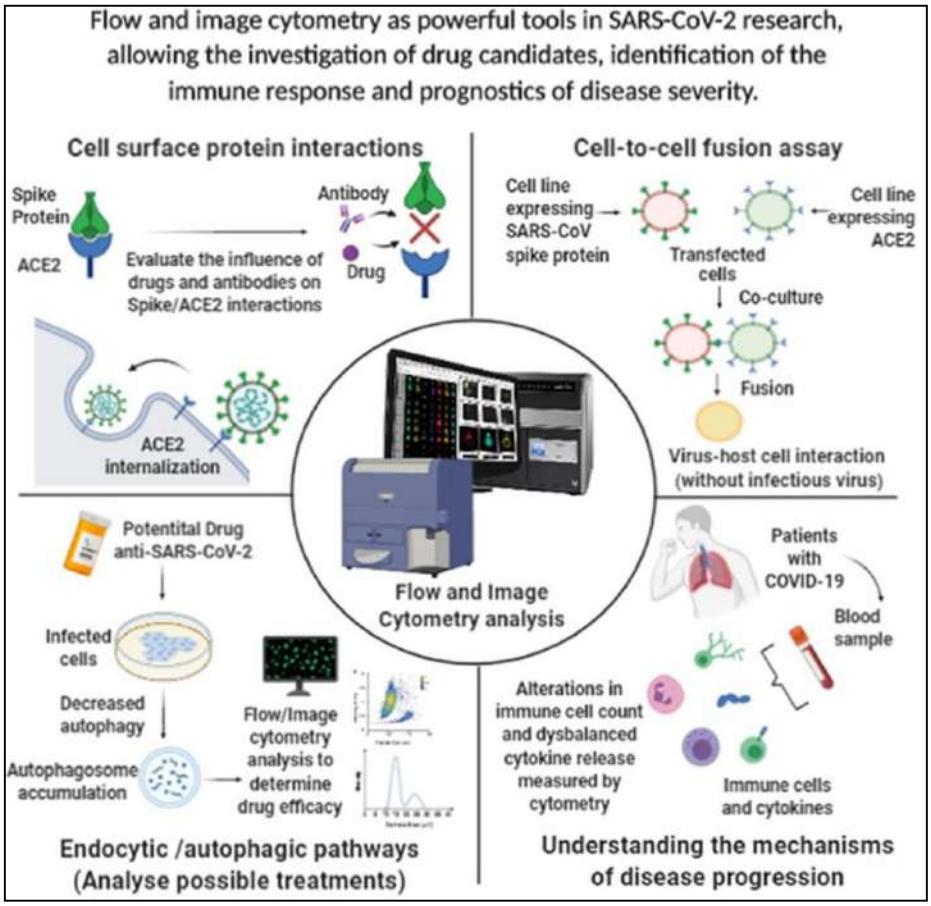


Figure 4 : Perspectives and Implications of Flow-Cytometric assays in COVID-19.

Discussion

Flow cytometry are used to characterize and count types of white blood cells in the evaluation of infectious diseases, autoimmune disorders or immunodeficiencies. It's also used to diagnose and classify leukemia or lymphoma. To detect COVID-19 infection flow cytometry is generally used as follow up testing after a complete blood count (CBC) or white blood cells scan (WBC). This is especially true if initial testing showed an increased number of lymphocytes, abnormal cell counts or the presence of immature blood cells. Healthcare providers use flow cytometry to predict the consequences of such viral infection and also how aggressive the cancer will be and to help determine if the cancer will respond to certain treatment.

Flow cytometry may be used whenever the healthcare provider needs to learn more about the cells inside the body. This type of testing can check the number of immune cells, assess cell cycle status, identify cancer cells or even analyze the DNA. Researchers use flow cytometry any time they want to learn more about the complexities of certain conditions and diseases.

Conclusion

As there are no approved drugs for treatment yet, COVID-19 has now become a major challenge all over the world. Several plant-derived alkaloids such as chloroquine, hydroxychloroquine, bidebiline E, bisnordihydrotoxiferine, thalifaberine, etc., are being studies for combating this virus. Studies across the globe over the last few months clearly show that the SARS-CoV-2 is highly contagious, transmitted by asymptomatic patients/individuals and the infection can be extremely severe for some individuals, which requires patients to be hospitalized with treatment in intensive care units. To detect the virus circulating within local communities, a quick, sensitive and accurate detection of the infection is highly desirable. Flow Cytometry method will not only help in quick and high-throughput detection of infection in densely populated habitats but will also help in reducing dependencies on qRT-PCR machines and reagents. Moreover, integration of this method with robotics liquid handling devices could also help in achieving human-free super high-throughput sample analysis. So adoption of flow cytometry methods will also be useful for scoring community infection studies in metropolitan cities, hospital staffs and the people who are associated with emergency services in this unprecedented time across the globe.

REFERENCE

1. Lippé R. Flow virometry: a powerful tool to functionally characterize viruses. J. Virol.
2. Aspland, A., Chew, C., Douagi, I., Galland, T., Marvin, J., Monts, J., Nance, D., Smith, A. L., & Solga, M. (2021). Risk awareness during operation of analytical flow cytometers and implications throughout the COVID-19 pandemic. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, *99*(1), 81–89. https://doi.org/10.1002/cyto.a.24282
3. Schmid, I., Lambert, C., Ambrozak, D., Marti, G. E., Moss, D. M., Perfetto, S. P., & International Society of Analytical Cytology (2007). International Society for Analytical Cytology biosafety standard for sorting of unfixed cells. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, *71*(6), 414–437. https://doi.org/10.1002/cyto.a.20390
4. Reifel, K. M., Swan, B. K., Jellison, E. R., Ambrozak, D., Baijer, J., Nguyen, R., Monard, S., Lyon, G., Fontes, B., & Perfetto, S. P. (2020). Procedures for Flow Cytometry-Based Sorting of Unfixed Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infected Cells and Other Infectious Agents. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, *97*(7), 674–680. https://doi.org/10.1002/cyto.a.24040
5. Chan K-H, Sridhar S, Zhang RR, Chu H, Fung AY-F, Chan G, et al. Fac-tors affecting stability and infectivity of SARS-CoV-2. J Hospital Infect. 2020;106(2):226–231.
6. International Society for Advancement of Cytometry. 2020 May2020 (Survey Results), SRL Newsletter. https://isac-net.org/page/SRL_Newsletter Accessed 15 Sep 2020.
7. Darzynkiewicz Z et al. Cytomtery. 1981.
8. FACS Multiset System. Becton Dickinson.2007.
9. Flow cytometry with plant cells analysis of genes, Chromosomes and genomes edited by Jaroslav Dolezel. 2007.
10. Givan AL. principles of flow cytometry: an overview.Methods cell Biol.2001.63:19-50.
11. Herr I and Debatin K-M. Blood 2001,98: 2603-2614.
12. Kim CN et al. cancer Res.1997, 57: 3115-3120.
13. Loken MR. Immunofluroscence techniques in flow cytometry and sorting. 1990, 341-53. Wiley.
14. McCoy P. Flow cytometry. In: McClatchey KD. Clinical laboratory Medicine 2nd ed Philadelphia. Lippincott, wlliams and Wilkins.2002.1401-1425.
15. Renz A et al. Blood .2001,98: 1542-1548.
16. Riley RS. The flow cytometer. Historical development and present applications. In: Ross W. Clinical Applications of flow cytometry, New York: lgaku-shoin Medical Publishers.1993,313.
17. Coronavirus. World Health Organization. Available at: https://www.who.int/health-topics/coronavirus#tab=tab_1
18. Coronavirus Disease 2019 Testing Basics. U.S. Food and Drug Administration. Available at: www.fda.gov/.../coronavirus-disease-2019-testing-basics
19. Coronavirus disease (COVID-19) pandemic. World Health Organization. Available at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019.

20. McKinnon, K., 2018. Flow Cytometry: An Overview. Current Protocols in Immunology,
21. Soni, N., Pai, P., Krishna Kumar, G., Prasad, V., Dasgupta, S. and Bhadra, B., 2020. A flow virometry process proposed for detection of SARS-CoV-2 and large-scale screening of COVID-19 cases. Future Virology.
22. Takahashi, K., H. Okamoto, S. Kishimoto, E. Munekata, K.
23. Tachibana, Y. Akahane, H. Yoshizawa, and S. Mishiro. 1992.
24. Demonstration of a hepatitis C virus-specific antigen predicted
25. from the putative core gene in the circulation of infected hosts. J.
26. Gen. Virol. 73:667-672.
27. Takizawa, T., S. Matsukawa, Y. Higuchi, S. Nakamura, Y.
28. Nakanishi, and R Fukuda. 1993. Induction of programmed cell
29. death (apoptosis) by influenza virus infection in tissue culture
30. cells. J. Gen. Virol. 74:2347-2355.
31. Thomas, M. S., M. Gao, D. M. Knipe, and K. L. Powell. 1992.
32. Association between the herpes simplex virus major DNAbinding protein and alkaline nuclease. J. Virol. 66:1152-1161.
33. Timm, E. A., and C. C. Stewart. 1992. Fluorescent in situ
34. hybridization en suspension (FISHES) using digoxigenin-labeled
35. probes and flow cytometry. BioTechniques 12:362-367.
36. Tsai, W.-P., and S. Oroszlan. 1988. Site-directed cytotoxic antibody against the C-terminal segment of the surface glycoprotein
37. gp9O of avian reticuloendotheliosis virus. Virology 166:608-609.
38. Ubol, S., and D. E. Griftfin. 1991. Identification of a putative
39. alphavirus receptor on mouse neural cells. J. Virol. 65:263-271.
40. van den Hurk, S., M. D. Parker, D. R Fitzpatrick, T. J. Zamb,
41. J. V. van den Hurk, M. Campos, R. Harland, and L. A. Babiuk.
42. 1991. Expression of bovine herpesvirus 1 glycoprotein gIV by
43. recombinant baculovirus and analysis of its immunogenic properties. J. Virol. 65:263-271.
44. Shukla, Jill. (2018). Flow cytometry: An introduction and application to cytology.

RAMAKRISHNA MISSION
VIVEKANANDA CENTENARY COLLEGE

Title: Disease X and COVID-19: global perspective and scenario

Submitted by: Nobendu Mukerjee

Registration No.: A01-1112-116-011-2019-20

Exam Roll No.: 2022151193

Semester: V

Subject: Microbiology (Hons.)

Guide: Dr. Bibhabasu Hazra, Department of Microbiology, Ramakrishna Mission Vivekananda Centenary College

Acknowledgement

This study and the research that went into it would not have been feasible without my supervisor, Dr. Bibhabasu Hazra's, unwavering support. From the first interaction with the final draft of this paper, his enthusiasm, knowledge, and meticulous attention to detail have been an inspiration and have kept my work on track. I am particularly grateful for Prof. Br. Parachaitanya, our Head of Department, for his unwavering support. The generosity and competence of each and every one of you has improved this study in countless ways and saved me from numerous mistakes; those that inevitably remain are only my responsibility. Authors are also grateful to DST-FIST support by Govt. of India to the Department of Microbiology, Ramakrishna Mission Vivekananda Centenary College, West Bengal, India.

Disease X and COVID-19: global perspective and scenario

Abstract

Disease X is caused by pathogen X, an infectious agent that is presently unknown but has the potential to trigger an epidemic or pandemic. Pathogen X might be any pathogen, including bacteria, viruses, parasites, fungi, and prions. Disease X has the potential to cause an epidemic or a pandemic breakout. To raise awareness and stimulate research, the WHO convenes an expert committee every year to update its list of extremely serious infectious illnesses with no viable treatments or vaccines. Because of the global public health system's complete failure during the current COVID-19 pandemic, there is an urgent need for an innovative global pandemic preparation system. The devastation inflicted by the COVID-19 pandemic has earned this virus the distinction of becoming the first Disease X. Lack of international cooperation, unequal distribution of healthcare knowledge and resources between developed and developing countries, censorship of data on case numbers and deaths, laxity in social distancing measures, and the spread of false information about vaccination and drugs all contribute to the uncontrolled spread of the first disease X throughout the world. During the first wave, India was somewhat effective in limiting case numbers; however, the second wave destroyed the whole nation due to a more virulent strain, lax preventative measures, and enormous gatherings for religious and political causes. It is critical that measures are made and appropriate regulations are put in place to mitigate the destruction caused by this virus before the situation worsens.

Keywords: Disease X, pandemic, COVID, Infectivity, pathogen, RNA virus

1. Introduction

X stands for unexpected. According to the World Health Organization (WHO), “Disease X represents the knowledge that a serious international epidemic could be caused by a pathogen currently unknown to cause human disease.” Pathogen X, an infectious agent that is presently unknown but has the potential to produce an epidemic or pandemic, causes Disease X. Any pathogen, including viruses, bacteria, fungi, parasites, and prions, could be pathogen X [1].

The WHO's research and development blueprint was launched in 2016 with the purpose of shortening the time it takes to discover, evaluate, and approve medicinal countermeasures for

the world's most lethal pathogens [1]. Disease X was a term chosen by the WHO in February 2019 to signify a future pandemic on their shortlist of high-priority diseases.

The knowledge of Disease X promotes the focus of research efforts on entire classes of viruses (e.g., Flaviviruses) rather than single strains (e.g., zika virus), improving their capacity to act to unforeseen strains [2]. It is recommended that the WHO and competent health authorities keep a watch on lately increased incidences of infectious illnesses such as Ebola, Zika, and Dengue, among others, since they have the potential to create devastation comparable to the present COVID-19 epidemic [3–7]. Thus, with each lesson learned from an epidemic or pandemic, global health services have improved preparation, management, and risk communication strategies, emphasizing the importance of taking appropriate action before the full clinical profile and etiological agent behind the new disease are fully understood.

2. Candidates of disease X and its clinical forms

Disease X holds the potential to outbreak as an epidemic or a pandemic. It is imperative to the public health department to list the emerging infectious diseases to promote accelerated vaccine development [1]. To raise awareness and stimulate research, the WHO convenes an expert committee every year to update its list of extremely serious infectious illnesses with no viable treatments or vaccines. The current list contains Rift Valley fever virus, Zika virus, MERS-CoV (middle east respiratory syndrome coronavirus), Marburg virus disease, Ebola virus disease, Crimean-Congo hemorrhagic fever virus, Lassa fever, severe acute respiratory syndrome (SARS) virus, Nipah virus, Henipaviral disease and Disease X [8].

During an annual review in 2017, WHO added the Zika virus to the list after causing an unexpected rise in cases of microcephaly [9]. The WHO announced in 2019 that the Wuhan pneumonia of unknown etiology should be recognized as the first Disease X [10]. The first Disease X now has a name, COVID-19, which was added to the list of conditions for research prioritization [11].

RNA viruses are more likely to be the infective viruses for newly emerging disease X due to their high potential to mutate into variant forms. The causative pathogen is not limited to viruses but includes bacteria, viruses, fungi, parasites, and prions. The zoonotic reservoirs of pathogens use insect vectors to infect the human population [1]. For instance, the outbreak of the H1N1 virus consisted of the genetic material of avian, human, and swine-origin involving

wildlife and migration between animals and farmworkers. The spillover of animals to humans in SARS-CoV-2 was not apparent, but the mutations in animals before transmission to humans have been supported for the emerging threat [8]. A possible bat origin was suggested after the whole genome sequence of the novel coronavirus SARS-CoV-2 matched with bat SARS-related coronavirus (SARSr-CoV-RaTG13) by 96% [10].

The 'Pathogen pyramid' model has been used to understand the origin of zoonotic disease in the human population **(Figure 1).** Four levels have been categorized to understand the origin and to group the severity of different origins. Level 1 includes direct animal to human transmission, mostly involving non-simian retroviruses. Level 2 represents zoonotic diseases overcoming species barriers to infect the human cells but holds the least chance of transmission from human to human. Examples are the Japanese encephalitis virus, rabies, and influenza A virus. At level 3, the zoonotic pathogens promote transmission from human to human, resulting in outbreaks that blow up and eventually settle down, likely involving viruses like the plague, Nipah, Ebola, and Marburg disease virus. Ultimately at level 4, the human-to-human transmission occurs, leading to epidemics and pandemics such as ongoing COVID-19 and its preceding forms of influenza, MERS-CoV, and SARS. HIV can also be considered at this level, given its epidemiology [12].

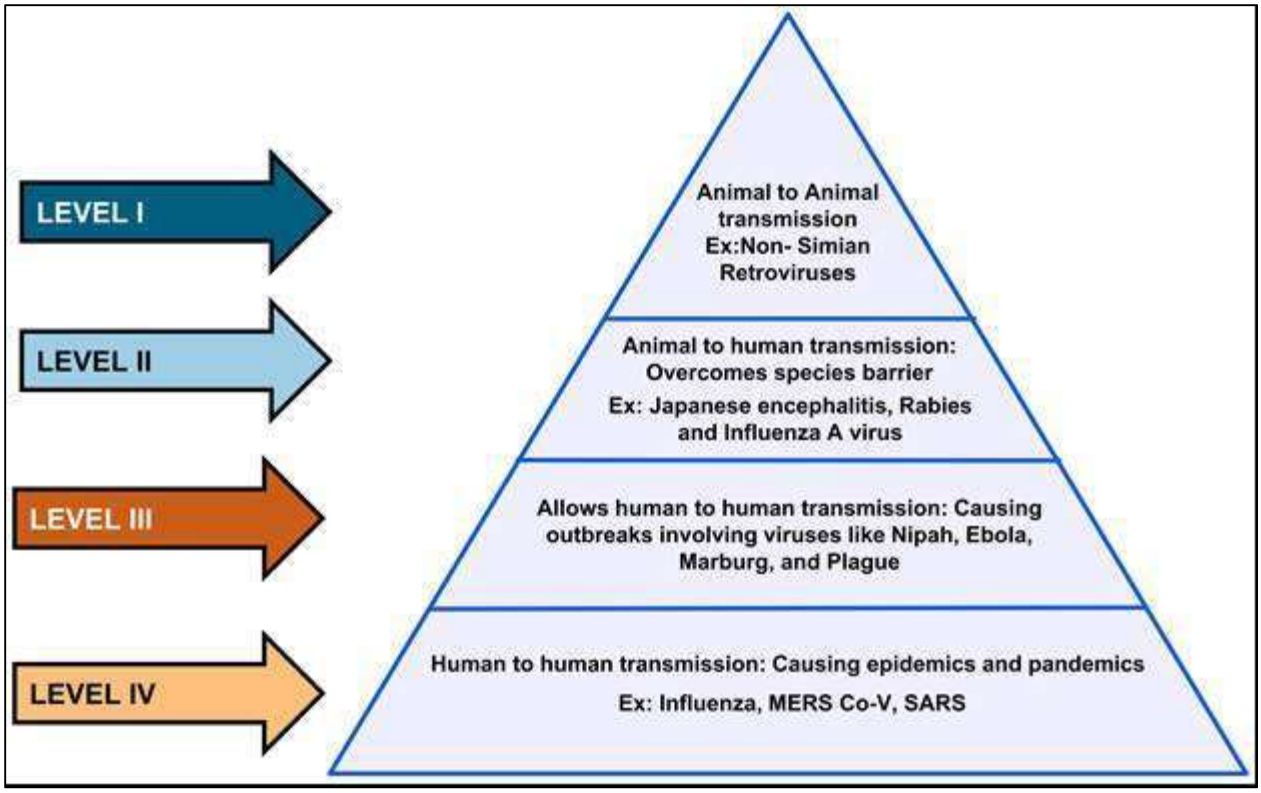


Figure 1. Pathogen Pyramid Model to understand the origin of zoonotic diseases in humans.

MERS Co-V: Middle Eastern Respiratory Syndrome Coronavirus; SARS: Severe Acute Respiratory Syndrome.

3. COVID-19 as the first Disease X and lesson learnt from the pandemic

3.1. Global impact and what went wrong:

Diverse strategies were planned and implemented across the globe for the containment of COVID-19. It is almost 2 years since the virus was first reported from China and although the pandemic does seem close to coming to an end, it's still far from reality. It is however still possible to map out certain important lessons that we can learn from COVID-19.

On the 31st of December 2019, the WHO head office was officially contacted for cases of "pneumonia of unknown etiology (unknown cause)" detected in the City of Wuhan, Hubei Province in China, yet according to reports, healthcare officials and scientists had informed the Chinese government bodies weeks earlier of a SARS virus like illness prevalent among their

patients [13]. Rather than creating an alert system to spread awareness to the rest of the province and the higher authorities, they choose to conceal case numbers and pressed charges of spreading false rumors against the physicians who spoke up, most notable being the now late Dr. Li Wenliang. Experts have suggested that if appropriate measures were implemented a mere few days in advance, the eventual spread of the SARS-CoV2 virus could have been lowered considerably. Countries have struggled to understand and explain the epidemiology of this novel viral disease, at times due to the challenges of being a densely populous nation, or establishing a consistent network for information outflow, sometimes due to internal disagreements and delays [13].

"Global solidarity and unity is the sole solution to this war against the COVID-19." This pandemic saw instances of poor international coordination and many powerful countries trying to obtain sole rights to the COVID-19 vaccine for themselves without any consideration for developing nations. There were strict legal lockdown and quarantine solutions were implemented in Japan and South Korea early in the pandemic helping to contain the virus immensely. In contrast, places with 'softer' initial responses had a sudden increase in cases subsequently leading to a major need to reassess policies and adopt increasingly severe measures to protect crumbling health systems from complete collapse [14].

Attempts at communication and collaboration at the highest inter and intra government levels have been a huge disappointment. The first virtual G7 and G20 summits took place months after the pandemic began. Even when the leaders came together and discussions did take place, there was a lack of consensus among world leaders. When the G7 summit happened in March, instead of discussing coordination and solidarity to fight the pandemic, disputes on which country was to be blamed took place and a post-meeting joint statement failed to be drafted [14].

3.2. Indian scenario of COVID-19 and what went wrong during the second wave of COVID-19:

Many reasons contributed to the disastrous second wave of Indian COVID-19, including the interaction of novel mutant strains, failure to follow COVID-appropriate procedures such as mask wearing, social separation, and other COVID-appropriate behaviour, and a delay in completing the vaccination push [15,16]. A new SARS-CoV-2 lineage B.1.617 emerged in India

and has been held responsible for the surge in cases due to a higher transmissibility of this strain having quickly spread to multiple countries [17].

Series of political campaigning for state elections in some of the most densely populated states like West Bengal, Tamil Nadu and religious mass gathering events including the gathering of approximate 9 million pilgrims at the Kumbh Mela festival ended up acting as a catalyst to begin the second wave [17,18].

Foregoing of all the social distancing and lockdown measures for propagation of political and religious agendas can be held accountable. Hesitancy to take vaccines, concealing the actual data of number of cases and deaths along with delays in the vaccination drive added to the case surge [17].

Public health policymakers were accustomed to less aggressive variants. There was a negligent attempt made after the first wave to study the wave, learn lessons from it, bridge the gaps in the Indian healthcare system, educate the masses to still follow the rules, and most critically prepare for the second wave in a better fashion [18].

4. Recommendations for Disease X

There is an imperative need for an innovative global pandemic preparedness system owing to the utter failure of the public health system worldwide during the current COVID-19 pandemic. [2]

Predicting the pandemic potential of novel microbes: New metagenomic technique can identify potential human infections in other organisms. This contains studies on the significance of host-relatedness.

Host relatedness: Evidence suggests that viruses from more closely related hosts (e.g., humans and chimpanzees) are more likely to spill over from one species to another [19,20].

Virus relatedness**:** is another predictor of pandemic potential as viruses in wildlife more similar to existing human pathogens are more likely to cause new diseases in people and hence need to be surveilled. The viruses with high mutation rates with no proofreading mechanisms pose a greater risk of infecting people [21].

Preventing Zoonotic Spillover and environmental protection: Scientists created a risk rating methodology and an interactive online application called "Spill Over" to predict a vulnerability index for wildlife viruses by comparing the danger of viruses with undetermined zoonotic spillover prospects to viruses that are documented to be zoonotic [22].

In environmentally chaotic places, we need to figure out where spillover is most likely to occur. The risk for spillover increases in areas where human habitat comes close to wildlife reserves, as with excess deforestation and wildlife habitat loss. The consequent rise in the populations of birds, rodents, and bats in human habitats increases people's exposure to zoonotic virus reservoirs. Hence conservation of rainforests and other wildlife resources is vital to reduce future pandemics.

Pre-pandemic preparation of medical countermeasures can be crucial: The COVID-19 pandemic showed us how concealing cases or delaying adequate personal protective measures can culminate tremendous human suffering and death. International Bodies like the WHO need to be alerted at the earliest to avert global catastrophes. Expanded database of pathogen sequences will help to swiftly triage and identify homologous pathogens to the future pathogen X. Hence, pre-pandemic medical countermeasures can be prepared regardless of the identity of pathogen X. Developed nations can help expand their diagnostic companies to low-and-middle-income countries to help them establish infrastructure for managing the burden of a pandemic in future. Regulatory measures and paperwork for starting the research on a new pathogen vaccine or small molecule for therapy against future pathogen X needs to be fast-tracked. This can be made possible by designating bodies in the government for pre-pandemic management by allocating funds to research on the pandemic potential of pathogens and preventive measures.

Integrating Services: Following the saying “prevention is better than cure” will never become more relevant than the current times. There have been pandemics in the past, and there certainly will be more pandemics in the future. The only solution is to learn from the mistakes, plan ahead and attempt to minimize the damage and loss by acting early. We need to be prepared for any impending pandemics by combining public health, healthcare, and emergency management services. Government and non-government entities will need to work hand in hand to make this possible.

Sample Transportation and diagnosis: Logistical difficulties in transporting blood and other serum samples are a huge barrier in expediting diagnoses of future disease X. Storing and transporting such hazardous samples from remote areas is inefficient and can be solved by setting up regional locations' biorepositories. A biorepository or a biological materials repository is used to collect, process, store, and distribute biospecimens to support future scientific diagnoses.

Data Sharing: Sharing data on emerging disease X pathogen sequences and lowering the time it takes to form contracts for intellectual property sharing between states or nations might accelerate research on potential treatments or vaccinations for a future pathogen X and save millions of lives. The pandemic Influenza Preparedness Framework is a ground-breaking project for increasing access to vaccinations, diagnostic kits, and antiviral medications, particularly in low-income countries [1].

Vaccine Development for emerging diseases: Vaccination is the best modality to prevent and control infectious diseases [23]. The wonder called 'mRNA' vaccines against infectious microbes can be manufactured as therapeutic or prophylactic agents. mRNA vaccines carrying antigens of virulent pathogen induce potent and robust immune responses. The manufacture of mRNA vaccines is a cell-free process, rapid, and straightforward vs other types of vaccines. This makes mRNA vaccines a promising biological tool to cover the gap between emerging pathogens and the dire need for potent vaccines against them. Large scale RNA production to accomplish commercialization is the pioneering step toward making mRNA vaccines. In 2017 several countries and international organizations came together to start a vaccine preparedness initiative. Germany, Japan, India, Norway, the Welcome Trust, World Economic Forum and the Bill & Melinda Gates Foundation, established the strategic Coalition for Epidemic Preparedness Innovations (CEPI) to grow support to fight significant health epidemic/pandemic threats through vaccine development [2].

Creating Safety Guidelines and pre-preparedness for outbreak management: During a pandemic, people require appropriate leadership and consistency to maintain public morale. As a result, leaders must be prepared to manage biological disasters such as the COVID-19 pandemic. Provide safety precautions. It is critical to educate the people on how to safeguard themselves

and their families. Some examples are social distancing legislation and proper mask etiquette. Travel restrictions and airport screening should be implemented. Support viral containment, restricting virus dissemination to new areas, testing, and active contact tracing. Make a stockpile of all required drugs and equipment.

Protect healthcare workers at significant risk for contracting the disease. An adequate supply of personal protective equipment and keeping stockpiles of all necessary medications in advance that may become indispensable during a pandemic.

Development of a global intervention target product profile (iTPP): The required qualities of a new product to meet a high unmet clinical demand are defined by a Target Product Profile (TPP). They guide drug or vaccine manufacturers to develop 'fit for purpose' products, thus allowing novel therapeutic or preventive agents to go faster from labs to patients [24].

Clinical leadership and consistency: They are observable and learnable sets of practices essential at all levels of healthcare. Individuals do not offer health care; instead, complex systems that function together, frequently involving vast numbers of people and organizations, do. Working in concert with large teams, including interprofessional collaboration, has become essential for 21st-century healthcare professionals, especially during this pandemic, to provide more effective and overall treatment for patients and the public.

Building trust and cooperation with the public: Medical professionals need to educate the masses to dispel speculations myths and curbing vaccine hesitancy among youth, and counter the spread of false information across various social media platforms.

5. Conclusion

Disease X is defined as a disease caused by a novel pathogen unknown to cause any disease in human beings before. The world has seen many incidents of major disease outbreaks like the plague, the Spanish flu, smallpox in the past, and swine flu, bird flu, dengue, and chikungunya virus epidemics during the present century, with COVID-19 being the most recent and severe disease. The lack of international cooperation, unequal distribution of healthcare knowledge and resources between developed and developing countries, censorship of data regarding case numbers and deaths, leniency in social distancing measures, along with the spread

of false information among people about vaccination and drugs lead to the uncontrolled spread of the first disease X all over the world. India was relatively successful in controlling case numbers during the first wave; however, the second wave devastated the whole nation due to a more virulent strain, leniency of precautionary measures, and mass gatherings for religious and political purposes. We recommend more research to study the spillover of viruses and other microbes from animals to humans, the development of mRNA vaccines, and a global iTPP to combat future disease X emergencies. Safety guidelines need to be set up and should be applicable beyond national borders along with sharing of intellectual property (like new sequences of disease X pathogen) between the developed and the developing or underdeveloped world. If we can carefully examine the mistakes made during the COVID-19 pandemic and use the lessons learned to implement a better pre-pandemic preparedness plan, we may be able to dodge another blow to the world's human and economic resources caused by the ongoing coronavirus pandemic.

References:

[1] S. Simpson, M.C. Kaufmann, V. Glozman, A. Chakrabarti, Disease X: accelerating the development of medical countermeasures for the next pandemic, Lancet Infect. Dis. 20 (2020) e108–e115. doi:10.1016/S1473-3099(20)30123-7/ATTACHMENT/019BBF14-2643-4ED0-A59D-867265351CC4/MMC1.PDF.

[2] C. Possas, E.T.A. Marques, J.B. Risi, Jr, A. Homma, COVID-19 and Future Disease X in Circular Economy Transition: Redesigning Pandemic Preparedness to Prevent a Global Disaster, Circ. Econ. Sustain. (2021) 1. doi:10.1007/S43615-021-00060-X.

[3] F.M.A. Khan, M.M. Hasan, Z. Kazmi, A.C. dos Santos Costa, A.T. Aborode, S. Ahmad, M.Y. Essar, Ebola and COVID-19 in Democratic Republic of Congo: grappling with two plagues at once, Trop. Med. Health. 49 (2021) 1–4. doi:10.1186/S41182-021-00356-6/METRICS.

[4] M. Bardhan, D. Pramanik, R. Riyaz, M.M. Hasan, M.Y. Essar, Dual burden of Zika and COVID-19 in India: challenges, opportunities and recommendations, Trop. Med. Health. 49 (2021) 1–4. doi:10.1186/S41182-021-00378-0/METRICS.

[5] A.C. dos S. Costa, M.M. Hasan, E. Xenophontos, P. Mohanan, E.E. Bassey, H.T. Hashim, S. Ahmad, M.Y. Essar, COVID-19 and Zika: An emerging dilemma for Brazil, J. Med.

Virol. 93 (2021) 4124–4126. doi:10.1002/JMV.27006.

[6] A.T. Aborode, A.C. Corriero, E.A. Fajemisin, M.M. Hasan, S.K. Kazmi, O. Olajiga, Dengue and Coronavirus disease (COVID-19) syndemic: Double threat to an overburdened healthcare system in Africa, Int. J. Health Plann. Manage. (2021). doi:10.1002/HPM.3334.

[7] A. Yousaf, F.M.A. Khan, M.M. Hasan, I. Ullah, M. Bardhan, Dengue, measles, and COVID-19: A threefold challenge to public health security in Pakistan, Ethics, Med. Public Heal. 19 (2021) 100704. doi:10.1016/J.JEMEP.2021.100704.

[8] K. V. Iserson, The Next Pandemic: Prepare for "Disease X," West. J. Emerg. Med. 21 (2020) 756. doi:10.5811/WESTJEM.2020.5.48215.

[9] T. Burki, Known unknowns and unknown unknowns, Lancet. Infect. Dis. 18 (2018) 385. doi:10.1016/S1473-3099(18)30181-6.

[10] S. Jiang, Z.L. Shi, The First Disease X is Caused by a Highly Transmissible Acute Respiratory Syndrome Coronavirus, Virol. Sin. 35 (2020) 263–265. doi:10.1007/S12250-020-00206-5.

[11] A.D.T. Barrett, Developing Zika vaccines: the lessons for disease X, Genome Med. 10 (2018). doi:10.1186/S13073-018-0561-2.

[12] P. Chatterjee, P. Nair, M. Chersich, Y. Terefe, A. Chauhan, F. Quesada, G. Simpson, One Health, "Disease X" & the challenge of "Unknown" Unknowns, Indian J. Med. Res. 153 (2021) 264–271. doi:10.4103/IJMR.IJMR_601_21.

[13] The Guardian. Chinese inquiry exonerates coronavirus, (2020). https://www.theguardian.com/world/2020/mar/20/chinese-inquiry-exonerates-coronavirus-whistleblower-doctor-li-wenliang (accessed November 22, 2021).

[14] M. French, T. Monahan, Dis-ease Surveillance: How Might Surveillance Studies Address COVID-19?, Surveill. Soc. 18 (2020) 1–11. doi:10.24908/ss.v18i1.13985.

[15] S. Ghosh, N. Moledina, M.M. Hasan, S. Jain, A. Ghosh, Colossal challenges to healthcare workers combating the second wave of coronavirus disease 2019 (COVID-19) in India, Infect. Control Hosp. Epidemiol. (2021) 1–2. doi:10.1017/ICE.2021.257.

[16] S. Rackimuthu, M.M. Hasan, M. Bardhan, M.Y. Essar, COVID-19 vaccination strategies and policies in India: The need for further re-evaluation is a pressing priority, Int. J. Health Plann. Manage. (2021). doi:10.1002/HPM.3321.

[17] O.P. Choudhary, Priyanka, I. Singh, A.J. Rodriguez-Morales, Second wave of COVID-19 in India: Dissection of the causes and lessons learnt, Travel Med. Infect. Dis. 43 (2021). doi:10.1016/J.TMAID.2021.102126.

[18] S.U. Kumar, D.T. Kumar, B.P. Christopher, C.G.P. Doss, The Rise and Impact of COVID-19 in India, Front. Med. 0 (2020) 250. doi:10.3389/FMED.2020.00250.

[19] D.G. Streicker, A.S. Turmelle, M.J. Vonhof, I. V. Kuzmin, G.F. McCracken, C.E. Rupprecht, Host phylogeny constrains cross-species emergence and establishment of rabies virus in bats, Science. 329 (2010) 676–679. doi:10.1126/SCIENCE.1188836.

[20] S.S. Morse, J.A.K. Mazet, M. Woolhouse, C.R. Parrish, D. Carroll, W.B. Karesh, C. Zambrana-Torrelio, W.I. Lipkin, P. Daszak, Prediction and prevention of the next pandemic zoonosis, Lancet (London, England). 380 (2012) 1956–1965. doi:10.1016/S0140-6736(12)61684-5.

[21] C.R. Parrish, E.C. Holmes, D.M. Morens, E.-C. Park, D.S. Burke, C.H. Calisher, C.A. Laughlin, L.J. Saif, P. Daszak, Cross-Species Virus Transmission and the Emergence of New Epidemic Diseases, Microbiol. Mol. Biol. Rev. 72 (2008) 457. doi:10.1128/MMBR.00004-08.

[22] Z.L. Grange, T. Goldstein, C.K. Johnson, S. Anthony, K. Gilardi, P. Daszak, K.J. Olival, T. O'Rourke, S. Murray, S.H. Olson, E. Togami, G. Vidal, J.A.K. Mazet, Ranking the risk of animal-to-human spillover for newly discovered viruses, Proc. Natl. Acad. Sci. U. S. A. 118 (2021). doi:10.1073/PNAS.2002324118/-/DCSUPPLEMENTAL.

[23] C. Zhang, G. Maruggi, H. Shan, J. Li, Advances in mRNA vaccines for infectious diseases, Front. Immunol. 10 (2019) 594. doi:10.3389/FIMMU.2019.00594/BIBTEX.

[24] P. Cocco, A. Ayaz-Shah, M.P. Messenger, R.M. West, B. Shinkins, Target Product Profiles for medical tests: A systematic review of current methods, BMC Med. 18 (2020) 1–12. doi:10.1186/S12916-020-01582-1/FIGURES/4.

An Update on: Severe combined immunodeficiencies (SCIDs)

Submitted by- Kousik Bhattacharya

Semester- V

Registration No.- A01-1112-116-026-2019(2019-20)

Exam Roll No.- 2022151207

Guided By- Dr. Sekhar Pal

Department of Microbiology

RKMVC College Rahara

Acknowledgement:

For acknowledgement section I first of all would like to acknowledge **Dr. Sekhar Pal sir**, Dept. Microbiology, Ramakrishna Mission Vivekananda Centenary College Rahara for his serious guidance. Next I would like to thank our departmental co-ordinator **Br. Parachaitanya Maharaj** and **all the teachers** of the department for selecting me in such a project work. A serious thanks also goes to my friend **Somnath Roy** for his suggestions and technical assistance.

Abstract

Severe combined immunodeficiencies (SCIDs) are a group of inherited disorders responsible for severe dysfunctions of the immune system. These diseases are life-threatening when the diagnosis is made too late; they are the most severe forms of primary immunodeficiency. SCID patients often die during the first two years of life if appropriate treatments to reconstitute their immune system are not undertaken. Conventionally, SCIDs are classified according either to the main pathway affected by the molecular defect or on the basis of the specific immunologic phenotype that reflects the stage where the blockage occurs during the differentiation process. However, during the last few years many new causative gene alterations have been associated with unusual clinical and immunological phenotypes. Many of these novel forms of SCID also show extra-hematopoietic alterations, leading to complex phenotypes characterized by a functional impairment of several organs, which may lead to a considerable delay in the diagnosis. Here we review the biological and clinical features of SCIDs paying particular attention to the most recently identified forms and to their unusual or extra-immunological clinical features.

SCID= severe combined immunodeficiency, CID= Combined immunodeficiencies

Introduction

Severe combined immunodeficiencies (SCIDs) are a group of inherited disorders responsible for severe dysfunctions of the immune system that lead to the absence or dysfunction of the T and B cells derived from the thymus gland and bone marrow, thus affecting both cellular and humoral adaptive immunity. Recently, Kwan et al., on the basis of data obtained from 11 U.S. newborn screening programs in the general population, reported an incidence of SCID of 1 in 58,000 live-births, an incidence much higher than the previous estimate of one in 100,000 based on retrospective clinical diagnosis of SCID.[1]. This group of diseases belongs to the most severe forms of primary immunodeficiency (PID), which are often fatal when the diagnosis is made too late.[2]. Even though children with SCID appear healthy at birth, they are predisposed to severe bacterial, viral, and fungal infections as the maternal transferred antibodies decline. During the first year of life, failure to thrive, diarrhoea, and oral candidiasis are common findings; *Pneumocystis jiroveci* may frequently cause a severe interstitial pneumopathy; and maternal engraftment of lymphocytes can cause graft-versus-host disease (GVHD).[3].SCID patients often die during the first two years of life if appropriate treatments to reconstitute their immune system are not undertaken.[4]. For most patients, the only curative treatment is the

allogeneic hematopoietic stem cell transplantation (HSCT).[5]. Gene therapy offers a cure for two specific forms of SCID and, although other SCID forms may become amenable to this treatment in the future, it is likely that HSCT will continue to be used for the majority of SCID patients.[6]. Conventionally, SCIDs can be classified according either to the main pathways affected by the molecular defect or on the basis of the specific immunologic phenotype related to that genetic defect, as T cell– deficient but normal B cell (T−B+) SCID and both T cell– and B cell–deficient (T−B−) SCID, with a further subdivision depending on the presence or absence of NK cells (NK+ or NK–, respectively).[2]. This classification, traditionally considered as representative of the stage where the blockage occurs during the differentiation process, was, until a few years ago, very useful in directing molecular studies toward a certain genetic alteration. However, during the last years many new causative gene alterations have been identified with peculiar clinical and immunological phenotypes. In a few cases, the genetic alteration allows for a normal T cell differentiation program but compromises T cell functionality by affecting the initial or final phase of intracellular signalling. These functional T cell disorders are characterized by immune dysregulation and cancer predisposition, as well as infections. In addition, hypomorphic mutations in some SCIDs genes make possible the development of non-functional oligoclonal T cells that are responsible for a complex of clinical conditions that may include hyperinflammation or autoimmunity. Many of the novel forms of SCID also show extra-hematopoietic alterations, leading to complex phenotypes characterized by functional impairment of organs different from primary lymphoid organs, which can make the diagnostic process very complex by standard methods. Taking this into account, the traditional international classification of SCIDs based on immunophenotype may no longer be optimal for clinical and research purposes [7],[8] —diagnostic criteria have to be continuously updated to take into account these unusual phenotypic presentations. In his work of 2014, Shearer emphasizes that currently there is no consensus among clinical immunologists on how best to diagnose and treat these rare disorders. It is not surprising that an important clinical dilemma concerns the distinction of SCIDs from other diseases such as combined immunodeficiencies (CIDs). Recently, it was proposed that patients who exhibit an absence or a severe reduction of T cells (CD3+ < 300/L), absence or severe reduction (<10% of the lower limit) of a proliferative response to phytohemagglutinin, or a maternal lymphocyte engraftment should be defined as having typical SCID.[5]. Moreover, the European Society for Immunodeficiency suggested as criteria for the diagnosis of CID the presence of one of the following parameters: one severe infection, an immunodysregulation disorder, cancer, familial CID associated with moderate age-related reduction of CD3+, CD4+, CD8+ T cells or of naive

T cells. However, a cut-off to distinguish SCID from CID has not yet been well defined. A main aim of this review is to report on the biological and clinical features of SCID, paying attention to the most recently identified forms and to the unusual or extra-immunological clinical features. An attempt to relate together pathogenetic mechanisms to specific clinical features is proposed.

Clinical Presentation

The clinical presentation of patients with SCID is variable. Usually, patients are asymptomatic at birth and present within their first weeks or months of life with infections and/or failure to thrive (FTT). Some patients may also present with signs of immune dysregulation caused by autologous (Omenn syndrome) **(Figure-1)** or allogeneic (maternal GvHD) T cells. Some genetic defects cause first year of life. Some patients may also present with leaky SCID, usually caused by hypomorphic mutations in classical SCID causing-genes, responsible for a less severe phenotype with infections and autoimmunity. (42).

Figure 1 Omenn Syndrome in a Sardinian 5-month-old female infant (absence of RAG1-RAG2 mutations, unidentified gene defect). “Leaky” mutations of practically all SCID genes (whose null mutations cause instead typical SCID) produce Omenn syndrome, in fact described in infants with defects of RAG1-RAG2, DCLRE1C Artemis, ADA, DNA Ligasi IV, RMRP-CHH, common gc, IL7Ra, WHNFOXN1, ZAP-70, and complete DiGeorge anomaly (DiGeorge Syndrome; CHARGE). In many infants with Omenn syndrome, that is clinically not leaky but very serious, genetic defect remains unidentified (several known, and probably also unknown, genes to be sequenced) [42]

Types of SCID

SCID due to defective survival of hematopoietic lineage precursors

Reticular dysgenesis (RD) is an autosomal recessive form of SCID characterized by both early myeloid lineage differentiation arrest and impaired lymphoid development.[9]. It is considered the most severe form of SCID, accounting for less than 2%. RD is caused by biallelic mutations in the adenylate kinase 2 gene (AK2), which cause the absence or the strong reduction of the

expression of AK2 protein. [9,10]. The syndrome is characterized by the absence of granulocytes and lymphocytes in peripheral blood.

SCID due to accumulation of toxic metabolites

Adenosine deaminase (ADA) deficiency and purine nucleoside phosphorylase (PNP) deficiency are inherited disorders of the purine metabolism characterized by abnormal accumulation of toxic nucleoside products.[11]. ADA deficiency is responsible for a T cell–, B cell–, and NK cell–deficient (T–B–NK–) form of SCID associated with thymic hypoplasia and absence of lymphocyte proliferative response.

It is particularly expressed in the lymphoid system, especially in the thymus, where it plays a key role in its differentiation and maturation. The absence of ADA activity is responsible for a massive accumulation of Ado and dAdo, in particular in thymocytes, lymphocytes, and erythrocytes. [11,13]. dAdo phosphorylation by nucleoside kinases leads to the production of deoxynucleotide triphosphates (dATP) whose accumulation, altering lymphocyte signalling pathways and serving as a danger signal, may cause the severe lymphopenia observed in ADA deficiency.

Purine nucleoside phosphorylase gene (PNP) mutations result in an extremely rare autosomal recessive disorder accounting for 4% of all form of SCIDs.[14]. Autoimmunity, recurrent infections, failure to thrive, and neurologic dysfunction are some of the main features of PNP deficiency. PNP maps to chromosome 14q13 and encodes a protein that catalyses the phosphorolysis of guanosine, deoxyguanosine, inosine, and deoxy inosine, to their respective purine bases. [11,15,16]. Mutations in the PNP pathways result in elevated deoxyguanosine triphosphate storage and in T cell toxicity due to the inhibition of the mechanisms of DNA synthesis and repair, resulting in an increased sensitivity to DNA damage and apoptosis, especially in T lymphocytes during selection within the thymus.. PNP deficiency can be suspected when lymphopenia is associated with reduced PNP enzymatic activity in red blood cells in a patient with recurrent respiratory infections and other typical manifestations.[18].

SCID due to cytokine signalling anomalies

In particular, SCIDs caused by defects of the common gamma chain, Janus kinase 3 (JAK3), or the IL-7 receptor α chain (IL-7Rα) are prototypic cytokine-associated disorders, accounting for 67– 74% of all cases of SCIDs. [19,20]. Mutations of γc gene cause X-linked SCID (X-SCID), one of the most common forms of SCID, accounting for 50% of all cases. The γc gene

(IL2RG) maps to chromosome Xq13.1 and encodes a transmembrane protein that is a component of several cytokine receptors, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, all critical for lymphocyte development and function.[21]. The γc interacts with the intracellular tyrosine kinase JAK3, which acts as a transducing element [22] indispensable for cell growth and control of hematopoietic cell development. Evidence indicates that γc is widely expressed in non-hematopoietic cells as well, even though its function in these cells has not yet been clearly elucidated. It has been reported that γc is implicated in the growth hormone receptor signalling, suggesting the existence of a subtle interaction between endocrine and immune systems. [23–27] interacts with the intracellular tyrosine kinase JAK3, which acts as a transducing element [22] indispensable for cell growth and control of hematopoietic cell development. Evidence indicates that γc is widely expressed in non-hematopoietic cells as well, even though its function in these cells has not yet been clearly elucidated. It has been reported that γc is implicated in the growth hormone receptor signalling, suggesting the existence of a subtle interaction between endocrine and immune systems. [23–27] JAK3, mainly expressed in lymphoid and myeloid cells, is essential for the differentiation of hematopoietic precursors; [28–30] its deficiency is responsible for an autosomal recessive SCID.

SCID due to V(D)J recombination and TCR abnormalities

V(D)J recombination is a complex process that occurs in early B and T cell development. It is responsible of the introduction of site-specific DNA double strand breaks (DSBs) by the recombination activating genes (RAG) 1 and 2. [31,64] The cleavage of the hairpin and the joining of these segments requires the DNA nonhomologous end joining (NHEJ) DNA repair factors, which generate the diversity through recombination of the V, D, and J segments and junction. NHEJ also plays a role in preserving the genomic stability of cells exposed to X-ray DNA damage. Consistent with these functions, it is not surprising that mice lacking NHEJ components exhibit a SCID phenotype and radiosensitivity (RS), a phenotype referred to as RS-SCID. In humans, several mutations in NHEJ genes have been identified, including mutations in genes for DNA ligase IV (LIG4), XLF/Cernunnos (NHEJ1), DNA-PKcs (PRKDC), and Artemis (DCLREIC), that are associated with SCID. [31–33] Of note, the increased radiosensitivity peculiar to these forms of SCID can be used as a diagnostic tool. [34,35].

SCID due to thymic abnormalities: from DiGeorge syndrome to nude/SCID

The prototype of athymic disorders caused by abnormalities of the stromal component of the thymus—the primary lymphoid organ for T cell differentiation—is the nude/SCID syndrome, described in humans in 1996.[36]. This form of SCID is the only one not primarily related to an intrinsic abnormality of the hematopoietic cell, but rather to a defect in hematopoietic cell–supporting thymic epithelial cells. [37–39] This human SCID is the equivalent of the murine nude/SCID phenotype described in 1966, although in humans the phenotype is more severe. It is one of the rarest forms of SCID, and only three mutations have been associated thus far with nude/SCID.[40] The gene responsible for the disease in humans is FOXN1, located on chromosome 17 [41].

New-born screening for SCID

Recently, T cell receptor excision circles (TREC)– based newborn screening has been implemented in several countries. Compared with patients identified by the clinical features, patients identified through newborn screening programs, similar to children identified because of a positive familial history, can receive an early and accurate diagnosis by one month of life and then undergo HSCT or gene therapy by 3 months of age, before the occurrence of severe complications. This results in a significantly improved outcome. [125,126]. The TREC assay, based on the detection of intracellular accumulation of products derived from process of T cell receptor gene splicing and rearrangement, is able to detect several defects, which result in either SCID or profound T cell lymphopenia that is also seen in patients affected with 22q11.2DS, CHH, CHARGE, and AT.[127]. However, one limitation of the TREC assay is that it is not able to identify all forms of CID or atypical SCID. Some genetic disorders, such as deficiency of ZAP70, late onset ADA, Nijmegen breakage syndrome, MHC class II deficiency, and many others, are likely to be missed because TRECs are usually found at normal levels. The identification of kappa-deleting recombination excision circles (KREC), a sensitive marker of newly formed B cells, increases the possibility of identifying other forms of SCID/CID that are associated with low numbers of B lymphocytes, such as NBS and late onset ADA. Furthermore, it has been reported that tandem mass spectrometry can easily identify abnormal purine metabolites in newborns with typical or late onset ADA and PNP deficiency,[12] thus increasing the spectrum of disorders detectable through newborn screening.

Facts

On January 1, 2008, Wisconsin (USA) became the first state in the world to screen all newborns for SCID through a method based on measurement of T cell receptor excision circles (TRECs)

by polymerase chain reaction (PCR), using DNA extracted from newborn dried blood spots (Guthrie cards); TRECs are by products generated during normal T cell maturation **(Figure 2)** and are consistently absent or present in very low numbers in newborns with SCID.

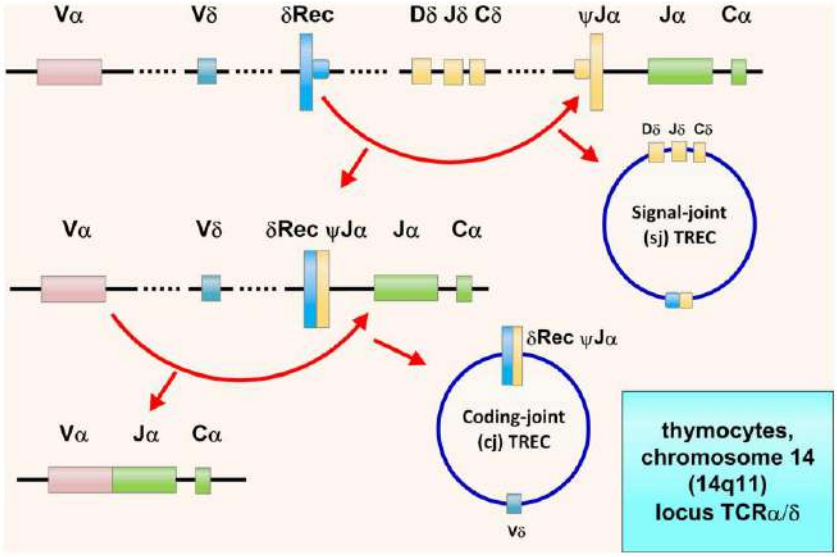


Figure 2 T cell Receptor Excision Circles (TRECs). TRECs are episomal DNA circles produced in thymocytes by excisional rearrangements of T cell receptor (TCR) genes; they are stable, not duplicated during mitosis, diluted out with each cell division, and therefore higher in thymocytes, recent thymic emigrants (RTEs) and naïve T cells. Quantitative polymerase chain reaction (PCR) of coding-joint (cj) δRec ψJa TREC, produced at TCRa/δ locus within chromosome 14 (14q11) by >70% of developing human α:β T cells, counts in the peripheral blood naïve α:β T lymphocytes recently dismetted by thymus: in newborn, values <25 TRECs/μL indicate SCID[43].

Wisconsin SCID screening poster **(Figure 3)** describes the fundamental features of SCID: children with SCID do not produce T lymphocytes (or, however, functional T lymphocytes), acquire multiple, persistent and severe viral, bacterial and fungal infections shortly after birth,

fail to thrive, and rarely reach their first birthday; SCID is a paediatric emergency.

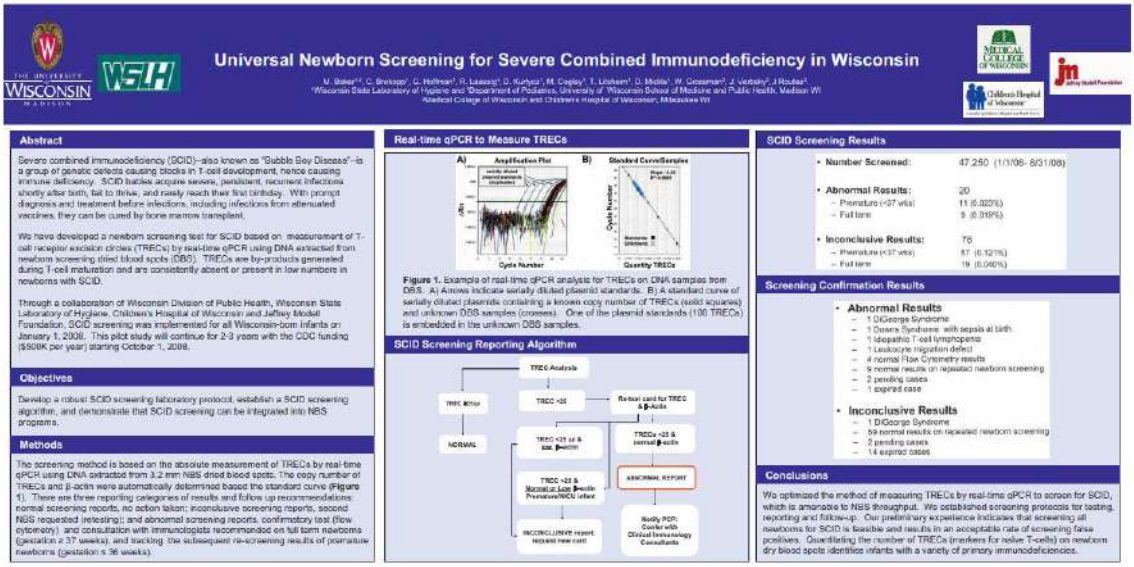


Figure 3 Wisconsin Newborn SCID screening poster. Reproduced with kind permission of the WI State Laboratory of Hygiene, http://www.slh.wisc.edu/posters/Baker102808.pdf.[42].

Conclusion and Future Perspective

SCIDs are a heterogeneous group of syndromes related to alterations of distinct genes that cause abnormalities in the maturation and/or function of T, B, and/or NK cells. Recently, advances in next generation DNA sequencing have allowed new gene identification through whole exome sequencing or whole genome sequencing of several forms of SCID and CID of unknown cause. The phenotypic and the molecular heterogeneity of SCIDs, as revealed by the expanding phenotypes observed, is making traditional classification of this group of disorders very intricate. Frequently, different mutations in the same gene can lead to different clinical phenotypes, such as OS, leaky SCID, or CID, that may even be inherited with different mechanisms. Here the discussion is mainly focused in detail on different forms of SCID and CID, paying attention to the distinctive peculiar clinical and laboratory features, in order to provide information to clinicians for recognizing and carefully managing these novel forms of PIDs.

Reference-

1. Kwan, A. et al. 2014. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. JAMA 312: 729–738.

2. Van der Burg, M. et al. 2011. The expanding clinical and immunological spectrum of severe combined immunodeficiency. Eur. J. Pediatr. 170: 561–571.

3. Denianke, K.S. et al. 2001. Cutaneous manifestations of maternal engraftment in patients with severe combined immunodeficiency: a clinicopathologic study. Bone Marrow Transplant. 28: 227–233.

4. Palamaro, L. et al. 2012. SCID-like phenotype associated with an inhibitory autoreactive immunoglobulin. J. Investig. Allergol. Clin. Immunol. 22: 67–70.

5. Shearer, W.T. et al. 2014. Establishing diagnostic criteria for severe combined immunodeficiency disease (SCID), leaky SCID, and Omenn syndrome: the primary immune deficiency treatment consortium experience.J. Allergy Clin. Immunol. 133: 1092–1098.

6. Gaspar, H.B. et al. 2013. How I treat severe combined immunodeficiency. Blood 122: 3749–3758.

7. Chinen, J. et al. 2014. Advances in basic and clinical immunology in 2013. J. Allergy Clin. Immunol. 133: 967–976.

8. Maggina, P. et al. 2013. Classification of primary immunodeficiencies: need for a revised approach? J. Allergy Clin. Immunol. 131: 292–294.

9. Pannicke, U. et al. 2009. Reticular dysgenesis (aleukocytosis) is caused by mutations in the gene encoding mitochondrial adenylate kinase 2. Nat. Genet. 41: 101–105.

10. Lagresle-Peyrou, C. et al. 2009. Human adenylate kinase 2 deficiency causes a profound hematopoietic defect associated with sensorineural deafness. Nat. Genet. 41: 106–111.

11. Nyhan, W.L. 2005. Disorders of purine and pyrimidine metabolism. Mol. Genet. Metab. 86: 25–33.

12. la Marca, G. et al. 2014. The inclusion of ADA-SCID in expanded newborn screening by tandem mass spectrometry. J. Pharm. Biomed. Anal. 88: 201–206

13. Malacarne, F.et al. 2005. Reduced thymic output, increased spontaneous apoptosis and oligoclonal B cells in polyethylene glycol-adenosine deaminase-treated patients. Eur. J. Immunol. 35: 3376–3386.

14. Somech, R.et al. 2013. Purine nucleoside phosphorylase deficiency presenting as severe combined immune deficiency.

Immunol. Res. 56: 150–154.

15. Aytekin, C. et al. 2010. Kostmann disease with developmental delay in three patients. Eur. J. Pediatr. 169:759–62.

16. Alangari, A. et al. 2009. Purine nucleoside phosphorylase deficiency in two unrelated Saudi patients. Ann. Saudi Med.29: 309–312.

17. Dror, Y. et al. 2004. Purine nucleoside phosphorylase deficiency associated with a dysplastic marrow morphology.

Pediatr. Res. 55: 472–477.

18. Grunebaum, E. et al. 2004. Novel mutations and hot-spots in patients with purine nucleoside phosphorylase deficiency. Nucleosides Nucleotides Nucleic Acids 23: 141

19. Puel, A. et al. 2000. Mutations in the gene for the IL-7 receptor result in T(-)B(+)NK(+) severe combined immunodeficiency disease. Curr. Opin. Immunol. 12: 468–473.

20. Puel, A. et al. 1998. Defective IL-7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. Nat. Genet. 20: 394–397.

21. Notarangelo, L.D.et al. 2009. Primary immunodeficiencies: 2009 update. J. Allergy Clin. Immunol. 124: 1161–1178.

22. Notarangelo, L.D.et al. 2000. Of genes and phenotypes: the immunological and molecular spectrum of combined immune deficiency: defects of the gamma(c)-JAK3 signaling pathway as a model.Immunol. Rev. 178: 39–48.

23. Du, C.et al. 2005. IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP. Kidney Int. 67: 1397–1409.

24. Ozawa, A. et al. 2004. Endogenous IL-15 sustains recruitment of IL-2Rbeta and common gamma and IL-2- mediated chemokine production in normal and inflamed human gingival fibroblast. J. Immunol. 173: 5180–5188.

25. Adriani, M. et al. 2006. Functional interaction of common gamma chain and growth hormone receptor signaling apparatus. J. Immunol. 177: 6889–6895.

26. Vigliano, I. et al. 2011.γ Chain transducing element: a shared pathway between endocrine and immune system. Cell Immunol. 269: 10–15.

27. Amorosi, S.et al. 2009. The cellular amount of the common g-chain influences spontaneous or induced cell proliferation. J. Immunol. 182: 3304–3309.

28. Kawamura, M. et al. 1994. Molecular cloning of L-JAK, a Janus family protein-tyrosine kinase expressed in natural killer cells and activated leukocytes. Proc. Natl. Acad. Sci. U.S.A. 91: 6374–6378.

29. Rane, S.G. et al. 1994. JAK3: a novel JAK kinase associated with terminal differentiation of hematopoietic cells. Oncogene 9: 2415–2423.

30. Notarangelo, L. 1996. Immunodeficiencies caused by genetic defects in protein kinases. Curr. Opin. Immunol. 8: 448–453.

31. Bassing, C.H. et al. 2002. The mechanism and regulation of chromosomal V(D)J recombination. Cell 109: S45–S55. 64. Woodbine, L. et al. 2013. PRKDC mutations in a SCID patient with profound neurological abnormalities. J. Clin. Invest. 123: 2969–2980.

32. Cagdas, D. et al. 2012. Two SCID cases with Cernunnos-XLF deficiency successfully treated by haematopoietic stem cell transplantation. Pediatr. Transplant. 16: 167–171.

33. Schwarz, K. et al. 2003. Human severe combined immune deficiency and DNA repair. Bioassays 25: 1061–1070.

34. Aloj, G. et al. 2012. Severe combined immunodeficiences: new and old scenarios. Int. Rev. Immunol. 31: 43–65.

35. Noordzij, J.G.et al. 2003. Radiosensitive SCID patients with Artemis gene mutation schow a complete B-cell differentiation arrest at the pre-B-cell receptor checkpoint in bone marrow. Blood 101: 1446–1452.

36. Pignata, C. et al. 1996. Progressive deficiencies in blood T cells associated with a 10p12-13 interstitial deletion. Clin. Immunol. Immopathol. 80: 9–15.

37. Pignata, C. 2002. A lesson to unraveling complex aspects of novel immunodeficiencies from the human equivalent of the nude/SCID phenotype. J. Hematother. Stem Cell Res. 11: 409–414.

38. Romano, R. et al. 2013. FOXN1: a master regulator gene of thymic epithelial development program. Front Immunol. 4: 187.

39. Palamaro, L.et al. 2014. FOXN1 in organ development and human diseases. Int. Rev. Immunol. 33: 83–93.

40. Chou, J.et al. 2014. A novel mutation in FOXN1 resulting in SCID: a case report and literature review. Clin. Immunol. 155: 30–32.

41. Romano, R. et al. 2012. From murine to human Nude/ SCID: the Thymus, T-cell development and the missing link. Clin. Dev. Immunol. 2012: 467101.

42. Haddad E and Hoenig M (2019) Hematopoietic Stem Cell Transplantation for Severe Combined Immunodeficiency (SCID). Front. Pediatr. 7:481. doi: 10.3389/fped.2019.00481.

43. Cossu: Genetics of SCID. Italian Journal of Pediatrics 2010 36:76.

trp RNA-binding attenuation protein (TRAP)-trp leader RNA interactions mediate translational

SUBMITTED BY: SUBRATA PATRA

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE

DEPARTMENT OF MICROBIOLOGY

REGISTRATION NO: A01-1112-116-024-2019

ROLL NO: 625

SEMESTER: V

GUIDED BY: Dr. SUBRATA KUNDU

DEPT. OF MICROBIOLOGY

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE

ACKNOWLEDGEMENT

I would like to acknowledge and give my warmest thanks to my supervisor Dr. Subrata Kundu who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator in charge; revered Bhrahamachari Parachaitanya Maharaj for allowing me to participate DSE project, which help me to venture relevant meaningful topics. Finally, I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

Abstract:

Survival and replication of most bacteria require the ability to synthesize the amino acid L-tryptophan whenever it is not available from the environment. In this article we describe the genes, operons, proteins, and reactions involved in tryptophan biosynthesis in bacteria, and the mechanisms they use in regulating tryptophan formation. We show that although the reactions of tryptophan biosynthesis are essentially identical, gene organization varies among species - from whole-pathway operons to completely dispersed genes. We also show that the regulatory mechanisms used for these genes vary greatly.

Introduction:

Extensive knowledge exists for the genes, enzymes, operons, and reactions of L-tryptophan (Trp) biosynthesis of bacteria, and on the mechanisms they use in regulating Trp formation [**1-5]. All organisms with this pathway use structurally similar enzymes, suggesting that the genes for this pathway evolved just once, probably late in the evolution of genes for amino acid biosynthesis. Trp is one of the rarest amino acids in most proteins, and it is the most costly to synthesize. It is generally encoded by a single codon, UGG, which may have served as a stop codon in early codon evolution. The 3D structures of all seven of the Trp biosynthetic enzymes are known, and most resemble structures of proteins catalyzing similar reactions in other pathways. The organization of trp genes within operons and the regulatory mechanisms used to control trp operon expression both vary greatly, undoubtedly reflecting organismal divergence in relationship to different metabolic contexts of Trp biosynthesis. Various trans-acting proteins and cis-acting sites and regions are used to regulate trp operon expression. The variety of mechanisms used suggests that groups of organisms experienced differing selective pressures in response to differing capabilities and needs. Intracellular concentrations of Trp and of charged and uncharged tRNATrp are the cues most often sensed in trp operon regulation. In this article we review and update knowledge on the distribution of the genes, enzymes, and regulatory mechanisms used by bacteria in Trp biosynthesis.

Phylogenetic distribution of trp gene organization and associated regulatory factors, elements, and mechanisms

Although some studies on trp gene regulation in Archaea have appeared recently [6-9], these are outside the scope of this review. Shown to the right are the trp-gene clustering, trp-gene fusions, and regulatory features. trp genes that have specialized functional roles other than primary Trp biosynthesis and which therefore are not regulated by Trp are not shown. A few pathogens (Haemophilus, Coxiella, and Propionibacterium) that appear to be in the process of

losing the Trp pathway [4] are also shown. The extensive variation in trp gene arrangements within operons and the repertoire of alternative mechanisms used in regulating transcription of these genes (Fig. 2), illustrate the plasticity that bacteria must have had for optimization of Trp biosynthesis.

Sensing Trp and tRNATrp in Gram-negative bacteria, Actinobacteridae, and Deinococci:

Not surprisingly, particular gene arrangements and regulatory strategies are often present in related phylogenetic groupings. For example, in Escherichia coli and other lowerGammaproteobacteria the seven trp biosynthetic genes are organized within a single transcriptional unit, the trp operon, which always has a trpCF gene fusion and which, in a smaller group, has a trpGD fusion (Fig. 1). In this group of bacteria two principal mechanisms are used for transcriptional regulation - one sensing Trp and a second sensing uncharged tRNATrp. Initiation of transcription of the trp operon is regulated by repression by the Trpactivated TrpR repressor protein (Figs. 2a-2b) [10,11]. Regulation by TrpR in Psychrobacter and Coxiella has been predicted by bioinformatic analysis, and regulation by TrpR has been demonstrated experimentally [12] in the phylogenetically distant Chlamydiales (which are not Gram-negative bacteria). Thus far, trpR generally appears to be autoregulated, and it exists predominantly in a monocistronic operon (Fig. 1). However, in some organisms trpR is co-transcribed with other trp genes, allowing coordinate expression, e.g., in the trpREG, trpRBA, and trpRDCFBA operons of Psychrobacter cryohalolentis, Chlamydia trachomatis and Chlamydophila caviae, respectively (Fig.1).

Uncharged tRNATrp is also sensed by transcription attenuation mechanisms as a regulatory signal (Figs. 2c-2d) [11,*13,14]. Interestingly, although trp operons containing all the trp genes are commonly subject to regulation by transcription attenuation, in some organisms this mechanism is used to regulate only a subset of the trp genes (Fig. 1). Thus, transcription attenuation is used to regulate the trpE and trpGDC operons of Pseudomonas aeruginosa, the trpEG operon of some Alphaproteobacteria (e.g., Rhizobium etli) and Actinobacteridae (e.g.,Streptomyces coelicolor), as well as the monocistronic trpE operon of Deinococci (e.g.,Deinococcus radiodurans) (Fig 1). In P. aeruginosa (and P. entomophila) transcription of the trpBA operon is activated by the TrpI protein in response to the accumulation of indoleglycerol phosphate (InGP), a Trp biosynthetic intermediate [15,16] (Figs. 2e-2f). InGP accumulates whenever the cellular Trp concentration is low. The trpI and trpBA operons of these organisms are divergently transcribed (Fig. 1) and their transcription is regulated by the binding of TrpI at their overlapping regulatory regions (Figs. 2e-2f). When the Trp concentration

is high, the InGP concentration is low, and TrpI binds near its promoter and autoregulates its own synthesis. When the Trp concentration is low and InGP accumulates, two molecules of activated TrpI are bound near the trpBA promoter, activating trpBA transcription (Figs. 2e-2f) [15,16].

Sensing Trp and tRNATrp in Gram-positive bacteria:

Although Gram-positive bacteria also sense both the intracellular level of free Trp and the availability of charged tRNATrp as regulatory signals, quite different strategies are used by these organisms in regulating the trp biosynthetic operon (Fig. 1). For example, in B. subtilis and some of its close relatives, coordinate expression of all seven trp genes is required for Trp biosynthesis. Six of these genes are clustered in the trp operon, trpEDCFBA, which is transcribed as part of a supraoperon also containing genes involved in the common aromatic pathway, and in phenylalanine, tyrosine, and histidine biosynthesis. The seventh trp gene, trpG, (also functioning as pabA) is located in the unlinked folate operon. Transcription of the trp operon of B. subtilis is regulated by attenuation by the Trp-activated trpRNA-binding attenuation protein, TRAP [17,18] (Figs. 2g-2h). In the presence of excess Trp, TRAPmediated transcription termination predominates, but those few transcripts that escape termination are subject to translational regulation via formation of a secondary structure that blocks ribosome access to the trpE ribosome-binding site [19]. Expression of trpG is coordinately regulated with the other trp genes by Trp-activated TRAP, which binds at the trpG mRNA ribosome-binding site, inhibiting its translation [20]. TRAP regulation of the trp operon also occurs in some Clostridia, although here trp operon organization varies significantly from that of B. subtilis. For example, in Syntrophomonas wolfei and Carboxydothermus hydrogenoformans all the trp genes are transcribed as part of a common operon. In the former, trpE and trpG are fused and located at the end of the operon while in the latter, the operon begins with the aroF gene, followed by unfused trpE and trpG genes(Fig. 1).

In B. subtilis the availability of uncharged tRNATrp is also sensed, and accumulation of uncharged tRNATrp regulates synthesis of AT, an anti-TRAP protein. AT binds to Trpactivated TRAP, inhibiting its function [21,22]. (Figs. 2i-2j). The structural gene for AT, rtpA, is transcriptionally regulated by the T box mechanism (see below), in response to the accumulation of uncharged tRNATrp [22]. AT synthesis is also regulated translationally by uncharged tRNATrp [21]. AT appears to have evolved very recently since it has only been identified in B. subtilis and the closely related B. licheniformis (Fig. 1 In the other TRAPcontaining organisms it is not known whether some other regulatory mechanism is used to sense uncharged tRNATrp accumulation.

In the vast majority of Gram-positive bacteria, TRAP is not present. Transcription of the trp operon is regulated in response to uncharged tRNATrp accumulation, by the T box mechanism. This mechanism involves leader RNA recognition of uncharged tRNATrp targeted with great

specificity and affinity in the absence of any protein factors. The T box was originally identified as the regulatory element used for many aminoacyl-tRNA synthetase operons in Gram-positive bacteria. It was later found to be associated with many other operons of amino acid metabolism, such as those containing transporter genes and biosynthesis genes. The T box elements regulating trp biosynthetic genes are often tandemly arranged in the upstream transcribed region of the trp operon. However, in some species only a single T box element is present. In organisms that use the T box mechanism to sense uncharged tRNATrp in regulating trp operon expression, the existence of a free-Trp sensing mechanism is unknown. In organisms with tandem T box elements, the quantitative advantages derived from employing tandem T boxes might compensate for the lack of a separate regulatory mechanism for sensing Trp. Regulation of the trp operon by the T box mechanism is almost exclusively confined to the Firmicutes.

Application:

A model for the tryptophan operon is formulated based on the genetic and biophysical data available on the structure of the operon and the nature of interactions between the represser and its ligands. Studies have been done, on wild-type, superrepressing, and loose-binding strains to identify conditions at which the stability of the system changes (i.e., evolves to a stable synthesis or periodic synthesis with increasing amplitude). Also, the factors that increase the yield of tryptophan are studied and predictions made, based on the results, for obtaining overproducing strains of tryptophan that can be used for the industrial production of this useful amino acid.

The Escherichia coli tryptophan (trp) promoter has been used extensively for the high level production of proteins on a small and large scale. This regulated promoter is readily available, relatively easy to turn on, and can be used in essentially and E. coli host background. This article gives a detailed use of the trp promoter including the design of expression vectors, subsequent culture conditions for promoter induction, and, finally, a protocol for the most common way of detecting the newly synthesized protein of interest. Its successful use for heterologous protein expression, however, sometimes requires consideration of parameters other than transcription such as translation initiation, translation elongation, and proteolysis. In this respect we offer guidance in getting through these post-transcriptional problems, which can occur with the use of any promoter.

Conclusion and future prospects:

Interest in the use of E. coli as a host strain in the hypersynthesis of foreign proteins is predicted to remain high. This is because its genetics and physiology are well understood, because it is readily manipulated using laboratory procedures that are proved and widely

practised, and because the final level of accumulated foreign protein can frequently reach 30% of the totaI~ with expression vectors generally available to the scientific community. The empirical approaches to protein overproduction that characterized the early work with E. coli have no\v given way to more systematic and fundamental studies aimed at identifying the factors that govern the final1evel of protein that can be recovered. For example, the many instances of cellulartoxicity and death as a consequence of protein overproduction have focused attention upon controlling the rate oftranscription initiation as a critical aspect of practical protein production. In this respect the tIp promoter continues to find favour. Transcription from this promoter is subject to manipulation through control over Trp repressor-operator interaction. Its signal strength is comparable to the other major promoters now in use, enabling the experimenter to drive the production of abundant amounts of many proteins. On high-copy-number plasmids the trp promoter tends to be incompletely regulated, a complication that has been overcome at least in part by increasing the intracellular level of Trp repressor by DNA manipulations that elevate the trpR gene dosage. As our understanding of structure~functionrelationships in the Trp repressor system continues to unfold t it may be possible to utilize mutationally altered Trp repressors to improve regulation at the trp promoter, making this process more amenable to the requirements of biotechnology. For example, if one could modify repressor-operator interaction by heating or cooling a fermentation vessel, thereby activating a trp promoter held in check by a thermolabile or cryolabile repressor, the sometimes undesirable use of indolylacrylic acid to activate expression could be abandoned. Since the previous survey of trp promoter-driven systems (Johnson and Somerville, 1985), the number of relevant literature reports has approximately quadrupled. It will be interesting to see whether the number of new production applications for this system continues to grow with an 18*month doubling time.

References:

1. Gutierrez-Preciado A, Jensen RA, Yanofsky C, Merino E. New insights into regulation of the tryptophan biosynthetic operon in Gram-positive bacteria. Trends Genet 2005;21:432–436.[PubMed:15953653]The extensive studies of Trp-pathway regulation in Gram-positive bacteria within the Bacillales Order provide an excellent comparative complement to what is known of Gram-negative bacteria related to Escherichia coli.

2. Xie G, Bonner CA, Jensen RA. Dynamic diversity of the tryptophan pathway in chlamydiae: reductive evolution and a novel operon for tryptophan recapture. Genome Biol 2002;3:research0051. [PubMed:12225590]

3. Xie G, Bonner CA, Song J, Keyhani NO, Jensen RA. Inter-genomic displacement via lateral gene transfer of bacterial trp operons in an overall context of vertical genealogy. BMC Biol 2004;2:15.[PubMed: 15214963]

4. Xie G, Keyhani NO, Bonner CA, Jensen RA. Ancient origin of the tryptophan operon and the dynamics of evolutionary change. Microbiol Mol Biol Rev 2003;67:303–342. [PubMed: 12966138]

5. Yanofsky C. Using studies on tryptophan metabolism to answer basic biological questions. J BiolChem 2003;278:10859–10878. [PubMed: 12556463]

6. Cubonova L, Sandman K, Karr EA, Cochran AJ, Reeve JN. Spontaneous trpY mutants and mutational analysis of the TrpY archaeal transcription regulator. J Bacteriol 2007;189:4338–4342. [PubMed:17400746]

7. Gast DA, Wasserfallen A, Pfister P, Ragettli S, Leisinger T. Characterization of Methanobacterium thermoautotrophicum Marburg mutants defective in regulation of L-tryptophan biosynthesis. J Bacteriol 1997;179:3664–3669. [PubMed: 9171414]

8. Tang X, Ezaki S, Fujiwara S, Takagi M, Atomi H, Imanaka T. The tryptophan biosynthesis gene cluster trpCDEGFBA from Pyrococcus kodakaraensis KOD1 is regulated at the transcriptional level and expressed as a single mRNA. Mol Gen Genet 1999;262:815–821. [PubMed: 10628865]

9. Xie Y, Reeve JN. Regulation of tryptophan operon expression in the archaeon Methanothermobacter thermautotrophicus. J Bacteriol 2005;187:6419–6429. [PubMed: 16159776]

10. Rose JK, Squires CL, Yanofsky C, Yang HL, Zubay G. Regulation of in vitro transcription of the tryptophan operon by purified RNA polymerase in the presence of partially purified repressor and tryptophan. Nat New Biol 1973;245:133–137. [PubMed: 4582892]

11. Yanofsky C. The different roles of tryptophan transfer RNA in regulating trp operon expression in E. coli versus B. subtilis. Trends Genet 2004;20:367–374. [PubMed: 15262409]

12. Akers JC, Tan M. Molecular mechanism of tryptophan-dependent transcriptional regulation in Chlamydia trachomatis. J Bacteriol 2006;188:4236–4243. [PubMed: 16740930]

*13. Merino E, Yanofsky C. Transcription attenuation: a highly conserved regulatory strategy used by bacteria. Trends Genet 2005;21:260–264. [PubMed: 15851059]Evidence is given that transcription attenuation is a frequently used mechanism of regulation.

14. Yanofsky C. Transcription attenuation: once viewed as a novel regulatory strategy. J Bacteriol 2000;182:1–8. [PubMed: 10613855]

15. Chang M, Crawford IP. The roles of indoleglycerol phosphate and the TrpI protein in the expression of trpBA from Pseudomonas aeruginosa. Nucleic Acids Res 1990;18:979–988. [PubMed: 2107533]

16. Olekhnovich I, Gussin GN. Effects of mutations in the Pseudomonas putida miaA gene: regulation of the trpE and trpGDC operons in P. putida by attenuation. J Bacteriol 2001;183:3256–3260. [PubMed: 11325956]

17. Babitzke P, Gollnick P, Yanofsky C. The mtrAB operon of Bacillus subtilis encodes GTP cyclohydrolase I (MtrA), an enzyme involved in folic acid biosynthesis, and MtrB, a regulator of tryptophan biosynthesis. J Bacteriol 1992;174:2059–2064. [PubMed: 1551827]

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE

Project Report on-

Production & Uses of the "fairy moss", Azolla

Bsc. Microbiology (Honours)

Submitted By- Rakesh Ghosh

Semester- V

Registration No- A01-1112-116-002-2019 of 2019-2020

Examination Roll No- 2022151184

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Guided By- Prof. Basudev Murmu

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

YEAR-2021-22

ACKNOWLEDGEMENT

I would like to acknowledge and give my warmest thanks to my supervisor Prof. Basudev Murmu sir, department of Microbiology, Ramakrishna Mission Vivekananda Centenary College, who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator; revered Br. Parachaitanya Maharaj for allowing me to participate in this DSE project, which help me to venture relevant meaningful topics. Finally, I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

Introduction

Azolla commonly known as mosquito fern, duckweed fern, fairy moss, and water fern, is a small free floating aquatic fern native to Asia, Africa, and the America. It grows in swamps, ditches, and even in lakes and rivers where the water is not turbulent (Lumpkin and Plucknett, 1980). The name *Azolla* is derived from the two Greek words, *Azo* (to dry) and *Ollyo* (to kill) thus reflecting that the fern is killed by drought. *Azolla-Anabaena* is a symbiotic complex in which the endophytic blue-green algae *Anabaena zollae* lives within the leaf cavities of the water fern *Azolla (*Lain). The endosymbiont, which is nitrogen-fixing, provides sufficient nitrogen for both itself and its host (Peters, 1978). The fern, on the other hand, provides a protected environment for the algae and also supplies it with a fixed carbon source. It has capability to fix atmospheric nitrogen as well as to produce biomass at a very high rate. A lot of research works was done on *Azolla* during the past and recent decades. *Azolla* is commonly used as biofertilizer as well as green manure in the paddy field. Now a days *Azolla* (either fresh or in dried) is also used as a feed ingredient for ruminants and nonruminants type of livestock. Besides its utilization as biofertilizer and livestock feed, *Azolla*, the 'green gold mine' of the nature is also used as medicine, water purifier, human food and for production of biogas.

Isolation of *Azolla:*

Fragmentation of the abscission layer of *Azolla* or *Azolla* frond is used for multiplication of *Azolla*. Under laboratory condition it is multiplied in small cement tanks (10 sq m). Following steps are to be followed in laboratory multiplication of *Azolla*:

(1) Maintain water level at 10 cm in tank/soil pond.

(2) Add 250 gm of fresh cow dung in tank water with 2.5 gm of super phosphate.

(3) Inoculate 200 gm of *Azolla* fronds in tank water.

(4) Apply furadan granules (2.5 gm) on the 7th day of fronds inoculation.

Similarly, frond based spore inoculums of *Azolla* (*A. microphylloa*) can be used. These frond based spore inoculums are pre-soaked in superphosphate solution (25 ppm) at a moisture saturation of 12 hours. Pre-soaked dried spores are released in 10 ml water and mixed well in tank water. Approximately, 1 gm of frond based spore inoculums is needed. The inoculated spores germinate well and sporelings emerge in 2 weeks period and multiply as *Azolla* . Growth of *Azolla* fronds. The frond based spore inoculum of *A. microphylla* is used at 2–3 kg/ha for inoculation in transplanted rice fi eld. The frond based spore inoculums are presoaked in superphosphate solution (25 ppm) at moisture saturation for 12 hours. Pre-soaked dried spores are released into 25 liters of water, mixed well and sprinkled in the main fi eld uniformly for 7–10 days after transplanting. The inoculated spores germinate well and the sporelings emerge in 2 weeks period and multiply well in

transplanted rice. *Azolla* biomass is effectively used as biofertilizer for rice. A layer of *Azolla* covering a hectare of rice fi eld contains about 15–25 t biomass. The dry matter of *Azolla* usually contains 3–6% nitrogen. *Azolla* is decomposed in flooded rice fi eld condition in 2–3 weeks period. *Azolla* can contribute 40–60 kg N/ha. The multiplication of *Azolla* along with rice crop also suppresses the aquatic weed populations. In addition to nitrogen release, *Azolla* also contribute potassium, phosphorus, calcium, sulphur, zinc and iron to rice soil. The dual culture method of growing *Azolla* with rice has perhaps the more widespread applicability because standing water is available in the field for growth of *Azolla* during the growth of rice from seedling to panicle initiation in most wetland rice fields. It grows harmoniously with rice plants and often remains green and healthy during such growth, being shaded from high light intensity by the rice canopy. Fresh biomass of *Azolla* is broadcasted in the main field 7–10 days after transplanting the rice. Inoculation of the fresh biomass of *A. microphylla* at 200 kg/ha could multiply faster and could cover the rice fi elds as a green mat in 2–3 weeks period with 15–25 t biomass accumulation. *Azolla* technology is very efficient in terms of N2 fixation and biomass accumulation during *rabi* season due to better environmental conditions (particularly, cloudy days coupled with low temperature favor its vegetative multiplication) prevailing during the second season rice. Growing *Azolla* along with rice crop is called dual culture and it does not affect the growth of rice crop in any way.

For application of dried frond based spore inoculums of *Azolla*, 100 kg of sporulated fresh fronds of *A. microphylla* is formed into a heap. This is covered with a thin layer of clay soil slurry (20%) and allowed to undergo decomposition for a period of 21 days. Well-dried frond material is called "frond based spore inoculums" of *Azolla*. The viability of the spore inoculum is good for 10–15 months, which can be applied in rice field at 3 kg/ha.

Factors influencing the biomass

Production of *Azolla*

Environment influences the productivity of *Azolla* in rice production system.The primary constraint to the use of *Azolla* is its requirement for an aquatic habitat. An individual *Azolla plant* can survive only for a few hours on a dry surface under a tropical condition. It can survive for only a few days or a week on rice soil that dries during intermittent irrigations. Some varieties of *Azolla can* survive indefinitely on moist and shaded mud, but they will not multiply to any useful extent. For example, when enough irrigation water is available, relative growth rate, total biomass accumulation and nitrogen concentration are higher in cool dry environments. Environmental factors, such as temperature, light intensity and humidity are important in controlling the growth, multiplication and

nitrogen fixation in *Azolla.*

Water

Azolla is very sensitive to dryness. It dies in a few hours if the soil becomes dry. *Azolla* growth is promoted by a fairly shallow depth of water (5 cm). Such a situation favors mineral nutrition since roots are near the soil and also reduces the negative effect on growth due to water turbulence. On the other hand, it should not allow rooting in the soil, which restricts growth, because it creates a tropic premature state of over population.

Temperature

The geographical distribution of *Azolla* clearly indicates that the genus is adapted to very different temperature conditions. Most of the *Azolla* species are widely distributed in temperate regions, as they are generally sensitive to the higher temperature of the tropics. Low tolerance to high temperature is one of the constraints to the multiplication of *Azolla* under field conditions. The optimum temperature range in which most of them grow well is 20–25°C. The temperature, particularly flood water temperature in rice fields, is a crucial factor, therefore the selection of strain for use in a given place at a given season must be done while taking temperature into special consideration. The influence of temperature on the growth of *Azolla* is dependent on many factors. Light intensity interacts with the effect of temperature. At higher light intensities, the optimum temperature shifts to higher temperature. The higher tolerance level of *A*. *microphylla* BR-GI and *Azolla* sp. – ST-SI to higher temperature is at 38 ± 1°C/25± 1°C day/night regime.

Humidity

The optimum relative humidity needed for normal *Azolla* growth is 85–91%. Low relative humidity below 60% causes *Azolla* fronds to dry up, turn fragile and become more susceptible to adverse conditions. High relative humidity causing a longer dew period results in susceptibility of the plant to diseases. In the tropics, high relative humidity during rainy season causes insect infestation.

Light intensity

Like other green plants, *Azolla* requires light for photosynthetic activity and the production of organic skeleton used in the cell synthesis reaction. In the tropics, direct sunlight during clear days at mid-day affects the growth and multiplication; while cloudy days provide a very favorable light exposure. Under high sunlight intensities, the fronds turn bricks-red which is a sign of physiological stress. Possibly to avoid maximum absorption of sunlight, the *Azolla* plant produces anthocyanin to protect its photosynthetic mechanism from damage. The growth and nitrogenase activity of *A. filculoides* changes with increasing light intensity. The growth increases with increasing light intensity to a maximum in 50% sunlight and CO2 fixation saturated at 8000 lux in *A. carolliniana*. The growth rate of *Azolla* is higher at 25% sunlight (24500 lux) than full sunlight. The nitrogenase

activity follows the same pattern, being highest in 50% sunlight and fractionally lower in 25% and 75% sunlight.

Wind

Wind tends to push all the fronds together on the same plant on the water surface. It is possible to reduce the influence of this factor by providing bunds and *Azolla*-rice intercrop.

Soil pH

The soil pH has a greater influence on the growth and multiplication of *Azolla* and the slightly acidic to neutral pH is found to be suitable for its growth. The very acidic soils of pH 3.0–3.8 do not support the growth. The soils having pH 5–7 support better growth than the soils of pH 8.0. *Azolla* can survive within a pH range of 3.5–10, but optimum growth is noticed at a pH range of 4.5–7.0. The growth of *Azolla* is reported to be good in nutrient solution at pH 5.5. The luxuriant growth of *Azolla* is produced at pH 5.5–6.6 in the irrigation water with soluble iron content of 0.56 mg/liter. It is also noticed that *Azolla* thrives at low pH in the presence of ferrous ions rather than that of ferric ions. An inverse relationship between pH and the temperature influences nitrate reduction and nitrogen fixation. Nitrogen reduction is optimum at pH 4.5 and 30°C, while nitrogen fixation is optimal at pH 6.0 and 20°C. The nitrogen fixation decreased at neutral pH.

Azolla as Biofertilizer in Rice Cultivation

Azolla is cultivated in the paddy field either as monocrop or as intercrop and incorporated into the mud/soil for increasing humus and nutrient content of the soil. This practice of *Azolla* cultivation is widely popular in the countries of south-east Asia like India, China, Phillipines, Indonesia etc. Peters (1978) reported that the use of *Azolla* increased rice yields by 112% over unfertilized controls when applied as a monocrop during the fallow season, by 23% when applied as an intercrop with rice, and by 216% when applied both as a monocrop and an intercrop. When *Azolla* used as a bio-fertilizer in paddy field it produces around 300 tons of green bio-hectare per year under normal subtropical climate which is comparable to 800 kg (1800 kgs of urea) of nitrogen (Wagner 1997). *Azolla* has quick decomposition rate in soil and thus it speeds up the efficient availability of its nitrogen to rice plant. The quick multiplication rate and rapid decomposing capacity of *Azolla* has become paramount important factor to use as green manure cum bio-fertilizer in rice field. The benefits of *Azolla* application in the rice field are the following: Basal application @ 10-12 tones/hectare increases soil nitrogen by 50-60 kg/ha and reduces 30-35 kg of nitrogenous fertilizer requirement of rice crop. Release of green *Azolla* twice as dual cropping in rice crop @ 500 kg/ha enriches soil

nitrogen by 50 kg/ha and reduces nitrogen requirement by 20-30 kg/ha. Use of *Azolla* increases rice yield by 20 to 30% (Raja *et al*., 2012).

Azolla in Reclamation of Saline Soils

Although, *Azolla* is relatively sensitive to salt, cultivation in saline environment for a period of two consecutive years decreased salt content from 0.35-0.15 and desalinate rate (71.4%) was 1.8 times faster than through water leaching and 2.1 times faster than *Sesbania* and also reduced the electrical conductivity, pH of acidic soil and increased calcium content of soil (Anjuli *et al.*, 2004).

Azolla as Mosquito Repellent

Azolla can also be used in the control of mosquitoes, for a thick *Azolla* mat on the water surface can prevent breeding and adult emergence. In a survey of pools, ponds, wells, rice fields and drains (Ansari and Sharma 1991) found that breeding by *Anopheles* spp. was almost completely suppressed in water bodies that were completely covered with *Azolla*.

Azolla in Production of Biogas

Anaerobic fermentation of *Azolla* (or a mixture of *Azolla* and rice straw) results in the production of methane gas which can be utilized as fuel and remaining effluent can be used as a fertilizer because it contains all the nutrients originally incorporated in plant tissues except for a small percentage of nitrogen lost as ammonia as suggested by Van Hove 1989. Das *et al.* 1994 mixed cow dung and *Azolla* residues and found that best ratio was 1:0.4, which gave a gas production 1.4 times that of cow dung alone.

Azolla in Bioremediation

It was found that *A. pinnata* and *Lamna minor* removed the heavy metals iron and copper from polluted water (Jain *et al.* 1989). The pollutants at low concentration could be treated by passing it through ponds and can be reused for Agriculture purpose. Recently Arora *et al.* (2006) found that tolerance and phyto-accumulation of chromium by three *Azolla* species and also results found by Cohen-Shoel *et al.* 2002 shows bio filtration of toxic elements by *Azolla* biomass. *Azolla* exhibits a remarkable ability to concentrate metals Cu, Cd, Cr, Ni, Pb and nutrients directly from pollutants or sewage water.

Azolla as a Component of Space Diet

Recent research by Katayama *et al.,* 2008 in collaboration with Space Agriculture Task Force suggested *Azolla* as a component of the space diet during habitation on Mars and found that *Azolla* was found to meet human nutritional requirements on Mars.

Azolla as a human food

Azolla is widely used as a livestock feed in India and the Far East, but its potential as a food for people is less well known. This is now changing and some of the impetus has come from studies into diets that could be used for space stations, space travel, and habitation on the Moon and Mars. *Azolla's* protein content is close to that of soybean. It is a rich source of minerals (10-15% of dry weight), essential amino acids (7-10% of dry weight), vitamins and carotenoids. 20 – 30% of *Azolla's* dry weight is protein, which is a lot for a vegetable. The quality of the protein in *Azolla* is good.

Reference:

1. Hall, D.O.; Markov, S.A.; Watanabe, Y. and Rao, K.K. 1995. The potential applications of cyanobacterial Photosynthesis for clean technologies, *Photosyn. Res.,* **46**: 159-167.

2. Jain, S.K.; Vasudevan, P. and Jha, N.K. 1989. Removal of some heavy metals from polluted water by aquatic plants: studies on duckweed and water valvet. *Biol. Wastes.,* **28**:115-126.

3. Katayama, N.; Masamichi, Y.; Yoshiro, K.; Chung, C.L.; Watanabe, I. and Hidenori, W. 2008. Space Ag. Task Force-*Azolla* as a component of the space diet during habitation on Mars, *Acta Astronautica.,* **63**:1093-1099

4. Lumpkin, T. A. and Plucknett, D. L. 1980. *Azolla:* Botany, physiology and use as a green manure. *Econ. Bot.* **34:** 111-153.

5. Murthy, T. N. K.; Ashok, M.; Thirumalesh, T.; Umesh, B. U. and Nataraju, O. R., 2013. Effect of partial replacement of *Azolla* for concentrate supplement on lactating crossbred cows. *Environment and Ecology*, **31** (2): 415-417

6. Parthasarathy, R.; Kadirvel, R. and Kathaperumal, V. 2002. *Azolla* as a partial replacement for fish meal in broiler rations. *Indian Vet.J*., **79** (2): 144-146

7. Peters, G. A. 1975. The *Azolla-Anabena Azollae* relationship III studies on metabolic capacities and a further characterization of the symbiont, *Arch. Microbiol.,* **103**: 113-122.

8. Peters, G. A. 1976. Studies on the *Azolla-Anabaena Azollae* symbiosis. In. W. E. Newton & C. L Nyman (ed.), Proceedings of the First International Symposium on Nitrogen Fixation. Vol. 2. Washington State University Press, Pullman pages 592-610

9. Peters, G. A. 1977. The *Azolla-Anabaena Azollae* symbiosis. *In* A. Hollaender (ed.),Genetic engineering for nitrogen fixation. Plenum Press, New York. pages 231-258 Peters, G. A. 1978. Blue-green algae and algal associations. BioScience **28**: 580-585.

10. Rai, R. B.; Dhama, K.; Damodaran, T.; Ali, H.; Rai S.; Singh, B. and Bhatt, P. 2012. Evaluation of *Azolla* (*Azolla pinnata*) as a poultry feed and its role in poverty alleviation among landless people in northern plains of India. *Vet. Pract.*, **13** (2): 250-254

11. Raja W.; Rathaur P.; John, S. A. and Ramteke, R.W. 2012. *Azolla*: an aquatic Pteridophyte with great potential *InternationalJournal of Research in Biological Sciences* **2**(2): 68-72

12. Seulthrope, C.D. 1967. The biology of aquatic vascular plants, *Edward Arnold (Pub) Ltd. London*., pp. 610- 615.

13. Tamang, Y. and Samanta, G., 1993. Feeding value of *Azolla* (*Azolla pinnata*) an aquatic fern in Black Bengal goats.

Indian J. Anim. Sci., **63** (2) : 188-191

6. Van Hove, C. 1989. *Azolla* and its multiple use with emphasis on Africa, *Food and Agriculture Organization, Rome,* **21**:112 -116.

14. Wagner, G.M. 1997. *Azolla:* A Review of Its Biology and Utilization. *The Botanical Review* **63**(1): 1-26.

A Review on Toxicity and Bioremediation of Heavy Metals Contaminated Ecosystem from Tannery Wastewater

Submitted by

Debarshi Paul

Registration number: A01-1152-116-008-2019
Roll number: 2022151190

Guided by: Dr. Sujoy Pal

Department of Microbiology,
Ramakrishna Mission Vivekananda Centenary College, Rahara,
Kolkata 700118

January, 2022

ACKNOWLEDGEMENT

I would like to acknowledge and give my warmest thanks to my supervisor Dr. Sujoy Pal who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator in charge; revered Brahmacharya Parachaitanya Maharaj for allowing me to participate in this project work, which help me to venture relevant meaningful topics.

Finally, I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

A Review on Toxicity and Bioremediation of Heavy Metals Contaminated Ecosystem from Tannery Wastewater

1. Abstract :

The discharge of untreated tannery wastewater containing bio toxic substances of heavy metals in the ecosystem is one of the most important environmental and health challenges in our society. Hence, there is a growing need for the development of novel, efficient, eco-friendly, and cost-effective approach for the remediation of inorganic metals (Cr, Hg, Cd, and Pb) released into the environment and to safeguard the ecosystem. In this regard, recent advances in microbes-base heavy metal have propelled bioremediation as a prospective alternative to conventional techniques. Heavy metals are non-biodegradable and could be toxic to microbes. Several microorganisms have evolved to develop detoxification mechanisms to counter the toxic effects of these inorganic metals. This present review offers a critical evaluation of bioremediation capacity of microorganisms, especially in the context of environmental protection. Furthermore, this article discussed the mechanism of detoxification for the removal of heavy metals. The use of biofilm has showed synergetic effects with many fold increase in the removal of heavy metals as sustainable environmental technology in the near future.

Keywords : Tannery wastewater, Biotoxic, Eco-friendly, Cost-effective, Bioremediation, Detoxification, Biofilm.

2. Introduction :

Industrial tannery wastewater is a major source of heavy metal contamination in our environment. Environmental pollution by heavy metals has become a serious threat to living organisms in an ecosystem [1]. Metal toxicity is of great environmental concern because of their bioaccumulation and non-biodegradability in nature [2]. Several inorganic metals like magnesium (Mg), nickel (Ni), chromium (Cr3+), copper (Cu), calcium (Ca), manganese (Mn), and sodium (Na) as well as zinc (Zn) are vital elements needed in small quantity for metabolic and redox functions. Heavy metals such as aluminium (Al), lead (Pb), cadmium (Cd), gold (Au), mercury (Hg), and silver (Ag) do not have any biological role and are toxic to living organisms [1,3]. Bioremediation is employed in order to transform toxic heavy metals into a less harmful state using microbes [4] or its enzymes to clean-up polluted environment. The technique is environmentally friendly and costeffective in the revitalization of the environment [5]. Bioremediation of heavy metals has limitations. Among these are production of toxic metabolites by microbes and non-biodegradability of heavy metals.

The direct use of microorganisms with distinctive features of catabolic potential and/or their products such as enzymes and bio surfactant is a novel approach to enhance and boost their remediation efficacy. For instance, the use of microbial fuel cell (MFC) to degrade recalcitrant heavy metals has been explored. Biofilmmediated bioremediation can be applied for cleaning up of heavy metal contaminated environment.

Microbial technologies are active and growing [6]. Long trajectory exists on how microbes and metals interact in both natural and man-made environments. Microbial-metal interactions is primarily focused on metals removal, i.e., remediation and depollution. The recent revival of the use of solid-state electrodes as electron donors or acceptors for microbial growth has brought innovative prospects, resulting to microbial-electrochemical technologies (METs). The application of microorganisms as a green approach for the synthesis of metallic nanoparticles (NPs) has been reported . Genetically modified microorganisms have also been used as a remediation technique [7]. Genetic engineering and chemical modification could alter the components of cells surface and can efficiently improve the adsorption capacity and selectivity to target-metal species.

Several factors which influences and limit bioremediation efficiency include temperature, pH, redox potential, nutritional status, moisture, and chemical composition of heavy metals [8]. The use of microbes alone has shown limited efficiency owing to various factors including poor competitiveness as well as excessive heavy metal concentrations. Effectiveness can be enhanced by several amendments with inorganic nutrients, bio-surfactants, bulking agents, and compost as well as biochar [9]. These adjustments have been comprehensively reviewed in recent studies.

There are several protection mechanisms of heavy metal resistance by microbial cells. These mechanisms are extracellular barrier, extracellular sequestration, and active transport of metal ions (efflux), intracellular sequestration, and reduction of metal ions [10].

This study therefore seeks to review the reports of previous investigators on the toxic effect and the use of microbial cell and their products, namely, bio-surfactants, to enhance remediation of heavy metals. It also discusses the factors that influence bioremediation of heavy metals along with their underlining mechanisms. The findings and analyses are presented in the following sections. Current research work on microbial bio sorption and detoxification is not only summarized but also future directions are suggested.

3. Toxicity of Heavy Metals to Microorganisms :

Toxicity of heavy metals is the ability of a metal to cause detrimental effects on micro-organisms, and it depends on the bioavailability of heavy metal and the absorbed dose [11]. Heavy metal toxicity involves several mechanisms, that is, breaking fatal enzymatic functions, reacting as redox catalysts in the production of reactive oxygen species (ROS), destructing ion regulation, and directly affecting the formation of DNA as well as protein [12]. The physiological and biochemical properties of microorganisms can be altered by the presence of heavy metals. Chromium (Cr) and cadmium (Cd) are capable of inducing oxidative damage and denaturation of microorganisms as well as weakening the bioremediation capacity of microbes.

Chromium Cr (III) may change the structure and activity of enzymes by reacting with their carboxyl and thiol groups [13]. Intracellular cationic Cr (III) complexes interact electrostatically with negatively charged phosphate groups of DNA, which could affect transcription, replication, and cause mutagenesis [13].

Heavy metals like copper (Cu (I) and Cu (II)) could catalyze the production of ROS via Fenton and Haber-Weis reactions, which will act as soluble electron carries. Tis can cause severe injury to cytoplasmic molecules, DNA, lipids, and other proteins [14]. Aluminium (Al) could stabilize superoxide radicals, which is responsible for DNA damage. Heavy metals could stop vital enzymatic functions by competitive or noncompetitive interactions with substrates that will cause configurational changes in enzymes [30]. Furthermore, it can also cause ion imbalance by adhering to the cell surface and entering through ion channels or trans-membrane carriers.

Cadmium (Cd) and lead (Pb) pose deleterious effect on microbes, damage cell membranes, and destroy the structure of DNA. This harmfulness is generated by the displacement of metals from their native binding sites or ligand interactions. The morphology, metabolism, and growth of microbes are affected by changing the nucleic acid structure, causing functional disturbance, disrupting cell membranes, inhibiting enzyme activity, and oxidative phosphorylation [15]

4. Mechanism of Microbial Detoxification of Heavy Metal :

Microorganisms adopt different mechanisms to interact and survive in the presence of inorganic metals. Various mechanisms used by microbes to survive metal toxicity are biotransformation, extrusion, use of enzymes, production of exo-polysaccharide (EPS) [16], and synthesis of metallothioneins. In response to metals in the environment, microorganisms have developed ingenious mechanisms of metal resistance and detoxification. The mechanism involves several procedures, together with electrostatic interaction, ion exchange, precipitation, redox process, and surface complexation [17]. The major mechanical means to resist heavy metals by microorganism are metal oxidation, methylation, enzymatic decrease, metal-organic complexion, metal decrease, metal ligand degradation, metal efflux pumps, demethylation, intracellular and extracellular metal sequestration, exclusion by permeability barrier, and production of metal chelators like metallothioneins and bio surfactants [18].

Microorganisms can decontaminate metals by valence conversion, volatilization, or extracellular chemical precipitation [18]. Microorganisms have negative charge on their cell surface because of the presence of anionic structures that empower the microbes to bind to metal cations [19]. The negatively charged sites of microbes involved in adsorption of metal are the hydroxyl, alcohol, phosphoryl, amine, carboxyl, ester, sulfhydryl, sulfonate, thioether, and thiol groups [19].

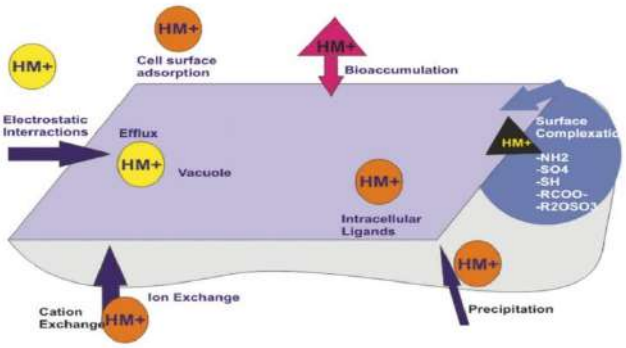


Figure : Mechanisms of heavy metal uptake by Microorganisms [17].

4.1. Bio Sorption Mechanism :

The uptake of heavy metals by microbial cells through bio sorption mechanisms can be classified into metabolism-independent bio sorption, which mostly occurs on the cells exterior and metabolism-dependent bioaccumulation, which comprises sequestration, redox reaction, and species-transformation methods [20]. Bio sorption can be carried out by dead biomass or living cells as passive uptake through surface complexation onto the cell wall and surface layers [21]. Bioaccumulation depends on a variety of chemical, physical, and biological mechanisms and these factors are intracellular and extracellular processes, where bio sorption plays a limited and ill-defined role [21].

4.2. Intracellular Sequestration :

Intracellular sequestration is the complexation of metal ions by various compounds in the cell cytoplasm. The concentration of metals within microbial cells can result from interaction with surface ligands followed by slow transport into the cell. The ability of bacterial cells to accumulate metals intracellular has been exploited in practices, predominantly in the treatment of effluent treatment. Cadmium-tolerant *P. putida* strain possessed the ability of intracellular sequestration of copper, cadmium, and zinc ions with the help of cysteine-rich low molecular weight proteins. Also, intracellular sequestration of cadmium ions by glutathione was revealed in *Rhizobium leguminosarum* cells [22].

The rigid cell wall of fungi is made up of chitin, mineral ions, lipids, nitrogen-containing polysaccharide, polyphosphates, and proteins. They can decontaminate metal ions by energetic uptake, extracellular and intracellular precipitation, and valence conversion, with several fungi accumulating metals to their mycelium and spores. The exterior of the cell wall of fungi behaves like a ligand used for labelling metal ions and brings about the elimination of inorganic metals [56–59]. Peptidoglycan, polysaccharide, and lipid are components of cell wall that are rich in metal-binding ligands (e.g., -OH, - COOH, -HPO42−, SO42− -RCOO−, R2OSO3−, -NH2, and -SH). Amine can be more active in metal uptake among these functional groups, as it binds to anionic metal species via electrostatic interaction and cationic metal species through surface complexation.

4.3. Extracellular Sequestration :

Extracellular sequestration is the accumulation of metal ions by cellular components in the periplasm or complexation of metal ions as insoluble compounds. Copper-resistant *Pseudomonas syringae* strains produced copper-inducible proteins CopA, CopB (periplasmic proteins), and CopC (outer membrane protein) which bind copper ions and microbial colonies [23]. Bacteria can eject metal ions from the cytoplasm to sequester the metal within the periplasm. Zinc ions can cross from the cytoplasm by efflux system where they are accumulated in the periplasm of Synechocystis PCC 6803 strain [24].

Metal precipitation is an extracellular sequestration. Iron reducing bacterium such as *Geobacter spp.* and sulfur reducing bacterium like *Desulfuromonas spp.* are capable of reducing harmful metals to less or nontoxic metals. *G. metallireducens*, a strict anaerobe, is capable of reducing manganese (Mn), from lethal Mn (IV) to Mn (II), and uranium (U), from poisonous U (VI) to U (IV) [49].*G. sulfur*reducens and *G. metallireducens* have the ability to decrease chromium (Cr) from the very lethal Cr (VI) to less toxic Cr (III) [25]. Sulfate-reducing bacteria generate large amounts of hydrogen sulfide that causes precipitation of metal cations [26].

Klebsiella planticola strain generates hydrogen sulfide from thiosulfate under anaerobic conditions and precipitated cadmium ions as insoluble sulfides. Also, cadmium was precipitated by *P. aeruginosa* strain under aerobic conditions. *Vibrio harveyi* strain precipitated soluble divalent lead as complex lead phosphate salt.

4.4. Extracellular Barrier of Preventing Metal Entry into Microbial cells :

Microbial plasma membrane, cell wall, or capsule could prevent metal ions from entering the cell. Bacteria can adsorb metal ions by ionizable groups of the cell wall (amino, carboxyl, phosphate, and hydroxyl groups). Pardo et al. [28], Taniguchi et al. [27], and observed high level of passive bio sorption of heavy metal ions for nonviable cells of *Pseudomonas putida, Brevibacterium sp., and Bacillus sp.*

Pseudomonas aeruginosa biofilm cells show higher resistance to ions of copper, lead, and zinc than planktonic cells, while cells located at the periphery of the biofilm were killed. Extracellular polymers of biofilm accumulated metal ions and then protect bacterial cells inside the biofilm.

4.5. Methylation of Metals :

Methylation increases metal toxicity as a result of increased lipophilicity and thus increased permeation across cell membranes. Microbial methylation plays a significant function in metal remediation. Methylated compounds are regularly explosive; for instance, Hg (II) can be bio methylated by some bacteria such as *Bacillus spp., Escherichia spp., Clostridium spp., and Pseudomonas spp.* to gaseous methyl mercury. Bio methylation of selenium (Se) to volatile dimethyl selenite and arsenic (As) to gaseous arsines as well as lead (Pb) to dimethyl lead was witnessed in polluted top soil [12].

4.6. Reduction of Heavy Metal Ions by Microbial Cell :

Microbial cells can convert metal ion from one oxidation state to another, hence reducing their harmfulness. Bacteria use metals and metalloids as electron donors or acceptors for energy generation. Metals in the oxidized form could serve as terminal acceptors of electrons during anaerobic respiration of bacteria. Reduction of metal ions through enzymatic activity could result in formation of less toxic form of mercury and chromium [29].

5. Bioremediation Capacity of Microorganisms on Heavy Metals :

The uptake of heavy metals by microorganisms occurs via bioaccumulation which is an active process and/or through adsorption, which is a passive process. Several microorganisms like bacteria, fungi, and algae have been used to clean up heavy metal contaminated environments [30]. The application of metal-resistant strains in single, consortium, and immobilized form for the remediation of heavy metals has yielded effective results while the immobilized form could have more chemo sorption sites to biosorb heavy metals.

5.1. Bacteria Remediation Capacity of Heavy Metal :

Microbial biomass has different biosorptive abilities, which also varies significantly among microbes. However, the bio sorption ability of each microbial cell depends on its pretreatment and the experimental conditions. Microbial cell must adapt to alteration of physical, chemical and bioreactor configuration to enhance bio sorption [23]. Bacteria are important bio sorbents due to their ubiquity, size, and ability to grow under controlled conditions and resilience to environmental conditions [31].

De Jaysankar and his coauthors [32] use mercuryresistant bacteria such as *Alcaligenes faecalis, Bacillus pumilus, Pseudomonas aeruginosa,* and *Brevibacterium iodinium* for the removal of cadmium (Cd) and lead (Pb). In this study, *P. aeruginosa* and *A. faecalis* removed 70 % and 75 % cadmium (Cd) with reduction of 1000 mg/L to 17.4 mg/L of cadmium (Cd) by *P. aeruginosa* and to 19.2 mg/L by *A. faecalis* in about 72hrs. *Brevibacterium iodinium* and *Bacillus pumilus* remove greater than 87 % and 88 % of lead (Pb) with a reduction of 1000 mg/L to 1.8 mg/L in 96 hours. In another study, uses indigenous facultative anaerobic *Bacillus cereus* to detoxify hexavalent chromium. *Bacillus cereus* has an excellent capacity of 72 % Cr (VI) removal at 1000 mg/mL chromate concentration. The bacteria were capable of reducing Cr (VI) under a wide range of temperatures (25 to 40◦ C) and pH (6 to 10) with optimum at 37◦ C and initial pH 8.0.

Several heavy metals have been tested using bacteria species like *Flavobacterium, Pseudomonas, Enterobacter, Bacillus, and Micrococcus sp.* Their great biosorption ability is due to high surface-to-volume ratios and the potential active chemosorption sites (teichoic acid) on the cell wall. Bacteria are more stable and survive better when they are in mixed culture. Therefore, consortia of cultures are

metabolically superior for biosorption of metals and are more appropriate for field application. De Jaysankar et al. [32] reported 78 % reduction of chromium (Cr) using bacteria consortium of Acinetobacter sp. and Arthrobacter sp. of 16 mg/L metal ion concentration. *Micrococcus luteus* was used to remove a huge quantity of Pb from a synthetic medium. Under ideal environments, the elimination ability was 1965 mg/g [33].

Abioye and his coworkers [34] investigated the biosorption of lead (Pb), chromium (Cr), and cadmium (Cd) in tannery effluent using *Bacillus subtilis, B. megaterium, Aspergillus niger, and Penicillium sp. B. megaterium* recorded the highest lead (Pb) reduction (2.13 to 0.03 mg/L), followed by *B. subtilis* (2.13-0.04 mg/L). *A. niger* show the highest ability to reduce the concentration of chromium (Cr) (1.38- 0.08 mg/L) followed by *Penicillium sp.* (1.38-0.13 mg/L) while *B. subtilis* exhibited the highest ability to reduce the concentration of cadmium (Cd) (0.4-0.03 mg/L) followed by *B. megaterium* (0.04-0.06 mg/L) afer 20 days. Kim and his coauthors [35], designed a batch system using zeoliteimmobilized *Desulfovibrio desulfuricans* for the removal of chromium (Cr6+), copper (Cu), and nickel (Ni) with removal efficiency of 99.8%, 98.2%, and 90.1%, respectively. Ashruta and his coworkers [36] reported efficient removal of chromium, zinc, cadmium, lead, copper, and cobalt by bacterial consortia at approximately 75 to 85% in less than two hours of contact duration.

5.2. Fungi Remediation Capacity of Heavy Metal :

Fungi are widely used as bio sorbents for the removal of toxic metals with excellent capacities for metal uptake and recovery. Most studies showed that active and lifeless fungal cells play a significant role in the adhesion of inorganic chemicals. Srivastava and Thakur [37] also reported the efficiency of *Aspergillus sp*. used for the removal of chromium in tannery waste water. 85% of chromium was removed at pH 6 in a bioreactor system from the synthetic medium, compared to a 65 % removal from the tannery effluent. Tis could be due to the presence of organic pollutants that hinder the growth of the organism.

Coprinopsis atramentaria is studied for its ability to bio accumulate 76 % of Cd2+, at a concentration of 1 mg L−1 of Cd2+, and 94.7% of Pb2+, at a concentration of 800 mg L−1 of Pb2+. Therefore, it has been documented as an effective accumulator of heavy metal ions for mycoremediation. Park and his coauthors [38] reported that dead fungal biomass of *Aspergillus niger*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *and Penicillium chrysogenum* could be used to convert toxic Cr (VI) to less toxic or nontoxic Cr (III). Luna et al. [39] also observed that *Candida sphaerica* produces bio surfactants with a removal efficiency of 95 %, 90 %, and 79 % for Fe (iron), zinc (Zn), and lead (Pb), respectively. These surfactants could form complexes with metal ions and interact directly with heavy metals before detachment from the soil. Candida spp. accumulate substantial quantity of nickel Ni (57–71%) and copper Cu (52– 68 %), but the process was affected by initial metal ion concentration and pH (optimum 3–5).

Bio surfactants have gained interest in recent years owing to their low toxicity, biodegradable nature, and diversity. Mulligan et al. [40] assessed the viability of using surfactin, rhamnolipid, and sophorolipid for the removal of heavy metals (Cu and Zn). A single washing with 0.5 % rhamnolipid removed 65 % of copper (Cu) and 18 % of the zinc (Zn), whereas 4% sophorolipid removed 25% of the copper (Cu) and

60% of zinc (Zn). Several strains of yeast such as *Hansenula polymorpha, S. cerevisiae, Yarrowia lipolytica, Rhodotorula pilimanae, Pichia guilliermondii,* and *Rhodotorula mucilage* have been used to bio-convert Cr (VI) to Cr (III).

5.3. Heavy Metal Removal Using Biofilm :

There are several reports on the application of biofilms for the removal of heavy metals. Biofilm acts as a proficient bioremediation tool as well as biological stabilization agent. Biofilms have very high tolerance against toxic inorganic elements even at a concentration that is lethal. It was revealed in a study conducted on *Rhodotorula mucilaginosa* that metal removal efficiency was from 4.79 to 10.25 % for planktonic cells and from 91.71 to 95.39 % for biofilm cells. Biofilms mechanisms of bioremediation could either be via bio sorbent or by exopolymeric substances present in biofilms which contain molecules with surfactant or emulsifier properties [41].

5.4. Algae Remediation Capacity of Heavy Metal :

Algae are autotrophic and hence require low nutrients and produce enormous biomass compared to other microbial bio sorbents. These bio sorbents have also been used for heavy metal removal with a high sorption capacity [12]. Algae biomass is used for bioremediation of heavy metal polluted effluent via adsorption or by integration into the cells. Phycoremediation is the use of various types of algae and cyanobacteria for the remediation of heavy metals by either removal or degradation of toxicant [41]. Algae have various chemical moieties on their surface such as hydroxyl, carboxyl, phosphate, and amide, which act as metal-binding sites [12].

Goher and his coauthors [42] used dead cells of Chlorella vulgaris to remove cadmium (Cd2+), copper (Cu2+), and lead (Pb2+) ions from aqueous solution under various conditions of pH, bio sorbent dosage, and contact time. The results suggested that the biomass of C. vulgaris is an extremely efficient bio sorbent for the removal of cadmium (Cd2+) , copper (Cu2+) and lead (Pb2+) at 95.5 %, 97.7 %, and 99.4 %, respectively, from mixed solution of 50 mg dm−3 of each metal ion.

6. Future Prospects :

Certain factors inhibiting the widespread application of this technology as identified by various researchers include difficulty in obtaining a reliable and inexpensive biomass and negative effects of coexisting metal ions on biosorptive capacity among others. Tannery effluent and bio sorbent characteristics need to be assessed prior to application. Keeping in focus the inhibitions of bioremediation technology, the future prospect looks promising on microbial genetic technologies and the development of increased specificity using biofilms which could be achieved by optimization process and immobilization techniques. Hence, more effort should be made in biofilms mediated bioremediation,

genetically modified microbes, and microbial fuel cell (MFC) in the bioremediation of heavy metals in the ecosystem.

7. Conclusion :

The current states of the bioremediation of heavy metal reviewed in this study show much promise for metal bio sorption and detoxification, especially from biofilm and genetically modified microbes. Biofilm-mediated techniques, microbial gene transfer, and microbial fuel cells-based techniques have come up as strong contenders in recent years. The peptidoglycan and polysaccharides component of the cell wall of the bio sorbents is an active binding site for higher metal uptake. This technique is cost-effective and a green technology that has advantages such as faster kinetics, high metal binding over a broad range of pH, and temperature. Tis review provides an opportunity to reveal the role of microbial cell, biofilm, and their metabolites towards remediation of heavy metals and environmental research. Further research area needs to be extended on the focus of gene transfer within biofilms for heavy metal remediation. These would facilitate the development of improved techniques for the bioremediation of heavy metals in the ecosystem.

8. References :

[1] S. Siddiquee, K. Rovina, and S. A. Azad, "Heavy metal contaminants removal from wastewater using the potential filamentous fungi biomass: a review," Journal of Microbial and Biochemical Technology, vol. 07, no. 06, pp. 384–393, 2015.

[2] R. K. Gautam, S. Soni, and M. C. Chattopadhyaya, "Functionalized magnetic nanoparticles for environmental remediation," Handbook of Research on Diverse Applications of Nanotechnology in Biomedicine, Chemistry, and Engineering, pp. 518–551, 2014.

[3] D. Lakherwal, "Adsorption of heavy metals: a review," International Journal of Environmental Research Development, vol. 4, pp. 41–48, 2014.

[4] R. J. Ndeddy Aka and O. O. Babalola, "Effect of bacterial inoculation of strains of *Pseudomonas aeruginosa, Alcaligenes feacalis* and *Bacillus subtilis* on germination, growth and heavy metal (Cd, Cr, and Ni) uptake of brassica juncea," International Journal of Phytoremediation, vol. 18, no. 2, pp. 200–209, 2016.

[5] Y. Ma, M. Rajkumar, C. Zhang, and H. Freitas, "Beneficial role of bacterial endophytes in heavy metal phytoremediation," Journal of Environmental Management, vol. 174, pp. 14–25, 2016.

[6] T. Hennebel, N. Boon, S. Maes, and M. Lenz, "Biotechnologies for critical raw material recovery from primary and secondary sources: R&D priorities and future perspectives," New Biotechnology, vol. 32, no. 1, pp. 121–127, 2015.

[7] V. Paliwal, S. Puranik, and H. J. Purohit, “Integrated perspective for effective bioremediation,” Applied Biochemistry and Biotechnology, vol. 166, no. 4, pp. 903–924, 2012.

[8] P. K. Shukla, S. Sharma, K. N. Singh, V. Singh, S. Bisht, and V. Kumar, Rhizoremediation: A Promising Rhizosphere Technology, Y. B. Patil and P. Rao, Eds., vol. 331, 2013.

[9] A. Wiszniewska, E. Hanus-Fajerska, E. MuszyNska, and K. Cia- ´ rkowska, “Natural organic amendments for improved phytoremediation of polluted soils: a review of recent progress,” Pedosphere, vol. 26, no. 1, pp. 1–12, 2016.

[10] M. R. Bruins, S. Kapil, and F. W. Oehme, “Microbial resistance to metals in the environment,” Ecotoxicology and Environmental Safety, vol. 45, no. 3, pp. 198–207, 2000.

[11] L. D. Rasmussen, S. J. Sørensen, R. R. Turner, and T. Barkay, “Application of a mer-lux biosensor for estimating bioavailable mercury in soil,” Soil Biology & Biochemistry, vol. 32, no. 5, pp. 639–646, 2000.

[12] U. Hildebrandt, M. Regvar, and H. Bothe, “Arbuscular mycorrhiza and heavy metal tolerance,” Phytochemistry, vol. 68, no. 1, pp. 139–146, 2007.

[13] C. Cervantes, J. Campos-Garc´ıa, S. Devars et al., “Interactions of chromium with microorganisms and plants,” FEMS Microbiology Reviews, vol. 25, no. 3, pp. 335–347, 2001.

[14] J. Giner-Lamia, L. L´opez-Maury, F. J. Florencio, and P. J. Janssen, “Global transcriptional profles of the copper responses in the cyanobacterium synechocystis sp. PCC 6803,” PLoS ONE, vol. 9, no. 9, p. e108912, 2014.

[15] M. Bissen and F. H. Frimmel, “Arsenic—a review. Part I: occurrence, toxicity, speciation, mobility,” Acta Hydrochimica et Hydrobiologica, vol. 31, no. 1, pp. 9–18, 2003.

[16] G. Wu, H. Kang, X. Zhang, H. Shao, L. Chu, and C. Ruan, “A critical review on the bio-removal of hazardous heavy metals from contaminated soils: Issues, progress, eco-environmental concerns and opportunities,” Journal of Hazardous Materials, vol. 174, no. 1-3, pp. 1–8, 2010.

[17] T. Yang, M. Chen, and J. Wang, “Genetic and chemical modification of cells for selective separation and analysis of heavy metals of biological or environmental significance,” TrAC Trends in Analytical Chemistry, vol. 66, pp. 90–102, 2015.

[18] K. Ramasamy, S. Kamaludeen, and B. Parwin, “Bioremediation of metals microbial processes and techniques,” in Environmental Bioremediation Technologies, S. N. Singh and R. D. Tripathi, Eds., pp. 173–187, Springer Publication, New York, NY, USA, 2006.

[19] M. Gavrilescu, “Removal of heavy metals from the environment by bio sorption,” Engineering in Life Sciences, vol. 4, no. 3, pp. 219–232, 2004.

[20] B. Godlewska-Zyłkiewicz, “Microorganisms in inorganic chemical analysis,” Analytical and Bioanalytical Chemistry, vol. 384, no. 1, pp. 114–123, 2006.

[21] M. Fomina and G. M. Gadd, “Biosorption: current perspectives on concept, definition and application,” Bioresource Technology, vol. 160, pp. 3–14, 2014.

[22] A. I. G. Lima, S. C. Corticeiro, and E. M. de Almeida Paula Figueira, “Glutathione-mediated cadmium sequestration in Rhizobium leguminosarum,” Enzyme and Microbial Technology, vol. 39, no. 4, pp. 763–769, 2006.

[23] J.-S. Cha and D. A. Cooksey, “Copper resistance in Pseudomonas syringae mediated by periplasmic and outer membrane proteins,” Proceedings of the National Acadamy of Sciences of the United States of America, vol. 88, no. 20, pp. 8915–8919, 1991.

[24] C. Telwell, N. J. Robinson, and J. S. Turner-Cavet, “An SmtBlike repressor from Synechocystis PCC 6803 regulates a zinc exporter,” Proceedings of the National Acadamy of Sciences of the United States of America, vol. 95, no. 18, pp. 10728–10733, 1998.

[25] M. Bruschi and G. Florence, “New bioremediation technologies to remove heavy metals and radionuclides using Fe(III)-, sulfate-and sulfur-reducing bacteria,” in Environmental Bioremediation Technologies, S. N. Singh and R. D. Tripathi, Eds., pp. 35–55, 2006.

[26] C. L. Wang, S. C. Ozuna, D. S. Clark, and J. D. Keasling, “A deep-sea hydrothermal vent isolate, Pseudomonas aeruginosa CW961, requires thiosulfate for Cd2+ tolerance and precipitation,” Biotechnology Letters, vol. 24, no. 8, pp. 637–641, 2002.

[27] J. Taniguchi, H. Hemmi, K. Tanahashi, N. Amano, T. Nakayama, and T. Nishino, “Zinc biosorption by a zinc-resistant bacterium, Brevibacterium sp. strain HZM-1,” Applied Microbiology and Biotechnology, vol. 54, no. 4, pp. 581–588, 2000.

[28] R. Pardo, M. Herguedas, E. Barrado, and M. Vega, “Biosorption of cadmium, copper, lead and zinc by inactive biomass of Pseudomonas putida,” Analytical and Bioanalytical Chemistry, vol. 376, no. 1, pp. 26–32, 2003.

[29] T. Barkay, S. M. Miller, and A. O. Summers, “Bacterial mercury resistance from atoms to ecosystems,” FEMS Microbiology Reviews, vol. 27, no. 2-3, pp. 355–384, 2003.

[30] S. Neha, V. Tuhina, and G. Rajeeva, “Detoxifcation of hexavalent chromium by an indigenous facultative anaerobic *Bacillus cereus* strain isolated from tannery effluent,” African Journal of Biotechnology, vol. 12, no. 10, 2013.

[31] S. Srivastava, S. B. Agrawal, and M. K. Mondal, “A review on progress of heavy metal removal using adsorbents of microbial and plant origin,” Environmental Science and Pollution Research, vol. 22, no. 20, pp. 15386–15415, 2015.

[32] J. De, N. Ramaiah, and L. Vardanyan, “Detoxifcation of toxic heavy metals by marine bacteria highly resistant to mercury,” Marine Biotechnology, vol. 10, no. 4, pp. 471–477, 2008.

[33] Z. M. Puyen, E. Villagrasa, J. Maldonado, E. Diestra, I. Esteve, and A. Sol´e, “Biosorption of lead and copper by heavy-metal tolerant Micrococcus luteus DE2008,” Bioresource Technology, vol. 126, pp. 233–237, 2012.

[34] O. P. Abioye, O. A. Oyewole, S. B. Oyeleke, M. O. Adeyemi, and A. A. Orukotan, "Biosorption of lead, chromium and cadmium in tannery efuent using indigenous microorganisms," Brazilian Journal of Biological Sciences, vol. 5, no. 9, pp. 25–32, 2018.

[35] I. H. Kim, J.-H. Choi, J. O. Joo, Y.-K. Kim, J.-W. Choi, and B.-K. Oh, "Development of a microbe-zeolite carrier for the effective elimination of heavy metals from seawater," Journal of Microbiology and Biotechnology, vol. 25, no. 9, pp. 1542–1546, 2015.

[36] G. A. Ashruta, V. Nanoty, and U. Bhalekar, "Biosorption of heavy metals from aqueous solution using bacterial EPS," International Journal of Life Science, vol. 2, no. 3, pp. 373–377, 2014.

[37] S. Srivastava and I. S. Takur, "Isolation and process parameter optimization of *Aspergillus sp.* for removal of chromium from tannery effluent," Bioresource Technology, vol. 97, no. 10, pp. 1167–1173, 2006.

[38] D. Park, Y.-S. Yun, J. H. Jo, and J. M. Park, "Mechanism of hexavalent chromium removal by dead fungal biomass of *Aspergillus niger*," Water Research, vol. 39, no. 4, pp. 533–540, 2005.

[39] J. M. Luna, R. D. Rufno, and L. A. Sarubbo, "Biosurfactant from *Candida sphaerica* UCP0995 exhibiting heavy metal remediation properties," Process Safety and Environmental Protection, vol. 102, pp. 558–566, 2016.

[40] C. N. Mulligan, R. N. Yong, and B. F. Gibbs, "Remediation technologies for metal-contaminated soils and groundwater: an evaluation," Engineering Geology, vol. 60, no. 1–4, pp. 193–207, 2001.

[41] M. H. El-Masry, E. El-Bestawy, and N. I. El-Adl, "Bioremediation of vegetable oil and grease from polluted wastewater using a sand biofilm system," World Journal of Microbiology and Biotechnology, vol. 20, no. 6, pp. 551–557, 2004.

[42] M. E. Goher, A. M. A. El-Monem, A. M. Abdel-Satar, M. H. Ali, A.-E. M. Hussian, and A. Napi´orkowska-Krzebietke, "Biosorption of some toxic metals from aqueous solution using non-living algal cells of Chlorella vulgaris," Journal of Elementology, vol. 21, no. 3, pp. 703–714, 2016.

Project Report on :

Emergence of Colistin Resistance in Enterobactericia Due to mcr-1 Gene and Drug Designing

Submitted by : Rounak Basu

Reg.no. : A01-1112-116-009-2019

Roll no. : 610 semester : v

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Guided by – Mr. Avijit Chakraborty

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Introduction : The polymyxins (polymyxin E (colistin) and polymyxin B) are a family of cationic polypeptide antibiotics with a lipophilic fatty acyl side chain . Although they belong to an old generation of antibiotics, polymyxins represent the last line of defense against lethal infections by gram-negative pathogens with pan-drug resistance . Unfortunately, certain species of the Enterobacteriaceae like K. pneumoniae have been recently showing an appreciable resistance to colistin. Indeed, colistin resistance (i.e., inefficient binding of polymyxins to the lipid A moiety of lipopolysaccharide) is mainly due to the 4'-phosphoethanolamine (PEA) modification of the lipid A on the LPS . This type of chemical modification on the bacterial lipid A can be attributed to either the chromosome-encoded machinery in K. pneumoniae or the plasmid-transferred mobilized colistin resistance (MCR-1) gene in certain species of Enterobacteriaceae like E. coli . For the former, two sets of bacterial two-component systems (pmrAB plus phoPQ) and the regulator mgrB are implicated, in which the lipid A of LPS is chemically modified and thereafter exhibits reduced affinity to polymyxin . The latter represents an unique mechanism for bacterial colistin resistance in that the mcr-1 gene product, annotated as a member of a family of phosphoethanolamine transferases, catalyzes the modification of lipid A moiety on bacterial LPS (Fig 1). To the best of our knowledge, the natural occurrence of the mcr-1 gene has been traced to no less than five species: E. coli, Salmonella enteric , K. pneumonia , Enterobacter aerogenes and E. Cloacae (of note, it was also experimentally spread/transmitted from E. coli to Pseudomonas aeruginosa by conjugation). Also, the range of host reservoirs with potential to carry the mcr-1-harbouring enterobacteria extends from poultry/livestock (chickens , pigs , dogs, and cattle) to humans , and published data from January-April 2016 suggests that the mcr-1 gene has been disseminated into no less than 18 countries. To acertain degree, the global spread of the mcr-1 gene might be related to a food-chain based dissemination pathway, which was shown by Zhu's group . Thus, they observed the paralleled existence of mcr-1 in meat/food samples and in the healthy human microbiome . Worryingly, the MCR-1 colistin resistance gene was strikingly shown to coexist with other multiple-drug resistance genes (i.e, carbapenem and extended-spectrum β-lactam), highlighting the possibility that micro-organisms with pan-drug resistances are emerging . For instance, a variant of the notorious NDM-1 was detected to coexist with MCR-1 in the Enterobacteriaceae (NDM-5 in K. pneumoniae and NDM-9 in a chicken meat isolate of E.coli). So far, most of the studies in this field focused on epidemiological investigations, which is in part due to the relatively limited availability of the genomic iinformation.Till now, the mechanism for transfer, origin, and biochemical analysis of the diversified plasmid-borne MCR-1 colistin resistance remains poorly understood.

Diversity and chemistry of colistin : Colistin, a polypeptide antibiotic of the polymyxin family, non-ribosomally synthesized with 1750 Da molecular weight, consisting of a cyclic heptapeptide with a tripeptide side chain acylated at the N terminus by a fatty acid tail (Figure 1). It is noteworthy that the hydrophobicity of the N-terminal fatty acyl segment is responsible for the inherent toxicity and greatly influences the antimicrobial activity of colistin. Polymyxin includes five different chemical compounds (polymyxins A, B, C, D, and E). It is mainly two of them, colistin A (polymyxin E1) and colistin B (polymyxin E2), that have been used in clinical practice; these differ only in their fatty acid tails. The difference between polymyxin B and colistin lies in the amino-acid components and both are polycations at physiological pH owing to the five L-a,g-diaminobutyric acid (Dab) residues. There are two different forms of colistin available commercially, colistin sulfate for oral and topical use and colistimethate sodium (CMS) for parenteral and aerosol therapy; both forms may be given by inhalation. Colistin (usually used as the sulfate salt) is a polycation, whereas colistimethate (used as the sodium salt) is a polyanion at physiological pH. CMS is an inactive prodrug of colistin and has no

intrinsic antibacterial activity. CMS produced by a sulfomethylation reaction in which the primary amine groups on a,g-diaminobutyric acid (Dab) are reacted with formaldehyde followed by sodium bisulfite. The composition must be converted to colistin in vivo, but this occurs slowly and incompletely. CMS is increasingly the last line of defense for multi-drug-resistant Gram-negative bacteria and is now being used as 'salvage' therapy in critically ill patients. CMS is less toxic than colistin when it is administered parenterally . As opposed to CMS which is rapidly hydrolyzed in plasma, colistin is stable for several days at room temperature. There are several commercial preparations of colistimethate, and their differences have undoubtedly contributed to confusion when evaluating dosing guidelines. 'Coly-Mycin M' Parenteral is produced by Parkedale Pharmaceuticals in the United States. Another preparation of colistimethate is 'Colomycin Injection', manufactured by Alpharma ApS (Denmark). Topical formulations of colistin sulfate and polymyxin B sulfate are also available in many countries. CMS is much more commonly used internationally (e.g. North America, South America, Asia, Europe and Australia) whereas parenteral polymyxin B is mainly available in the USA, Brazil and Singapore. Nowadays, novel derivatives of polymyxins have been developed. Some of them, including NAB739, are directly antibacterial whereas others, including NAB7061, lack the direct activity but sensitize bacteria to other antibiotics.

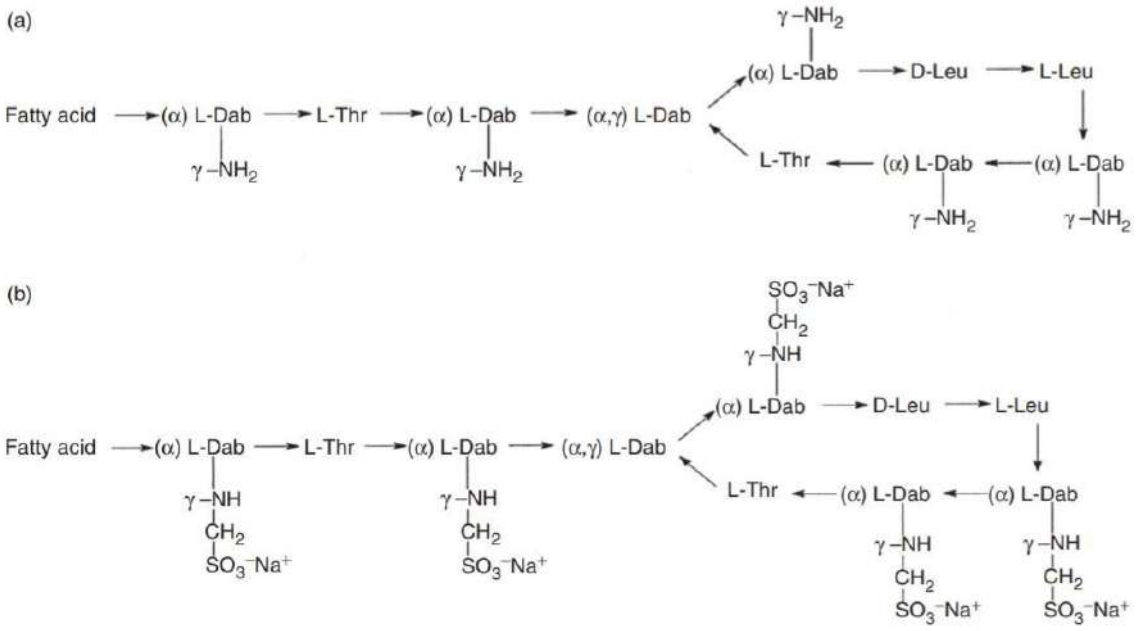


Figure 1. Colistin structures. (A) Structures of colistin A and B. (B) Structures of colistimethate A and B. Fatty acid: 6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B; Thr ¼ threonine; Leu ¼ leucine; Dab ¼ a,g-diaminobutyric acid; a and g indicate the respective amino groups involved in the peptide linkage. (Modified from Li et al.4).

Mechanism of action : The bactericidal effect of colistin is extremely rapid but the exact mechanism by which colistin can kill bacterial cells is currently unclear. Between colistin and polymyxin B, colistin is used more extensively worldwide due to its better availability. Colistin resistance is not

dependent upon bacterial metabolic activity and acquired resistance is rare. Gram-negative bacteria are characterized by the presence of an outer membrane. The protective function of the outer membrane mainly relies on the presence of lipo-polysaccharide (LPS) constituents at the surface of the cell, which limit the penetration of hydrophobic and/or large antibiotics. The polyanionic bacterial LPS is the initial target, it bears negative charge and this LPS confers to the integrity and stability of the bacterial outer membrane. But polymyxins having positive charge is critical for their interaction with the hydrophobic lipid A component of LPS. Their antibacterial effect on Gram-negative bacteria acts through a detergent-like effect, via a two-step mechanism. It comprises initial binding with electrostatic interactions between the polycationic ring of colistin to cell envelope components, causing the displacement, in a competitive fashion, of the calcium (Ca2þ) and magnesium (Mg2þ) ions from the phosphate groups of LPS that act as membrane stabilizers, leading to disruption of the outer

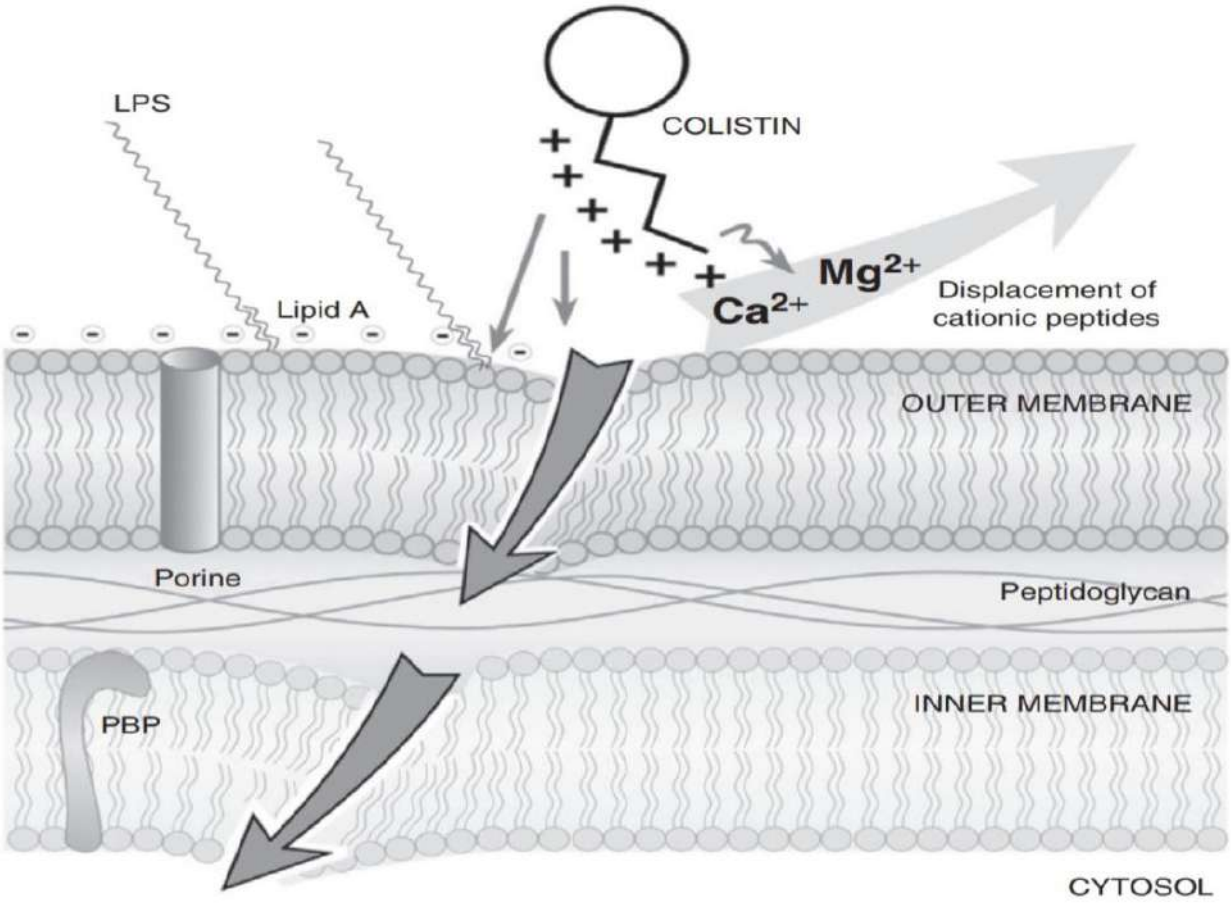


Figure 2. Action of colistin on bacterial membrane. The cationic cyclic decapeptide structure of colistin binds with the anionic LPS molecules by displacing calcium and magnesium from the outer cell membrane of Gram-negative bacteria, leading to permeability changes in the cell envelope and leakage of cell contents. By binding to the lipid A portion of LPS, colistin also has an anti-endotoxin activity. Disruption of the membranes should promote permeability for more conventional anti-pseudomonals. LPS: lipopolysaccharides; PBP: penicillin-binding protein.(From Martis et al.).

membrane and to the loss of cellular contents, thus killing the bacterium (Figure 2) . This process is independent of the entry of polymyxins into the cell, and seems to be inhibited in the presence of these bivalent cations. The killing process with colistin is not dependent upon bacterial metabolic activity, and this may be a significant contributing factor towards the slow development of resistance, a resistance which develops more slowly than that to tobramycin. Besides leading to cytoplasmic leakage, this binding can have a neutralizing effect on the biological properties of endotoxins25. The endotoxin of Gram-negative bacteria is the lipid A portion of LPS molecules, and colistin binds and neutralizes LPS. The significance of this mechanism for in vivo antimicrobial action, namely prevention of the endotoxin's ability to induce shock through the release of cytokines, is not clear, because plasma endotoxin is immediately bound by LPS-binding protein, and the complex is quickly bound to cell-surface CD14. More recently, the effects of slow-releasing colistin microspheres suggest that higher blood concentrations of colistin reduce the levels of endotoxin and cytokines in endotoxin-induced sepsis, and lead to decreased toxicity58. An alternative mechanism has recently been presented, demonstrating that polymyxins induce rapid cell death through hydroxyl radical production. Notably, it was demonstrated that this mechanism of killing occurs in multi-drug-resistant clinical isolates of A. baumannii and that this response is not induced in a polymyxin-resistant isolate.

Mechanism underlying polymixin resistance : The mechanisms underlying polymyxins resistance in GNB are complex and not completely understood until now. Generally, GNB can develop resistance to polymyxins through intrinsic, mutation or adaptation mechanisms, besides the horizontallyacquired resistance mediated via the mcr-1 gene and its variants. Cross-resistance between colistin and polymyxin B has been reported. Although the underlying mechanisms of resistance are common among GNB, they may differ between different species.

The main polymyxins resistance mechanisms can be summarized as follows:

(i) modifications of the LPS moiety via the addition of cationic groups to the LPS;

(ii) mutations that lead to the loss of the LPS;

(iii) porin mutations and overexpression of efflux pump systems;

(iv) over-production of capsular polysaccharide (CPS) in someGNB that hide the polymyxin binding sites and the release of CPS trapping polymyxins;

(v) enzymatic inactivation of colistin

- This mechanisms can be followed by either chromosomally encoding colistin resistance or plasmid mediated colistin resistance. Mobile Colistin Resistance (MCR) gene is responsible for plasmid mediated colistin resistance, that is emergent as because horizontal transfer of resistance gene.

- **Plasmid mediated resistance of Polymyxins :** In addition to the mutations-based mechanisms of resistance mentioned above, the horizontal transfer of a plasmid-borne gene; mcr-1 (standing for mobile colistin resistance) has become a significant cause for the dissemination of polymyxin resistance among various GNB . Indeed, the emanation of the MCR enzymes could be tracked down to the 1980s in China and 2005 in France, in pathogens isolated from poultry and veal calves, respectively . In late 2015, the plasmid-mediated mcr-1 gene was first described in an E. colistrain isolated from food animals in China . Since then, dissemination of mcr-1 among different Enterobacteriaceae strains, including *E. coli, K.*

pneumoniae, Enterobacter cloacae, Enterobacter aerogenes, Cronobacter sakazakii, S. enterica, Raoultella ornithinolytica, Citrobacter freundii, Citrobacter braakii, Shigella sonnei,Kluyvera ascorbata, and *Moraxella* spp. has been reported worldwide in over 30 countries across five continents in farm and wild animals, food (meat and vegetables), humans (colonized and infected), aquatic environments, hospital sewage , wild birds , and vector insects (housefly/blowfly). Although several reports have proposed that flies may serve as intermediate vectors for the transmission of mcr-1 between animals and humans, the exact path for the circulation/ spread of mcr-1 remains ambiguous . This global dissemination of the mcr-1 gene suggests that the use of colistin in veterinary medicine has probably sped up that dissemination among animals and humans, and this is consistent with the hypothesis that livestock, primarily pigs are most likely the primary source of MCR-1 producers . MCR-1 is a phosphoethanolamine lipid A transferase enzyme, belonging to the "YhjW/YjdB/YijP" alkaline phosphatase superfamily . The mechanism by which mcr-1 can mediate colistin resistance does not differ from that found in intrinsically resistant GNB. MCR-1 encodes a PEtN transferase leading to the addition of a PEtN moiety to the lipid A of LPS, increasing the cationic charges on LPS, and consequently, decreases the binding of colistin to LPS . This action is attributed to the chemical structure of the PEtN transferase. The N-terminal region of PEtN transferase is inserted in the inner membrane, while the C-terminal catalytic sulfatase domain is found periplasmic. The latter process is responsible for the transfer of a pEtN moiety from its physiological donor phosphatidylethanolamine to the Kdo of LPS . Structure-guided functional studies have confirmed this mechanism of mcr-1 and revealed that the enzymatic activity of mcr-1 renders the recipient strains resistant to polymyxin (Figure 3). A previous study reported that the mcr-1 gene leads to 4- to 8-fold increase in the MICs of colistin in E. coli, which indicates that the mcr-1 alone without other resistance mechanisms is enough to provide resistance against colistin in E. coli and other Enterobacteriaceae . An in silico analysis of the amino acid sequence of the mcr-1 gene showed that it is closely related to the PEtN transferases (pmrC) found in *Paenibacillus* spp., as well as to other enzymes from GNB, some of which are intrinsically resistant to colistin . Currently, 22 functional genetic variants of mcr-1 have been assigned , including mcr-1.1 ,mcr-1.2 , mcr-1.3 , mcr-1.4 , mcr-1.5 , mcr-1.6 , mcr-1.7 , mcr-1.8, mcr-1.9 , mcr-1.10 , mcr-1.11 , mcr-1.12 , mcr-1.13 , mcr-1.14 , and mcr-1.15 , while the other genetic variants from mcr-1.16 to mcr-1.22, were uploaded to NCBI GenBank (https://www.ncbi.nlm.nih.gov/nuccore/NG_065944.1). These variants differ from mcr-1 by one or a few amino acids. Therefore, they all share high nucleotide and amino acid identity (∼99%), and thus confer a similar effect on colistin resistance .Intriguingly, the determinants of transferable colistin resistance have extended further away mcr-1 to include a number of novel mcr-1 alleles . Up to now, nine mcr alleles have been reported including mcr-1 , namely; mcr-2 (1617 bp) , mcr-3 (1626 bp) , mcr-4 (1626 bp), mcr-5 (1644 bp) , mcr-6 (1617 bp) , mcr-7 (1620 bp) , mcr-8 (1698 bp) , and the most recently detected mcr-9 (2661 bp) (Table 2). Although, all these alleles have been characterized as PEtN transferases, sharing conserved amino acid groups; but, the degree of similarity in amino acid sequences between them is variable, thus reflecting different genetic origins. Investigations on the genetic environment of mcr genes revealed that mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, and mcr-8 share only 81%, 34%, 33%, 31%, 82%, 29%, and 31% amino acid sequence identity with mcr-1, respectively. Regarding the recently identified mcr-9 gene, the three-dimensional (3D) structural models related to all the nine mcr homologues (mcr-1 to −9) showed that mcr-3, mcr-4, mcr-7, and mcr-9, are sharing a high degree of similarity at the structural level . Indeed, the mcr-1 gene is the most prevalent among Enterobacteriaceae isolated from human samples . The analysis of the protein structure of MCR-1 showed the presence of two PEtN

transferases, namely; LptA and EptC (or cptA) from *Neisseria meningitidis* and *Campylobacter jejuni*, respectively, both are intrinsically resistant to polymyxins . Of note, mcr-2 and mcr-5 are viewed as two infrequent members of the MCR-like protein family.

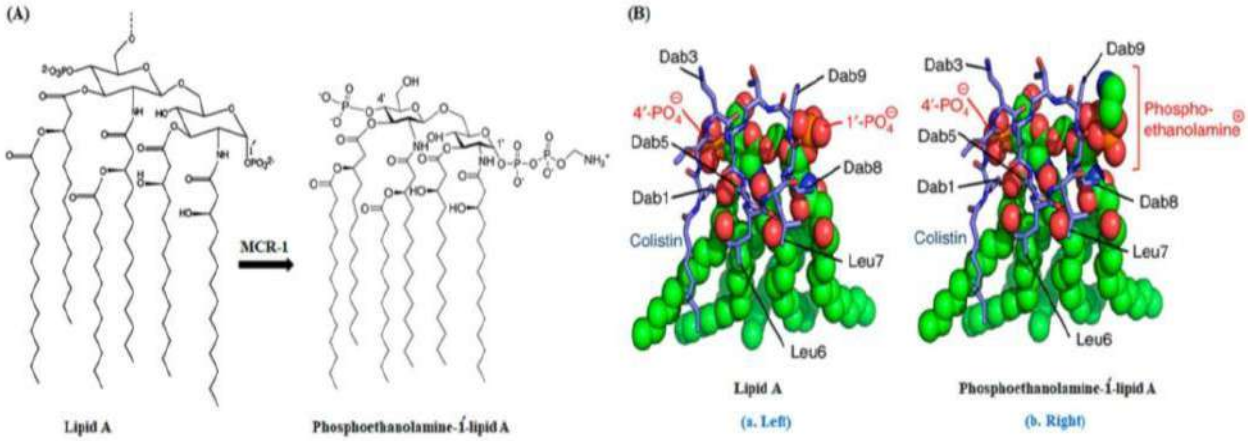


Figure-3 . Scheme of colistin binding to lipid A. (A) a Schematic of the transfer of phosphoethanolamine to the 1-PO4 group of Hexa-acylated lipid A in the presence of MCR-1. (**B**) Models of colistin (blue sticks) binding to lipid A (left) or phosphoethanolamine-1′-lipid A (right) (spheres coloured green, red, blue, and orange for C, O, N, and P atoms, respectively). a (left), The positively charged Dab colistin residues interact with the negatively-charged 1′ and 4′ phosphate groups of lipid A, reducing the net-negative charge of lipid A. The hydrophobic leucine residues and tail of colistin A bind with the fatty acid tails of lipid A, allowing the uptake of colistin A, and disrupt, the bacterial OM. b (right), a model of colistin binding to phosphoethanolamine-1′-lipid A indicates the addition of positively charged phosphoethanolamine onto the 1′-PO4 of lipid A likely interferes with the interaction of positively charged Dab8 and Dab9 side chains with the phosphate group, preventing colistin binding to the outer membrane of GNB. The model **B** is adapted from Yang et al. .

Mcr-1 harbouring plasmid reservoir : A serious concern regarding mcr genes is their location on transferable plasmids. The mcr-1 gene was identified for the first time in an IncI2 plasmid named pHNSHP45 (64 105 bp) . Following this initial plasmid detection, several mcr-1-carrying plasmidshave been reported belonging to different incompatibility groups with various sizes (58–251 kb) ; IncI2 , IncHI2 , IncX4 , IncP , IncY, IncF, IncFI, IncFII, IncFIB, IncK2 , IncN, and IncQ plasmids.

Moreover, many other replicon types of plasmids were specified to harbour mcr-like genes, indicating that the mcr-1-like variants might have been circulated worldwide by multiple plasmids. As a result of the global spreading rate of the other mcr-like variants, the replicon types of plasmids harbouring these mcr-like variants are very scarce. Intriguingly, it has been demonstrated that two different mcr-1-harbouring plasmids can coexist in a single colistin-resistant E. coli isolate, such as the IncI2-type plasmid, pGD65-3, and the IncX4-like plasmid, ppGD6-4.

Sequence analysis of mcr genes showed that the mcr-1 gene is often accompanied by an ISApl1 insertion sequence (IS), which is located upstream. It has been

reported that the ISApl1, which is located downstream of mcr-1 is not as stable as it does in the upstream of mcr-1. Generally, ISApl1 is flanked with mcr-1 and contributes to its transposition. Besides, another IS, namely IS1 might also appear upstream of the mcr-1 gene .

Intriguingly, Poirel et al. [23] revealed that the mcr-1 gene is located within a 2,600-bp genetic structure, called the "mcr-1 cassette," that might have been mobilized by transposition. The cassette was noticed carrying its promoter sequences leading to the mcr-1 expression.

The mcr genes have altered the scenario of colistin resistance since they have become a probable menace to public health. Furthermore, some, but not all plasmids-harbouring the mcr-1 gene can encode other antibiotic resistance genes, such as blaCTX-M, floR and/or qnr, which can encode resistance to various antibiotic classes, including polymyxins, β-lactams, quinolones , tetracyclines , and amphenicols .

Of note, the position of the mcr-1 gene on MDR-plasmids is worrisome, since upon the use of antibiotics other than polymyxins this will lead to co-selection for the isolates that harbour mcr-1 and facilitate its dissemination . More worryingly, is the integration of the mcr-1 gene into the bacterial chromosome, which has been discovered to occur in some strains . For instance, in Switzerland, the integration of the mcr-1 gene has been detected on the chromosome of an E. coli strain, which indicates that the mcr-1 gene might be integrated and consequently, stabilized in the genome of some isolates.

The higher occurrence of the mcr-1 gene in bacteria carrying genes coding for carbapenemases and/or ESBLs (e.g. CTX-M-15 and CTX-M-55) is most probably due to various and complex genetic events selected under antibiotic pressure . For instance, in a previous study, the co-transfer of mcr-1 and blaCTX-M-1 genes, which are located on the IncHI2 plasmid of *S. enterica* isolated from retail swine meat by horizontal gene transfer under colistin selection has been reported. Indeed, this hinders the therapeutic options for the treatment of *S. enterica* infections.

Besides, the existence of the mcr-1 gene has been reported in high drug-resistant Enterobacteriaceae isolates harbouring plasmids encoding different carbapenemase genes (blaNDM-1, blaNDM-5, blaNDM-9, blaOXA- 48, blaKPC-2, and blaVIM-1). For instance, the simultaneous presence of mcr-1 and the Metallo-β-lactamase NDM-5 has been detected in K. Pneumoniae clinical isolate . These findings reflect the possibility of emerging a severe public health crisis due to Enterobacteriaceae isolates harbouring both mcr-1 and carbapenemase-encoding genes.

Gene	No. of alleles	Associated plasmids and other mobile elements	Coexistence of other resistance genes	Host bacterial species	Potential origin of *mcr* genes
mcr-1	22	IncI2, IncX4, IncHI2/HI2A, IncHI1 IncF, IncN, IncP, IncQ, IncX, IncY, IncPO111 Mainly associated with IS*Apl1*, Tn*6330* transposon IS*26*-like element Occasionally chromosomal	$bla_{CTX\text{-}M\text{-}55/14/15/65/1/2/8/9/27}$, $bla_{NDM\text{-}1/5/9}$ *ampC*, $bla_{IMP\text{-}8}$, $bla_{SHV12/110}$, $bla_{KPC\text{-}2}$, $bla_{TEM\text{-}1/10/52/135/195}$, $bla_{CMY\text{-}2}$, $bla_{OXA\text{-}1/48}$, $bla_{VIM\text{-}1}$ *aph(3')-Ia/Ib/Iv, aac(3)-Iva, aph(3")-Ib, aph(4)-Ia, aac(6')Ib-cr, aph(6)- Id, aac(6")-Ib-cr, aadA1/A2, strA, strB sul1, sul2, sul3, tet*(A), *tet*(B) *fosA3, PER, qnrB, qnrS, floR catA, cmlA, dfrA1, dfrA12, qoxAB, arr-3 mcr-3, mcr-4, mcr-5*	*E. coli* *K. pneumoniae* *Salmonella* spp. *Enterobacter* spp. *Shigella* spp. *Citrobacter* spp. *Moraxella* spp. *K. ascorbata* *Providencia alcalifaciens*	*Moraxella porci*

Table 1 . Main characteristics of mcr-1 gene related to polymyxin resistance.

Functional dissection of MCR-1 colistin resistance : functional details of the MCR-1 protein are poorly understood. Here, the attempt is taken to address this issue. Philius

Transmembrane Prediction Server (http://www.yeastrc.org/philius/pages/philius/runPhilius.jsp) suggested that the MCR-1 protein is an

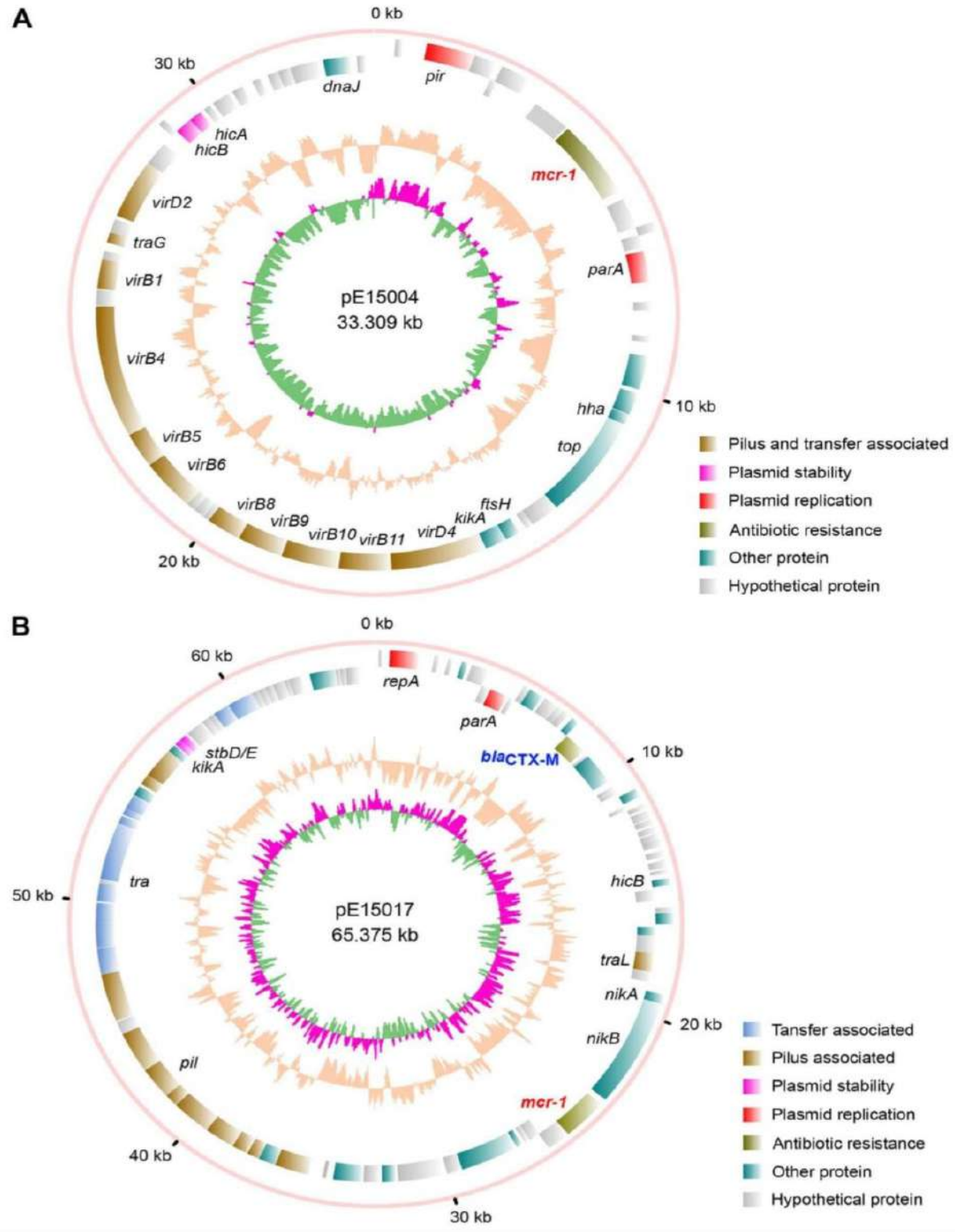


Fig: 4. Scheme for the two mcr-1-harbouring plasmids pE15004 and pE15017. A. Genomic map of the mcr-1 containing IncX4-type plasmid pE15004 from the human gut microbiota. B. Genomic map of the MCR-1 and ESBL-coproducing IncI2-type plasmid pE15017 from the human gut microbiota. Circles from inside to outside indicate the GC screw, GC content and the open-reading frames in different

DNA strands. The plasmid sequences were annotated by RAST, and the maps were generated using Circos program.

integral membrane protein with five trans-membrane regions (S3A Fig). Similar to the LptA (EptA) of Neisseria, the multiple sequence alignments indicated that the MCR-1 protein also belongs to a family of phosphoethanolaminelipid A (PEA) transferases with putative conserved sites (E246, T285, H395, D465 and H466) required for its catalytic activity, i.e., the addition of PEA to lipid A from phosphatidylethanolamine . Because the fact that the nascent LPS in cytoplasm is flipped by the ABC transporter MsbA into periplasm and the covalent modification of the lipid A component on LPS occurs in periplasm , it is speculated that the trans-membrane regions ensures the correct anchoring of the MCR-1 enzyme to the periplasmic face of the cytoplasmic membrane attached to the catalytic domain of PEA transferase. While, experimental evidence for this hypothesis is lacking.

Implications for drug design : Currently, multiple approaches are under development for the treatment of antibiotic-resistant superbugs, including polymyxin-resistant bacteria (Daly et al. 2017). To the best of our knowledge, there are three primary approaches being investigated to reduce MCR-1-associated colistin resistance. The first solution is the development of novel antibiotics against MCR-positive organisms, such as eravacycline (Fyfe et al. 2016), plazomicin (Denervaud-Tendon et al. 2017), and artilysin (Schirmeier et al. 2018). Another method appears to be the mainstream approach involving the effective administration of colistin as well as the potential use of combination therapies with additional agents to generate synergistic associations. These agents can include antibiotics that are typically restricted for use against gram-positive bacteria, such as amikacin (Bulman et al. 2017; Zhou et al. 2017), aztreonam (Bulman et al. 2017), rifampin (Brennan-Krohn et al. 2018; Li et al. 2018), azithromycin (Brennan-Krohn et al. 2018; LiM et al. 2018), clarithromycin (MacNair et al. 2018), linezolid (Brennan-Krohn et al. 2018), azidothymidine (Hu et al. 2019), and derivatives of tryptamine (Barker et al. 2019). Alternatively, natural products acting as adjuvants can be used, some of which can interact with lipopolysaccharides to perturb the outer bacterial membrane, such as pentamidine (Stokes et al. 2017) and meridianin D analogs (Huggins et al. 2018). In contrast, other adjuvants do not have specific roles insofar as we know, such as resveratrol (Cannatelli et al. 2018), pterostilbene (Zhou et al. 2018), osthole (Zhou et al. 2019), and niclosamide (Domalaon et al. 2019). The last but most important direction is to identify specific drugs targeting MCR. Several approaches have been reported to reduce MCR expression at the gene level, such as the use of peptide- conjugated phosphorodiamidate morpholino oligomers (PPMOs) to target mcr-1 mRNA (Daly et al. 2017), peptide nucleic acid against the mcr-1 gene (Nezhadi et al. 2019), and the CRISPR/Cas9 system to target mcr-1-harboring plasmids (Dong et al. 2019). However, few studies have investigated specific drugs targeting MCR, with promising results only having been observed for 1-phenyl-2-(phenylamino) ethanone derivatives (Lan et al. 2019) and the lipid A analog ethanolamine (Wei et al. 2018), both of which bind the cavity pocket. With the identification of the Zn2+-dependent catalytic core and two putative substrate-binding pockets, the use of targeted drug design has become a highly promising approach, especially with the detailed information of how substrate analogs and other small molecules bind to cMCR-1 (Son et al.2018).First, whether the Zn2+-dependent catalytic core and two putative substrate-binding pockets are important enough as drug target sites was investigated. The importance of the Zn2+-dependent catalytic core has been repeatedly confirmed by our lab and others through MIC measurements of strains carrying MCR mutations in residues around the catalytic core. Among these mutations, T285 has been shown to be particularly important, as MCR activity was almost completely abolished when T285 was mutated to alanine. To determine whether the substrate-binding site plays an important role in the activity of MCR-1, the mutation of residues

around these sites has also been tested. The full- length membrane protein MCR-1 was extracted using an appropriate concentration of the detergent n-dodecyl-beta-D-maltoside (DDM) to maintaining its activity. Because both substrates of MCR-1 are water-insoluble substances, it is difficult to detect this enzyme activity in vitro, which is also a bottleneck in this field. Recently, researchers have used a fluorescently labeled substrate, 1-acyl-2-{12-([7-nitro-2-1,3-benzoxadiazol-4-yl) amino]dodecanoyl}-sn-glycero-3-phosphoethanolamine (NBD-PEA), to detect the semi-enzymatic activity of MCR-1 (Anandan et al. 2017). This assay was also used in mutation analyses of potentially important amino acids involved in binding phosphatidylethanolamine (ethanolamine-bound sites). The results showed that MCR-1 harboring amino acid mutations around the ethanolamine-binding pocket could not the substrate NBD-PEA. Since the substrate analog ethanolamine likely occupies the binding pocket of the substrate phosphatidylethanolamine, the addition of ethanolamine could block the enzyme activity of MCR. Therefore, the activity of this enzyme was assessed after adding ethanolamine, the results of which showed that 10 mM ethanolamine could completely block the enzymatic reaction. An effort has also been made to determine the affinity of substrate analogs toward MCR-1 in vitro. For instance, the microscale thermophoresis (MST) method was used to determine the affinity of ethanolamine toward cMCR-1, with the results showing that ethanolamine could only bind cMCR-1 at a low affinity of Kd = 605 + 93.3 μM. These results indicate that further modification of the ethanolamine structure is needed to improve its affinity to this protein. Attempts have also been made to further determine the affinity of D-glucose toward cMCR-1, but after many experiments, no adequate results have been obtained. Therefore, a careful re-evaluation of the cMCR-1 crystal structure data was performed. The results showed that there was a clear electron density at the D-glucose-binding pocket in the high-resolution structure of cMCR-1 in the Apo form without soaking the crystals in any small molecules, and none of the small molecules in the crystallization solutions could fit the electron density map (Fig.5). Despite the little that is known about the origin of the observed electron density, these results demonstrated that the pocket may have become occupied by a substance in bacteria prior to protein crystallization. Interestingly, only when a high concentration of D-glucose was used in the crystal soaking assay was the unknown substance in the pocket removed and replaced by D-glucose.To further confirm that ethanolamine can be used as an inhibitor of MCR-1 activity, the inhibitory effect

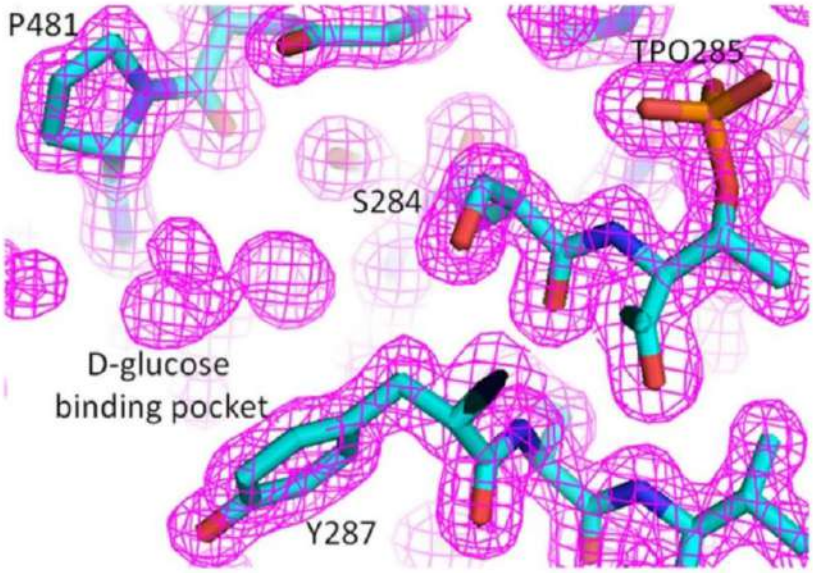


Fig : 5 Unknown electron densityin the D-glucose-binding pocket. Specific small molecules in the D-glucose-binding pocket in the 2Fo-Fc electron density map. The map is contoured at a 1.0 σ level. Blue, nitrogen; red, oxygen

of ethanolamine toward bacteria containing MCR-1 was also tested in vivo. The results clearly showed that ethanolamine could inhibit the expression of MCR-1 in a concentration-dependent manner under in the presence of 4 mg/mL polymyxin B, further confirming that ethanolamine can be used as an inhibitor of MCR-1 activity. Considering the structural paradigm and functional unification within MCR family, we assume that ethanolamine would also act as inhibitor of other MCR members, although this would require verification through further experimentation. Since another MCR substrate analog is D-glucose, which can be used as an energy source by bacteria, it was unclear whether it could inhibit MCR-1 activity in vivo. Thus, an inhibition assay using D-glucose was also performed, the results of which showed that it could not inhibit the growth of bacteria encoding MCR-1.

Conclusion : The emergence of the plasmid-mediated mcr-1 gene encoding for colistin resistance in GNB, which is transferable between different bacterial species has highlighted the possibility of losing colistin efficiency against MDR GNB in humans. Up to now, 22 new genetic variants of mcr-1 have been identified in different countries, indicating the possibility of continuous evolution. Besides, a number of novel mcr-1 alleles have been reported including mcr-1, namely; mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8, and the very most recently detected mcr-9.

Therefore, prospective surveillance and epidemiological studies should be implemented to detect the rate of dissemination of this resistant-gene in humans as well as in animals.

In summary, most of the studies performed to date have focused on the detection of the mcr-1 gene and assessing the characteristics of the plasmid-carrying bacteria, with only a few studies having investigated the structure and molecular mechanism of MCR-1. Due to the important potential hazard of mcr-1, studies on the mechanism of action and drug design for MCR-1 are urgently needed. Many research groups have made concerted efforts to study the substrate-binding sites of MCR-1 and identify specific MCR-1 inhibitors. Through a series of painstaking efforts by many research groups, several versions of the high-resolution structures of cMCR-1 alone and in complex with two substrate analogs were obtained in a very short time. In addition, the enzymatic activity of this protein was tested in vitro and in vivo using these two substrate analogs, one of which was shown to have the function of MCR-1-inhibiting activity. These results have provided a potential drug prototype that could provide great theoretical guidance for the further design of MCR-1 inhibitors.

Acknowledgement : would like to acknowledge and give my warmest thanks to my supervisor **Avijit Chakraborty** who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator in charge; revered **Bhrahamachari Parachaitanya Maharaj** for giving me this opportunity, which help me to venture relevant meaningful topics. Finally, I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

Reference :

[1] Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clin Infect Dis. 2005;40(9):1333–1341.

[2] Sun J, Zhang H, Liu YH, et al. Towards understanding MCR-like colistin resistance. Trends Microbiol. 2018;26(9):794–808.

[3] Jeannot K, Bolard A, Plesiat P. Resistance to polymyxins in Gram-negative organisms. Int J Antimicrob Agents. 2017;49(5):526–535.

[4] Srinivas P, Rivard K. Polymyxin resistance in Gram-negative pathogens. Curr Infect Dis Rep. 2017;19 (11):38.

[5] Sherry N, Howden B. Emerging Gram negative resistance to last-line antimicrobial agents fosfomycin, colistin and ceftazidime-avibactam - epidemiology,laboratory detection and treatment implications.Expert Rev Anti Infect Ther. 2018;16(4):289–306.

[6] Velkov T, Roberts KD, Nation RL, et al. Pharmacology of polymyxins: new insights into an 'old' class of antibiotics. Future Microbiol. 2013;8 (6):711–724.

[7] Talbot GH, Bradley J, Edwards JE, Jr., et al. Bad bugs need drugs: an update on the development pipeline from the antimicrobial availability task force of the infectious diseases society of America. Clin Infect Dis. 2006;42(5):657–668.

[8] Bialvaei AZ, Samadi KH. Colistin, mechanisms and prevalence of resistance. Curr Med Res Opin. 2015;31(4):707–721.

[9] Lim LM, Ly N, Anderson D, et al. Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. Pharmacotherapy. 2010;30 (12):1279–1291.

[10] Dijkmans AC, Wilms EB, Kamerling IM, et al. Colistin: revival of an old polymyxin antibiotic. Ther Drug Monit. 2015;37(4):419–427.

[11] Son SJ, Huang R, Squire CJ, et al. MCR-1: a promising target for structure-based design of inhibitors to tackle polymyxin resistance. Drug Discov Today. 2019;24(1):206–216.

[12] Koyama Y. A new antibiotic "colistin" produced by spore-forming soil bacteria. J Antibiot. 1950 1950;3:457–458.

[13] Kaye KS, Pogue JM, Tran TB, et al. Agents of last resort: polymyxin resistance. Infect Dis Clin North Am. 2016;30(2):391–414.

[14] Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. Drug Resist Updat. 2010;13 (4–5):132–138.

[15] Gurjar M. Colistin for lung infection: an update. J Intensive Care. 2015;3(1):3.

[16] Li J, Nation RL, Milne RW, et al. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. Int J Antimicrob Agents. 2005;25(1):11–25.

[17] Antonic V, Stojadinovic A, Zhang B, et al.Pseudomonas aeruginosa induces pigment production and enhances virulence in a white phenotypic variant of Staphylococcus aureus. Infect Drug Resist. 2013;6:175–186.

[18] Mlynarcik P, Kolar M. Molecular mechanisms of polymyxin resistance and detection of mcr genes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2019;163(1):28–38.

[19] Abachin E, Poyart C, Pellegrini E, et al. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of Listeria monocytogenes. Mol Microbiol. 2002;43(1):1–14.

[20] Gregoire N, Aranzana-Climent V, Magreault S, et al. Clinical Pharmacokinetics and Pharmacodynamics of colistin. Clin Pharmacokinet. 2017;56(12):1441– 1460.

[21] Liberati A, Altman DG, Tetzlaff J, et al. The PRISMA statement for reporting systematic reviews and metaanalyses of studies that evaluate health care interventions: explanation and elaboration. PLoS Med. 2009;6 (7):e1000100.

[22] Rhouma M, Beaudry F, Theriault W, et al. Colistin in Pig production: chemistry, mechanism of antibacterial action, microbial resistance emergence, and one health perspectives. Front Microbiol. 2016;7:1789.

[23] Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev. 2017;30(2):557–596.

[24] Yu Z, Qin W, Lin J, et al. Antibacterial mechanisms of polymyxin and bacterial resistance. Biomed Res Int. 2015;2015:679109.

[25] Yu Z, Zhu Y, Fu J, et al. Enhanced NADH metabolism involves colistin-induced killing of Bacillus subtilis and Paenibacillus polymyxa. Molecules. 2019;24(3):387.

[26] Mogi T, Murase Y, Mori M, et al. Polymyxin B identified as an inhibitor of alternative NADH dehydrogenase and malate: quinone oxidoreductase from the Gram-positive bacterium Mycobacterium smegmatis. J Biochem. 2009;146(4):491–499.

[27] Deris ZZ, Akter J, Sivanesan S, et al. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. J Antibiot. 2014;67(2):147– 151.

[28] Martis N, Leroy S, Blanc V. Colistin in multi-drug resistant Pseudomonas aeruginosa blood-stream infections: a narrative review for the clinician. J Infect. 2014;69(1):1–12.

[29] Grau-Campistany A, Manresa A, Pujol M, et al. Tryptophan-containing lipopeptide antibiotics derived from polymyxin B with activity against Gram positive and Gram negative bacteria. Biochim Biophys Acta. 2016;1858(2):333–343.

[30] Domalaon R, Berry L, Tays Q, et al. Development of dilipid polymyxins: Investigation on the effect of hydrophobicity through its fatty acyl component. Bioorg Chem. 2018;80:639–648.

[31] Rose F, Heuer KU, Sibelius U, et al. Targeting lipopolysaccharides by the nontoxic polymyxin B nonapeptide sensitizes resistant Escherichia coli to the bactericidal effect of human neutrophils. J Infect Dis. 2000;182(1):191–199.

[32] Vaara M. Polymyxin derivatives that sensitize Gram negative bacteria to other antibiotics. Molecules. 2019;24(2):249.

[33] MacNair CR, Stokes JM, Carfrae LA, et al. Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. Nat Commun. 2018;9(1):458.

[34] Hood MI, Becker KW, Roux CM, et al. . genetic determinants of intrinsic colistin tolerance in Acinetobacter baumannii. Infect Immun. 2013;81 (2):542–551.

[35] Liu YY, Wang Y, Walsh TR, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016;16(2):161–168.

[36] Lima T, Domingues S, Da Silva GJ. Plasmid-mediated colistin resistance in Salmonella enterica: A review. Microorganisms. 2019;7(2):55.

[37] Cannatelli A, Giani T, D'Andrea MM, et al. Mgrb inactivation is a common mechanism of colistin resistance in KPC-producing Klebsiella pneumoniae of clinical origin. Antimicrob Agents Chemother. 2014;58(10):5696–5703.

[38] Lippa AM, Goulian M. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. PLoS Genet. 2009;5(12):e1000788.

[39] Cheng YH, Lin TL, Lin YT, et al. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in Klebsiella pneumoniae. Antimicrob Agents Chemother. 2016;60(6):3709–3716.

[40] Perez F, El Chakhtoura NG, Yasmin M, et al. Polymyxins: To Combine or Not to Combine? Antibiotics. 2019;8(2):38.

[41] Fresno S, Jimenez N, Izquierdo L, et al. The ionic interaction of Klebsiella pneumoniae K2 capsule and core lipopolysaccharide. Microbiology. 2006;152(Pt 6):1807–1818.

[42] Lima WG, Alves MC, Cruz WS, et al. Chromosomally

encoded and plasmid-mediated polymyxins resistance in Acinetobacter baumannii: a huge public health threat. Eur J Clin Microbiol Infect Dis. 2018;37(6):1009–1019.

[43] Feng Y. Transferability of MCR-1/2 polymyxin resistance: complex dissemination and genetic mechanism. ACS Infect Dis. 2018;4(3):291–300.

[44] Snitkin ES, Zelazny AM, Gupta J, et al. Genomic insights into the fate of colistin resistance and Acinetobacter baumannii during patient treatment. Genome Res. 2013;23(7):1155–1162.

[45] Adams MD, Nickel GC, Bajaksouzian S, et al. Resistance to colistin in Acinetobacter baumannii associated with mutations in the PmrAB two-component system. Antimicrob Agents Chemother. 2009;53(9):3628–3634.

[46] Cheah SE, Johnson MD, Zhu Y, et al. Polymyxin resistance in Acinetobacter baumannii: genetic mutations and transcriptomic changes in response to clinically relevant dosage regimens. Sci Rep. 2016;6:26233.

[47] Moffatt JH, Harper M, Harrison P, et al. Colistin resistance in Acinetobacter baumannii is mediated by complete loss of lipopolysaccharide production. Antimicrob Agents Chemother. 2010;54(12):4971–4977.

[48] Bojkovic J, Richie DL, Six DA, et al. Characterization of an Acinetobacter baumannii lptD deletion strain: permeability defects and response to inhibition of lipopolysaccharide and fatty acid biosynthesis. J Bacteriol. 2015;198(4):731–741.

[49] Whitfield C, Trent MS. Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem. 2014;83:99–128.

[50] Thi Khanh NN, Riordan DW, Do Hoang NT, et al. The induction and identification of novel colistin resistance mutations in Acinetobacter baumannii and their implications. Sci Rep. 2016;6:28291.

[51] Lin MF, Lin YY, Lan CY. Contribution of EmrAB efflux pumps to colistin resistance in Acinetobacter baumannii. J Microbiol. 2017;55(2):130–136.

[52] Trebosc V, Gartenmann S, Totzl M, et al. Dissecting colistin resistance mechanisms in extensively drugresistant Acinetobacter baumannii clinical isolates. MBio. 2019;10(4):e01083-19.

[53] Muller C, Plesiat P, Jeannot K. A two-component

regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2011;55(3):1211–1221.

[54] Miller AK, Brannon MK, Stevens L, et al. Phoq mutations promote lipid A modification and polymyxin resistance of Pseudomonas aeruginosa found in colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother. 2011;55(12):5761–5769.

[55] Zhao F, Feng Y, Lu X, et al. Incp plasmid carrying colistin resistance gene mcr-1 in Klebsiella pneumoniae from hospital sewage. Antimicrob Agents Chemother. 2017;61(2):e02229-16.

[56] Lee JY, Na IY, Park YK, et al. Genomic variations between colistin-susceptible and -resistant Pseudomonas aeruginosa clinical isolates and their effects on colistin resistance. J Antimicrob Chemother. 2014;69(5):1248–1256.

[57] Morey P, Viadas C, Euba B, et al. Relative contributions of lipooligosaccharide inner and outer core modifications to nontypeable Haemophilus influenzae pathogenesis. Infect Immun. 2013;81(11):4100–4111.

[58] Quiroga C, Nastro M, Di CJ. Current scenario of plasmid-mediated colistin resistance in Latin America. Rev Argent Microbiol. 2019;51(1):93–100.

[59] Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003;67(4):593–656.

[60] Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol. 2014;5: 643.

[61] Aghapour Z, Gholizadeh P, Ganbarov K, et al. Molecular mechanisms related to colistin resistance in Enterobacteriaceae. Infect Drug Resist. 2019;12:965–975.

[62] Cannatelli A, D'Andrea MM, Giani T, et al. In vivo emergence of colistin resistance in Klebsiella pneumoniae producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. Antimicrob Agents Chemother. 2013;57(11):5521–5526.

[63] Mills G, Dumigan A, Kidd T, et al. Identification and characterization of two Klebsiella pneumoniae lpxL lipid A late Acyltransferases and their role in Virulence. Infect Immun. 2017;85(9):e00068-17.

[64] Gefen O, Chekol B, Strahilevitz J, et al. TDtest: easy detection of bacterial tolerance and persistence in clinical isolates by a modified disk-diffusion assay. Sci Rep. 2017;7:41284.

[65] Herrera CM, Hankins JV, Trent MS. Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. Mol Microbiol. 2010;76(6):1444–1460.

[66] Trimble MJ, Mlynarcik P, Kolar M, et al. Polymyxin: alternative mechanisms of action and resistance. Cold Spring Harb Perspect Med. 2016;6(10):a025288.

[67] Da Silva GJ, Domingues S. Interplay between colistin resistance, virulence and fitness in Acinetobacter baumannii. Antibiotics. 2017;6(4):28.

[68] Chambers JR, Sauer K. The MerR-like regulator BrlR impairs Pseudomonas aeruginosa biofilm tolerance to colistin by repressing PhoPQ. J Bacteriol. 2013;195 (20):4678–4688.

[69] Haenni M, Poirel L, Kieffer N, et al. Co-occurrence of extended spectrum beta lactamase and MCR-1 encoding genes on plasmids. Lancet Infect Dis. 2016;16(3):281–282.

[70] Wang Q, Sun J, Li J, et al. Expanding landscapes of the diversified mcr-1-bearing plasmid reservoirs. Microbiome. 2017;5(1):70.

[71] Wang Y, Hu Y, Cao J, et al. Antibiotic resistance gene reservoir in live poultry markets. J Infect. 2019;78 (6):445–453.

[72] Yang RS, Feng Y, Lv XY, et al. Emergence of NDM-5- and MCR-1-producing Escherichia coli Clones ST648 and ST156 from a single Muscovy Duck (Cairina moschata). Antimicrob Agents Chemother. 2016;60 (11):6899–6902.

[73] Gao R, Hu Y, Li Z, et al. Dissemination and mechanism for the MCR-1 colistin resistance. PLoS Pathog. 2016;12(11):e1005957.

[74] Yang Q, Li M, Spiller OB, et al. Balancing mcr-1 expression and bacterial survival is a delicate equilibrium between essential cellular defence mechanisms. Nat Commun. 2017;8(1):2054.

[75] AbuOun M, Stubberfield EJ, Duggett NA, et al. . mcr-1 and mcr-2 variant genes identified in Moraxella species isolated from pigs in Great Britain from 2014 to 2015. J Antimicrob Chemother. 2017;72 (10):2745–2749.

[76] Borowiak M, Fischer J, Hammerl JA, et al. Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting Salmonella enterica subsp. enterica serovar Paratyphi B. J Antimicrob Chemother. 2017;72 (12):3317–3324.

[77] Carattoli A, Villa L, Feudi C, et al. Novel plasmid mediated colistin resistance mcr-4 gene in Salmonella and Escherichia coli, Italy 2013, Spain and Belgium, 2015 to 2016. Euro Surveill. 2017;22 (31):30589.

[78] Wang X, Wang Y, Zhou Y, et al. Emergence of a novel mobile colistin resistance gene, mcr-8, in NDM-producing Klebsiella pneumoniae. Emerg Microbes Infect. 2018;7(1):122.

[79] Xavier BB, Lammens C, Ruhal R, et al. Identification of a novel plasmid-mediated colistin-resistance gene,mcr-2, in Escherichia coli, Belgium, June 2016. Euro Surveill. 2016;21(27):30280.

[80] Yang F, Shen C, Zheng X, et al. Plasmid-mediated colistin resistance gene mcr-1 in Escherichia coli and Klebsiella pneumoniae isolated from market retail fruits in Guangzhou, China. Infect Drug Resist. 2019;12:385–389.

[81] Yang YQ, Li YX, Lei CW, et al. Novel plasmid- mediated colistin resistance gene mcr-7.1 in Klebsiella pneumoniae. J Antimicrob Chemother. 2018;73(7):1791–1795.

[82] Yin W, Li H, Shen Y, et al. Novel plasmid-mediated colistin resistance gene mcr-3 in Escherichia coli. MBio. 2017;8(3):e00543-17.

[83] Di P V, Arena F, Tascini C, et al. . mcr-1.2, a new mcr variant carried on a transferable plasmid from a colistin-resistant KPC carbapenemase-producing *Klebsiella pneumoniae* strain of sequence type 512. Antimicrob Agents Chemother. 2016;60(9):5612–5615.

[84] Yang YQ, Li YX, Song T, et al. Colistin resistance gene mcr-1 and its variant in Escherichia coli isolates from chickens in China. Antimicrob Agents Chemother. 2017;61(5):e01204-16.

[85] Zhao F, Feng Y, Lu X, et al. Remarkable diversity of Escherichia coli carrying mcr-1 from hospital sewage with the identification of two New mcr-1 variants. Front Microbiol. 2017;8:2094.

[86] Tijet N, Faccone D, Rapoport M, et al. Molecular characteristics of mcr-1-carrying plasmids and new mcr-1 variant recovered from polyclonal clinical *Escherichia coli* from Argentina and Canada. PLoS One. 2017;12(7):e0180347.

[87] Lu X, Hu Y, Luo M, et al. MCR-1.6, a new MCR variant carried by an IncP plasmid in a colistin-resistant Salmonella enterica serovar Typhimurium isolate from a healthy individual. Antimicrob Agents Chemother. 2017;61(5):e02632-16.

[88] Manageiro V, Clemente L, Romao R, et al. Incx4 plasmid carrying the New mcr-1.9 gene variant in a CTX- M-8-producing Escherichia coli isolate recovered from swine. Front Microbiol. 2019;10:367.

[89] Deshpande LM, Hubler C, Davis AP, et al. Updated prevalence of mcr-like genes among Escherichia coli and Klebsiella pneumoniae in the SENTRY program and characterization of mcr-1.11 variant. Antimicrob Agents Chemother. 2019;63(4):e02450-18.

[90] Alba P, Leekitcharoenphon P, Franco A, et al. Molecular epidemiology of mcr-encoded colistin resistance in enterobacteriaceae from food-producing animals in Italy revealed through the EU harmonized antimicrobial resistance Monitoring. Front Microbiol. 2018;9:1217.

[91] Ma F, Shen C, Zheng X, et al. Identification of a novel plasmid carries mcr-4.3 in *Acinetobacter baumannii* from China. Antimicrob Agents Chemother. 2019;63(6):e00133-19.

[92] Xu Y, Zhong LL, Srinivas S, et al. Spread of MCR-3 colistin resistance in China: An epidemiological, Genomic and Mechanistic study. EBioMedicine. 2018;34:139–157.

[93] Carroll LM, Gaballa A, Guldimann C, et al.Identification of novel mobilized colistin resistance gene mcr-9 in a multidrug-resistant, colistin-susceptible Salmonella enterica Serotype Typhimurium isolate. MBio. 2019;10(3):e00853-19.

[94] Litrup E, Kiil K, Hammerum AM, et al. Plasmid- borne colistin resistance gene mcr-3 in Salmonella isolates from human infections, Denmark, 2009–17. Euro Surveill. 2017;22(31):30587.

The role of lac operon and lac repressor in the induction using lactose for the expression of periplasmic human interferon-α2b by Escherichia coli.

Submitted By: - SRINJAY PARAY

Registration Number: - A01-1122-116-014-2019

Exam Roll number: - 2022151196

Semester: - V

Subject: - UGC PROJECT

Guided by: - DR. SUBRATA KUNDU

DEPART MENT OF MICROBIOLOGY,

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE, RAHARA

ACKNOWLEDGEMENT

I would like to acknowledge and give heartiest thanks to my supervisor and project guide Dr. Subrata Kundu who gave me the opportunity to do a project on this topic. He also gave me creative ideas and guided me through this project to make this project possible. His guidance and advice carried me through all the stages of writing this project.

I am also thankful to our Departmental Coordinator in charge, Br. Parachaitanya Maharaj for giving me the opportunity to do this project, which helped me a lot to venture relevant topics.

Finally, I would like to thank my Parents, family members and friends. They all kept me going, and this paper would not have been possible without their cordial support.

The role of lac operon and lac repressor in the induction using lactose for the expression of periplasmic human interferon-α2b by Escherichia coli.

Abstract: -

The effect of lac operon in the induction using lactose for the expression of periplasmic human interferon-α2b (PrIFN-α2b) was studied in shake flask culture. Escherichia coli strains Rosetta2 (DE3) [R2 (DE3)] containing the lac operon and Rosetta-gami2 (DE3) [RG2 (DE3)] containing the deletion of entire lac operon with high level of lac repressor were used. R2 (DE3) over-expressed PrIFN-α2b at substantial levels (270–380 μg/L) in lactose-induced media. In spite of the deletion of lac operon in RG2 (DE3), the cells exposed to lactose produced PrIFN-α2b albeit in less quantity (18–20 μg/L). Under similar conditions, the percentage of IFN-α2b translocated into periplasm for cells induced with lactose was 43–57 and 15–30% in R2 (DE3) and RG2 (DE3), respectively. The PrIFN-α2b expressed by RG2 (DE3) grown in control medium and terrific broth was 290.3 and 134.7 μg/L, respectively. The basal expression levels obtained in R2 (DE3) strain were 10-fold higher than those obtained in RG2 (DE3) strain. The target proteins expressed in uninduced state did not affect the growth, indicating that IFN-α2b was non-toxic to the bacterial cells. Equivalent level of PrIFN-α2b expression was obtained in RG2 (DE3) induced by IPTG and in R2 (DE3) induced by lactose.

Introduction: -

Escherichia coli is often selected as the host for recombinant protein production because of its suitability for large-scale cultivation at reduced cost. The regulation of E. coli lac operon and its control elements has been one of the most widely studied in the aspect of molecular system since the 1960s (Grossman et al. 1998; Hansen et al. 1998; Jacob and Monod 1961; Kuhlman et al. 2007). pET vectors have been extensively applied for the expression of recombinant proteins in E. coli (Makrides 1996; Grossman et al. 1998; Ou et al. 2004). The gene of interest is placed under the control of T7 lac promoter in many of these vectors, and its expression is regulated by T7 RNA polymerase. Expression of recombinant protein can be induced by the addition of either lactose or a synthetic inducer into the recombinant bacterial culture. By utilizing the phenomenon of catabolite repression, autoinduction medium is designed for

lactose-inducible bacterial expression systems, in which protein expression is induced automatically from lac promoter without the need to monitor cell growth (Studier 2005).

Lactose is often used as an inducer for induction of T7 lac promoter due to low cost and because it is non-toxic to the bacterial cells. To initiate a transcription, lactose is transferred into cells by functional lac permease for active transport of lactose into bacteria (encoded by lacY gene) (Mieschendahl et al. 1981). A portion of transported lactose is converted into allolactose by the β-galactosidase enzyme (encoded by lacZ gene) (Kalnins et al. 1983) to perform transgalactosidation reactions (Shukla and Wierzbicki 1975). β-galactoside transacetylase (encoded by lacA gene) is an enzyme that transfers an acetyl group from acetyl-CoA to β-galactosides and is responsible for lactose catabolism.

The analogue of lactose, isopropyl β-D-thiogalactoside (IPTG) is also widely used as an inducer. IPTG can be transfered into the cells by lac permease or by diffusion and then allosterically interacts with repressor molecules without any modifications (Mahoney 1998). Functional allolactose and IPTG, which bind to the lac repressor, will cause a structural change in the repressor resulting in loss in affinity for the operator. Thus, T7 RNA polymerase can bind to the promoter and transcribes the targeted gene. However, there is a drawback in a lac induction expression system where a problem of uninduced expression may occur. To overcome this problem, the pET system provided some strains with mutation or deletion of the lac operon with a high level of repressor production.

Lac control elements and repressors are important in protein synthesis (Blommel et al. 2007). A high level of the lac repressor ensures stringent repression in the uninduced state, but this approach may result in the elevated repression of T7 RNA polymerase synthesis. Transcription can be strongly repressed by the lac repressor, but T7 RNA polymerase initiates transcription very actively in the absence of the repressor, or in the presence of repressor plus inducer (Dubendorff and Studier 1991). Target protein expression levels in lacY and lacZ mutant strains may become elevated when the strains are allowed to grow until the early stationary phase and the expression was greatly influenced by the medium composition and cell growth phase (Grossman et al. 1998).

Several induction approaches have been proposed for protein expression, which include manipulation of induction time (Neubauer et al. 1992; Vila et al. 1997), concentration of the inducer, and the composition of the growth medium (Donovan et al. 1996; Nancib et al. 1991). It is noted that evaluation of the effect of the lac operon on the expression level of IFN-α2b in

the periplasmic region of E. coli using lactose has not previously been conducted. The objective of the present study was to determine the effect of the lac operon and lac repressor in the induction using lactose for the expression of periplasmic human interferon-α2b (PrIFN-α2b) by recombinant E. coli.

Materials and Methods: -

Source of materials

Glucose, ammonium sulphate, magnesium sulphate, yeast extracts, K2HPO4, glycerol and Terrific broth were purchased from Merck, Germany. Peptone and lactose were purchased from Laboratorios Conda, Spain. Potassium phosphate was purchased from J.T. Baker, Mexico. Sodium phosphate was purchased from Sigma, Germany, while sodium chloride was purchased from Fisher Scientific, UK.

Characteristics of recombinant Rosetta2 (DE3) and Rosetta-gami2 (DE3): -

Rosetta2 (DE3) [R2 (DE3)] host strain [F− ompT hsdSB(rB− mB−) gal dcm (DE3) pRARE2] and Rosetta-gami 2 (DE3) [RG2 (DE3)] host strain [Δ(ara − leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F'[lac + lacIq pro] gor522::Tn10 trxB pRARE2] were used in this study. The main reason of choosing R2 (DE3) is to obtain more soluble product (not producing high level of repressors), to improve the recovery of intact recombinant proteins (lacking an outer membrane protease), and to reduce metabolic burden of the host cell (less formation of disulfide bonds in cytoplasm) according to its preferred genotypes. The main difference between these two strains is the lac operon for the transcription and the lac repressor for the basal expression. RG2 (DE3) contains a lactose mutant gene and has the deletion of the lac operon with a high level expression of the lac repressor while R2 (DE3) is a derivative from strain BL21 with the functional lac operon. Both strains carried seven codons rarely used in E. coli.

Preparation of recombinant R2 (DE3) strains: -

The details of the construction of the recombinant pET26b-IFNα2b plasmid and RG2 (DE3) strain have been described in our previous study (Ramanan et al. 2010b). The expression vector which contains T7 lac promoter, pelB signal sequence and IFN-α2b gene was transformed into R2 (DE3) competent cells, to obtain the recombinant strain (R2 (DE3)/pET26b-IFNα2b) for the production of the target protein. R2 (DE3) and RG 2(DE3) have similar expression hosts,

containing the plasmid that carries the genes for seven codons, rarely used in E. coli. This approach can be used to enhance the expression level of heterologous proteins by overcoming codon bias (Schumann and Ferreira 2004; Sørensen and Mortensen 2005). Additionally, R2 (DE3) contains a lamda prophage which has the gene for T7 RNA polymerase.

Fermentations: -

The stock cultures of R2 (DE3) and RG2 (DE3) were cultivated at 37°C until an early log phase was reached (OD = 0.4–0.6). The cells were revived by modulating 1 mL of stock culture into 50 mL TB medium supplemented with 30 mg/L kanamycin and 34 mg/L chloramphenicol. The culture was grown at 37°C with a constant shaking at 250 rpm for 16 h. To start the fermentation, 8% (v/v) of inoculum was added into 250-mL baffled shake flasks containing 50 mL of sterilized production medium. The flasks were incubated at 37°C in an incubator shaker (Certomat® BS-1 B; Braun, Germany), agitated at 250 rpm. The cultures were induced with lactose (0.2–4%) or IPTG (1 mM) for different periods of cultivation according to the need of each experiment, and then the temperature was switched to 30°C. Samples were withdrawn at timed intervals after the induction for analysis.

Medium and induction: -

The optimized medium for the expression of PrIFN-α2b using IPTG by recombinant *E. coli* as proposed by Tan et al. (2009) was used in this study for lactose-induced culture. This medium consisted of 5.5 g/L glucose, 10 mL/L glycerol, 55.2 g/L yeast extract and 42.3 g/L peptone, and 10 mL/L of 0.1 M of potassium buffer. Initial pH of the medium was adjusted to 7 using either 1 M HCl or 1 N NaOH. To study the effect of lactose as an inducer in the optimized medium, 4% (w/v) lactose (LacM) was added to the medium at 4 h of fermentation to replace 1 mM IPTG. Fermentations using IPTG as an inducer were also carried out as controls. For comparison, medium formulations as reported in the literature such as ZYP-5052 (Studier 2005) and Kotik (Kotik et al. 2004) were modified and also tested for the induction of PrIFN-α2b by lactose. The modified ZYP-5052 medium consisted of 10 g/L casamino acid, 5 g/L yeast extract, 50 mM disodiumphosphate, 50 mM potassium diphosphate, 2.5 M ammonium sulphate, 50 mM magnesium sulphate, 5 g/L glycerol, 0.5 g/L glucose, and 2 g/L lactose. The modified Kotik medium consisted of 13 g/L glycerol, 18 g/L peptone casein, 3 g/L lactose, 14.6 g/L di-sodium phosphate, 3 g/L potassium diphosphate, 0.5 g/L sodium chloride, 1 g/L ammonium sulphate, and 0.25 g/L magnesium sulphate.

Analytical methods: -

Cell concentration was quantified by optical density (OD) and dry cell weight (DCW). The OD was measured at 600 nm using spectrophotometer (Lambda 25; Perkin Elmer). The relationship between DCW and OD_{600} can be expressed as; DCW (g/L) = 3.59 OD_{600}.

The culture sample was centrifuged (rotor model 1619, Universal 32R centrifuge; Hettich, Switzerland) at 8,000*g* for 10 min at 4°C. The supernatant was used for glucose analysis and the cell pellet was disrupted for the extraction of PrIFN-α2b using the osmotic shock method as described by Ramanan et al. (2009). The shrunk cells after osmotic shock extraction were disrupted with glass bead shaking as described elsewhere (Ramanan et al. 2008) for 25 min. The disrupted cells were then centrifuged at 8,000*g* for 10 min at 4°C, where the supernatant was used for the determination of soluble cytoplasmic IFN-α2b (cIFN-α2b). Sodium dodecyl sulphate (SDS) (0.05%) was added to the cell pellet for 5 min and extracted as inclusion bodies IFN-α2b (IbIFN-α2b).

The concentration of glucose was analyzed using Biochemistry Analyzer (Model 2700 Select; YSI). The quantity of IFN-α2b was determined using the fully automated Surface Plasmon Resonance detection system (BIAcore 3000; GE HealthCare) according to the method described by Ramanan et al. (2010a). In brief, the supernatant extracted was mixed with HBS-EP buffer in 1:1 ratio and the resultant solution was passed to the reference and anti-IFN flow cells for 1 min at a flow rate of 5 µL/min with a measurement unit of Response Unit (RU). After each measurement, the surface was regenerated with a 1-min pulse of 10 mM glycine (pH 2.5) at 5 µL/min. The relationship between IFN-α2b and RU can be expressed as; IFN-α2b (µg/L) = 0.363 RU. All experiments were carried out at 25°C with the CM5 chip and the results were further analyzed using BIA evaluation software (v.4.1).

Results: -

Effect of the lac operon on protein expression and translocation of soluble IFN-α2b to the periplasmic space of E. coli

A series of media with different nutrient constituents were used to cultivate R2 (DE3) and RG2 (DE3) to perform the subsequent induction phase aimed to achieve high levels of PrIFNα2b expression using IPTG and lactose as the inducer molecules. R2 (DE3) expressed significant levels of PrIFN-α2b (267–375 µg/L) in all lactose- and IPTG-induced media. The lac mutant

strain RG2 (DE3) expressed PrIFN-α2b at a very low level when lactose was used as inducer, where the maximum expression level was 20.4 μg/L, and this gave the specific yield of only 2.7 μg/g DCW. When IPTG was used as an inducer, RG2 (DE3) expressed PrIFN-α2b up to a level of 290.3 μg/L, which was ten times higher than that obtained when lactose was used. Although the total IFN-α2b expression level in R2 (DE3) was two-fold higher than the RG2 (DE3) cells in the control medium, the periplasmic secretion was only slightly increased (1.26-fold). In terms of specific yield, the value obtained by RG2 (DE3) in IPTG-induced culture (40.9 μg/g DCW in control medium) was about 13 times higher than the values for lactose-induced culture (2.7 μg/g DCW in LacM medium). In lactose-induced culture, the percentage of IFN-α2b translocated into periplasmic region by R2 (DE3) was ranged from 43 to 57%, while the IFN-α2b secreted in periplasmic space was greatly reduced to 15–30% in RG2 (DE3). However, the secretion rate of RG2 (DE3) was increased by 2-fold (58%) when IPTG was used as an inducer.

The role of lactose in retaining the expression of target gene

The growth of both R2 (DE3) and RG2 (DE3) was higher in lactose-induced media as compared to IPTG-induced media (Fig. 1). In both strains, the expression of PrIFN-α2b ceased markedly over time after 4 h of induction by IPTG in both terrific broth and optimized medium. In contrast, the expression of PrIFN-α2b was retained for a longer duration in R2 (DE3) culture induced with lactose. In ZYP-5052 medium, PrIFN-α2b only began to decrease at a slow rate after the maximum expression was achieved at 16 h of fermentation. In Kotik medium, the expression of PrIFN-2b was increased up to 24 h of fermentation. However, PrIFN-α2b was not significantly expressed (18–20 μg/L) in RG2 (DE3) culture induced using lactose as compared to R2 (DE3) culture due to the Δ(lac)X74 and lac genetic marker which signifies the deletion of the lac operon from the chromosome and unable to utilize lactose.

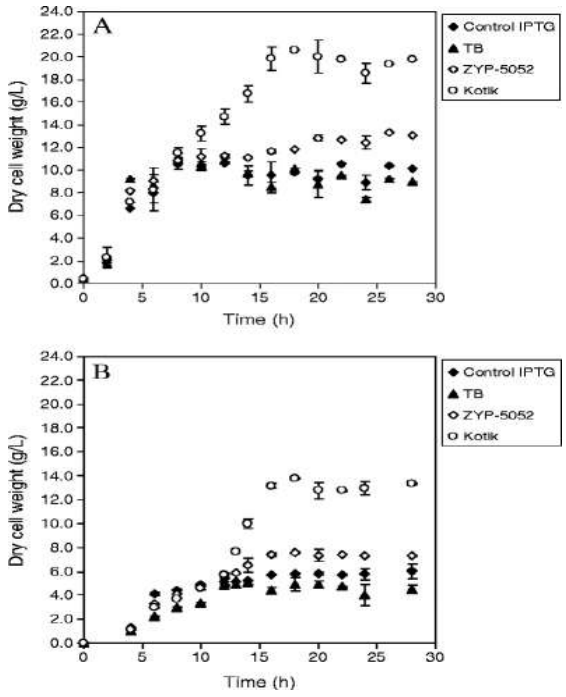


Fig 1: - *Growth profile, measured as dry cell weight, of a R2 (DE3) and b RG2 (DE3) in different media of fermentation.*

Basal expression

Basal expressions of PrIFN-α2b for both the strains were analysed at 4 h of fermentation prior to IPTG induction (Fig. 2). The expression of PrIFN-α2b (range 150–200 μg/L) by R2 (DE3) occurred at the early stages of fermentation using all media, prior to induction. This is contrary to the result in which very low basal expression (10–30 μg/L) of target genes was achieved in RG2 (DE3), but the usual high levels of expression were obtained upon induction. The basal expression rate of PrIFN-α2b in R2 (DE3) was approximately 10-fold higher than that obtained in RG2 (DE3), which contains the lacIq genotype (the gene responsible for producing high levels of lac repressor) in suppressing the basal expression.

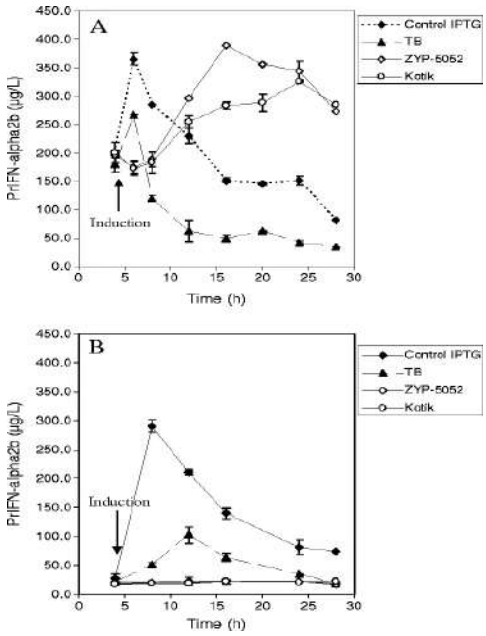


Fig 2: - *Expression of PrIFN-α2b during the cultivation of a R2 (DE3) and b RG2 (DE3) in different media.*

Effect of lactose concentration on the expression of PrIFN-α2b

The concentration of lactose in ZYP-5052 medium was modified according to the design of experiment at a range from 0.2–4% (w/v) for the R2 (DE3) strain. With pET26b–IFNα2b as the expression vector, the periplasmic protein expression level was triggered by adding different concentrations of lactose to the culture. The expression of PrIFN-α2b was gradually increased from 20 to 25% with increasing lactose concentration from 0.2 to 4% (w/v) during induction. However, the concentration of lactose did not exert a significant influence on the growth of E. coli in ZYP-5052 medium. The total expression of IFN-α2b increased gradually with increasing lactose concentration up to 4% (w/v), which was similar to the profile of the expression of PrIFN-α2b. Maximum expressions of PrIFN-α2b (413.0 µg/L) and cIFN-α2b (612.2 µg/L) were obtained when 4% (w/v) lactose was used as an inducer.

The effect of induction point on the expression by lac operon

The effect of the induction point was evaluated by adding 4% lactose at the exponential growth phase (4 h induction point) and at the stationary phase (12 h induction point). In all fermentations, glucose was exhausted when the exponential growth phase was achieved (data not shown). For 12 h induction, growth of R2 (DE3) was abruptly arrested at DCW of 8.07 g/L and reached a stationary growth phase after 8 h of fermentation. On the other hand, R2 (DE3) continued to grow markedly for 4 h induction. The growth of RG2 (DE3) was about 10% higher

at 4 h induction as compared to 12 h induction, yet both induction phases yielded similar level of PrIFN-α2b (10–20 μg/L). With different induction points, the highest value of PrIFN-α2b (380.7 μg/L) was observed in R2 (DE3) when induced with lactose at the middle of exponential growth phase, where the translocation rate reached its maximum value (55.7 %). PrIFN-α2b decreased rapidly when lactose was added at a stationary phase (107.7 μg/L), suggesting that induction point was the important factor for efficient expression of periplasmic protein in E. coli. A very low percentage of soluble IFN-α2b translocated to the periplasmic space was observed (13.6 and 16.5% for induction point at 4 and 12 h, respectively) when RG2 (DE3) was induced with lactose.

Discussion: -

The expression of PrIFN-α2b can be regulated by lactose to a significant level by controlling the expression of the lac operon gene to regulate its rate of transcription. In the absence of the lac operon, the expression of PrIFN-α2b cannot be regulated by lactose to a significant level, and failure in the lac operon induction process resulted in significant reduction in the formation of allolactose, the true inducer of the lac operon. This was presumably due to a defect in lactose accumulation (Flagg and Wilson 1976). Therefore, the present study was performed to strengthen the above observation by examining to what extent the lac operon would affect recombinant IFN-α2b production in periplasm.

Basal expression can be controlled in an inducible T7 expression system by blocking the target T7 promoter with the lac repressor (Dubendorff and Studier 1991). The availability of the lacIq gene in RG2 (DE3) encodes high levels of repressor that exhibits tight regulation of the lac promoter, and this high level repressor ensures stringent repression in the uninduced state. The presence of high levels of lac repressor in RG2 (DE3) strongly inhibited the transcription by the lac operator (Cooper and Magasanik 1974). However, when lactose or IPTG is added to the culture medium, it caused the repressor to be released from the operator to enable RNA polymerase to initiate the transcription (Reznikoff and Miller 1978; Dubendorff and Studier 1991). In R2 (DE3) cultures, basal expression did not affect the cell growth and expression of PrIFN-α2b. This shows that the IFN-α2b expressed by these strains did not exert toxicity on the cells during the fermentation.

With the deletion of the lac operon, lactose is not effectively utilized as inducer, which resulted in a high level of the repressor molecule in RG2 (DE3) bound to upstream cis activated operator and reduced the expression of T7 RNA polymerase (Schumann and Ferreira 2004). Unlike

lactose, IPTG is not metabolized by the cell (Donovan et al. 1996), but transported into the cell by methods other than lac permease and hence directly bound to the lac repressor reducing its affinity for the operator (Mahoney 1998; Lewis 2005). This action enhanced the synthesis of T7 RNA polymerase and subsequently induced the transcription and translation of IFN-α2b in RG2 (DE3).

A high concentration of inducer would increase the level of transcription in the cells, thus enhancing the expression and/or translocation of some secreted proteins (Donovan et al. 1996). However, a higher rate of expression would affect the rate of translocation in periplasmic expression (Rosenberg 1998), and the excess of expressed recombinant protein is likely to accumulate in inclusion bodies (Mergulhao et al. 2005; Mergulhão and Monteiro 2007). Thus, some soluble recombinant proteins were expressed in high levels using lower concentrations of IPTG as inducer (Azaman et al. 2010). On the other hand, the rate of translocation was also reduced in the cultures expressing a lesser amount of soluble IFN-α2b probably due to the competition of periplasmic host proteins. It should be noted that the high level of periplasmic expression could be achieved in a narrow range of IPTG inducer concentration where the rate of production should match the rate of translocation of the protein (Azaman et al. 2010). In the case of lactose, the increase in the amount of lactose did not greatly influence expression of PrIFN-α2b. As the lactose could be used as a carbon source for cell metabolism other than the inducer, lactose provides flexibility of choosing the preferred level of concentration without having any deleterious effect on the translocation of expressed protein.

Leakage of periplasmic protein can occur during cell division, auto cell lysis (Somerville et al. 1994) and due to the accumulation of recombinant protein in the periplasm. In the last case, the accumulation of protein in periplasm would increase the osmotic pressure and hence drive the protein across the outer membrane (Hasenwinkle et al. 1997). Additionally, perturbations in the membrane (Pugsley et al. 1997) occurred during the production of proteins and thus increased its selective permeability (Slos et al. 1994). Leakage of protein might also occur due to the use of IPTG as inducer which would cause the metabolic burden to the cell (Kilikian et al. 2000; Baneyx 1999). For example, leakage of active subtilisin E in E. coli into the culture occurred at 6 h after induction with IPTG (Takagi et al. 1988). In our case, a drastic decrease in PrIFN-α2b level could also be due to the leakage of protein into the culture or due to the degradation of protein in prolonged fermentation induced with IPTG. It is important to note that this phenomenon was not observed in lactose-induced culture. Lactose appeared to

enhance the solubility and retained the secreted proteins and benefited greatly from the lower transcription rates as compared to IPTG.

In the process of recombinant protein production by E. coli, lactose induction is a switching point between cell growth and recombinant protein synthesis. The transcription of the foreign gene on the plasmid begins with the addition of lactose and in consequence brings great changes to the metabolism of the host cell by initiating the translation of heterologous protein. With the presence of glucose at the beginning of fermentation, there is no induction of the lac operon by lactose as growth on glucose causes inducer exclusion, which means that lactose is unable to enter the cell. This situation occurred due the potentiation of growth on glucose, which is inhibitory to lacY transport and for other sugar transport systems (Osumi and Saier 1982). Growth on glucose also reduced the levels of cAMP in the cells, which in turn reduced the ability to induce the expression of the lac operon. cAMP promoted the transcription of the lac operon by binding to CAP (catabolite gene activator protein) and caused an allosteric transformation (Lee et al. 2005; Wong et al. 1997). Lactose added after 4 h of fermentation in R2 (DE3) was immediately utilized for cell maintenance and protein expression after the exhaustion of the primary carbon source due to catabolite repression (Magasanik 1961). When the culture was induced at a stationary growth phase, R2 (DE3) was unable to express PrIFN-α2b at high levels. Reduced expression was mainly due to glucose starvation which abruptly stopped RNA accumulation in the cells (Chaloner-Larsson and Yamazaki 1978), and subsequently repressed the expression of PrIFN-α2b. The availability of glucose in the growth medium had no impact on the expression of PrIFN-α2b in by RG2 (DE3) as lactose was incapable of inducing the T7lac promoter. The expression of PrIFN-α2b by R2 (DE3) obtained at the 4 h induction point was significantly higher than that obtained at 12 h induction when lactose was used as an inducer. This is because the nutrient required for gene expression is more than sufficient in the earlier stages as compared to the later stages of fermentation (Donovan et al. 1996). Results from this study indicate that the selection of an appropriate induction time greatly influenced the lac operon induction, which subsequently affected the expression of PrIFN-α2b.

In this study, the availability of the lacIq gene in RG2 (DE3) encoded high levels of repressor that ensures stringent repression in the uninduced state. The basal expression in R2 (DE3) was shown to be non-toxic towards E. coli cells. In addition, lactose is a cheap inducer and does not exert a toxic effect on the cells which may affect cell growth and expression of the proteins.

Thus, it would be advantageous to use lactose as inducer to obtain the same level of transcription as IPTG.

Conclusion: -

In spite of the deletion of the lac operon, a low level of expression of IFN-α2b by RG2 (DE3) was observed in the lactose-induced cultures. On the other hand, R2 (DE3) over-expressed PrIFN-α2b at substantial levels (270–410 μg/L) in lactose-induced media. Lactose-induced culture greatly enhanced (~18 times) the expression of PrIFN-α2b in the presence of the lac operon gene. The percentage of IFN-α2b translocated into periplasm induced with lactose was 43–67% in R2 (DE3). The basal expression levels obtained in R2 (DE3) strain were 10-fold higher than those obtained in RG2 (DE3) strain. Lactose induction in R2 (DE3) has an equivalent amount of expression to RG2 (DE3) induced by IPTG. In addition, maximum production of PrIFN-α2b (380.7 μg/L) was observed in R2 (DE3) when induced by lactose at the middle of the exponential growth phase. Thus, selection of the host strain via suitable genetic markers is essential in the induction of lactose for expression of the target gene.

Future prospectus: -

As from the results it is found that recombinant *E. coli* R2 (DE3) can produce more amount of PrIFN-α2b in lactose containing culture then Whey which is a discarded material in Paneer production can be used as a preferred substrate for a large-scale production of PrIFN-α2b. We know that PrIFN-α2b has an effective role in suppressing many cancers and are able to treat viral diseases. So, if large production of PrIFN-α2b is made then the cost of defeating disease using immunotherapy will become less. Hence would be helpful for human community.

Reference: -

Azaman S, Ramakrishnan N, Tan J, Rahim R, Abdullah M, Ariff AB (2010) Optimization of an induction strategy for improving interferon-alpha2b production in the periplasm of Escherichia coli using response surface methodology. Biotechnol Appl Biochem 56:141–150

Baneyx F (1999) Recombinant protein expression in Escherichia coli. Curr Opin Biotechnol 10(5):411–421

Blommel PG, Becker KJ, Duvnjak P, Fox BG (2007) Enhanced bacterial protein expression during auto-induction obtained by alteration of lac repressor dosage and medium composition. Biotechnol Prog 23(3):585–598

Chaloner-Larsson G, Yamazaki H (1978) Effects of the spoT and relA mutation on the synthesis and accumulation of ppGpp and RNA during glucose starvation. Biochem Cell Biol 56(4):264–272

Cooper TG, Magasanik B (1974) Transcription of the lac operon of Escherichia coli. J Biol Chem 249(20):6556

Donovan RS, Robinson CW, Glick BR (1996) Review: Optimizing inducer and culture conditions for expression of foreign proteins under the control of the lac promoter. J Ind Microbiol Biotechnol 16(3):145–154

Dubendorff JW, Studier FW (1991) Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. J Mol Biol 219(1):45

Flagg JL, Wilson TH (1976) lacY mutant of Escherichia coli with altered physiology of lactose induction. J Bacteriol 128(3):701

Grossman TH, Kawasaki ES, Punreddy SR, Osburne MS (1998) Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. Gene 209(1–2):95–103

Hansen LH, Knudsen S, Sorensen SJ (1998) The effect of the lacY gene on the induction of IPTG inducible promoters, studied in Escherichia coli and Pseudomonas fluorescens. Curr Microbiol 36(6):341–347

Hasenwinkle D, Jervis E, Kops O, Liu C, Lesnicki G, Haynes CA, Kilburn DG (1997) Very high-level production and export in Escherichia coli of a cellulose binding domain for use in a generic secretion-affinity fusion system. Biotechnol Bioeng 55(6):854–863

Jacob F, Monod J (1961) Genetic regulatory mechanisms in the synthesis of proteins. J Mol Biol 3:318–356

Kalnins A, Otto K, Ruther U, Muller-Hill B (1983) Sequence of the lacZ gene of Escherichia coli. EMBO J 2(4):593

Kilikian BV, Suarez ID, Liria CW, Gombert AK (2000) Process strategies to improve heterologous protein production in Escherichia coli under lactose or IPTG induction. Process Biochem 35(9):1019–1025

Kotik M, Kocanová M, Maresová H, Kyslík P (2004) High-level expression of a fungal pyranose oxidase in high cell-density fed-batch cultivations of Escherichia coli using lactose as inducer. Protein Expr Purif 36(1):61–69

Kuhlman T, Zhang Z, Saier MH Jr, Hwa T (2007) Combinatorial transcriptional control of the lactose operon of Escherichia coli. Proc Natl Acad Sci USA 104(14):6043–6048

Lee SK, Newman JD, Keasling JD (2005) Catabolite repression of the propionate catabolic genes in Escherichia coli and Salmonella enterica: evidence for involvement of the cyclic AMP receptor protein. J Bacteriol 187(8):2793

Lewis M (2005) The lac repressor. C R Biol 328(6):521–548

Magasanik B (1961) Catabolite repression. Cold Spring Harb Symp Quant Biol 26:249–256

Mahoney RR (1998) Galactosyl-oligosaccharide formation during lactose hydrolysis: A review. Food Chem 63(2):147–154

Makrides SC (1996) Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Mol Biol Rev 60(3):512

Mergulhão FJ, Monteiro GA (2007) Periplasmic targeting of recombinant proteins in Escherichia coli. Meth Mol Biol Clifton Then Totowa 390:47

Mergulhao FJM, Summers DK, Monteiro GA (2005) Recombinant protein secretion in Escherichia coli. Biotechnol Adv 23(3):177–202

Mieschendahl M, Bfichel D, Bocklage H, Mfiller-Hill B (1981) Mutations in the lacY gene of Escherichia coli define functional organization of lactose permease. Proc Natl Acad Sci USA 78(12):7652

Nancib N, Branlant C, Boudrant J (1991) Metabolic roles of peptone and yeast extract for the culture of a recombinant strain of Escherichia coli. J Ind Microbiol Biotechnol 8(3):165–169

Neubauer P, Hofmann K, Holst O, Mattiasson B, Kruschke P (1992) Maximizing the expression of a recombinant gene in Escherichia coli by manipulation of induction time using lactose as inducer. Appl Microbiol Biotechnol 36(6):739–744

Osumi T, Saier MH (1982) Regulation of lactose permease activity by the phosphoenolpyruvate: sugar phosphotransferase system: evidence for direct binding of the glucose-specific enzyme III to the lactose permease. Proc Natl Acad Sci USA 79(5):1457

Ou J, Wang L, Ding X, Du J, Zhang Y, Chen H, Xu A (2004) Stationary phase protein overproduction is a fundamental capability of Escherichia coli. Biochem Biophys Res Commun 314(1):174–180

Pugsley AP, Francetic O, Possot OM, Sauvonnet N, Hardie KR (1997) Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria-a review. Gene 192(1):13–19

Ramanan RN, Ling TC, Ariff AB (2008) The performance of a glass bead shaking technique for the disruption of Escherichia coli cells. Biotechnol Bioprocess Eng 13(5):613–623

Ramanan RN, Tey BT, Ling TC, Ariff AB (2009) Classification of pressure range based on the characterization of Escherichia coli cell disruption in high pressure homogenizer. Am J Biochem Biotechnol 5(1):21–29

Ramanan RN, Ling TC, Tey BT, Ariff AB (2010a) A simple method for quantification of interferon-α2b through surface plasmon resonance technique. Afr J Biotechnol 9(11):1680–1689

Ramanan RN, Tik WB, Memari HR, Azaman SNA, Ling TC, Tey BT, Lila MAM, Abdullah MP, Rahim RA, Ariff AB (2010b) Effect of promoter strength and signal sequence on the periplasmic expression of human interferon-α2b in Escherichia coli. Afr J Biotechnol 9(3):285–292

Reznikoff WS, Miller JH (1978) The operon. Z Allg Mikrobiol 20:298

Rosenberg HF (1998) Isolation of recombinant secretory proteins by limited induction and quantitative harvest. Biotechniques 24(2):188–192

Schumann W, Ferreira LCS (2004) Production of recombinant proteins in Escherichia coli. Genet Mol Biol 27:442–453

Shukla TP, Wierzbicki LE (1975) Beta-galactosidase technology: A solution to the lactose problem. Crit Rev Food Sci Nutr 5(3):325–356

Slos P, Speck D, Accart N, Kolbe HVJ, Schubnel D, Bouchon B, Bischoff R, Kieny MP (1994) Recombinant cholera toxin B-subunit in Escherichia coli: high-level secretion, purification, and characterization. Protein Expr Purif 5(5):518–526

Somerville JE, Goshorn SC, Fell HP, Darveau RP (1994) Bacterial aspects associated with the expression of a single-chain antibody fragment in Escherichia coli. Appl Microbiol Biotechnol 42(4):595–603

Sørensen HP, Mortensen KK (2005) Advanced genetic strategies for recombinant protein expression in Escherichia coli. J Biotechnol 115(2):113–128

Studier FW (2005) Protein production by auto-induction in high density shaking cultures. Protein Expr Purif 41(1):207–234

Takagi H, Morinaga Y, Tsuchiya M, Ikemura H, Inouyea M (1988) Control of folding of proteins secreted by a high expression secretion vector, pIN-III-ompA: 16-fold increase in production of active subtilisin E in Escherichia coli. Nat Biotechnol 6(8):948–950

Tan JS, Ramanan RN, Azaman SNA, Ling TC, Shuhaimi M, Ariff AB (2009) Enhanced interferon-α2b production in periplasmic space of Escherichia coli through medium optimization using response surface method. J Open Biotechnol 3:103–110

Vila P, Luis Corchero J, Cubarsi R, Villaverde A (1997) Enhanced fitness of recombinant protein synthesis in the stationary phase of Escherichia coli batch cultures. Biotechnol Lett 19(3):225–228

Wong P, Gladney S, Keasling JD (1997) Mathematical model of the lac operon: inducer exclusion, catabolite repression, and diauxic growth on glucose and lactose. Biotechnol Prog 13(2):132–143

Gene disruption of *plasmodium falciparum* P52 results in attenuation of malaria liver stage development

SUBMITTED BY: SUBHRADEEP ROY

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE

DEPARTMENT OF MICROBIOLOGY

REGISTRATION NO: A01-1152-116-017-2019

ROLL NO: 2022151199

SEMESTER: V

GUIDED BY: MR. AVIJIT CHAKRABORTY (Guest Faculty)

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE

DEPT. OF MICROBIOLOGY

Gene disruption of *plasmodium falciparum* p52 results in attenuation of malaria liver stage development

Abstract

Malaria is a global infectious disease that remains a leading cause of morbidity and mortality in the developing world. Severe and fatal malaria is predominantly caused by *Plasmodium falciparum*. Its management and prognosis depend on the awareness of a possible diagnosis in travelers returning from endemic areas, early recognition, and timely effective treatment. The proteins p52 and p36 are expressed in the sporozoite stage of the malaria parasite responsible for replication inducing merozoite formation which in turn trigger invasion of hepatocytes. These proteins result in the formation of parasitophorous vacuole (PV), within which the parasite undergoes intracellular replication as a liver stage. Parasites with a single p52 or p36 gene deletion can arrest this stage by disrupting parasitophorous vacuole(PV) formation and P52 and P36 maturation gets inhibited also indicates that both proteins are equally important in the establishment of PV and act in the same pathway. Gene deletion presented here offers the genetic homogeneity, standardization, batch to batch consistency can become a weapon for attenuated malaria vaccine and might provide a safe and reproducible platform to develop an efficacious whole cell malaria vaccine that prevents infection at the pre-erythrocytic stage.

Key words: *Plasmodium falciparum*, p52, p36, parasitophorous vacuole, genetic homogeneity.

Introduction

Malaria is a mosquito-borne disease caused by five protozoa: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale,* and most recently implicated *P.knowlesi*. Infection with *P. falciparum* is being accounted for more than 90% of the world's malaria mortality and therefore remains an important threat to public health on a global scale. Malaria is a formidable global health problem, affecting 300 million to 500 million people worldwide annually. The resulting 1 million deaths per year are mainly caused by plasmodium falciparum infections. Eradication of malaria will in large part depend on an effective vaccine that prevents infection by plasmodium falciparum, but such vaccine

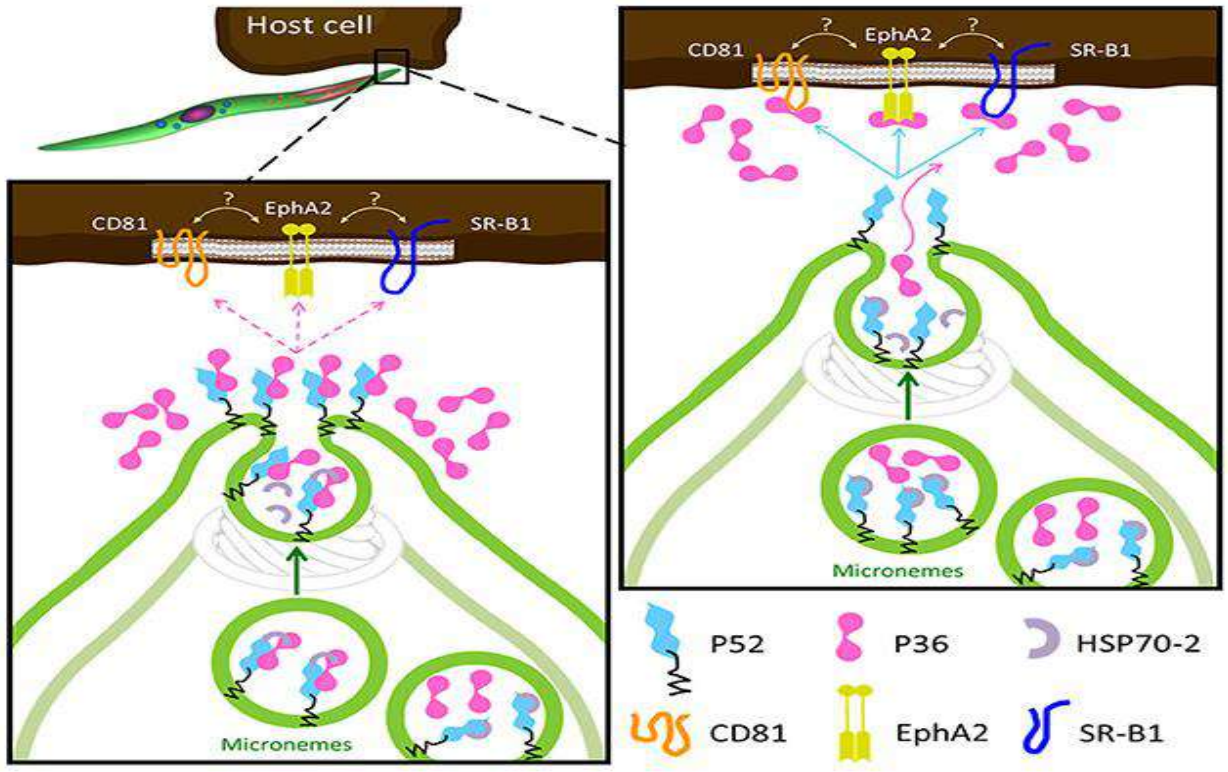
has remained elusive. The complexity of both the parasites life cycle and host immune responses to infection have contributed to the slow progress in the development of a vaccine that can induce efficient and long lasting protective immune responses.*Plasmodium* transmission occurs when sporozoites are deposited in the skin by a feeding, infected Anopheles mosquito. By means of gliding motility and cell traversal, sporozoite cross skin tissue and enter blood capillaries which allow their transport to the liver where they invade hepatocytes and form liver stages. Following parasite growth and replication within a hepatocyte, tens of thousands of merozoites will be released into the blood stream initiating the asexual blood cycle, leading to symptomatic malaria disease and possibly death. Before successfully establishing a liver infection, sporozoite will traverse several cell types including hepatocytes within transient vacuoles, searching for a suitable host hepatocyte. Upon encountering such a cell, sporozoites switch to "infection mode" and enter by creating the replication-permissive parasitophorous vacuole(PV). The PV plays a pivotal role in cell survival and normal progression of liver stage development as it separates the parasite from the host cell cytoplasm. The conserved Plasmodium proteins P36 and P52 have crucial functions in parasite fertilization and immune evasion. The P52 and P36 have been linked to the establishment and maintenance of the PV following dual deletion of these genes do not display a PVM. This intervention can be analyzed by electron microscopy.

Targeting two sporozoite-expressed loci in *P. falciparum*-p52 and p36- deletion indicates that they are important for productive liver infection. Analysis of the human parasite pre-erythrocytic stages has been challenging because no practical model for in-vivo evaluation of liver infection exists. Nonetheless, liver infection can be modeled in cell culture. HC-04 is a continuous hepatocytic cell line can support complete liver stage development of *P.falciparum*. To delete p52 and p36 from the parasite genome,positive–negative selection strategy. Double-cross-over homologous recombination between targeting sequences in transfection can beconstructed and the endogenous genes resulted in replacement of p52 and p36 geneindividually with the human dihydrofolate reductase (dhfr) selectable marker. Firstly, for each targeted locus gametocyte-producing clone 3D7 needs to be generated. Transfectant parasites will be appeared between days 21 and 35 under positive selection with WR92210 followed by negative selection cytosine deaminase-uracil phosphoribosyl transferase with 5-fluorocytosine. Transfectant will be then analyzed by southern blotting to detect gene deletion. So, disrupting these key genes cannot elicit a productive infection

in the liver stage. Arrest gene at this position highlights importance of being able to generate vaccine that are capable of inducing strong cell- mediated immunity.

P52 AND P36 GENE

During their lifecycle *plasmodium* rely upon some proteins that establish key interactions with the host or vector and between parasite sexual stages, with the purpose of ensuring infection, reproduction and proliferation. Among these is a group of secreted or membrane-anchored proteins known as six cysteine(6-cys) family.P52 and P36 genes both are micronemal plasmodium proteins act in concert to establish replication-permissive compartment within infected hepatocytes. P52 and P36(named according to molecular mass), having two s48/45 structural domains each, cysteine rich proteinbelongs to the conserved plasmodium 6-cys s48/45 family comprising proteins with crucial functions in parasite fertilization and immune evasion. P52 and P36 are arranged in tandem in the genome and while both have a secretory signal sequence, only P52 is predicted to be GPI-anchored. While both P36 and P52 are important for sporozoite invasion, it remained unknown how these proteins interact with the hepatocyte surface. EphA2, a host cell receptor is involved in hepatocyte susceptibility to infection. In the presence of P36, interaction of EphA2 with its natural



ligand EphrinA1 is disrupted leading to formation of parasitophorous vacuole(PV). This is an intriguing finding in light of the structural similarity of s48/45 domain with the Ephrin fold. Except EphA2, there are alternate host cell receptors including SR-B1, CD81 are required for productive invasion to various degree. P52 acts as scaffold which forms complex with P36 for their interaction with hepatocyte receptor. P52 was first identified in the comprehensive sporozoite transcriptome study. P52 having paralog of P36 is predicted to have a signal sequence and two 6-cys domains. Immunofluorescence and immune electron microscopy can localize P52 to the sporozoite micronemes. P52 was reported to be translocated to the surface of the sporozoite during gliding motility. P52/P36 complex could also establish contact with the EphA2 receptor on the hepatocyte surface also leading to formation of PV. However, a direct interaction between these two proteins has not been reported. It is possible that two proteins function in separate, but closely related.

Changes over P52/P36 gene

Plasmodium falciparum possesses multiple families of adhesion proteins, which have evolved either with the apicomplexan parasite lineages since their descent from free living ancestors or which were acquired by the parasite from an ancestor host through horizontal gene transfer. Many of these proteins mediate key interactions by the parasite's extracellular invasive stages and intracellular replicative stages either with host cells.Targeting two sporozoite-expressed loci in *P. falciparum*-p52 and p36- deletion indicates that they are important for productive liver infection. Analysis of the human parasite pre-erythrocytic stages has been challenging because no practical model for in-vivo evaluation of liver infection exist. Nonetheless, liver infection can be modeled in cell culture. HC-04 continuous hepatocytic cell line can support complete liver stage development of *P.falciparum*. To delete p52 and p36 from the parasite genome, positive–negative selection strategy. Double-cross-over homologous recombination between targeting sequences in transfection can be constructed and the endogenous genes resulted in replacement of p52 and p36 geneindividually with the human dihydrofolate reductase (dhfr) selectable marker. Firstly, for each targeted locus gametocyte-producing clone 3D7 needs to be generated. Transfectant parasites will be appeared between days 21 and 35 under positive selection with WR92210 followed by negative selection cytosine deaminase-uracil phosphoribosyl transferase with 5-fluorocytosine. Transfectant will be then analyzed by southern blotting to detect gene deletion. So disrupting these key genes cannot elicit a productive infection in the liver stage. Deletion of these two genes in the

erythrocytic stage does not result in any observable defect during blood stage replication, indicating that these gene have no apparent critical function during this part of parasite life cycle. Using gametocyte culture by membrane feeding to infect mosquito followed by evaluation of midgut oocyst infection in mosquito showed no discernable changes between wild type and P52 and P36 knockout lines.This indicates that gene deletion does not affect the sexual stages of the parasite. The gene deletions do not affect parasite replication, mosquito infection, and sporozoite production rates.Arrest gene at this position highlights importance of being able to generate vaccine that are capable of inducing strong cell- mediated immunity.

RESULT:

Deletion of the orthologous genes in the rodent malaria models indicate that they are important for productive liver infection. Analysis of the human parasite pre-erythrocytic stages has been challenging because no practical model for in vivo evaluation of liver infection exists. Nonetheless, liver infection can be modeled in cell culture, using of HC-04 continuous hepatocytic cell line can support complete liver stage development of *P.falciparum*. Cell infection rates of the P52 and P36 lines are comparable with those of wild type *P.falciparum* indicating that the corresponding protein function does not support host cell entry. P52 gene disruption can also be achieved by single cross over integration. Recent study mainly focusses on double cross over recombination gene replacement, which makes impossible for the parasite to genetically restore the wild type locus. P52/P36 parasite lines carrying deletions show double gene knockout. The phenotype which shows double gene knockout exhibit more profound intra-hepatocytic growth arrest in vitro compared to single gene knockout. The findings in conjunction with the in vitro observations demonstrate that knockout parasites remain parasite but do not develop and do not persist in the host cell. So, at the end, we can elucidate *p.falciparum* can be attenuated by disrupting a single gene is a first essential step in the development of malarial attenuated vaccine. Further optimization of these parasites will likely use double cross over recombination to avoid reversion of wild type genotype, disruption of multiple genes each of which may generate arrested or protective parasites thereby creating a parasite which contains successive obstacles for the restoration of parasite growth, and removal of foreign DNA from the transgenic parasite genome which can ease the transition of genetically modified organisms for human use. A significant breakthrough towards feasibility of a live attenuated malaria vaccine is the demonstration of genetic attenuation by gene deletions.

CONCLUSION:

Malaria effects millions of people throughout the world and becoming most life-threatening disease day by day. To restrict such life-threatening disease, a vaccine is necessary for fighting against malaria. So my project was just an approach that how we can make a suitable malarial attenuated vaccine by focusing on some particular plasmodium micronemal protein deletion. We can conclude that P36 and P52 act as a complex within the same sporozoite infection pathway leading to host cell engagement and subsequent induction of PVM formation, which is a prerequisite for productive infection of hepatocytes. The P52 and P36 have been linked to the establishment and maintenance of the PV following dual deletion of these genes do not display a PVM These intervention can be analyzed by electron microscopy. Gene deletion presented here offers the genetic homogeneity, standardization, batch to batch consistency can become a weapon for attenuated malaria vaccine. Arrest gene at this position highlights importance of being able to generate vaccine that are capable of inducing strong cell- mediated immunity.

REFERENCES:

1. Arredondo SA, Swearingen KE, Martinson T, Steel R, Dankwa DA, Harupa A, Camargo N, Betz W, Vigdorovich V, Oliver BG, Kangwanrangsan N, Ishino T, Sather N, Mikolajczak S, Vaughan AM, Torii M, Moritz RL and Kappe SHI(2018) The Micronemal Plasmodium Proteins P36 and P52 Act in Concert to Establish the Replication-Permissive Compartment Within Infected Hepatocytes Front. Cell. Infect. Microbiol. 8:413. doi: 10.3389/fcimb.2018.00413
2. VanBuskirk, K. M.; O'Neill, M. T.; De La Vega, P.; Maier, A. G.; Krzych, U.; Williams, J.; Dowler, M. G.; Sacci, J. B.; Kangwanrangsan, N.; Tsuboi, T.; Kneteman, N. M.; Heppner, D. G.; Murdock, B. A.; Mikolajczak, S. A.; Aly, A. S. I.; Cowman, A. F.; Kappe, S. H. I. (2009). Preerythrocytic, live-attenuated Plasmodium falciparum vaccine candidates by design. Proceedings of the National Academy of Sciences, *106(31), 13004–13009.* doi:10.1073/pnas.0906387106.
3. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI (2005) The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 434:214–217.

4. Luke TC, Hoffman SL (2003) Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated Plasmodium falciparum sporozoite vaccine. J Exp Biol 206:3803–3808.
5. Arredondo, S. A., and Kappe, S. H. (2016). The s48/45 six-cysteine proteins:mediators of interaction throughout the Plasmodium life cycle. Int. J. Parasitol.47, 409–423. doi: 10.1016/j.ijpara.2016.10.002
6. Fidock DA, Wellems TE (1997) Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc Natl Acad Sci USA 94:10931–10936.
7. VanBuskirk, K. M.; O'Neill, M. T.; De La Vega, P.; Maier, A. G.; Krzych, U.; Williams, J.; Dowler, M. G.; Sacci, J. B.; Kangwanrangsan, N.; Tsuboi, T.; Kneteman, N. M.; Heppner, D. G.; Murdock, B. A.; Mikolajczak, S. A.; Aly, A. S. I.; Cowman, A. F.; Kappe, S. H. I. (2009). *Preerythrocytic, live-attenuated Plasmodium falciparum vaccine candidates by design. Proceedings of the National Academy of Sciences, 106(31), 13004–13009.* doi:10.1073/pnas.0906387106.
8. van Schaijk, B. C., Janse, C. J., van Gemert, G. J., van Dijk, M. R., Gego, A.,Franetich, J. F., et al. (2008). Gene disruption of Plasmodium falciparump52 results in attenuation of malaria liver stage development in culturedprimary human hepatocytes. PLoS ONE 3:e3549. doi: 10.1371/journal.pone.0003549.

Application of magnetosomes synthesized by magnetotactic bacteria in medicine

Submitted by

Shaswata Modak

Registration number: A01-1112-116-013-2019

Roll number: 2022151195

Paper code: MCBA DSE II

Guided by: Dr. Sujoy Pal

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata 700118

January,2022

ACKNOWLEDGEMENT

I would like to acknowledge and give my warmest thanks to my supervisor Dr. Sujoy Pal who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator in charge; revered Bhrahamachari Parachaitanya Maharaj for allowing me to participate in this skill development project, which help me to venture relevant meaningful topics.

Finally, I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

Applications of Magnetosomes Synthesized by Magnetotactic Bacteria in Medicine

Introduction:

Magnetosomes are intracellular structures produced by magnetotactic bacteria, which comprise magnetic nanoparticles surrounded by lipid bilayer membrane. They have attracted much attention for biotechnological applications. This is due to a series of appealing properties summarized below that are not usually found in chemically synthesized nanoparticles.

The magnetosomes are magnetic nanoparticles, which possess a narrow size distribution and uniform morphology when the magnetotactic bacteria are cultivated in optimum conditions, i.e., essentially using a low oxygen concentration (varied between 0.25 and 10 mbar, Heyen and Schüler, 2003) during the growth. In these conditions, the magnetosome size distribution can be as small as ~10 nm with magnetosome sizes typically lying between 45 and 55 nm for the most commonly studied species of magnetotactic bacteria (AMB-1 and MSR-1) (Bazylinski and Frankel, 2004; Taylor and Barry, 2004).

Magnetosomes:

Magnetosomes are usually large single magnetic domain nanoparticles. This leads to a magnetic moment that is thermally stable at physiological temperature. Therefore, it produces better magnetic properties than those found in chemically synthesized iron oxide nanoparticles that are usually superparamagnetic and possess a thermally unstable magnetic moment. It also yields high values of the coercivity (Hc ~ 20–40 mT) and ratio between the remanent and saturation magnetization (Mr/Ms ~ 0.4–0.5) (Pan et al., 2005; Alphandéry et al., 2008). In specific conditions described below, these magnetic properties result in higher heating capacities and better magnetic resonance imaging (MRI) contrast agents for the magnetosomes than for chemically synthesized nanoparticles.

Magnetosomes are usually arranged in chains inside the bacteria. This arrangement is stable enough to be preserved even after disrupting the bacteria to isolate the magnetosomes. Such arrangement is appealing since it prevents aggregation and yields a high rate of internalization within human cells, two properties that are usually desired for medical applications (Alphandéry et al., 2011b, 2012a).

The magnetosomes are covered by biological material made of a majority of lipids and a minority of proteins. This biological coating results in negatively charged magnetosomes with a good dispersion in water (Alphandéry et al., 2011b, 2012a). By contrast, chemically synthesized

nanoparticles are not naturally coated and need to be stabilized, for example, by being covered with dextran or PEG molecules. This usually makes their synthesis more complicated than that of the magnetosomes. They can easily be functionalized, due to the presence of various chemical groups at their surface (Sun et al., 2011).

Production:

Methods have been published that enable to produce a large quantity of magnetosomes up to 170 mg/L/day of magnetosomes (Matsunaga et al., 1990, 1996; Yang et al., 2001; Heyen and Schüler, 2003; Sun et al., 2008a; Liu et al., 2010; Zhang et al., 2011; Alphandéry et al., 2012b). When they are prepared in specific conditions, the magnetosomes possess a high biocompatibility and a low toxicity (Xiang et al., 2007; Sun et al., 2010). They are obtained by cultivating magnetotactic bacteria in a growth medium, which is not toxic (for example, ATCC medium 1653 for the AMB-1 species). This contrasts with the use of toxic products often used during the preparation of chemically synthesized nanoparticles.

Applications of Magnetosomes in MRI and Magnetic Hyperthermia:

Several studies report the use of bacterial magnetosomes as positive or negative contrast agents. Benoit et al. (2009) have shown using MRI that magnetotactic bacteria have a natural tendency to target tumors in mice when they are administered intravenously. In this study, magnetotactic bacteria were visualized in tumors using MRI. A portion of the magnetosomes was shown to generate T1 (longitudinal relaxation times)-weighted positive contrast, improving the visualization of the magnetotactic bacteria in the tumors. Another interesting aspect of this report resides in the finding that small magnetosomes of ~25 nm in mean sizes produce a positive contrast while large magnetosomes of mean sizes ~50 nm do not produce such contrast. For the small magnetosomes, the T1-weighted MRI signal is also found to increase with increasing bacterial concentrations provided the bacterial concentration remains below a threshold of 0.5 1010 cells/mL. Above 0.5×1010 cells/mL, the T1-weighted MRI signal decreases due to the competing T2 (transverse relaxation times) effect. In general, good contrast agents are characterized by very high relaxivities (the inverse of the T2 relaxation time, usually designated as r2) and very short values of T2. Such high values of r2 can be reached with the magnetosomes. Indeed, it has been shown that both magnetosomes enclosed in a gel and ferrimagnetic nanoparticles different from ferridex and with similar properties than the magnetosomes possess values of r2 as high as 1175 and 324 mM s−1, respectively (Hu et al., 2010; Lee et al., 2011). These two values are larger than the value of r2 ~ 133 mM s−1 found for

chemically synthesized nanoparticles ferridex, which are currently approved and tested in the clinic as contrast agents for MRI application (Lee et al., 2011)

Magnetic hyperthermia:

Magnetosomes are also good candidates to treat cancers using magnetic hyperthermia. Magnetic hyperthermia is a technique in which magnetic nanoparticles are administered (or sent) to tumors and then heated under the application of an alternating magnetic field. The heat induces anti-tumor activity. In order to be efficient for magnetic hyperthermia, the nanoparticles therefore need to produce a large amount of heat.

The magnetosomes possess good heating properties essentially due to their large sizes, their ferromagnetic behavior at physiological temperature and their high level of crystallinity. For ferrimagnetic nanoparticles, the quantity of heat generated under the application of an alternating magnetic field is essentially proportional to the area of their hysteresis loop, which increases with increasing nanoparticle sizes. Indeed, in most cases, Hc and Mr/Ms that are proportional to the area of the hysteresis loops, increase with increasing nanoparticle sizes. The amount of heat produced by the magnetosomes has been estimated by measuring magnetosome losses per cycles, which are defined as the magnetosome SAR (specific absorption rates) divided by the frequency of the applied magnetic field.

There are essentially two mechanisms that can produce heat when magnetosomes are exposed to an alternating magnetic field. They are either due to the reversal of the magnetosome magnetic moment or to the physical rotation of the magnetosomes under the application of an alternating magnetic field. In order to eliminate the contribution of the rotation to the heating mechanism of the magnetosomes, suspensions of whole magnetotactic bacteria that do not produce heat by rotation have been exposed to an alternating magnetic field (Alphandéry et al., 2011a).

In order to evaluate the anti-tumor activity of the magnetosomes (Alphandéry et al., 2011b), 100 μL of suspensions containing either individual magnetosomes or chains of magnetosomes at a concentration of 10 mg/mL were administered at the center of MDA-MB-231 breast tumors xeno-grafted under the skin of mice following the protocol illustrated in the schematic diagram of Figure 2. The mice were then exposed to an alternating magnetic field of average field strength ~20 mT and frequency 198 kHz three times during 20 min.

This produced an increase in the tumor temperature up to ~43°C. The treatment with the chains of magnetosomes yielded the total disappearance of the tumor 30 days following the treatment in several mice, while that using the individual magnetosomes did not produce significant anti-tumor activity (Alphandéry et al., 2011b). The efficacy of the treatment was attributed on the one

hand to the internalization of the chains of magnetosomes inside the tumor cells that enabled intracellular heating and hence efficient tumor cell destruction. On the other hand, the efficacy of the chains of magnetosomes was reported to arise from their homogenous distribution throughout the tumor, which is mostly due to their low level of aggregation.

Future prospects:

The future perspectives for magnetosomes are optimistic as it can be used in various medical applications of bacterial magnetosomes, for example, to detect nucleotide polymorphism, which is useful to diagnose diseases such as cancer, hypertension, or diabetes, to separate cells or to detect DNA (Arakaki et al., 2008). To separate cells, magnetic beads or SPION have been tested. However, these two types of magnetic materials present drawbacks. Magnetic beads are large and hence prevent cells from dividing and proliferating correctly. SPION on the other hand are only weakly magnetic due to their unstable magnetic moment at physiological and room temperatures, which makes them poorly efficient to separate cells. By contrast, the magnetosomes are of smaller sizes than the magnetic beads and are more strongly magnetic than the SPION due to their ferrimagnetic properties.

This makes them ideal candidates for applications in cell separation (Arakaki et al., 2008). The magnetosomes have also been used for immunoassays, for example, to detect small molecules such as pollutant, hormones, or toxic detergents. These molecules have been attached to the magnetosome surface using antibodies that specifically bind to them. The complex formed by the magnetosomes and these molecules has then been detected (Arakaki et al., 2008). Finally, magnetosomes have been used to extract DNA. For that, they have been modified and covered with layers of aminosilanes that link DNA. The complex formed by the magnetosomes and DNA has been bound to a magnetic column and DNA has been collected by elution with a phosphate buffer (Arakaki et al., 2008).

Conclusion:

In conclusion, I have presented in this review several medical applications of the magnetosomes and have also briefly described a few methods that can be used to prepare the magnetosomes for these applications.

References:

Alphandéry, E., Faure, S., Raison, L., Duguet, E., Howse, P. A., and Bazylinski, D. A. (2011a). Heat production by bacterial magnetosomes exposed to an oscillating magnetic field. J. Phys. Chem. C 115, 18–22. doi:10.1021/jp104580t

Alphandéry, E., Faure, S., Seksek, O., Guyot, F., and Chebbi, I. (2011b). Chains of magnetosomes extracted from AMB-1 magnetotactic bacteria for applica- tion in alternative magnetic field cancer therapy. ACS Nano 5, 6279–6296. doi:10.1021/nn201290k

Arakaki, A., Nakazawa, H., Nemoto, M., Mori, T., and Matsunaga, T. (2008). Forma- tion of magnetite by bacteria and its application. J. R. Soc. Interface 5, 977–999. doi:10.1098/rsif.2008.0170

Bazylinski, D. A., and Frankel, R. (2004). Magnetosome formation in prokaryotes. Nat. Rev. Microbiol. 2, 217–230. doi:10.1038/nrmicro842

Benoit, M., Mayer, D., Barak, Y., Chen, I. Y., Hu, W., Cheng, Z., et al. (2009). Visual- izing implanted tumors in mice with magnetic resonance imaging using magne- totactic bacteria. Clin. Cancer Res. 15, 5170–5177. doi:10.1158/1078-0432.CCR- 08- 3206

Heyen, U., and Schüler, D. (2003). Growth and magnetosome formation by microaerophilic Magnetospirillum strains in oxygen-controlled fermentor. Appl. Microbiol. Biotechnol. 61, 536–544. doi:10.1007/s00253-002-1219-x

Heyen, U., and Schüler, D. (2003). Growth and magnetosome formation by microaerophilic Magnetospirillum strains in oxygen-controlled fermentor. Appl. Microbiol. Biotechnol. 61, 536–544. doi:10.1007/s00253-002-1219-x

Lee, N., Kim, H., Choi, S. H., Park, M., Kim, D., Kim, H.-C., et al. (2011). Magnetosome-like ferromagnetic iron oxide nanocubes for highly sensitive MRI of single cells and transplanted pancreatic islets. Proc. Natl. Acad. Sci. U.S.A. 108, 2662–2667. doi:10.1073/pnas.1016409108
Matsunaga, T., Tadokora, F., and Nakamura, N. (1990). Mass culture of magnetic bacteria and their application to flow type immunoassays. IEEE Trans. Magn. 26, 1557–1559. doi:10.1109/20.104444

Matsunaga, T., Tsujimura, N., and Kamiya, S. (1996). Enhancement of magnetic particle production by nitrate and succinate fed-batched culture of Magnetospir- illum sp. AMB-1. Biotechnol. Tech. 10, 495–500. doi:10.1007/BF00159513

Pan, Y., Peterson, N., Winklhofer, M., Davila, A. F., Liu, Q., Frederichs, T., et al. (2005). Rock magnetic properties of uncultured magnetotactic bacteria. Earth Planet. Sci. Lett. 237, 311–325. doi:10.1016/j.epsl.2005.06.029

Sun, J., Li, Y., Liang, X.-J., and Wang, P. C. (2011). Bacterial magnetosome: a novel biogenetic magnetic targeted drug carrier with potential multifunctions. J. Nano- mater. 2011:469031, 13 p. doi:10.1155/2011/469031

Taylor, A. P., and Barry, J. C. (2004). Magnetosomal matrix: ultrafine structure may template biomineralization of magnetosomes. J. Microscopy 213, 180–197. doi:10.1111/j.1365-2818.2004.01287.x

Xiang, L., Wei, J., Jianbo, S., Gulli, W., Feng, G., and Ying, L. (2007). Purified and sterilized magnetosomes from Magnetospirillum gryphiswaldense MSR-1 were not toxic to mouse fibroblasts in vitro. Lett. Appl. Microbiol. 45, 75–81. doi:10.1111/j.1472- 765X.2007.02143.x

A project report on
Polyploidy and its application on breeding

By Soham Changdar

Reg No: A01-1112-116-018-2019

Exam Roll No: 2022151200

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Guided by – Dr. Sekhar Pal

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Acknowledgement:

For acknowledgement section I first of all would like to acknowledge Dr. Sekhar Pal sir, Dept. Microbiology, Ramakrishna Mission Vivekananda Centenary College Rahara for his serious guidance. Next I would like to thank our departmental co-ordinator Br. Parachaitanya Maharaj and all the teachers of the department for selecting me in such a project work. A serious thanks also goes to my friend Somnath Roy and Koushik Bhattacharaya for his suggestions and technical assistance.

Polyploidy and its application on breeding

Introduction: Polyploidy refers to the presence of more than two complete sets of chromosomes per cell nucleus, which has been considered a ubiquitous phenomenon in plant evolution and diversification. Occasionally whole set of chromosomes fail to separate in meiosis or mitosis, leading to polyploidy, the presence of more than two genomic set of chromosomes. Polyploidy include triploid(3n), tetraploid(4n), pentaploid(5n) even higher number of chromosomes set.

Polyploidy is common in plants and is a major mechanism by which new plant species have evolve approximately 40% of flowering plant species and form 70% - 80% of grasses are polyploids [1]. They include a number of agriculturally important plants such as wheats, oats, cotton, potato, sugar cane. polyploidy is less common in animals but is found in some invertebrates, fishes, salamander, frogs and lizards.

Polyploidy classes and modes of origin: There are two major types of polyploidy :

a. Autopolyploidy
b. Allopolyploidy

Autopolyploid : Autopoliploidy is due to accidentelly of mitosis or miosis that produce extra sets of chromosome ,all derived from a single species. Nondisjunction of all chromosome in mitosis is an early 2n for example daubles the chromosome number and produce an autotetraploid(4n) , as depicted in Fig:-1(a)

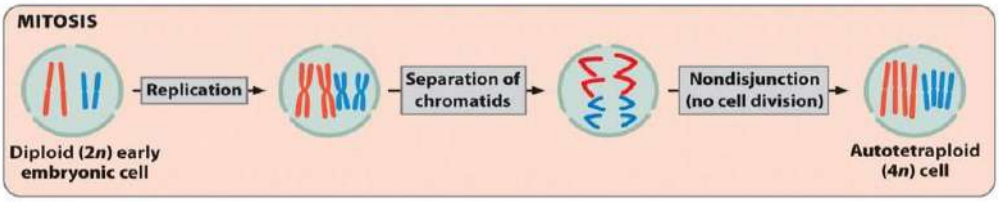


Fig 1(a) Autopolyploidy through mitosis [1]

An autotriploid (3n) may arise when non-disjunction in meiosis produce a diploid gamate that then fuse with a normal haploid gamate to produce a triploid zygote.(fig;1b) Alteranively triploids may arise from a cross between an autotetraploid that produce 2n gametes and a diploid that produce 1n gamates.

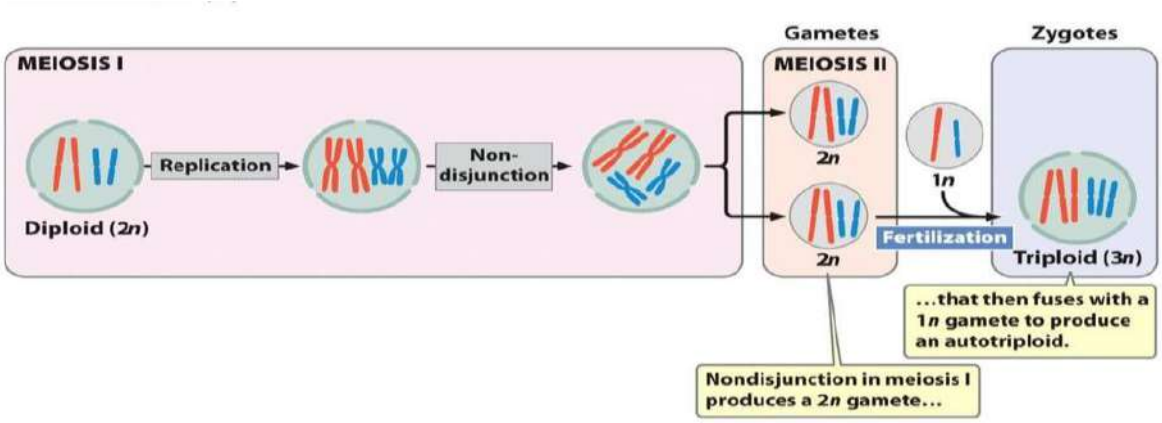
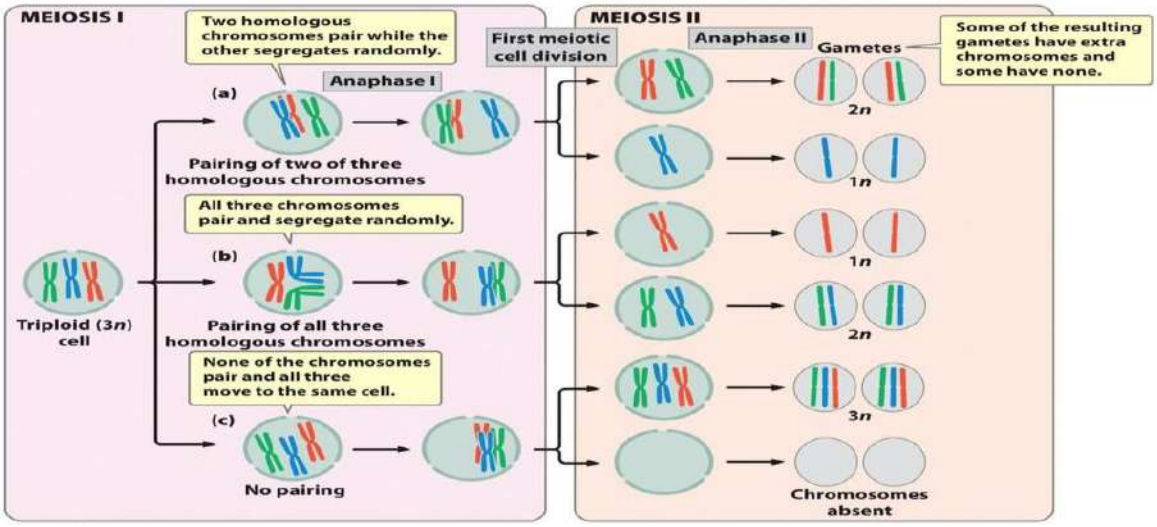


Fig 1(b) Autopolyploidy through meiosis [1]

Because all the chromosome set in autopolyploid are from the same, they are homologous and try to align in prophase-1 of mitosis which usually results in sterility. Consider meiosis in an autotriploid (fig-2). In meiosis in a diploid cell, two chromosome homologs pair and align, but in autotriploids, three homologs are present.



(Fig-2**) In meiosis of an autotriploid , homologous chromosome can pairor not pair in three ways.** This example illustrates the pairing and segregation of a single homologous set of chromosomes.[1]

The sterility that usually accompanies autoploidy has been exploited in agriculture. Wild diploid banana (2n =22), for example produce seeds that are hard and inedible, but triploid bananas (3n=33) are sterile and produce no seeds.

Allopolyploid:

Allopolyploid arises from hybridization between two species; the resulting polyploid carries chromosome sets derived from two or more species. Allopolyploids are often divided into two sub-classes: true and segmental allopolyploids. The formation of true allopolyploids involves hybridization between distantly related species. In this case, the divergent chromosome complements do not pair with each other, resulting in the formation of bivalents during meiosis and in a disomic inheritance pattern. On the other hand, segmental related species with partially differentiated genomes. Therefore, segmental allopolyploids may undergo univalent, bivalent and/or multivalent pairing during meiosis, being considered intermediate types between true allopolyploids and autoployploids.

Different mechanisms have been proposed to explain how polyploids arise in nature. Two major pathways are known to lead to polyploidy in plants: somatic doubling and formation of unreduced reproductive cells. Somatic doubling is associated with mitotic events such as endomitosis or endoreduplication, which may occur either in a zygote cell or in apical meristematic tissues, giving rise to mixoploids or even completely polyploid organisms. Despite being constantly used to attain artificial polyploids, somatic doubling is supposed to have a minor role in the origin of natural polyploid organisms.

The production and fusion of unreduced reproductive cells have been pointed out as the most predominant pathway leading to polyploidy in plants. The capacity of producing unreduced gametes is a heritable feature evidenced in many plant species. Besides

genetic control, environmental factors such as temperature, herbivory, wounding, water deficit and nutrients shortage influence the production of unreduced gametes. Once formed, the unreduced gamete can fuse either with another unreduced gamete (bilateral polyploidization) or with a reduced one (unilateral polyploidization).

The genetic effect of polyploidy:

Changes to cell and body size: Accompanying genome doubling and increases in genetic materials, the cell volumes of polyploids usually enlarge. Plants and animals may employ different strategies to cope with the increase in cell size associated with polyploidy [4,5] Polyploid plants maintain the same number of cells as diploids and thus develop larger organs and body sizes. By contrast, many animal polyploids reduce the overall number of cells and maintain a similar organ and body size to their diploid progenitors.

Genomic change after polyploid formation: Genomic instability and genomic rapid recombination are the most characteristic features of the new polyploid, in an effort to achieve the harmonious co-existence of multiple genomes within one nucleus [6,7]. For instance, in artificial synthetic polyploid Brassica hybrids, extensive genomic rearrangements and fragment loss within five generations were observed [8]. The structural changes to the genome consist of deletion, insertion, duplication, translocation, and transposition [7]. Using chromosome painting techniques, Kenton et al. found at least nine intergenomic chromosomal rearrangements in allotetraploid tobacco (Nicotiana tabacum) [9]. Similarly, by genomic in situ hybridization (GISH), Jellen et al. detected five intergenomic translocations in allotetraploid oat (Avena), and approximately 18 such rearrangements in the allohexaploid [10]. Liu et al. confirmed that the allotetraploid offspring of red crucian carp×common carp possess the parental genes, and demonstrated the hybridity by fluorescence in situ hybridization (FISH). Furthermore, genome recombination was found in allotetraploids at both the genome and transcriptome level, and it is hypothesized that the autosyndesis and allosyndesis of chromosomes coexist in meiosis of the germ cells.

Changes promote the evolution of polyploids: The abundant gene redundancy shields polyploids from the deleterious effects of mutation. Polyploids can mask recessive alleles by expressing dominant wild-type alleles, and diversify gene function by altering redundant copies of important or essential gene [2]. Furthermore, by fixing of different genomes, hybrid polyploids can integrate good parental traits and further display heterosis, showing better environmental adaptability compared with closely related diploid organisms [11]. By fast or slow genomic and gene expression changes, polyploids innovate and improve their function to achieve speciation and evolutionary success.

Application of polyploidy breeding in plants:

Studies on the growth and biochemical characters of natural polyploid plants showed that polyploids, especially allopolyploids, have many obvious advantages, such as bigger nutritive organs, faster metabolism, more secondary metabolites, and increased stress resistance [12]. Artificial polyploid breeding is becoming more and more common in plants. For vegetable crops [3], fruit trees [13], horticultural plants [14], medicinal plants [15], by the application of artificial selective breeding, distinct hybridization, tissue culture, physicochemical factors induced breeding, protoplast culture, and somatic hybridization, many polyploid lines are created and widely applied.

The triploid seedless watermelon is one of the best application polyploidy breeding [16]. The key to cultivating triploid watermelon is the acquisition of tetraploid parents. The most widely used method is by colchicine-treatment. When treating the seeds and seedlings of diploid watermelon, colchicine hinders the formation of spindles. Thus, the genome is doubled and tetraploid watermelons are produced. The triploid seeds were obtained by crossing tetraploid (♀) with diploid (♂). The triploid plants cannot generate seeds because of meiotic abnormalities, while the ovary can develop into triploid fruit by the stimulation of diploid pollen. By integrating the dual advantages of polyploidization and origin, the triploid seedless watermelons gain obvious advantages, such as higher sugar content, increased stress resistance, higher yield, larger fruit which are valued by the growers and consumers. China is now the biggest producer of seedless watermelon.

Many other polyploid lines have been obtained by selective breeding and hybridization in Malus (X=17), Pyrus (X=17), Euvitis (X=19), Prunus (X=8), Fragria (X=7). By inducing chromosome duplication with artificial induction, tissue culture, and hybridization, polyploid cabbage (*Brassica rapa pekinensis*), lettuce (*Lactuca sativa*), capsicum (*Chili pepper*), lily (*Lilium brownie*), and cucumber (*Cucumis sativus*) were obtained, some of which have been widely planted and applied [3]. Polyploid medicinal plant with higher output and increased stress resistance were formed in *Savia miltiorrhiza, Isatis tinctoria, Lonicera japonica, Glycyrrhiza pallidilora*[15].

Application of polyploidy breeding in animal:

Studies on animal polyploid breeding have played a significant role in the field of aquatic organisms, and the breeding of polyploids in crustacea, shellfish, marine fish, and freshwater fish has made tremendous progress. Many kinds of polyploids were obtained by biological (distant hybridization, nuclear transfer, cell fusion), physical (temperature shock, hydrostatic pressure shock), or chemical (chemical inducers such as cytochalasin, caffeine, polyethylene glycol and colchicine) methods [17]. Among the polyploid animals**,** sterile triploids could translate the energy of gonad development into increased energy for flesh development, and display obvious advantages in growth rate, taste, survival rate, and disease resistance [18]. Moreover, triploids are not only a good way to control the density of the cultured fish, but also an effective approach to safeguard fish genetic resources [19]. There were two main pathways to produce triploid fish. One is direct induction and the other is indirect production by crossing a tetraploid with a diploid.

Artificial induction of aquatic organism polyploids by physical and chemical methods is based on the retention of the first or the second polar body of the oocytes, or the inhibition of the first cleavage of the fertilized eggs, thus producing a triploid or tetraploid [20].

Polyploid breeding in crustacea and shellfish often makes use of cytochalasin or temperature shock treatment, which generally cause high mortality at the early embryo stage or during larva development [21,22]. As a result of this mortality, large-scale production by these methods is difficult. Temperature shock and hydrostatic pressure shock have been used to induce polyploid fish. Gui *et al.* sucessfully achieved a triploid C. auratus transparent colored variety by hydrostatic pressure shock, and further obtained tetraploid embryos by a

combination of hydrostatic pressure shock and cold shock [23]. These methods are subject to the control of treatment conditions and sample size, and cannot guarantee the production of 100% triploid offspring. While suppressing the first mitosis of the fertilized eggs, there have been no reports on the formation of bisexual fertile tetraploids. Acquisition of each generation of tetraploid broodstock must be handled manually, which increases costs. However, treatments by physical and chemical factors are different from the conditions in nature, which may result in aneuploids. The chemical agents used to induce polyploids often react with genetic materials or other structures in the cytoplasm, influencing the individual's development and activity, and may produce malformation and chimeras.

Conclusions and future perspectives:

Polyploidy has been widely studied in the last century and is arguably one of the most important mechanisms of adaptation and speciation in plants. Moreover, the fact that many of the most relevant crop species are polyploid has proven that polyploidy is also of great relevance for humans. The several consequences of polyploidy observed in natural populations have attracted great attention of plant breeders for the application of artificial polyploidy as a tool for crop improvement. The ‘‘gigas’’ effect is one of those direct consequences and, when occurring in organs of commercial interest, is a valuable feature for crop improvement. The phenomena of genome ‘‘buffering’’, heterozygosity and heterosis (hybrid vigor) deserve attention in plant breeding programs, as they may lead to the higher vigor displayed by polyploid organisms when compared to their diploid relatives.

Several protocols have been developed for polyploidy induction in a wide range of crop species. Since the discovery of colchicine, in vitro polyploidization using this antimitotic agent has been one of the most important applications for artificial polyploidy induction. Sexual polyploidization through the fusion of unreduced reproductive cells is also applied, especially when high levels of heterozygosity are required.

The initial expectations regarding polyploidy induction for plant improvement were extremely optimistic, especially after the discovery of colchicine. In the present days, only few successfully induced autopolyploids have commercial relevance. Nevertheless, polyploidy has become a highly valuable tool in plant breeding programs, and its application is not restricted to yielding improvement by the ‘‘gigas’’ effect promoted by autopolyploidy induction. Polyploids are also important as bridges for genetic transfer between species in which the direct cross is not possible, as well as to reestablish the fertility of sterile hybrids. Therefore, the application of polyploidy as a tool by plant breeders has allowed the development of increasingly productive and adapted cultivars.

References

1. Genetics, A Conceptual Approach (4^{th} edition) Benjamin A. Pierce
2. Comai L. The advantages and disadvantages of being polyploid. Nat Rev Genet, 2005, 6: 836–845
3. Yang Y G, Zhuang Y, Chen L Z, et al. Vegetable polyploid and polyploidy breeding (in Chinese). Acta Agricult Univ Jiangxi, 2006, 28: 534–538
4. Gregory T R, Mable B K. Polyploidy in Animals. In: Gregory T R, ed. The Evolution of the Genome. San Diego: Elsevier, 2005. 427–517

5. Mable B K. ‘Why polyploidy is rarer in animals than in plants’: myths and mechanisms. Biol J Linn Soc, 2004, 82: 453–466
6. Comai L. Genetic and epigenetic interactions in allopolyploid plants. Plant Mol Biol, 2000, 43: 387–399
7. Chen Z J, Ni Z F. Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. Bioessays, 2006, 28: 240–252
8. Song K, Lu P, Tang K, et al. Rapid genome change in synthetic polyploids of Brassica and its implications for polyploid evolution. Proc Natl Acad Sci USA, 1995, 92: 7719–7723
9. Kenton A, Parokonny A S, Gleba Y Y, et al. Characterization of the Nicotiana tabacum L. Genome by molecular cytogenetics. Mol Gen Genet, 1993, 240: 159–169
10. Jellen E N, Gill B S, Cox T S. Genomic in situ hybridization differentiates between A/D-and C-genome chromatin and detects intergenomic translocations in polyploid oat species (genus Avena). Genome,1994, 37: 613–618
11. De Bodt S, Maere S, Van de Peer Y. Genome duplication and the origin of angiosperms. Trends Ecol Evol, 2005, 20: 591–597
12. Ma H Y, Zhang J F, Li Z D. Research advances on plant polyploidy breeding techniques (in Chinese). Protect Forest Sci Technol, 2008, 1: 43–46
13. Wang T K, Zhang J Z, Qi Y S, et al. Advances on polyploid breeding of fruit crops in China (in Chinese). J Fruit Sci, 2004, 21: 592–597
14. Shun M H, Zhang S N. The application of polyploidy breeding in horticultural crops (in Chinese). Jiangsu Agricult Sci, 2004, 1: 68–72
15. Zhang X Y, Liu J F, Wang L P. Polyploidy breeding and its application research progress of medicinal plants (in Chinese). J Jilin Normal Univ (Nat Sci Ed), 2009, 4: 128–131
16. Yuan J M, Dang X M, Zhan Y F. Advances on polyploid breeding in watermelon (in Chinese). Chin J Tropical Agricult, 2009, 29: 65–70
17. Shen A L, Yao W Z. The proceeding on triploid breeding of aquatic animals (in Chinese). Reserv Fish, 2004, 24: 1–3
18. Liu Y, Liu S J, Sun Y D, et al. Polyploid hybrids of crucian carp×common carp (in Chinese). Rev China Agricult Sci Technol, 2003, 5: 3–6
19. Wu P. Research progress of fish polyploid breeding in China (in Chinese). J Shanghai Fish Univ, 2005, 14: 72–78
20. Hu L L, Li J E. The review of fish polyploid breeding research (in Chinese). Fish Sci Technol, 2009, 7–10
21. Yuan B J, Jiang N C, Lu J P, et al. A review of decapod crustacean multiploid breeding (in Chinese). Donghai Marine Sci, 1998, 16: 64– 68
22. Wang Z P, Li K J, Yu R H, et al. Progress of tetraploid breeding in mollusks (in Chinese). J Ocean Univ China, 2004, 34: 195–200
23. Gui J F, Liang S C, Sun J M, et al. Studies on genome manipulation in fish I. Induction of triploid transparent colored crucian carp (Carassius auratus transparent colored variety) by hydrostatic pressure (in Chinese). Acta Hydrobiol Sin, 1990, 14: 336–344

COMMON PROJECT REPORT ON

Telomerase aging and Cancer

Submitted By- Somnath Roy

Reg No.- A01-1112-116-004-2019

Exam Roll No.- 2022151186

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College, Rahara

Guided By- Dr. Sekhar Pal

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College, Rahara

Acknowledgement

I would like to acknowledge and give my warmest thanks to my supervisor Dr. Sekhar Pal sir, who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator in charge; revered Bhrahamachari Parachaitanya Maharaj for allowing me to participate in this project, which help me to venture relevant meaningful topics.

Finally, I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

INTRODUCTION:

Telomeres and Telomerase

Telomeres are specific DNA-protein structures that form protective caps at the ends of the linear chromosomes to protect that DNA from end-to-end fusion and exonucleolytic degradation. These structures are consisting of tandem repeats of TTAGGG at the tip of the linear chromosomes. DNA of telomeric region has an average length of 5-15 kb and shortens around 25-200 bp per human somatic cell division. Telomeres enable cells to distinguish chromosome ends from double strand breaks (DSBs) in the genome[1,2,4].

Now, because DNA synthesis occurs unidirectionally (5'→3') and requires an RNA primer for initiation, telomeres are not fully replicated by the conventional DNA polymerase complex.[3] To overcome this 'end-replication' problem, most eukaryotic cells utilize a novel DNA polymerase, telomerase. This enzyme adds DNA sequence repeats ("TTAGGG" in all vertebrates) to the 3' end of DNA strands in the telomere regions, which are found at the ends of eukaryotic chromosomes. Activity of telomerase is required to prevent telomere shortening and thus to ensure cell and organism survival. [5,6,7] Telomerase can synthesize telomeres *de novo* and consists of 2 essential components, an RNA component serving as a template for telomere sequence synthesis and a reverse transcriptase (RT). Telomerase encompasses a catalytic subunit with reverse transcriptase activity (Tert), an RNA component (Terc) that acts as a template for DNA synthesis and the protein dyskerin (Dkc1), which binds and stabilizes Terc. Robust telomerase expression is a feature of pluri-potent stem cells and early stages of embryonic development, although telomerase activity is also present in adult stem cell compartments, however its not sufficient to prevent telomere shortening associated with ageing. Mutations in the different components of telomerase (Tert, Terc and Dkc1), as well as in some shelterins (Tin2), have been linked to rare human genetic diseases, such as dyskeratosis congenita, aplastic anaemia and idiopathic pulmonary fibrosis. These diseases are associated with the presence of short/dysfunctional telomeres and they all exhibit a characteristic failure in the regenerative capacity of tissues (such as the bone marrow) and severe skin hyperpigmentation. [8,9]

Ageing and Evolution

Ageing is defined as the progressive functional decline of tissue function that eventually results in death. This type of functional decline can result from the loss or diminished function of postmitotic cells or from failure to replace such cells by a functional decline in the ability of (stem) cells to sustain replication and cell divisions. Aging is not a disease, and the biology of aging, which varies between individuals, is best understood in the context of evolution. The Disposable Soma model [10] provides a useful framework for such consideration. This model proposes that an increase in longevity in mammals is due to a concomitant reduction in the rates of growth and reproduction and an increase in the accuracy of synthesis of macromolecules. The notion that the fidelity of DNA repair is subject to selective forces and not necessarily better than (strictly) needed for a particular cell type, tissue, or species is not easily grasped. Differences in the fidelity of DNA repair pathways between cells of the germ-line and somatic (stem) cells and between comparable somatic cells from small, short-lived animals and large, long-lived species greatly complicate generalizations about the molecular mechanism of aging across different species. Limitations

in the use of model organisms to study the role of telomeres in human aging are perhaps best illustrated by the different consequences of telomerase deficiencies in humans and various model organisms [11,12,13]

TELOMERE STRUCTURE AND FUNCTION: OVERVIEW

Telomere Structure and Function

Linear chromosomes pose a general challenge: how to protect the natural ends of chromosomes from breakdown and degradation and avoid recognition and processing as double-strand breaks. There are many different solutions to this problem, ranging from covalently closed hairpin ends in some viruses, bacteria, and phages [14,16,19] to specific transposable elements in certain insects [15,18]. However, in organisms as diverse as protozoan, fungi, mammals, and plants, telomeres consist of G-rich repetitive DNA maintained by a specialized reverse transcriptase enzyme called telomerase. A detailed discussion of the structure and function of telomeres and telomerase in model organisms is outside the scope of this review, and the reader is referred elsewhere [17]. While many excellent reviews also exist regarding telomeres and telomerase in mammals [13,15], some further discussion is needed here to provide a context for understanding the consequences of telomere shortening and telomerase deficiencies in humans.

1. Telomere binding proteins

The DNA component of telomeres is characterized in all vertebrates by tandem repeats of (TTAGGG/CCCTAA) n [20]. Telomeric DNA typically ends in a single-strand G-rich overhang of between 50 and 300 nucleotides at the 3’ end, which has been proposed to fold back onto duplex telomeric DNA forming a “T-loop” structure [21]. The length of the repeats varies between chromosomes and between species. In humans and mice, the length of telomere repeats at individual chromosome ends in individual cells is strikingly variable [22,23]. Human chromosome ends are typically capped with between 0.5 and 15 kilobase (kb) pairs of detectable telomere repeats depending on the type of tissue, the age of the donor, and the replicative history of the cells. Individual ends of human chromosomes show marked variation in telomere length (Fig. 1) and the average length varies between chromosome ends. For example, chromosome 17p typically has

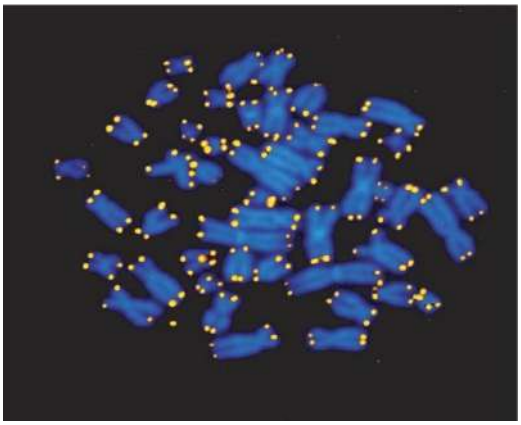


FIG. 1. The length of telomere repeats at individual chromosome ends is highly variable. Telomere repeats in a normal human lymphocyte are visualized using quantitative fluorescence in situ hybridization (Q-FISH) using peptide nucleic acid probes [22]. Telomeres are shown in yellow, whereas the DNA of chromosomes, counterstained with DAPI, is shown in blue. Note that the fluorescence on sister chromatid telomeres is typically of equal intensity in line with expectations for quantitative hybridization.

shorter telomeres than most other chromosome ends [24]. In human nucleated blood cells, the average telomere length shows a highly significant decline with age that is most pronounced for the cells of the immune system. Telomeres prevent the ends of linear chromosomes from appearing as DNA double strand (ds) breaks and protect chromosome ends from degradation and fusion. It has been proposed that telomeres can switch between an open state (in principle allowing elongation by telomerase) and a closed state (inaccessible to telomerase) with the likelihood of the open state

inversely related to the length of the repeat tract [25,26]. A model of how telomeres and telomerase interact in a telomere length-dependent manner is shown in Figure 2. This model is supported by data in yeast [27]. Recent studies in this model organism suggest that the timing of telomere replication is important for elongation by telomerase [29].

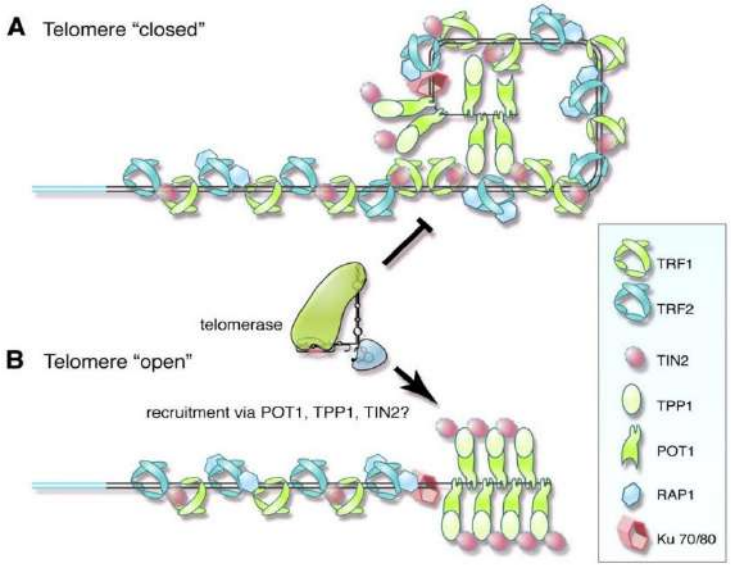


FIG. 2. Telomere function is linked to telomere length via the proteins that interact with double-strand telomere repeats (homodimers of TRF1 and TRF2), proteins that bind to the 3' single strand G-rich overhang present at the very end of chromosomes (POT1), and proteins that interact with these proteins such as RAP1, TPP1, and TIN2 as well as the Ku70/80 complex present at the junction of single- and double-stranded telomeric DNA. A: telomere "closed". TPP1 and POT1 form a complex with telomeric DNA via TIN2 and TRF1/2 if the length of telomere repeat tract is sufficiently long. TRF1 and -2 are known to bend telomeric DNA [27], and telomerase access is proposed to be blocked in a repeat length-dependent manner. B: telomere "open." The TPP1/POT1 recruits and stimulates enzymatic activity of telomerase [28] preferentially at short telomeres [29].

2. Telomerase

Telomerase is a specialized reverse transcriptase capable of extending the 3' end of chromosomes by adding TTAGGG repeats [28,29]. The human core enzyme consists of a reverse transcriptase protein (TERT) of 1,132 amino acids encoded by the hTERT gene [31], located on chromosome 5p15.33 and telomerase RNA containing 451 nucleotides (including the CAAUCCCAAUC telomere template) encoded by the telomerase RNA gene hTERC [32,33,34], located on chromosome 3q21-q28 (see Fig 2). The ribonucleoprotein dyskerin (encoded by the DKC1 gene on the X chromosome) is required for proper folding and stability of telomerase RNA [35] and was recently found to be part of the basic human telomerase enzyme complex [18,32]. Both the reverse transcriptase and telomerase RNA are expressed at very low levels, and haplo-insufficiency for either gene or mutations in DKC1 can give rise to various clinical manifestations. Telomerase levels are regulated at multiple levels including transcription, alternative splicing, assembly, subcellular localization, and posttranslational modifications of various components and of the enzyme complex itself. Expression of TERT is stimulated by c-Myc and estrogen and suppressed by Rb and p21. Multiple splice forms of TERT have been described with some having a dominant negative effect on telomerase activity [31]. Many questions about the efficiency of the assembly of fully functional as well as inactive telomerase complexes and the regulation of the subcellular trafficking of such complexes by posttranslational modification also remain largely unexplored. As a result, the relative importance of the such factors that have been proposed to affect the activity of telomerase at telomeres is difficult to discern, and the relative importance of such factors could vary between cell types. Another complicating factor is that the likelihood of a functional interaction between telomerase and repetitive DNA at telomeres is almost certainly also regulated at the level of telomere chromatin, an emerging research topic of much interest.

3. Telomeres and DNA damage responses

When the telomere pioneer Barbara McClintock received the Nobel prize in 1983 for her work on transposable genetic elements in maize, she referred in her acceptance speech to the importance of

responses of the genome to challenges [33,35]. She concluded her lecture with: “In the future attention undoubtedly will be centred on the genome, and with greater appreciation of its significance as a highly sensitive organ of the cell, monitoring genomic activities and correcting common errors, sensing the unusual and unexpected events, and responding to them, often by restructuring the genome. We know about the components of genomes that could be made available for such restructuring. We know nothing, however, about how the cell senses danger and instigates responses to it that often are truly remarkable.” While studies in the general areas of DNA repair, DNA damage responses, and apoptosis have all progressed tremendously, it is doubtful whether we are very much closer to an integrated view of the role of the genome in general and telomeres in particular in relation to how cells respond to stress of various kinds. Studies on p53, one of the major components of the response to stress, have highlighted that this protein has a very broad role in normal development and tumor formation, life expectancy, and overall fitness [31,39]. DNA damage signals are known to originate from short telomeres [27,38] and contribute to p53 activation and the cellular responses to stress. The telomere binding protein TRF2 binds to ataxia telangiectasia mutated (ATM) kinase and can inhibit its function [16,19,37], yet DNA damage signals appear to originate from telomeres with each replication cycle [35]. It has been proposed that telomeres switch between closed and open states [38] as is illustrated in Figures 2 and 3: perhaps the likelihood of the open state is proportional to the overall telomere length of the repeat tract [39]. As telomere length decreases with age, the amount of DNA damage signals originating from short telomeres is expected to increase (Fig. 3).

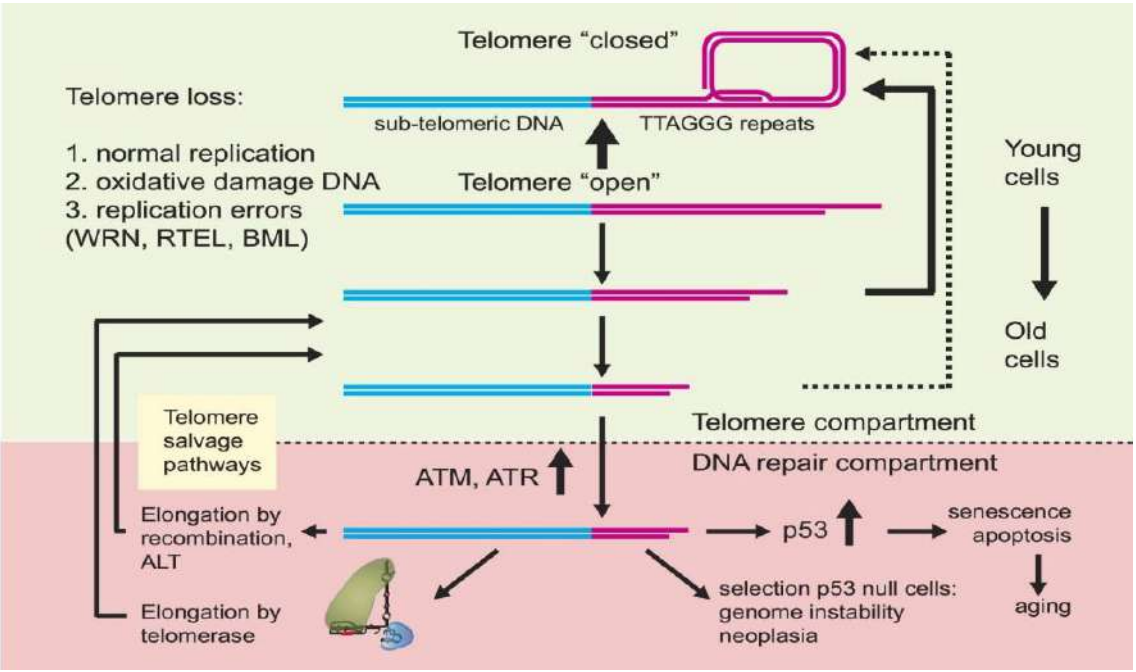


FIG. 3. Diagram of factors affecting the telomere length in primary somatic cells from human tissues. According to the model shown, telomeres in “young” somatic cells have long tracts of telomere repeats that favour folding into a “closed” structure that is invisible to the DNA damage response pathways and telomerase. As the telomere length at individual chromosome ends decreases, the likelihood that telomeres remain “closed” also decreases (see also Fig. 2). At one-point telomeres become too short and indistinguishable from broken ends. Such ends will be processed by enzymes in the DNA repair compartment (proposed to occupy a different nuclear domain than long telomeres). Depending on the cell type and the genes that are expressed in the cell, a limited number of short ends can be elongated by limiting levels of telomerase or recombination. However, with continued cell division and telomere loss, eventually too many short ends accumulate for the limited capacity of these “telomere salvage pathways.” At this point, defective telomeres will trigger levels of DNA damage signals such as p53 to which cells respond by either apoptosis or senescence. Rare (mutant) cells that do not upregulate functional DNA damage responses (e.g., by loss of functional p53) continue cell divisions in the presence of dysfunctional telomeres causing genome instability via chromosome fusions, chromosome breaks, and repetitive break-fusion bridge cycles. [37]

Higher “background” levels of activated p53 could decrease the threshold for activation of senescence or apoptosis in “old” cells, in line with the increased sensitivity to stress and more fragile nature of cells and tissues from the elderly. The role of telomeres in cellular aging relative to other proposed molecular mechanisms of aging including oxidative stress resulting from mitochondrial dysfunction or loss of ribosomal function remains to be precisely delineated. The development of an integrated view of the various molecular mechanisms of aging that have been proposed remains as formidable a challenge. However, it has become clear that telomeres are directly responsible for sustained DNA damage signals in senescent cells [17,37], and DNA damage foci originating from telomeres in senescent cells can readily be detected in vivo [21].

TELOMERE FUNCTION AND HUMAN AGING-RELATED DISEASE

Telomere Dysfunction

Telomerase deficiencies

Telomerase deficiencies were first implicated [29,32] in the inherited genetic disorder dyskeratosis congenita (DC) with the discovery of mutations in the dyskerin gene (DKC1) associated with the X-linked inheritance form of the disease [36,40]. Mutations in DKC1 are responsible for symptoms that include pancytopenia, abnormal skin pigmentation, nail dystrophy, leukoplakia, and bone marrow failure or pulmonary fibrosis which ultimately causes death in these patients, with a probability of bone marrow failure by age 20 exceeding 80% [37]. Dyskerin is a nucleolar protein that has been involved in the modification of specific small RNA molecules, specifically ribosomal RNAs and the telomerase template RNA or hTERC. Dyskeratosis can present in a variety of inheritance patterns that include X-linked, autosomal dominant, and autosomal recessive, implying other genetic causes for this disease as well as a possible link with other bone marrow failure syndromes such as constitutional aplastic anemia (AA), paroxysmal nocturnal hemoglobinuria (PNH), or myelodysplastic syndrome (MDS). AA and MDS reflect ineffective hematopoiesis and are more prevalent in elderly individuals, but most cases are not attributed to telomerase deficiencies despite showing telomere shortening [28]. In view of the studies implicating mutations in DKC1 with telomerase deficiencies and short telomere lengths [38], the telomerase genes were natural candidates for further investigations of younger patients presenting with DC or bone marrow failure syndromes. hTERC was the focus of initial efforts due to its relatively short size providing for relatively easy sequencing compared with hTERT, encoded by a fairly large gene. DC patients with hTERC mutations were indeed identified and were found to have reduced telomerase activity down to half of what can be measured in controls [23]. This gene-dose effect suggests that levels of telomerase RNA are tightly regulated. Strikingly, telomerase RNA levels also seem to be limiting in mice [18] and yeast [19], indicating that biallelic expression of the telomerase RNA gene is required in a broad range of organisms. In human cell lines, concomitant overexpression of hTERT and hTERC was necessary to substantially increase telomerase activity and elongate telomere length [36]. Apart from dyskerin, required for proper folding and stability of telomerase RNA, many other proteins are expected to modulate telomerase RNA levels, and deficiency of such proteins could result in reduced telomerase levels and reduced telomere length. An example of such a protein could be the protein encoded by the SBDS gene, which is deficient in the Shwachman-Diamond syndrome [25], although this protein must have other functions as well to explain the disease phenotype that involves specific cell types and tissues. Various mutations in hTERC have now been described, and many of these were shown to inhibit telomere elongation in telomerase reconstitution experiments

[26,29]. Detection of hTERC mutations have been detected in up to 15% of AA or MDS, showing that a clear DC phenotype is not always observed in patients that are telomerase deficient [11,12]. Conversely, hTERC mutations were not observed in a subset of patients with short telomeres that were diagnosed with Fanconi anemia [18]. Since hTERC mutations in marrow failure patient groups remain relatively infrequent, the screening of a large number of samples is required, or additional subset selection such as telomere length can be used to identify novel mutations [16,17]. More recently, substantial efforts have been made to screen for possible hTERT mutations, and a number of polymorphisms as well as mutations that cause telomerase deficiencies have been described [31]. Both telomerase genes are inked to the autosomal dominant form of DC, as well as cases of AA, PNH, and MDS [38,39].

Telomeres and Increased Cell Proliferation

In cellular in vitro models, for example, in the case of CD8 positive T cells, hTERT overexpression significantly enhances proliferation and cell survival [40]. Similar observations have been made with many different cell types. In vivo findings in animal tumor models showed that mTERC was upregulated early in tumorigenesis and that telomerase became activated in late stages of tumor progression [37]. These studies led to the examination of what the effects of constitutive expression or overexpression of TERT would be. mTert overexpression was shown to be associated with spontaneous mammary epithelial neoplasia and invasive carcinoma in aged mice [38], while constitutive expression of mTert in thymocytes promotes T-cell lymphoma [22]. More recently, work on targeted overexpression in specific tissues showed faster wound healing and increased tumorigenesis in the skin of K5- mTert mice (where mTerc is required for the tumor promoting effect) [26]. In addition, conditional induction (using a tetracycline-inducible system) in a mouse model showed that mTert causes the proliferation and mobilization of hair follicle stem cells [29]. This was visualized in situ as well as through the observation of exacerbated hair growth and faster hair regrowth in a manner independent from telomere synthesis. How TERT protein can also modulate the proliferation of stem cells in the skin even in the absence of telomerase RNA is currently not understood.

Cancer

The link between telomere biology and oncogenesis was first proposed when telomerase expression was found to be a hallmark of human cancer: telomerase expression or re-expression and activity can be detected in 90% of tumor samples [32]. Telomerase deficiencies and cancer appear to lie at opposite ends of a spectrum similar to p53: loss of p53 is observed in most tumors and is tumor promoting in mouse models, whereas mice with enhanced p53 responses exhibit increased cancer resistance, a shortened life span, and a number of early aging-associated phenotypes [34]. In both models aging appears to be driven in part by a gradual depletion of the functional capacity of stem cells. The link between p53 and telomeres is further illustrated in Li-Fraumeni syndrome (LFS), a cancer predisposition syndrome associated with germ line TP53 mutations. It was shown that the progressive earlier age of cancer onset (disease anticipation) in LFS is related to a measurable decrease in telomere length, with each generation providing the first rational biological marker for clinical monitoring of LFS patients [23]. Ectopic hTERT expression can allow post senescent cells to proliferate beyond crisis, in a process that could to be independent of catalytic activity [27]. Tumorigenesis is often associated with the upregulation of c-Myc that can be induced by retroviral insertion or translocation. c-Myc binding sequences are described within the hTERT promoter, and the MYC protein stimulates hTERT transcription [40], which may in turn contribute to tumorigenesis

or tumor progression. The flip side of continued expression or re-expression of hTERT in genetically stable primary cells and in animal models is enhanced longevity and a delay of senescence during in vitro culture [40]. However, sustained (over)expression of telomerase in CD4- or CD8-positive T cells over longer periods in culture was shown to promote genomic instability [39]. This may be directly due to hTERT overexpression or may be a consequence of extended proliferation and replication errors that may be exacerbated by culture conditions. In addition, gain of expression of hTERC due to the presence of multiple gene copies has also been recently associated with cervical dysplasia and invasive cancer progression [40].

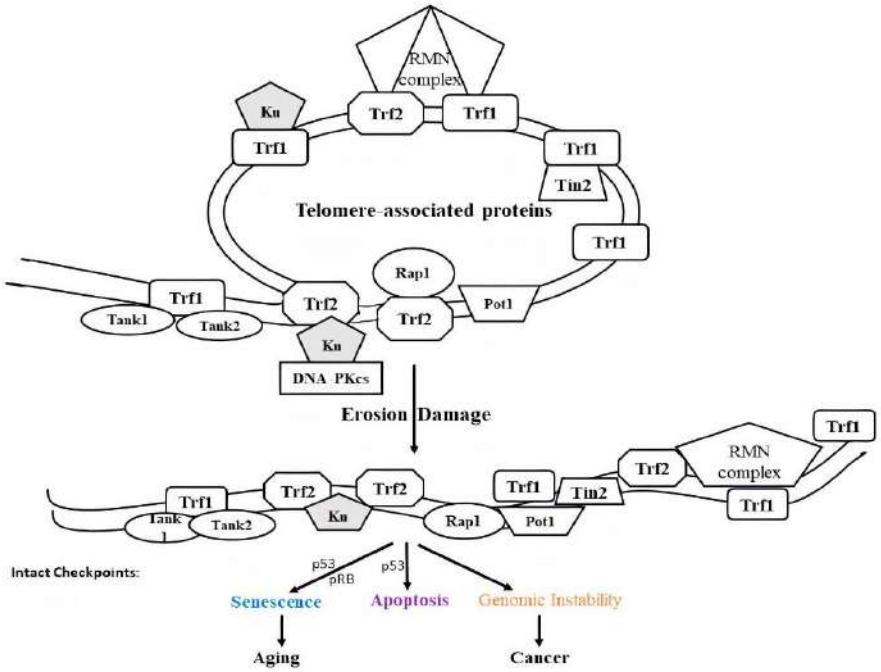


Fig4. Cellular consequences of telomere dysfunction. Telomere structure can be disrupted by direct damage, critical shortening or defects in telomere-associated proteins. In general, normal cells, with intact p53 and pRB checkpoints, respond to a dysfunctional telomere by undergoing cellular senescence, which may contribute to certain aging phenotypes. If only the p53 checkpoint is intact, telomere dysfunction generally promotes p53-mediated cell death. If neither the p53 nor pRB pathway is intact, cells may survive with genomic rearrangements and instability, which can lead to cancer.

Future prospects

With the recent cloning of human and mouse telomerase RNA, the identification of the proteins that associate with telomerase RNA, the construction of telomerase-deficient mice and the study of the normal cellular genes that regulate telomerase activity are all hot areas of current research. Identification of telomerase inhibitors and their use in clinical trials are clearly on the horizon. The pathways connecting telomere shortening, cellular senescence, telomerase expression and human cancer are presently under intense investigation and many surprises are likely to emerge. In addition to telomerase inhibition as a novel cancer therapy, manipulation of telomere length could, at least theoretically, change the rate of appearance of the aberrant pattern of gene expression associated with aging or other degenerative disease processes. For example, assuming that senescence is limiting in certain diseases then, for example, *ex vivo* elongation of telomeres ('winding up the clock') in normal T cells could have a major impact on immune dysfunction, such as in acquired immune deficiency syndrome (AIDS) when T cells are in 'decline'. Other potential examples include

wound healing, where an increase in cell proliferation might be beneficial, and cardiovascular disease, where plaque formation may be prevented if endothelial cells are encouraged to remain small and intact. Also, slowing the rate of telomere shortening in normal cells and tissues may profoundly affect the age of onset and severity of many age-associated illnesses. In conclusion, 'targeting telomeres or telomerase' fulfils many of the criteria for useful cancer therapy, and while there are still several important unanswered questions, the optimism surrounding the telomere hypothesis and its implications for the clinic appears justified.

CONCLUSIONS

Accumulated data support the notion that the loss of telomere repeats in (stem) cells and lymphocytes contributes to human aging. This notion is not widely accepted, primarily because the gradual loss of telomere repeats with age in cells of various tissues is not easily measured and because the average telomere length shows a lot of variation between species and between individuals of the same age. However, studies of model organisms as well as patients with telomerase mutations have shown that short telomeres result in dire consequences. It seems plausible that, with age, the proliferation of an increasing number of cells in normal individuals is compromised by progressive telomere loss. This is not necessarily a bad thing, as restrictions in the proliferation of somatic cells pose a barrier for the growth of aspiring tumor cells. Unfortunately, the telomere mechanism that limits the growth of premalignant cells also provides strong selection for cells that no longer respond to the DNA damage signals originating from short telomeres. Such cells are genetically unstable and have greatly increased ability to acquire genetic rearrangements that provide further growth advantages. The intricate involvement of telomeres in both aging and cancer ensures that pathways involving telomeres and telomerase will remain subject to intensive studies for many years to come.

REFERENCES

1. Alexander B, Coppola G, Perrault SD, Peura TT, Betts DH, King WA. Telomere length status of somatic cell sheep clones and their offspring. Mol Reprod Dev 74: 1525–1537, 2007.

2. Allsopp RC, Morin GB, DePinho R, Harley CB, Weissman IL. Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. Blood 102: 517–520, 2003.

3. Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acad Sci USA 89: 10114–10118, 1992.

4. Alter BP, Baerlocher GM, Savage SA, Chanock SJ, Weksler BB, Willner JP, Peters JA, Giri N, Lansdorp PM. Very short telomere length by flow FISH identifies patients with Dyskeratosis Congenita. Blood 110: 1439–1447, 2007.

5. Amiard S, Doudeau M, Pinte S, Poulet A, Lenain C, FaivreMoskalenko C, Angelov D, Hug N, Vindigni A, Bouvet P, Paoletti J, Gilson E, Giraud-Panis MJ. A topological mechanism for TRF2-enhanced strand invasion. Nat Struct Mol Biol 14: 147– 154, 2007.

6. Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie M, Vulto I, Phillips JA 3rd, Lansdorp PM, Greider CW, Loyd JE. Telomerase mutations in families with idiopathic pulmonary fibrosis. N Engl J Med 356: 1317– 1326, 2007.

7. Artandi SE, Alson S, Tietze MK, Sharpless NE, Ye S, Greenberg RA, Castrillon DH, Horner JW, Weiler SR, Carrasco RD, DePinho RA. Constitutive telomerase expression promotes mammary carcinomas in aging mice. Proc Natl Acad Sci USA 99: 8191– 8196, 2002.

8. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, DePinho RA. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 406: 641–645, 2000.

9. Artandi SE, DePinho RA. A critical role for telomeres in suppressing and facilitating carcinogenesis. Curr Opin Genet Dev 10: 39–46, 2000.

10. Autexier C, Lue NF. The structure and function of telomerase reverse transcriptase. Annu Rev Biochem 75: 493–517, 2006.

11. Baerlocher GM, Lansdorp PM. Telomere length measurements in leukocyte subsets by automated multicolor flow-FISH. Cytometry A 55: 1–6, 2003.

12. Baerlocher GM, Rice K, Vulto I, Lansdorp PM. Longitudinal data on telomere length in leukocytes from newborn baboons support a marked drop in stem cell turnover around 1 year of age. Aging Cell 6: 121–123, 2007.

13. Baerlocher GM, Vulto I, de Jong G, Lansdorp PM. Flow cytometry and FISH to measure the average length of telomeres (flow FISH). Nat Protocol 1: 2365–2376, 2006.

14. Baird DM, Davis T, Rowson J, Jones CJ, Kipling D. Normal telomere erosion rates at the single cell level in Werner syndrome fibroblast cells. Hum Mol Genet 13: 1515–1524, 2004.

15. Baird DM, Rowson J, Wynford-Thomas D, Kipling D. Extensive allelic variation and ultrashort telomeres in senescent human cells. Nat Genet 33: 203–207, 2003.

16. Baldwin JG Jr. Hematopoietic function in the elderly. Arch Intern Med 148: 2544–2546, 1988.

17. Baumann P, Cech TR. Pot1, the putative telomere end-binding protein in fission yeast and humans. Science 292: 1171–1175, 2001.

18. Bianchi A, Shore D. Early replication of short telomeres in budding yeast. Cell 128: 1051–1062, 2007.

19. Bianchi A, Smith S, Chong L, Elias P, de Lange T. TRF1 is a dimer and bends telomeric DNA. EMBO J 16: 1785–1794, 1997.

20. Bilaud T, Brun C, Ancelin K, Koering CE, Laroche T, Gilson E. Telomeric localization of TRF2, a novel human telobox protein. Nat Genet 17: 236–239, 1997.

21. Blackburn EH. Switching and signaling at the telomere. Cell 106: 661–673, 2001.

22. Blasco MA. Mouse models to study the role of telomeres in cancer, aging and DNA repair. Eur J Cancer 38: 2222–2228, 2002.

23. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 91: 25–34, 1997.

24. Blasco MA, Rizen M, Greider CW, Hanahan D. Differential regulation of telomerase activity and telomerase RNA during multistage tumorigenesis. Nat Genet 12: 200–204, 1996.

25. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. Extension of life-span by introduction of telomerase into normal human cells. Science 279: 349–352, 1998.

26. Britt-Compton B, Rowson J, Locke M, Mackenzie I, Kipling D, Baird DM. Structural stability and chromosome-specific telomere length is governed by cis-acting determinants in humans. Hum Mol Genet 15: 725–733, 2006.

27. Broccoli D, Smogorzewska A, Chong L, de Lange T. Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. Nat Genet 17: 231–235, 1997.

28. Brummendorf TH, Holyoake TL, Rufer N, Barnett MJ, Schulzer M, Eaves CJ, Eaves AC, Lansdorp PM. Prognostic implications of differences in telomere length between normal and malignant cells from patients with chronic myeloid leukemia measured by flow cytometry. Blood 95: 1883–1890, 2000.

29. Brummendorf TH, Maciejewski JP, Mak J, Young NS, Lansdorp PM. Telomere length in leukocyte subpopulations of patients with aplastic anemia. Blood 97: 895–900, 2001.

30. Brummendorf TH, Rufer N, Holyoake TL, Maciejewski J, Barnett MJ, Eaves CJ, Eaves AC, Young N, Lansdorp PM. Telomere length dynamics in normal individuals and in patients with hematopoietic stem cell-associated disorders. Ann NY Acad Sci 938: 293–303, 2001.

31. Calado RT, Graf SA, Wilkerson KL, Kajigaya S, Ancliff PJ, Dror Y, Chanock SJ, Lansdorp PM, Young NS. Mutations in the SBDS gene in acquired aplastic anemia. Blood 110: 1141–1146, 2007.

32. Calado RT, Pintao MC, Rocha V, Falcao RP, Bitencourt MA, Silva WA Jr, Gluckman E, Pasquini R, Zago MA. Lack of mutations in the human telomerase RNA component (hTERC) gene in Fanconi's anemia. Haematologica 89: 1012–1013, 2004.

33. Canela A, Martin-Caballero J, Flores JM, Blasco MA. Constitutive expression of tert in thymocytes leads to increased incidence and dissemination of T-cell lymphoma in Lck-Tert mice. Mol Cell Biol 24: 4275–4293, 2004.

34. Cao H, Hegele RA. LMNA is mutated in Hutchinson-Gilford progeria (MIM 176670) but not in Wiedemann-Rautenstrauch progeroid syndrome (MIM 264090). J Hum Genet 48: 271–274, 2003.

35. Cawthon RM.Telomere measurement by quantitative PCR. Nucleic Acids Res 30: e47, 2002.

36. Counter, C. et at, (1995) Telomerase activity in normal leukoeytes and in hematologic malignancies. Blood 85, 2315-2330

37. Hiyama, K. et al. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells, J. hnmnnoL (in press)

38. Piatyszek, M.A. et aL (1995) Detection of telomerase activity in human cells and tumors by a telomerie repeat amplification protocol (TRAP), Methods CellSci. 17, 1-15

39. Feng, J. et al. (1995) The RNA component of human telomerase, Science 269, 1236--1241

40. Blasco, M.A., Funk, W., Villeponteau, B. and Greider, C.W. (1995) Functional characterization and developmental regulation of mouse telomerase RNA, Science 269, 1267-1270

Novel Approaches To Combat Polymicrobial Infections With Pentadecanal And Pentadecanoic Acid Anti-Biofilm Coatings

Submitted by- **Pinaki Ghosh**

Reg. no.- Ao1-1112-116-005-2019

Exam roll no.- 2022151187

Ramakrishna Mission Vivekananda Centenary College

Department of Microbiology

Guided by- **Dr. Soumyadip Paul**

Introduction

Bacterial biofilms are formed by communities that are embedded in a self-produced matrix of extracellular polymeric substances (EPS). Bacterial biofilms can be considered

to be an emergent form of bacterial life, in which communal life is completely different from bacteria that live as free-living cells. Microorganisms with the potential to create biofilms can colonize a range of surfaces, including host tissues and medical devices, resulting in infections that are resistant to standard medicines. Emergent properties of bacterial biofilms include social cooperation, resource capture and enhanced survival following exposure to antimicrobials, and cannot be understood and predicted through the study of free-living bacterial cells. This is especially important in polymicrobial biofilms including both fungus and bacteria, therefore novel and effective anti-biofilm techniques are required to remove such severe diseases. The capacity of fatty acids to serve as anti-biofilm agents has prompted researchers to investigate their application against mixed biofilms of *Candida albicans* and *Klebsiella pneumoniae*[1]. This short report will demonstrate the ability of pentadecanal and pentadecanoic acid to prevent the formation of biofilm and in the process, highlight the value of an ecological perspective in the study of the emergent properties of biofilms, which enables an appreciation of the ecological success of biofilms as habitat formers and more generally, as a bacterial lifestyle.

Formation of Biofilms

Biofilms are three-dimensional colonies of microorganisms that adhere to a surface and are wrapped in an extracellular polymeric substance (EPS) that protects them. The creation of a biofilm occurs in five stages. Individual planktonic cells migrate and adhere to a surface in stage one. These adherent cells commence biofilm development on the surface and become enclosed in modest amounts of exopolymeric material if the right conditions are available. Cell aggregation and matrix formation occur in stage two, when adherent cells secrete an extracellular polymeric substance (EPS) and become irrevocably attached to the surface. The biofilm matures in stage three by forming microcolonies and water channel architecture, as well as becoming much more layered. The fully mature biofilm reaches its maximal cell density in stage four and is now referred to as a three-dimensional community.

Figure 1 : Scanning electron micrograph of a Staphylococcus aureus biofilm growing in an in vitro wound model.

The completed biofilm releases microcolonies of cells from the main community in stage five, allowing them to travel to other surfaces and disseminate the infection to new areas (Fig. 2)[2].

The extracellular matrix, also known as the EPS, is a complex mixture of proteins, lipids, nucleic acids (extracellular-DNA), and polysaccharides that surrounds the cells in a biofilm. These components not only help the biofilm adhere to the surface, but they also trap nutrients, offer structural support, and protect the biofilm against host immune responses and antimicrobial therapies[3]. In addition to the tasks listed above, the EPS

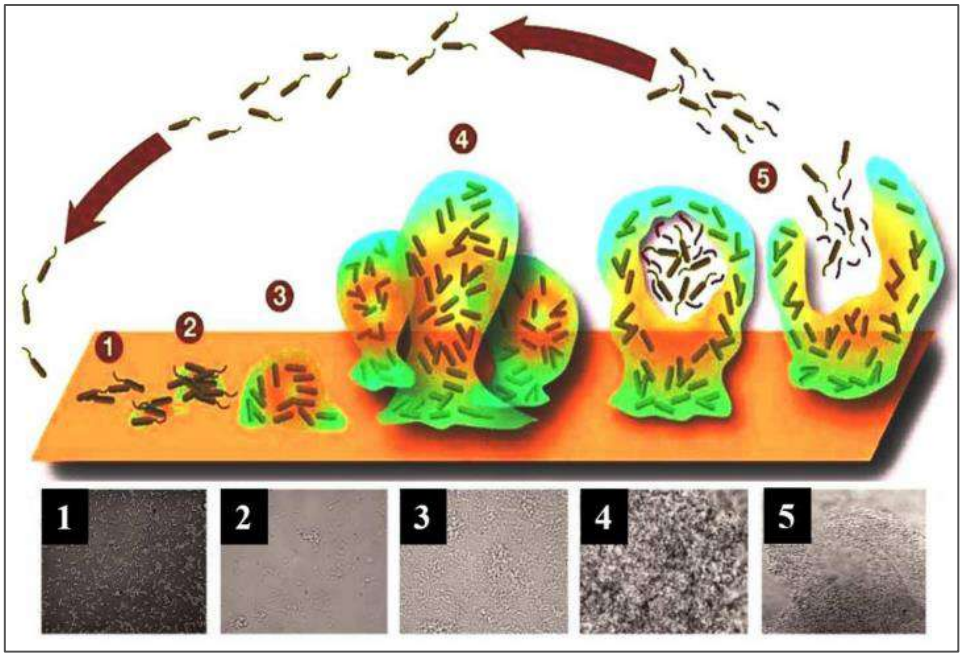


Figure 2: Different developmental stages of Pseudomonas aeruginosa biofilm

is also responsible for keeping the community of biofilm cells close together, allowing cell-to-cell communication (quorum sensing) and facilitating the interchange of genetic material via horizontal gene transfer[4].

Biofilm Inhibition Strategies

The material matrix of implanted medical devices and biomaterials provides an excellent environment for bacterial adherence, which promotes the production of mature biofilms[5]. As a result, measures that prevent bacteria from adhering to these materials are a clear preventative strategy. Surface modification is the most prevalent strategy for preventing bacterial adherence. The outside surface of the implanted medical device or biomaterial is altered, either directly or through the application of a coating, to create a bacteria-resistant barrier[6]. This method has demonstrated to be effective in reducing biofilm-related infections caused by orthopaedic implants. As a result, surface modification to inhibit biofilm formation is a broad field, with numerous extensive evaluations currently available[5], [7].

Another method for preventing biofilm formation is to utilize small molecule biofilm inhibitors. A biofilm inhibitor's anti-biofilm characteristics are frequently used to passivate the surface of an implanted medical device or biomaterial. Biofilm inhibitors

are one of the most researched areas in biofilm remediation, with a variety of different biofilm inhibitors now being characterized (e.g., phenols, imidazoles, furanone, indole, bromopyrrole, etc.). As a result, there are a plethora of extensive evaluations on biofilm inhibition drugs[8].

Biofilm Eradication Agents

Biofilm eradication agents are antibiotics that can be used alone to target and eliminate biofilm-residing cells. The design and development of BEAs is a new area of research in biofilm clean up[9]. Oritavancin, tris-QAC-10, glycerol monolaurate, docosahexaenoic acid, XF-70, and other promising BEAs have previously been created, and their activity, design, and possible useshave all been thoroughly investigated[10], [11]. Compared to the other organic molecules, lipids or fatty acids (i.e., aldehyde &ester derived) are the most influential BEA[12](Fig. 3).

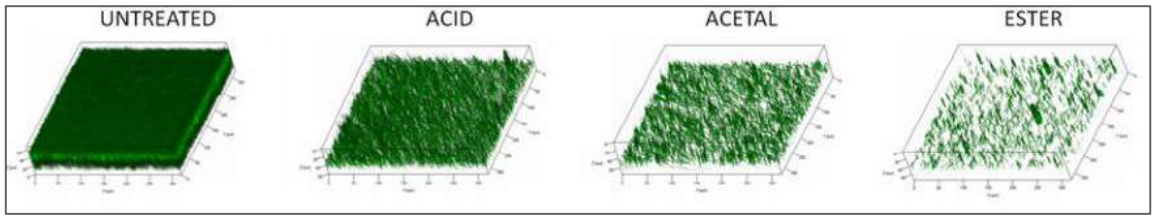


Figure 3: Analysis of the effect of different BEA on *Staphylococcus epidermidis*RP62Abiofilm structur

Targeting Biofilms with Fatty Acids

Several FAs have been reported to specifically inhibit or disrupt biofilm formation by diverse microbial pathogens, including *Staphylococcus aureus* [13]*, Pseudomonas aeruginosa, Candida albicans* [14]*, Serratia marcescens, Burkholderia cenocepacia,* and *Vibrio spp.*[15]. Only a few reviews have been published in the past, to our knowledge, concentrating mostly on the antibacterial properties of FAs and the probable mechanisms involved. The diversity of FAs, as well as their ability to prevent pathogenic colonization and virulence, is remarkable. They appear to work by reducing the expression of QS-regulated genes, particularly those involved in virulence (e.g., toxin manufacturing, fimbriae, hyphae, and so on), as well as non-QS targets (proteins involved in efflux pumps, oxidative stress, and ergosterol synthesis)[16]. FAs have been shown to inhibit swarming motility, adhesion, pathogenicity, and flagellar activity, as well as activate stress regulons that cause biofilm dispersion, making them vulnerable to antimicrobial action[17]. FAs operate as antibiofilm agents below the minimum inhibitory concentration (MIC, usually sub millimolar values), whereas at higher levels, they act on various cellular targets and have nonspecific broad-spectrum antimicrobial effects. The underlying processes of such unequal actions, however, remain a mystery.

Pentadecanal and pentadecanoic Acid as Anti-biofilm Agents

Pentadecanal is a long-chain fatty aldehyde that is pentadecane carrying an oxo substituent at position 1. It is a component of essential oils from plants like *Solanum erianthum* and *Cassia siamea*. It has a role as an antimicrobial agent, a volatile oil component and a plant metabolite. It is a long-chain fatty aldehyde and a 2,3-saturated fatty aldehyde (Fig. 4).

Pentadecanoic acid is a straight-chain saturated fatty acid containing fifteen-carbon atoms. It has a role as a plant metabolite, a food component, a *Daphnia magna* metabolite, a human blood serum metabolite and an algal metabolite. It is a long-chain fatty acid and a straight-chain saturated fatty acid (Fig. 4). It is a conjugate acid of a pentadecanoate.

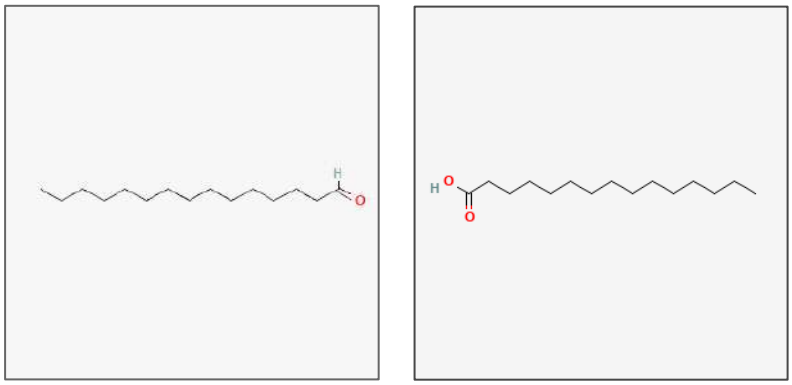


Figure 4: Structure of Pentadecanal & Pentadecanoic acid

Pentadecanal and pentadecanoic acid were shown to destabilize both single and dual-species biofilms by E. Galdiero et al [1]. The effect of pentadecanoic acid on dual-species biofilm was studied in depth; the anti-biofilm molecule displayed a preference for bacterial cells, and exposure to pentadecanoic acid significantly reduced the number of *Klebsiella pneumoniae*cells in the mixed biofilm. Quantification of cells in treated mixed biofilms indicated this preferential action effect, which was validated by CLSM (Confocal Laser Scanning Microscopy) analysis. In fact, the composition of the mixed biofilm developed in the presence of pentadecanoic acid differed from that of the dual-species biofilm formed in the absence of the molecule. When added to a mature biofilm or present throughout biofilm development, pentadecanoic acid had a significant impact on biofilm structure. The biofilm was characterized by lower biomass and an increased roughness coefficient, which is a direct indicator of biofilm heterogeneity, demonstrating that the treatment with pentadecanoic acid led to the formation of an inhomogeneous and unstructured biofilm [12].

The effect of pentadecanal and pentadecanoic acid on prefabricated single and mixed biofilms was documented in the eradication assay. Both molecules had the same eradication activity, however it was stronger in the case of *K. pneumoniae*single biofilms than in the case of *Candida albicans*single biofilms. The eradication achieved by using pentadecanoic acid resulted in a decrease in biofilm biomass and thickness.

Furthermore, cells exposed to pentadecanoic acid were alive (green indicates viable cells, red indicates dead or injured cells)(Fig. 5), indicating that the molecule showed no bactericidal effect against both *C. albicans*and *K. pneumoniae*in the mixed biofilm at the concentrations studied[18]. It's worth noting that both chemicals were able to eliminate up to 40-50 percent of the polymicrobial biofilm's total biomass (Fig. 6)[19].

Since pentadecanoic acid inhibits somewhat better than Pentadecanal (Fig. 7)and is chemically more stable (the pentadecanal is an aldehyde that could easily undergo oxidation reaction)[1].

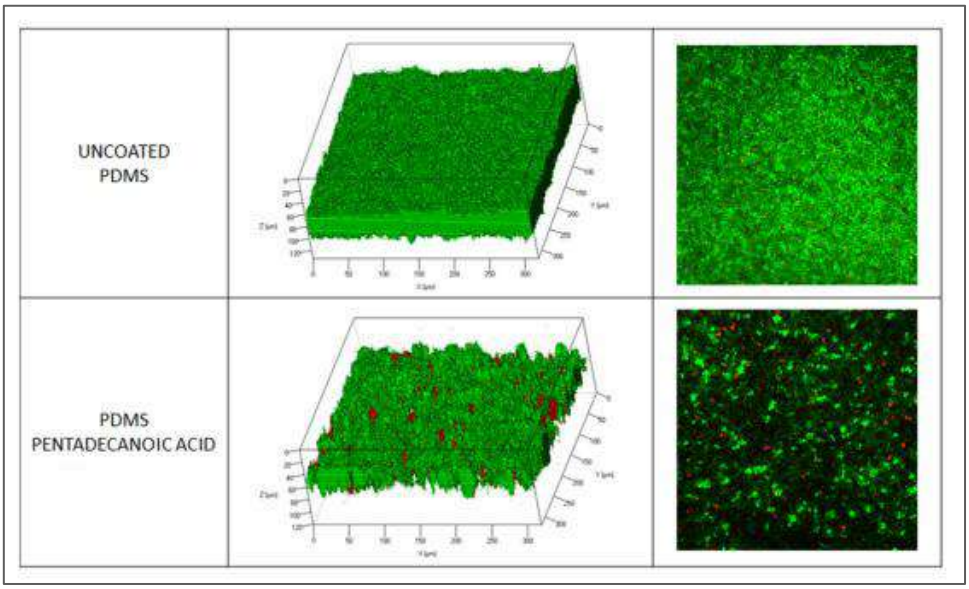


Figure 5: CLSM analysis of the biofilm-inhibiting effect of the pentadecanoic acid coating on *C. albicans/K. pneumoniae* mixed biofilm structures[1].

	Height
Uncoated PDMS	28.9 ± 18.1 nm
Pentadecanal coating	6.8 ± 0.3 μm
Pentadecanoic acid coating	6.4 ± 0.4 μm

Figure 6: The height data type of surface parameter with and without the desired coating clearly indicates that it can reduce more than 50% of biofilms' total biomass[19].

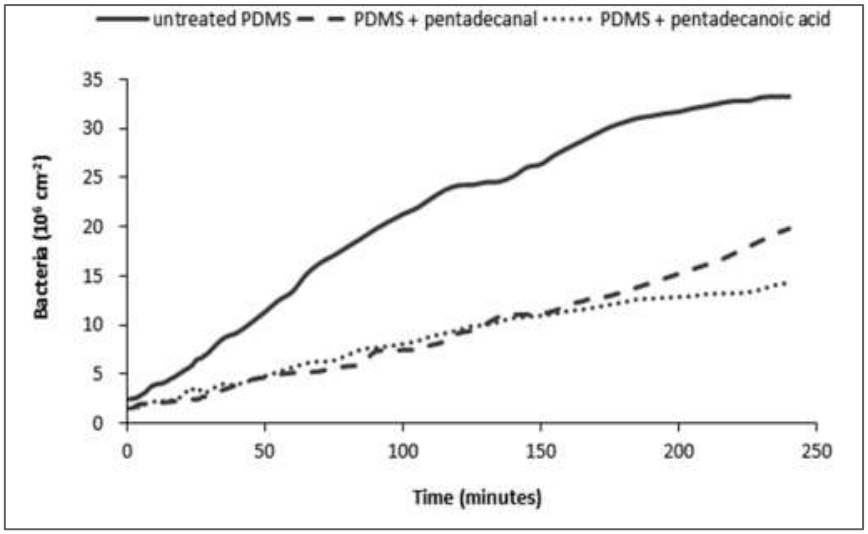


Figure 7: Growth kinetics of *S. epidermidis* RP62A on PDMS with and without the adsorbed anti-biofilm molecules[19].

Discussion & Future Perspectives

Biofilm resistance to antimicrobials is a complex phenomenon, not only correlated merely with the genetic resistance that arises from mutations, although the increased microbial cell density may help the transfer of resistance genes. Indeed, other mechanisms are involved, such as: (1) the low penetration of the antimicrobial agent due to the barrier function performed by the biofilm matrix; (2) the presence of persister cells exhibiting a high multidrug tolerance; and (3) a reduced susceptibility to antibiotics as a consequence of stress adaptive responses or changes in the chemical biofilm microenvironment. Accordingly, the strategies adopted to treat these challenging infections are rapidly changing . Although the precise molecular mechanism underlying aldehyde derivatives' anti-biofilm activity is still unknown, their impact on the *S. epidermidis* biofilm structure is clear[20], as the biofilm formed in their presence is significantly reduced and characterized by a porous structure with numerous channels and voids. These findings suggested that the aldehyde and its derivatives could be used in conjunction with antibiotics to treat biofilm infections[21].

Identification of FA specificity and their related molecular action will be useful in the control of pathogenic diseases in the near future. Because microbial interactions abound in the real world, it's critical to look into the impact of pentadecanal-derived lipids on polymicrobial (mixed culture) biofilms. Increasing our understanding of FA-based antibiofilm compounds could lead to the development of new tools in the fight against microbial diseases[22].

Conclusion

In conclusion, this study indicates the use of pentadecanal and its derivatives as valuable compounds for the development of novel biofilm prevention strategies[23]. However, these molecules may be effective in fighting existing infections when combined with an appropriate antibiotic therapy, suggesting that using pentadecanoic acid in combination with vancomycin may improve treatment efficacy for *S. epidermidis* biofilm-associated infections[12].

REFERENCES:

[1] E. Galdiero *et al.*, "Pentadecanoic acid against Candida albicans-Klebsiella pneumoniae biofilm: towards the development of an anti-biofilm coating to prevent polymicrobial infections," *Research in Microbiology*, vol. 172, no. 7–8, Nov. 2021, doi: 10.1016/j.resmic.2021.103880.

[2] A. D. Verderosa, M. Totsika, and K. E. Fairfull-Smith, "Bacterial Biofilm Eradication Agents: A Current Review," *Frontiers in Chemistry*, vol. 7. Frontiers Media S.A., Nov. 28, 2019. doi: 10.3389/fchem.2019.00824.

[3] P. Stoodley, K. Sauer, D. G. Davies, and J. W. Costerton, "Biofilms as complex differentiated communities," *Annual Review of Microbiology*, vol. 56. pp. 187–209, 2002. doi: 10.1146/annurev.micro.56.012302.160705.

[4] C. C. C. R. de Carvalho and M. M. R. da Fonseca, "Preventing biofilm formation: Promoting cell separation with terpenes," *FEMS Microbiology Ecology*, vol. 61, no. 3, pp. 406–413, Sep. 2007, doi: 10.1111/j.1574-6941.2007.00352.x.

[5] C. R. Arciola, D. Campoccia, P. Speziale, L. Montanaro, and J. W. Costerton, "Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials," *Biomaterials*, vol. 33, no. 26. pp. 5967–5982, Sep. 2012. doi: 10.1016/j.biomaterials.2012.05.031.

[6] K. Bazaka, M. v. Jacob, R. J. Crawford, and E. P. Ivanova, "Efficient surface modification of biomaterial to prevent biofilm formation and the attachment of microorganisms.," *Applied microbiology and biotechnology*, vol. 95, no. 2. pp. 299–311, 2012. doi: 10.1007/s00253-012-4144-7.

[7] D. Campoccia, L. Montanaro, and C. R. Arciola, "A review of the biomaterials technologies for infection-resistant surfaces," *Biomaterials*, vol. 34, no. 34. pp. 8533–8554, Nov. 2013. doi: 10.1016/j.biomaterials.2013.07.089.

[8] N. Rabin, Y. Zheng, C. Opoku-Temeng, Y. Du, E. Bonsu, and H. O. Sintim, "Agents that inhibit bacterial biofilm formation," *Future Medicinal Chemistry*, vol. 7, no. 5. Future Science, pp. 647–671, Apr. 01, 2015. doi: 10.4155/fmc.15.7.

[9] R. Roy, M. Tiwari, G. Donelli, and V. Tiwari, “Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action,” *Virulence*, vol. 9, no. 1. Taylor and Francis Inc., pp. 522–554, Jan. 01, 2018. doi: 10.1080/21505594.2017.1313372.

[10] H. Wolfmeier, D. Pletzer, S. C. Mansour, and R. E. W. Hancock, “New Perspectives in Biofilm Eradication,” *ACS Infectious Diseases*, vol. 4, no. 2. American Chemical Society, pp. 93–106, Feb. 09, 2018. doi: 10.1021/acsinfecdis.7b00170.

[11] J. L. del Pozo and R. Patel, “The challenge of treating biofilm-associated bacterial infections,” *Clinical Pharmacology and Therapeutics*, vol. 82, no. 2. pp. 204–209, Aug. 2007. doi: 10.1038/sj.clpt.6100247.

[12] A. Ricciardelli *et al.*, “Pentadecanal inspired molecules as new anti-biofilm agents against Staphylococcus epidermidis,” *Biofouling*, vol. 34, no. 10, pp. 1110–1120, Nov. 2018, doi: 10.1080/08927014.2018.1544246.

[13] Y. G. Kim, J. H. Lee, C. J. Raorane, S. T. Oh, J. G. Park, and J. Lee, “Herring oil and omega fatty acids inhibit Staphylococcus aureus biofilm formation and virulence,” *Frontiers in Microbiology*, vol. 9, no. JUN, Jun. 2018, doi: 10.3389/fmicb.2018.01241.

[14] K. G. Prasath, S. Sethupathy, and S. K. Pandian, “Proteomic analysis uncovers the modulation of ergosterol, sphingolipid and oxidative stress pathway by myristic acid impeding biofilm and virulence in Candida albicans,” *Journal of Proteomics*, vol. 208, Sep. 2019, doi: 10.1016/j.jprot.2019.103503.

[15] S. Ramanathan, D. Ravindran, K. Arunachalam, and V. R. Arumugam, “Inhibition of quorum sensing-dependent biofilm and virulence genes expression in environmental pathogen Serratia marcescens by petroselinic acid,” *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, vol. 111, no. 4, pp. 501–515, Apr. 2018, doi: 10.1007/s10482-017-0971-y.

[16] M. J. Nicol, “The kinetics of the dissolution of malachite in acid solutions,” *Hydrometallurgy*, vol. 177, pp. 214–217, May 2018, doi: 10.1016/j.hydromet.2018.03.017.

[17] D. Teper, Y. Zhang, and N. Wang, “TfmR, a novel TetR-family transcriptional regulator, modulates the virulence of Xanthomonas citri in response to fatty acids,” 2019.

[18] A. Heydorn *et al.*, “Quantification of biofilm structures by the novel computer program COMSTAT,” 2015. [Online]. Available: www.sgmjournals.org

[19] A. Ricciardelli, A. Casillo, M. M. Corsaro, M. L. Tutino, E. Parrilli, and H. C. van der Mei, “Pentadecanal and pentadecanoic acid coatings reduce biofilm formation of Staphylococcus epidermidis on PDMS,” *Pathogens and Disease*, vol. 78, no. 3, Mar. 2020, doi: 10.1093/femspd/ftaa012.

[20] J. P. O'Gara, "ica and beyond: Biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus," *FEMS Microbiology Letters*, vol. 270, no. 2. pp. 179–188, May 2007. doi: 10.1111/j.1574-6968.2007.00688.x.

[21] B. Parrino, D. Carbone, G. Cirrincione, P. Diana, and S. Cascioferro, "Inhibitors of antibiotic resistance mechanisms: clinical applications and future perspectives," *Future Medicinal Chemistry*, vol. 12, no. 5. Future Medicine Ltd., pp. 357–359, Mar. 01, 2020. doi: 10.4155/fmc-2019-0326.

[22] C. Kranjec *et al.*, "antibiotics Staphylococcal Biofilms: Challenges and Novel Therapeutic Perspectives," 2021, doi: 10.3390/antibiotics.

[23] P. Kumar, J. H. Lee, H. Beyenal, and J. Lee, "Fatty Acids as Antibiofilm and Antivirulence Agents," *Trends in Microbiology*, vol. 28, no. 9. Elsevier Ltd, pp. 753–768, Sep. 01, 2020. doi: 10.1016/j.tim.2020.03.014.

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE RAHARA

Project on: *Clostridium botulinum:* A foodborne pathogen.

Submitted by – Mourya Mondal

Department – Microbiology

Semester – V

Registration No – A01-1112-116-010-2019 Roll No – 2022151192

Guided by – Prof. Basudev Murmu sir.

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College Rahara

ACKNOWLEDGEMENT

I would like to acknowledge and give my warmest thanks to my supervisor Prof. Basudev Murmu who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this paper.

I would also like to thank our departmental coordinator in charge revered Br. Parachaitanya Maharaj for giving me the opportunity to do this project, which have helped to venture relevant meaningful topics.

Finally I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going and this paper would not have been possible without their cordial support.

INTRODUCTION

Foodborne pathogens (e.g. bacteria, virus and other parasites) are biological agents that can cause a foodborne illness event. A foodborne disease outbreak is defined as the occurrence of two or more cases of similar illness resulting from ingestion of the common food. Foodborne illness occurs when a pathogen is ingested with food and the pathogen multiplies itself inside the host or a toxigenic pathogen establish itself in a food product and establish itself and produces toxin, and then the toxin is ingested along with the food. Thus foodborne

illness is classified into (a) foodborne illness due to infection and (b) foodborne illness due to intoxication.

An example of foodborne disease is botulism caused by bacteria *Clostridium botulinum.* Foodborne botulism is a serious potentially fatal although rare disease. It is an intoxication caused by ingestion of a potent neurotoxin, the botulinum toxins produced by the bacteria in the contaminated food. The toxin causes flaccid paralysis. Person to person transmission of the disease does not occur.

BRIEF DESCRIPTION

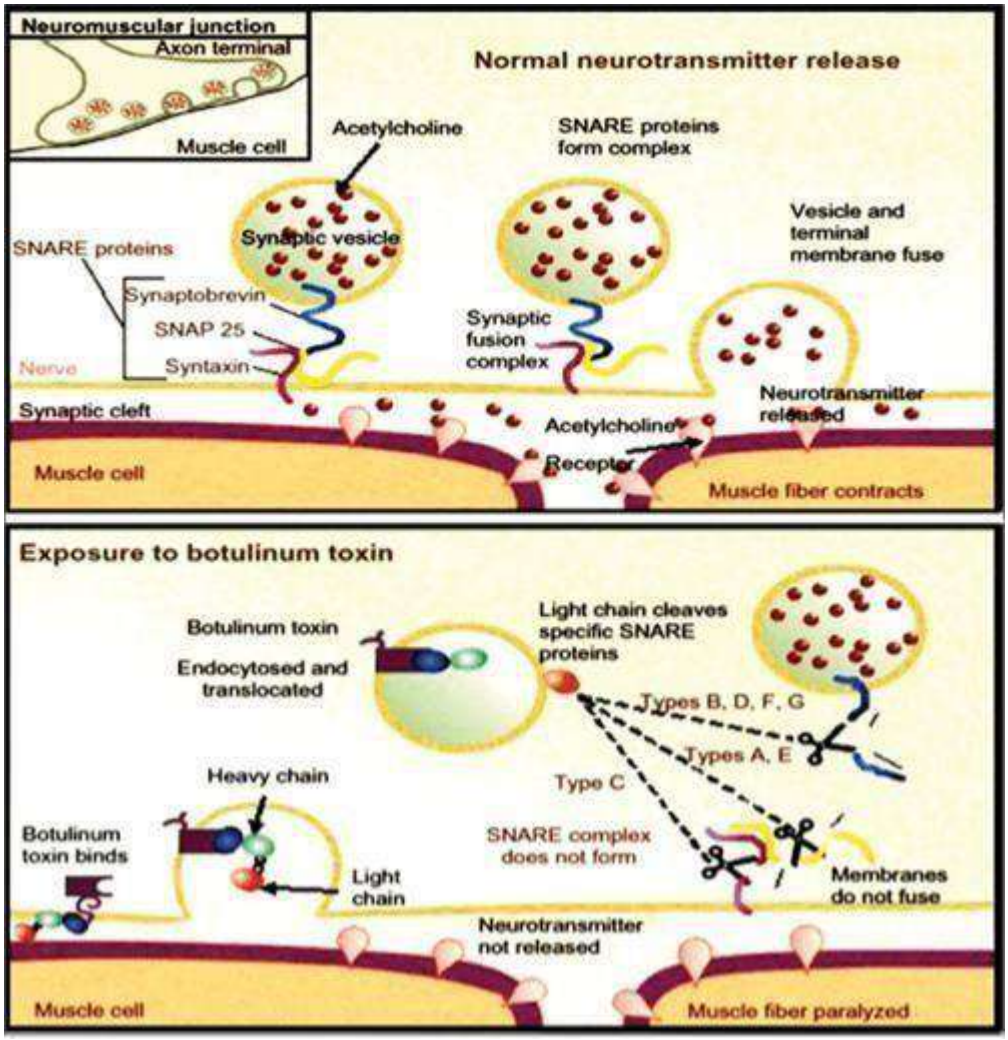
Clostridium botulinum is a gram positive, spore forming rod shaped bacterium. It is an anaerobic bacterium. Although the bacteria can tolerate slight amount of oxygen due to presence of the enzyme superoxide dismutase. *Clostridium botulinum* is able to produce a neurotoxin botulinum in anaerobic condition only during spore formation.

Botulinum toxin:

Botulinum toxin is a neurotoxin and it is one of the most lethal biological substance known. Till now eight type of toxins have been identified and these are allocated with letter A to H. Out of these toxins toxin A, B, E and H cause disease in humans. Toxin A, B and E are associated with foodborne disease. Toxin H has no cure, so it is the deadliest poison available. 1 nano gram per body weight of these enzyme is sufficient to kill an adult human.

The toxin released from the bacterium as a single chain. It is activated when it is cleaved. First, the toxin binds specifically to nerves who use the neurotransmitter acetylcholine. Once bound to the axon of the neuron the neuron takes up the toxin by receptor mediated endocytosis. As the vesicle moves the pH of the vesicle reduces and the toxin becomes activated. A portion of the toxin push the toxin out of the vesicle to cytoplasm. In cytoplasm the toxin cleaves the snare protein specific for acetylcholine. Thus it prevents binding of acetylcholine vesicle with muscle cells and hence cause paralysis.

Fig: Mechanism of action of botulinum toxin below.



PATHOLOGY:

There are several types of botulism.

- Foodborne botulism: Botulism associated with food.
- Infant botulism: Botulism occurs in infants, associated with honey.
- Inhalation botulism: Botulism occurs due to inhalation of botulinum toxin from air.

- Wound botulism: This occurs due to injection of drugs from syringes with contaminated spores.

Here, I will discuss about the botulism associated with food.

Foodborne botulism:

Clostridium botulinum is an anaerobic bacteria which grows in low acid environments. Foodborne botulism occurs due to growth of the bacteria in the food and production of toxin prior to consumption. Foods that are contaminated with botulinum toxin are mostly low acid foods, and improperly sterilized or preserved foods and canned foods. The botulinum toxin has been found in low acid vegetables like green beans, mushroom, beet, spinach. It is also found in meat, salted fish, smoked fish and canned food. Ready to eat foods with low oxygen foods are also susceptible to botulinum contamination. After ingestion of the toxin botulism occurs.

Symptoms:

The symptoms generally starts to appear 12 to 36 hours after consumption of the toxin. The symptoms are:

- Flaccid paralysis.

- Blurred vision.
- Dropping eyelids.
- Troubled breathing.
- Difficulty in swallowing.
- Muscle weakness.

Infant botulism:

Infant botulism occurs mostly in infants younger than six months. This type of botulism occurs due to ingestion of *Clostridium botulinum* spores. The stomach of a six month older or younger infant is not developed enough, the pH of stomach remains almost 4.5 rather than normal 1.5 in adults. This pH is not sufficient to stop the germination of spores. During spore germination the bacteria release the toxin and botulism occurs. This happens mostly due to consumption of honey which can be contaminated with spores.

Symptoms:

- Flaccid paralysis.
- Weak cry.
- Drooling.
- Dropping eyelids.

- Difficulty in feeding.

Diagnosis:

Diagnosis of botulism is mostly done by examining presence of botulinum toxin in blood or presence of the bacteria *Clostridium botulinum* in stool. The symptoms of the disease is somewhat similar to diseases like myasthenia gravis , meningitis and Gullian Barre syndrome. So often misdiagnosis occurs. For further clarification sometimes other measures are taken like brain scan, spinal fluid examination, and electromyogrphy.

After confirming the disease it is necessary to identify the type of infection. For this mice bioassay is done. Monoclonal antibody specific for different type of toxins are also used for identifying the type of toxin.

Treatment:

Botulism although rare is a fatal disease. The disease is mostly treated with antitoxins. The antitoxin neutralize the toxin but it does not cure the harm that is already done. Patients are needed to put in ventilator in case of respiratory problems. Patients with problem in swallowing are feed through feeding tube and saline water is given.

Delaying in treatment often results in death. People who have survived botulism may have shortness of breath and fatigue for years after recovery. Additional therapy may be needed.

Prevention:

Prevention of botulism is based good practice of food preparation particularly during sterilizing and canning. The spores of *Clostridium botulinum* is heat and radiation resistant, so it is difficult to sterilize them. However heating at 100°C for 15 minutes can destroy the spores. The spore can not germinate in high acid condition, high salt concentration and at low temperature. So it is good to preserve low acid foods at high salt concentration and low temperature.

Food from old or bulged cans should not be eaten as these type of cans can be contaminated with spores. The low oxygen condition gives an ideal environment for food germination. After germination the bacteria produces toxi and gas by fermentation. So the can becomes bulged. This types of cans should be avoided.

Infant botulism occurs from honey. So honey should not be given to infants younger than six months old.

Apart from these good practice of hygiene and spreading awareness is necessary to prevent botulism. According to W.H.O. the five key to safer food are:

- Keep clean
- Separate raw and cooked.
- Cook thoroughly.
- Keep food at safe temperature.
- Use safe water and raw materials.

APPLICATION

Although botulinum is a potential lethal toxin it has some uses in medication and cosmetics.

- Botulinum toxin is used for treatment of blepharospasm and strabismus.
- The toxin is used for treatment of other diseases like upper motor neuron syndrome, sialorrhea, cervical dystonia and chronic migraine.
- It is also used in cosmetic products to correct lining of eyebrows.

- Botox is a drug made from the toxin. Botox therapy is done for smoothening facial muscles and for removing wrinkles.

If the toxin is used at a safe dose in these treatments tge the side effects are rare and easily treatable.

FUTURE PERSPECTION

Research is going on for further use of botulinum toxin as a toxin. Trials are going on for treating patients with depression by using botulinum toxin.

Although the disease is rare, it is often fatal. So close monitoring during food processing is necessary to prevent any type of outbreak.

Treatment facilities for botulism should be improved.

Research is going on to develop a vaccine against botulinum toxin.

The severe lethality of the toxin has made itself a potential agent for bioweapon. It should be ensures that the toxin does not go to any wrong hand.

DISCUSSION

The mortality rate of botulism is high. So care should be given during food processing to stop any kind of

contamination. Adequate facilities should be present in hospitals to treat patients.

Failing to properly sterilize foods have led to outbreaks of botulism like in 2006 in Thailand botulism outbreak occurred due to inadequate sterilizing of soma canned bamboo shoot. In U.S.A. botulism outbreak occurred in 2007 due to wrong sterilization of a hot dog tomato sauce. In India infant botulism occurred in 2009.

So, to prevent further any outbreak spreading awareness is necessary as well as advanced ways of sterilizing techniques.

REFERENCE

1. Shukla, H.D.; Sharma, S.K. Clostridium botulinum: A bug with beauty and Weapon. Crit. Rev. Microbiol. 2005, 31, 11–18. [CrossRef]

2. Dutta, S.R.; Passi, D.; Singh, M.; Singh, P.; Sharma, S.; Sharma, A. Botulinum toxin the poison that heals: A brief review. Natl. J.

Maxillofac Surg. 2016, 7, 10–16.

3. Poulain, B.; Popoff, M.R. Why are botulinum neurotoxin-producing bacteria so diverse and botulinum neurotoxins so toxic?

Toxins 2019, 11, 34. [CrossRef] [PubMed]

4. Sobel, J. Botulism. Clin. Infect. Dis. 2005, 41, 1167–1173. [CrossRef] [PubMed]

5. Gu, S.; Rumpel, S.; Zhou, J.; Strotmeier, J.; Bigalke, H.; Perry, K.; Shoemaker, C.B.; Rummel, A.; Jin, R. Botulinum neurotoxin is shielded by NTNHA in an interlocked complex. Science 2012, 335, 977–981. [CrossRef] [PubMed]

6. Zhang, S.; Masuyer, G.; Zhang, J.; Shen, Y.; Lundin, D.; Henrik son, L.; Miyashita, S.; Martrnez-Carranza, M.; Dong, M.; Stenmark,

P. Identification and characterization of a novel botulinum neurotoxin. Nat. Commun. 2017, 8, 14130. [CrossRef]

7. Hatheway, C.L. Closridium botulinum: Ecology and Control in Foods; Hauschild, A.H.W., Dodds, K.L., Eds.; Marcel Dekker, Inc.:

New York, NY, USA, 1993; pp. 3–20.

8. Peck, M.W.; Smith, T.J.; Anniballi, F.; Austin, J.W.; Bano, L.; Bradshaw, M.; Cuervo, P.; Cheng, L.W.; Derman, Y.; Dorner, B.G.; et al.

Historical perspectives and guidelines for botulinum neurotoxin subtype nomenclature. Toxins 2017, 9, 38. [CrossRef]

9. Kukreja, R.V.; Singh, B.R. Comparative role of neurotoxin-associated proteins in the structural stability and endopeptidase activity of botulinum neurotoxin complex types A and E. Biochemistry 2007, 46, 14316–14324. [CrossRef]

10. Popoff, M.R. Ecology of neurotoxigenic strains of clostridia. Curr. Top. Microbiol. Immunol. 1995, 195, 1–29.

11. Hatheway, C.L. Toxigenic clostridia. Clin. Microbiol. Rev. 1990, 3, 66–98. [CrossRef]

12. Chellapandi, P.; Prisilla, A. Clostridium botulinum type A-virulome-gut interactions: A systems biology insight. Hum. Microbiome J.

2018, 7–8, 15–22. [CrossRef]

13. Cagan, E.; Peker, E.; Dogan, M.; Caksen, H. Infant botulism. Eur. J. Med. 2010, 42, 92–94. [CrossRef]

14. Xin, W.; Huang, Y.; Ji, B.; Li, P.; Wu, Y.; Liu, J.; Wang, X.; Yang, H.; Kang, L.; Gao, S.; et al. Identification and characterization of

Clorstridium botulinum strains associated with an infant botulism case in China. Anaerobe 2019, 55, 1–7. [CrossRef]

15. Grenda, T.; Kwiatek, K. Application of molecularbiology methods to the diagnosis of botulism in mallard ducks. Bull. Vet. Inst.

Pulawy 2009, 53, 365–368.

16. Grenda, T.; Kukier, E.; Goldsztejn, M.; Kozieł, N.; Kwiatek, K. Botulism: Current problem in veterinary medicine. Med. Weter.

2016, 72, 152–156.

17. Son, K.; Kim, Y.K.; Woo, C.; Wang, S.J.; Kim, Y.; Oem, J.K.; Jheong, W.; Jeong, J. Minimizing an outbreak of avian botulism

(Clostridium botulinum type C) in Incheon, South Korea. J. Vet. Med. Sci. 2018, 80, 553–556. [CrossRef] [PubMed]

18. Matovi´c, K.; Miši´c, D.; Karabasil, N.; Nedi´c, N.; Dmitri´c, M.; Jevti´c, G. Clostridium botulinum spores in European honey bees from

Serbia. J. Apic. Res. 2019, 58, 420–426. [CrossRef]

19. Grenda, T.; Grabczak, M.; Sieradzki, Z.; Kwiatek, K.; Pohorecka, K.; Skubida, M.; Bober, A. Clostridium botulinum spores in Polish honey samples. J. Vet. Sci. 2018, 19, 635–642. [CrossRef]

20. Mustafina, R.; Maikanov, B.; Wi´sniewski, J.; Tracz, M.; Anusz, K.; Grenda, T.; Kukier, E.; Goldsztejn, M.; Kwiatek, K. Contamination of honey produced in the Republic of Kazakhstan with Clostridium botulinum. Bull. Vet. Inst. Pulawy 2015, 59, 241–246. [CrossRef]

21. Maier, M.B.; Schweiger, T.; Lenz, C.A.; Vogel, R.F. Inactivation of non-proteolytic Clostridium botulinum type E in low-acid foods and phosphate buffer by heat and pressure. PLoS ONE 2018, 13, e0200102. [CrossRef] [PubMed]

22. Bhardwaj, T.; Haque, S.; Somvanshi, P. In silico identification of molecular mimics involved in the pathogenesis of Clostridium botulinum ATCC 3502 strain. Microb. Pathog. 2018, 121, 238–244.

[CrossRef]

23. Bhardwaj, T.; Somvanshi, P. A computational approach using mathematical modeling to assess the peptidoglycan biosynthesis of

Clostridium botulinum ATCC 3502 for potential drug targets. Gene Rep. 2018, 12, 179–186. [CrossRef]

24. Davies, J.R.; Rees, J.; Liu, S.M.; Acharya, K.R. High resolution crystal structures of Clostridium botulinum neurotoxin A3 and A4 binding domains. J. Struct. Biol. 2018, 202, 113–117. [CrossRef] [PubMed]

25. Eivazzadeh-Keihan, R.; Pashazadeh-Panahi, P.; Baradaran, B.; de la Guardia, M.; Hejazi, M.; Sohrabi, H.; Mokhtarzadeh, A.;

Maleki, A. Recent progress in optical and electrochemical biosensors for sensing of Clostridium botulinum neurotoxin. Trends Anal.

Chem. 2018, 103, 184–197. [CrossRef]

26. Walper, S.A.; Aragone´s, G.L.; Sapsford, K.E.; Brown, C.W., III; Rowland, C.E.; Breger, J.C.; Medintz, I.L. Detecting biothreat agents:

From current diagnostics to developing sensor technologies. ACS Sens. 2018, 3, 1894–2024. [CrossRef]

27. Wangroongsarb, P.; Kohda, T.; Jittaprasartsin, C.; Suthivarakom, K.; Kamthalang, T.; Umeda, K.; Sawanpanyalert, P.; Kozaki, S.;

Ikuta, K. Molecular characterization of Clostridium botulinum isolates from foodborne outbreaks in Thailand, 2010. PLoS ONE

2014, 9, e77792. [CrossRef] [PubMed]

DSE-II Project report on :

" Comparative study of aspectsbiopesticides and synthetic pesticides on the environmental and agricultural aspects"

By – Arijit Bag

Registration No : A01-1112-116-003-2019

Exam Roll No :2022151185

Depertment of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Gudided By - Prof.Basudev Murmu

Depertment of microbiology

Ramakrishna Mission Vivekananda Centenary College

Year – 2021-2022

Acknowledgement

I would like to acknowledge and give my warmest thanks to my supervisor Prof. Basudev Murmu who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator in charge; revered Brahmachari Parachaitanya Maharaj for allowing me to participate DSE project, which help me to venture relevant meaningful topics.

Finally, I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

Biopesticides is a good choice then synthetic pesticides in agriculture and environment

The loss of yields from agricultural production due to the presence of pests has been treated over the years with synthetic pesticides, but the use of these substances negatively affects the environment and presents health risks for consumers and animals. The development of agroecological systems using biopesticides represents a safe alternative that contributes to the reduction of agrochemical use and sustainable agriculture. Microalgae are able to biosynthesize a number of metabolites with potential biopesticidal action and can be considered potential biological agents for the control of harmful organisms to soils and plants. The present work aims to provide a critical perspective on the consequences of using synthetic pesticides, offering as an alternative the biopesticides obtained from microalgal biomass, which can be used together with the implementation of environmentally friendly agricultural systems.

Advantages of biopesticides

- Biopesticides are usually inherently less toxic than conventional pesticides.
- Biopesticides generally affect only the target pest and closely related organisms, in contrast to broad spectrum, conventional pesticides that may affect organisms as different as birds, insects and mammals.
- Biopesticides often are effective in very small quantities and often decompose quickly, resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides.
- When used as a component of Integrated Pest Management (IPM) programs, biopesticides can greatly reduce the use of conventional pesticides, while crop yields remain high.

Effects of synthetic pesticides and biopesticides on food, public health and the environment

In countries of the European Union, South and North America for compound to be considered biopesticide and be released for use in organic crops must undergo a series of toxicological tests. Most of the pesticides approved for organic agriculture are of comparatively low toxicological concern for consumers because they are not associated with any identified toxicity, this because the raw materials used for the production of biopesticides are part of the human diet (for example, iron and potassium bicarbonate, peppermint oil, among others).In addition, biopesticides are typically designed to affect only the target pest or groups of specific organisms and they are different from the synthetic pesticides that do not exhibit specificity in their performance. Therefore, synthetic pesticides besides present toxicity to the pests and pathogens contaminants of plant crops can also affect negatively humans, animals and the environment.The toxicity of synthetic

pesticides, biochemically, is given by inhibition of enzymes, modification in the signaling system, disturbance in electrolytic equilibrium, osmotic or pH, degradation of lipophilic membranes and pH gradients across membranes, in addition to the generation of free radicals and other substances that can destroy tissues, DNA and proteins of organisms. Due to the mechanisms of action of synthetic pesticides and the increased exposure of humans to these substances, in recent years the use of these products has been linked to the increase of some diseases, including Parkinson's disease, type 2 diabetes, certain types of cancers, endocrine disruption, neurotoxicity and even obesity .The most of the currently used pesticides are rapidly excreted, but there are still some pesticides used that accumulate in the human body at every meal consumed, besides this there are proven cases of workers exposed directly to the spray of pesticides who are intoxicated by these substances. The environment has also been affected by the use of synthetic pesticides, since less than 5% of the products applied in the plantations reach the target organisms, which put at risk the sustainability of the ecosystems. Pesticide residues can leach the subsoil and contaminate groundwater, as well as accumulate toxic levels in the soil by destroying natural vegetation and reducing populations of non-target organisms such as bees, fish, wildlife and livestock. It is also important to emphasize that the continuous use of 2 J. A. V. COSTA ET AL. synthetic pesticides makes them more resistant pests, necessitating the use of higher concentrations of product and consequently greater toxicity, resurgence and outbreak of new pests. Although biopesticides have considerable advantages under synthetic pesticides, since they pose less of a threat to the environment and human health, it is important to be careful about their use. There are some products that are approved for use as biopesticide and yet pose some health risk to the population. As for example, the use of copper, which is an essential nutrient in the diet of mammals and plants, but in high concentrations of consumption presents toxicity, besides being toxic for fish. In addition, plant species, algae and microalgae such as Chrysanthemum spp coronopifolia Gracilaria , Microcystis aeruginosa, among others, are toxic and may ., present metabolites with toxicity. Although the current organic certification and control systems provide the guarantee of a low level of pesticide contamination,organic foods treated with biopesticides alone do not have complete food safety. This is due to the contamination of organic crops treated with biopesticides by synthetic pesticides by spraying or volatilizing synthetic pesticides from areas adjacent to organic crops, their fraudulent use and the contamination of biopesticides during transportation and storage, among other forms.

Biopesticides vs. synthetic pesticides

In recent years, restrictions on the use of many synthetic pesticides such as organochlorines, organophosphates, carbamates and organophthaloids have led to increased demand for biopesticides. However, the use of synthetic pesticides still has advantages over biopesticides in relation to the low cost of production, higher yields and availability of fruits, vegetables and grains.These advantages attributed to the use of synthetic pesticides make their use still predominate in agriculture, while biopesticides are used almost exclusively in organic agriculture. However, because biopesticides are less harmful to the health of the population and the environment than synthetic pesticides, their use has been encouraged to reduce the use of agrochemicals and their side effects. Also, it is important to note that

biopesticides can replace the use of conventional pesticides and the crop yields remain high. The increased use of biopesticide is designed to overcome the use of chemical pesticides with a compound annual growth rate of more than 15%. For this, strategies are being studied, such as the proposal by Grovermann et al. (2017), which suggest that an incentive to the use of biopesticides be made through subsidies that reduce their costs. In addition to the use of taxes on synthetic pesticides according to the degree of toxicity of the same, so that the more toxic the product, the higher the taxes on it. However, the advantages related to the use of biopesticides can, at the same time, limit their market potential. An example of this is the high specificity of biopesticides, which prevents contamination of non-target organisms, but may also limit their action, such as biopesticides based on the fungus Coniothyrium minitans that targets only a single genus of pathogen (Sclerotinia spp.), and those based on the bacterium Serratia entomophila, which control only a single insect pest. The biodegradability characteristic of biopesticides reduces environmental problems, but results in the difficulty of maintaining product activity. In general, the persistence of the biopesticides activity is 14 days, while the ideal would be 21 days, which still few products can reach. Another difficulty is the production price of a biopesticide, since the cost of fermentation of the microorganisms is often greater than the cost of producing a synthetic chemical, so to be competitive in the market, microbial biopesticides must have high power against the pest or high productivity. Also, one should consider that biopesticides are usually living organisms or metabolites of these, due to this, one must be careful about the particularities of each product. The same metabolite extracted from different organisms may have different toxicity. However, this problem can be solved by demonstrating to the most effective method of using the product. Despite the difficulties encountered, the use of biopesticides should be prioritized by the fact that synthetic pesticides generate residues that cause diverse environmental consequences,as well as causing diverse health consequences for farmers who handle them.Thus, there is a need for research to optimize the production of biopesticides, increasing their use so that the use of synthetic pesticides can be totally replaced, making vegetable cultivations more sustainable, as the biopesticides capacity to maintain the crop yield and, at the same time, their efficiency in low concentrations and rapid decomposition, which makes them less harmful to the environment and increase profitability in production. Most chemical pesticides have a unique mode of action, in which pests develop resistance to the chemical components present in the pesticide quickly. Considering the great investment in developing a chemical pesticide, companies are concerned with developing management strategies to delay pesticide resistance. This includes mixtures of chemicals in agitation tanks with some type of biopesticide. Most biopesticides have complex modes of action, living microorganisms that infect and kill a pest, or microorganisms and plant extracts that contain mixtures of multiple classes of chemical compounds, providing greater durability. This complex mode of action may delay the development of resistance to chemical pesticides when used in conjunction with these, collaborating to reduce synthetic pesticides.Biopesticides developed in recent years are more effective, easier to use, more economical and have a longer shelf life than biopesticides from decades ago. Farmers are increasingly concerned about incorporating naturally occurring pest control products to contribute to the type of sustainable agriculture. Farmers and food companies are increasingly emphasizing consumer demand

for low or no-waste products, ensuring food security and preserving natural JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH, PART B 3 resources, which may be greater with the use of biopesticides.

Feature aspects

Biopesticides are attracting global attention as safer strategy to manage pest populations such as weeds, plant pathogens and insects while posing less risk to human being and the environment. If the global market increases further in the future, the biopesticides can play a vital role in substituting chemical pesticides reducing the current over-reliance on these ecologically harmful chemicals.

Conclusion

The desire of the public to avoid the use of synthetic compounds in agriculture in favor of environmentally friendly and sustainable practices has generated a demand for new agrochemicals to replace synthetic pesticides. As microalgae are potential producers of compounds with antifungal, antibiotic and toxic actions, the development of biopesticides based on these microorganisms is one of the major challenges for researchers because the microalgal metabolic pathways for producing these metabolites, as well as their mechanisms of action, remain poorly explored. The use of secondary metabolites produced by microalgae, or even of microalgal biomass, as a biopesticide should be further explored as a promising possibility for the replacement of chemical pesticides

References

[1] Gomiero, T. Agriculture and degrowth: state of the art and assessment of organic and biotech-based agriculture from a degrowth perspective. J. Clean. Prod. 2018, 197, 1823–1839. DOI: 10.1016/j.jclepro.2017.03.237.

[2] Mostafalou, S.; Abdollahi, M. Pesticides: an update of human exposure and toxicity. Arch. Toxicol. 2017, 91, 549–599. DOI: 10.1007/s00204-016-1849-x.

[3] Albuquerque, N. C. P.; Carr˜ao, D. B.; Habenschus, M. D.; Oliveira, A. R. M. Metabolism studies of chiral pesticides: a critical review. J. Pharm. Biomed. Anal. 2018, 147, 89–109. DOI: 10.1016/j.jpba.2017.08.011.

[4] Gomiero, T. Food quality assessment in organic vs. conventional agricultural produce: findings and issues. Appl. Soil Ecol. 2018, 123, 714–728. DOI: 10.1016/j.apsoil.2017.10.014.

[5] Marutescu, L.; Popa, M.; Saviuc, C.; Lazar, V.; Chifiriuc, M. C. Botanical pesticides with virucidal, bactericidal, and fungicidal activity. In New Pesticides and Soil Sensors, 1st ed.; Grumezescu, A., Ed.; Academic Press: London, 2017; pp 311–335.

[6] Richmond, A. Handbook of Microalgal Culture. Blackwell Publishing Ltd: Oxford, UK, 2003; p 239.

[7] Tassara, C.; Zaccaro, M. C.; Storni, M. M.; Palma, M.; Zulpa, G. Biological control of lettuce white mold with cyanobacteria. Int. J. Agric. Biol 2008, 10, 487–492.

[8] Wuang, S. C.; Khin, M. C.; Chua, P. Q. D.; Luo, Y. D. Use of Spirulina biomass produced from treatment of aquaculture wastewater as agricultural fertilizers. Algal Res. 2016, 15, 59–64. DOI: 10.1016/j.algal.2016.02.009.

[9] Castro, J. S.; Calijuri, M. L.; Assemany, P. P.; Cecon, P. R.; Assis, I. R.; Ribeiro, V. J. Microalgae biofilm in soil: greenhouse gas emissions, ammonia volatilization and plant growth. Sci. Total Environ. 2017, 574, 1640–1648. DOI: 10.1016/ j.scitotenv.2016.08.205.

[10] Abdel-Raouf, N. Agricultural Importance of Algae. Afr. J. Biotechnol. 2012, 11, 11648–11658.

[11] Manjunath, M.; Kanchan, A.; Ranjan, K.; Venkatachalam, S.; Prasanna, R.; Ramakrishnan, B.; Hossain, F.; Nain, L.; Shivay, Y. S.; Rai, A. B.; Singh, B. Beneficial cyanobacteria and eubacteria synergistically enhance bioavailability of soil nutrients and yield of okra. Heliyon 2016, 2, e00066. DOI: 10.1016/ j.heliyon.2016.e00066.

[12] Quadros, P. D.; Zhalnina, K.; Davis-Richardson, A. G.; Drew, J. C.; Menezes, F. B.; Camargo, F. A. O.; Triplett, E. W. Coal mining practices reduce the microbial biomass, richness and diversity of soil. Appl. Soil Ecol. 2016, 98, 195–203. DOI: 10.1016/j.apsoil.2015.10.016.

[13] Kheirfam, H.; Sadeghi, S. H.; Homaee, M.; Zarei, D. B. Quality improvement of an erosion-prone soil through microbial enrichment. Soil Tillage Res. 2017, 165, 230–238. DOI: 10.1016/j.still.2016.08.021.

[14] Bidyarani, N.; Prasanna, R.; Babu, S.; Hossain, F.; Saxena, A. K. Enhancement of plant growth and yields in chickpea (Cicer Arietinum L.) through novel cyanobacterial and biofilmed inoculants. Microbiol. Res. 2016, 188–189, 97–105. DOI:

10.1016/j.micres.2016.04.005. [15] Rossi, F.; Olguın, E. J.; Diels, L.; P

Project report on :

An overview of Ramachandran plot

Submitted by : **Himadri Mukherjee**

Reg.no. : **A01-1112-116-016-2019**

Roll no. : **617** semester : **v**

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Guided by – **Dr. Subrata Kundu**

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Introduction:- Ramachandran plot also known as a Ramachandran diagram or [Φ , Ψ] plot was originally developed in 1963 by G. N. Ramachandran, C. Ramakrishnan and V. Sasisekharan. Ramachandran plot provides a simple two-dimensional graphic representation of all possible protein structures in terms of torsion angles. The use of torsion angles to describe polypeptide and protein conformation was developed by Sasisekharan as part of his studies of the structure of collagen chains during his work as a graduate student in the research group of G.N. Ramachandran (1). Although the plot was developed using theoretical methods, mathematical calculations and models building, once the protein structure began to discover, the importance of plot was realized. The power of this approach was readily apparent and its use quickly became widespread. Even after 55 years of its discovery, as more and more protein structures are being discovered, it remains an important tool to confirm the accuracy of the structures. in order to know more about Ramachandran plot, let us first understand the basic nature of peptide bond and torsion angles.

Planer nature of peptide bond

proteins are most commonly, linear and unbranched polymers composed of amino acids linked together by peptide bonds. Peptide bonds are amide linkages formed between α-amino group of one amino acid and the α-carboxyl group of adjacent

amino acid. This reaction is a condensation reaction, in which a water molecule is released and the linked amino acids are referred to as amino acid residues.

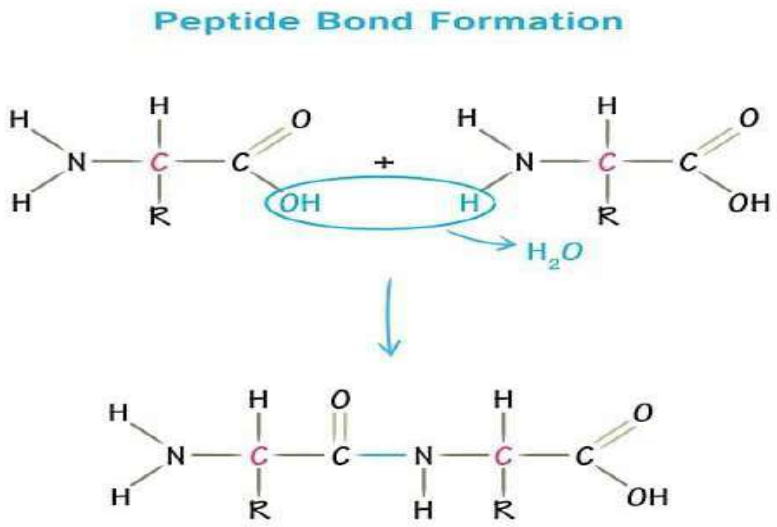


Figure 1:- The formation of a peptide bond (also called an amide bond) between the α-carboxyl group of one amino acid and the α-amino group of another amino acid is accompanied by the loss of a water molecule.

The peptide C—N bond has a partial double bond character. Consequently, the peptide bond length is only 1.33 Å, which is between the values expected for a C—N single bond (1.49 Å) and a C = N double bond (1.27 Å). The peptide bond appears to have approximately 40 percent double-bonded character due to resonance. The oxygen has a partial negative charge and the nitrogen has a partial positive charge, setting up a small electric dipole. The partial double bond character keeps the peptide

bond in a rigid planar configuration. Hence, for a pair of amino acids linked by a peptide bond, six atoms (Cα, C, O, N, H and Cα) lie in the same plane. (2)

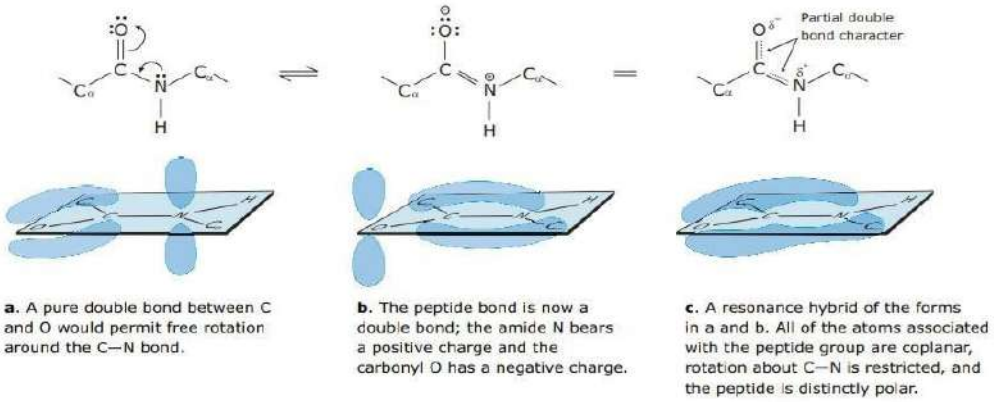


Figure 2:- Peptide-bond resonance structures. Each peptide bond has some double-bond character due to resonance. It can be written as a resonance hybrid of two structures. The peptide bond is essentially planar. For a pair of amino acids linked by a peptide bond, the six atoms of the peptide group lie in a single plane: the -carbon atom and CO group of the first amino acid and the NH group and -carbon atom of the second amino acid.

Torsion angle and peptide bond

A torsion angle, also known as a dihedral angle, are defined by four points in space. This is represented in the figure below by the structure A-B-C-D (second panel; note the distinction between bond angle and torsion angle). Imagine looking along the bond between atoms B and C (as indicated by the arrow). This is the central bond of the three bonds defined. At first, we

see the B-C bond in the plane of the figure, with the B-A bond pointing to the left and up out of the plane, and the C-D bond pointing down and to the right. Then we pick up this structure, and begin to turn it so that we can look straight down the B-C bond (third panel).

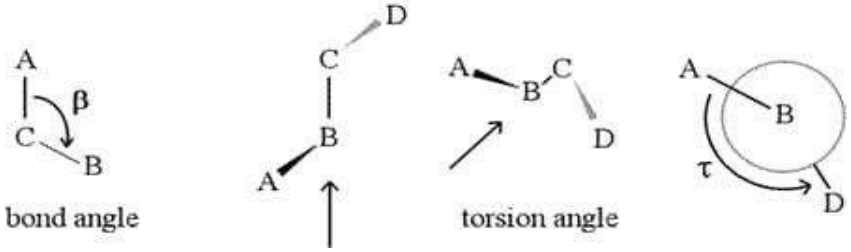


Figure 3:- A torsion angle is the angle at the intersection of two planes. A, B, C and D illustrate the position of the four atoms used to defi ne the torsion angle. The rotation takes place around the central B—C bond.

Eventually, our view is equivalent to the Newman projection in the last panel. From this view, the dihedral angle τ is the apparent angle subtended by D relative to A, as shown. We define the range of values of dihedral angles to be [−180°, +180°], and the value of τ is about -150° (The negative part of the range corresponds to when D is counterclockwise relative to A.) (3)

Of course, other atoms may be attached to B and C - and usually are. We would have to specify which atoms correspond to A, B, C, and D to unambiguously define dihedral angle. For long unbranched polymers, like proteins, it makes sense to require that A through D are all main chain atoms. Below is a molecular graphics image that corresponds to the above sketch and

illustrates the definition of the polypeptide main chain dihedral angle Phi (ϕ).

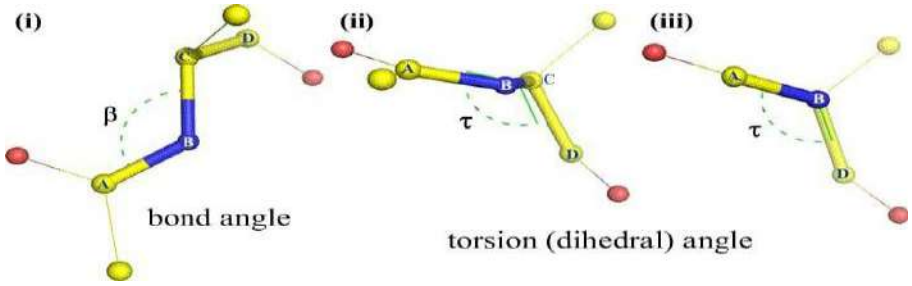


Figure 4 :- Molecular graphics image 0f bond angle and torsion angle

The bonds between the nitrogen of amino group and the α-carbon atom (i.e. N—Cα bond) and between the α-carbon atom and the carbon of carbonyl group (i.e. Cα—C bond) are pure single bonds. The two adjacent rigid peptide units can rotate about these bonds, acquiring various orientations. The rotations about these bonds can be specified by torsion angles Φ (phi) and Ψ (psi). The torsion angle about the bond between the amino nitrogen and the α-carbon atoms is called Φ whereas, the torsion angle about the bond between the α-carbon and the carbonyl carbon atoms is called Ψ (Figure 5). (4)

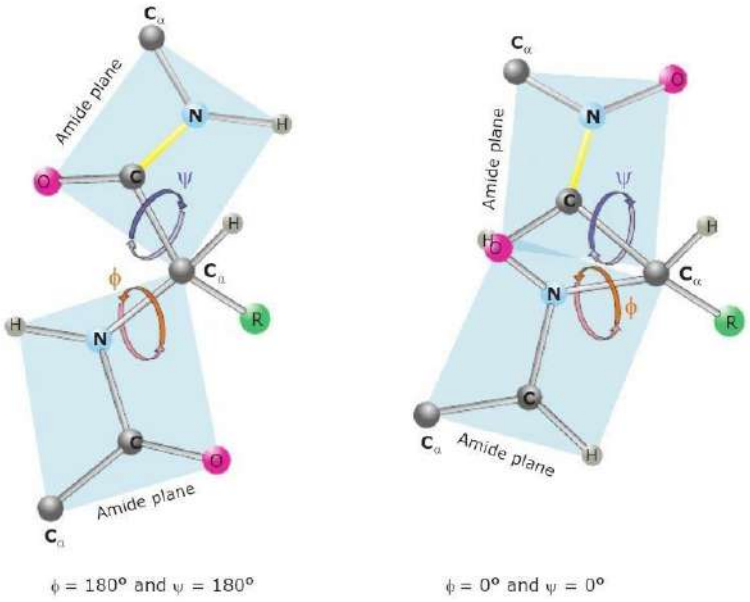


Figure 5 :- The bonds between the amino nitrogen and the α-carbon atom (i.e. N—Cα bond) and between the α-carbon atom and the carbonyl carbon (i.e. Cα—C bond) are pure single bonds. Hence, rotation can occur about these bonds. The rotations about these bonds can be specified by torsion angles Φ (phi) and Ψ (psi). The torsion angle about the N—Cα bond is called Φ and that about the Cα— C bond is Ψ . The rotations about torsion angles Φ and Ψ angles can vary over range of 0 to 360°.

Understanding the Ramachandran plot

Theoretically, Φ and Ψ can have any value between +180° and –180°, (i.e. 360° of rotation for each). However, not all combinations are possible in reality due to the physical clashes of atoms in 3-dimensional space. Atoms take up space and two atoms cannot occupy the same space at the same time. These physical clashes are called steric interference. Most values of Φ and Ψ are therefore not allowed due to steric interference between non-bonded atoms. The permitted values for Φ and Ψ

were first determined by G. N. Ramachandran. These permitted values can be visualized on a two-dimensional plot called a Ramachandran plot plotted between Φ and Ψ on x-axis and y-axis, respectively. (5) Polypeptide conformations are defined by the values of Φ and Ψ . Most values of Φ and Ψ are not allowed due to steric interference between non-bonded atoms. Hence, most areas of the Ramachandran plot (i.e., most combinations of Φ and Ψ) Represent statically disallowed conformations of a polypeptide chain because of steric collisions between side chains and main chain. In the figure 6, the white areas correspond to statically disallowed conformations in which any non-bonding interatomic distance is less than its corresponding van der Waals radii. These regions are statically disallowed for all amino acids except glycine which is unique as it lacks a side chain. The dark blue regions called allowed regions correspond to conformations where there are no steric interferences.

Most of Φ and Ψ values of a polypeptide chain fall within these allowed regions of the Ramachandran plot. There are, however, some notable exceptions. Glycine, the amino acid with a smallest side chain, is much less statically restricted than the other amino acid residues. Hence, its allowed range of Φ and Ψ covers a larger area of the Ramachandran plot. At glycine residues, polypeptide chains often assume conformations that are statically disallowed to other residues. The cyclic side chain of proline limits its range of Φ values to angles of around –60°,

making it most conformation ally restricted amino acid residue. Light blue regions correspond to conformation having outer limit van der Waals distances i.e., the atoms are allowed to come a little closer together. Since the D- and L-form of the amino acids have their side chain oriented differently with respect to the CO group, hence, they have different allowed Φ and Ψ angles. If polypeptides built from D-amino acids, they have different Φ and Ψ angles than those that are exclusively made up of L-amino acids. For example, a β-sheet made up of L-amino acids occupies upper left quadrant whereas, β-sheet made up of same amino acids of D-enantiomeric form would occupy lower right quadrant. (6)

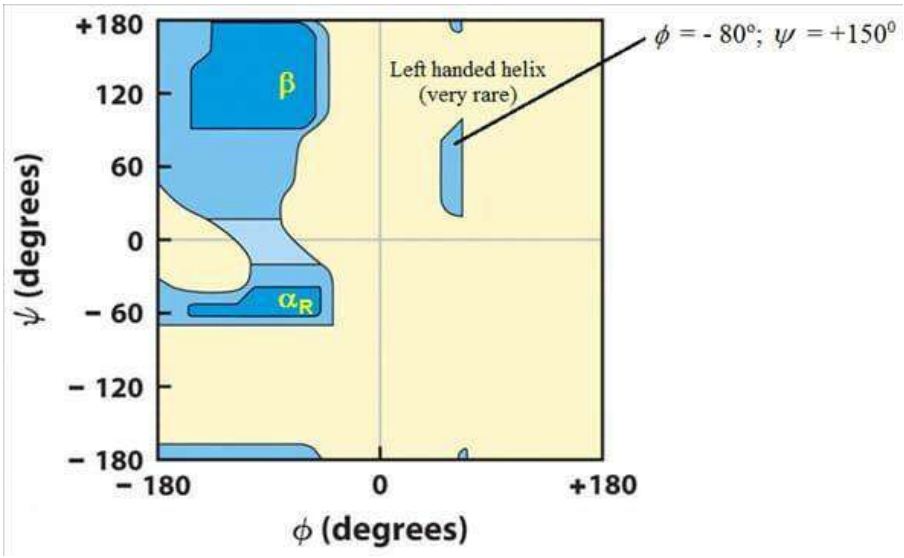


Figure 6: - 1, Dark blue: -Bond angle is easily allowed. This region bond value is max.

2, Intermediate: -List allow values are there.

3, Light blue: -Vary difficult to have the bond angle list favorable condition

Application of the Ramachandran plot: -

Ramachandran plot is mainly use to identify-

1) Predicting secondary structure from Ramachandran plot.

Every amino acid residue in a polypeptide can have specific set of Φ and Ψ angles, therefore, each residue can be represented as a point on Ramachandran plot with corresponding Φ and Ψ angles as x and y coordinates, respectively.

Polypeptides, when adopting secondary structures, rotate at specific torsional angles each time so as to form regular repetitive structures such as α-helix and β-sheet. (7) Therefore, on plotting these torsional angles to Ramachandran plot, we obtain, a very restricted area on the plot which can be used to identify and check the secondary structure in a given polypeptide. The backbone torsion angles for right-handed helix are approximately $\Phi = -57°$ and $\Psi = -47°$ and therefore, occupies small area on lower-left quadrant. β-sheet is made up of almost fully extended strands, with Φ and Ψ angles falling in the upper-left quadrant of the Ramachandran plot (figure 7).

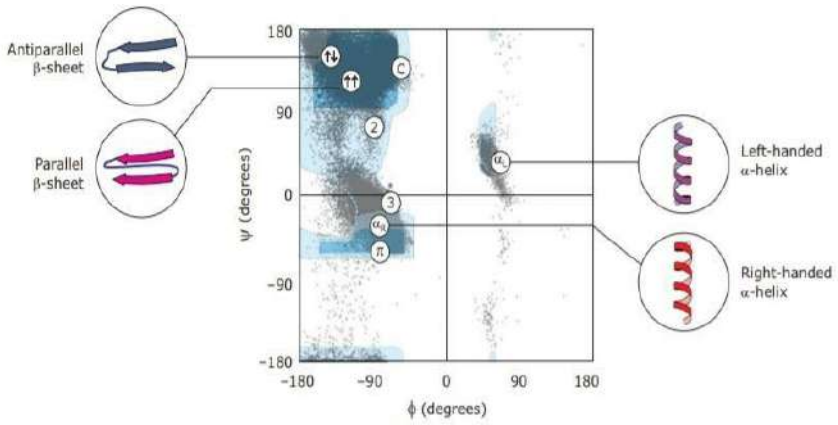


Figure 7: - Ramachandran plots showing a variety of secondary structures. The values of Φ and Ψ for various allowed secondary structures are overlaid on the plot.

2) Predicting quality of protein structure using Ramachandran plot.

The most important application of Ramachandran plot is the prediction of the quality of various protein structure determined using experimental methods (X-ray crystallography, NMR and (Cryo-EM). A good quality structure contains all the set of torsional angles in the allowed area whereas, a bad quality (low resolution) protein structure is reflected as a number of torsional angles falling in the forbidden region. Besides experimental methods, protein structure obtained using homology modeling or ab-initio methods are also routinely checked by plotting Ramachandran plot. (8)

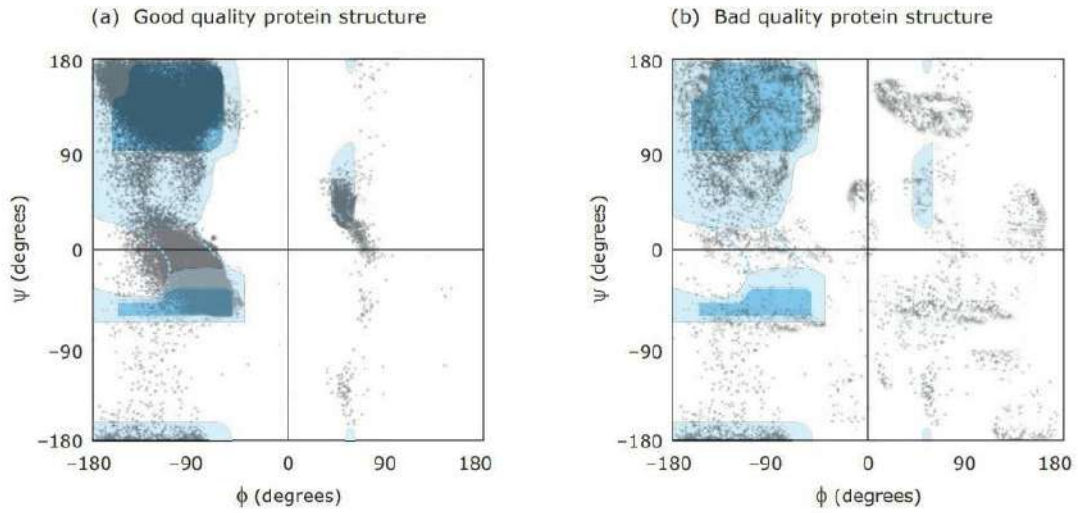


Figure 8: - Use of Ramachandran plot to predict the quality of protein structure. (a) A good quality Ramachandran plot contains most torsional angles in allowed region. (b) Bad quality or low-resolution protein structure shows a large number of torsional angles in the forbidden region.

Future Prospective: -

Making the Ramachandran plot easier and useful will save both time and money on drug discovery.

By using it, we can fight more actively against new infections.

Above all, its use has led to improvement in both commercial and medical fields.

Conclusion: -

With high-fidelity Ramachandran plot availability, the time is ripe for the completion of a definitive analysis of the “protein parts list” conceptualized by Fitzkee and coworkers (9). The study of linear groups by Hollingsworth begins this process, and extension to the analysis of all of the most common structures

will be the next step. Within a few years we should have such a complete and accurate list of all protein parts, and this will mostly impact our understanding and pave the way for fundamental, first principles understanding of the energetics behind the conformational preferences. It will also stimulate great improvements in both validation tools and the accuracy with which we can model and predict protein tertiary structure either de novo from its sequence or by template-based modeling.

References: -

1) Sasisekharan V. Stereochemical criteria for polypeptide and protein structures. In: Ramanathan N, editor. Collagen. Wiley & Sons; New York: 1962. pp. 39–78. [Google Scholar] 2) Branden CI and Tooze J (1999), Introduction to Protein Structure, 2nd edition, CRC Press.

3) Brown W, Foote C, Iverson B and Anslyn E (2009), Organic Chemistry, 5th edition, Brooks Cole.

4) StryerL.et.al. (2015), Biochemistry, 8th edition, W. H. Freeman and Company.

5) Ramachandran GN, Ramakrishnan C and Sasisekharan V (1963), Stereochemistry of polypeptide chain configurations, Journal of Molecular Biology, 7: 95–9.

6) Hollingsworth SA and Karplus PA (2010), A fresh look at the Ramachandran plot and the occurrence of standard structures in proteins, Biomol Concepts, 1(3-4): 271–283.

7) Ramachandran GN and Sasisekharan V (1968), Conformation of polypeptides and proteins, Advances in Protein Chemistry, Advances in Protein Chemistry, 23: 283–437.

8) Kendrew JC, Dickerson RE, Strandberg BE, Hart RG, Davies DR, Phillips DC and Shore VC
(1960), Structure of myoglobin: a three-dimensional Fourier synthesis at 2Å resolution, Nature, 185 (4711)

9) Fitzkee NC, Fleming PJ, Gong H, Panasik N, Jr, Street TO, Rose GD. Are proteins made from a limited parts list? Trends Biochem Sci. 2005; 30:73–80. [PubMed] [Google Scholar]

Project Title

REAL TIME RT-PCR IN DETECTION OF COVID-19

Submitted by

Ritam Mondal

Registration no.: A01-1112-116-012-2019
Semester - V ; Roll no. - 613
Department of Microbiology
Ramakrishna Mission Vivekananda Centenary College

Guided by

Dr. Soumyadip Paul

Introduction

Coronaviruses are a group of related RNA viruses that cause diseases in mammals and birds. In humans and birds, they cause respiratory tract infections that can range from mild to lethal. Mild illnesses in humans include some cases of the common cold (which is also caused by other viruses, predominantly rhinoviruses), while more lethal varieties can cause SARS, MERS and COVID-19, which is causing an ongoing pandemic. In cows and pigs they cause diarrhea, while in mice they cause hepatitis and encephalomyelitis.

Coronaviruses constitute the subfamily ***Orthocoronavirinae***, in the family *Coronaviridae*, order *Nidovirales* and realm *Riboviria*. They are enveloped viruses with a positive-sense single-stranded RNA genome and a nucleocapsid of helical symmetry. The genome size of coronaviruses ranges from approximately 26 to 32 kilobases, one of the largest among RNA viruses.[4] They have characteristic club-shaped spikes that project from their surface, which in electron micrographs create an image reminiscent of the solar corona, from which their name derives. Coronavirus (SARS-CoV-2), which causes COVID-19, only contain RNA, which means that they rely on infiltrating healthy cells to multiply and survive. Once inside the cell, the virus uses its own genetic code — RNA in the case of the COVID-19 virus — to take control of and 'reprogramme' the cells, turning them into virus-making factories.

In order for a virus like the COVID-19 virus to be detected early in the body using real time RT–PCR, scientists need to convert the RNA to DNA. This is a process called 'reverse transcription'. They do this because only DNA can be copied — or amplified — which is a key part of the real time RT–PCR process for detecting viruses.

Scientists amplify a specific part of the transcribed viral DNA hundreds of thousands of times. Amplification is important so that, instead of trying to spot a minuscule amount of the virus among millions of strands of genetic information, scientists have a large enough quantity of the target sections of viral DNA to accurately confirm that the virus is present.

What is real time RT–PCR?

Real time RT–PCR is a nuclear-derived method for detecting the presence of specific genetic material in any pathogen, including a virus. Originally, the method used radioactive isotope markers to detect targeted genetic materials, but subsequent refining has led to the replacement of isotopic labelling with special markers, most frequently fluorescent dyes. This technique allows scientists to see the results almost immediately while the process is still ongoing, whereas conventional RT–PCR only provides results at the end of the process. Real time RT–PCR is one of the most widely used laboratory methods for detecting the COVID-19

virus. While many countries have used real time RT–PCR for diagnosing other diseases, such as Ebola virus and Zika virus, many need support in adapting this method for the COVID-19 virus, as well as in increasing their national testing capacities.

Why use real time RT–PCR?

The real time RT–PCR technique is highly sensitive and specific and can deliver a reliable diagnosis in as little as three hours, though laboratories take on average between six and eight hours. Compared to other available virus isolation methods, real time RT–PCR is significantly faster and has a lower potential for contamination or errors, as the entire process can be carried out within a closed tube. It continues to be the most accurate method available for the detection of the COVID-19 virus. However, real time RT–PCR cannot be used to detect past infections, which is important for understanding the development and spread of the virus, as viruses are only present in the body for a specific window of time. Other methods are necessary to detect, track and study past infections, particularly those which may have developed and spread without symptoms.

How real time RT–PCR differs from general PCR?

RT–PCR is a variation of PCR, or polymerase chain reaction. The two techniques use the same process except that RT–PCR has an added step of reverse transcription of RNA to DNA, or RT, to allow for amplification.

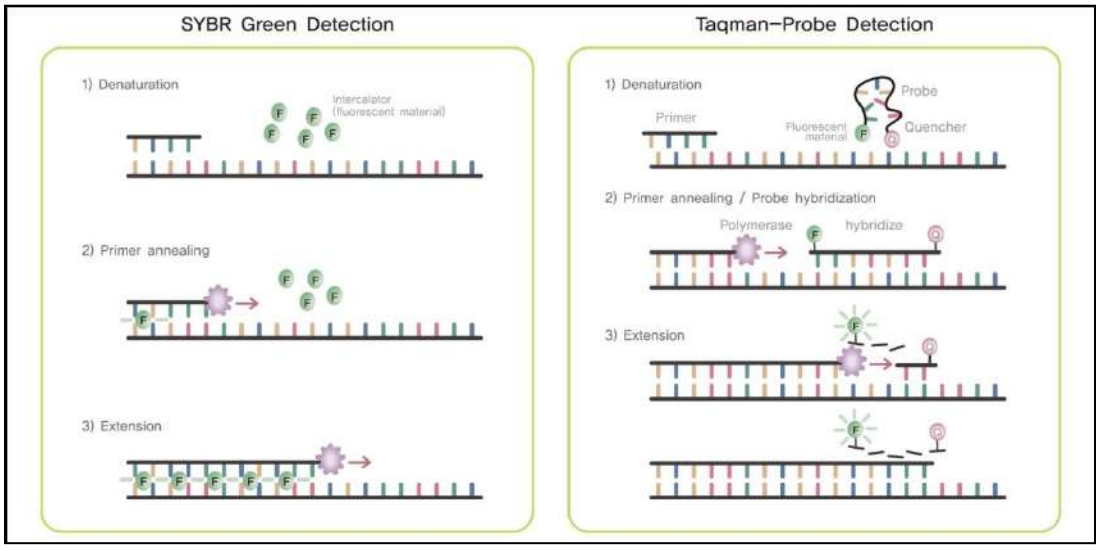


Figure 1 : Different types of markers used in Real Time PCR

This means PCR is used for pathogens, such as viruses and bacteria, that already contain DNA for amplification, while RT–PCR is used for those containing RNA that needs to be transcribed

to DNA for amplification. Both techniques can be performed in 'real time', which means results are visible almost immediately, while when used 'conventionally', results are only visible at the end of the reaction. PCR is one of the most widely used diagnostic tests for detecting pathogens, including viruses, that cause diseases such as Ebola, African swine fever and foot-and-mouth disease. Since the COVID-19 virus only contains RNA, real time or conventional RT–PCR is used to detect it.

How does real time RT–PCR work with the COVID-19 virus?

Broadly, two methods of tests are available for the novel coronavirus (SARS-CoV-2) that causes the COVID-19 disease: Molecular, which looks for the presence of the virus or its genetic material in the sample of nasal mucous or saliva and serological, which looks for the presence of antibodies in blood.
The first one is based on a routine lab technique, reverse transcription polymerase chain reaction (RT-PCR), which amplifies the minuscule amount genetic material in a pathogen and helps identify it. The technique has to be customised as per the disease by using primers, or short nucleic acid sequences, specific to the pathogen's genetic material.
Typically, the procedure involves sticking a swab, similar to an earbud, but uses nylon instead of cotton, deep into the nose or throat, retrieving mucous sample, placing the swab in buffer solution to transport and isolate the virus, replicating its genetic material using chemicals or reagents like primers, enzymes and nucleotides and then detecting it with fluorescent probes. Once the sample reaches lab, the entire process takes six hours.

A sample is collected from the parts of the body where the COVID-19 virus gathers, such as a person's nose or throat. The sample is treated with several chemical solutions that remove substances such as proteins and fats and that extract only the RNA present in the sample. This extracted RNA is a mix of the person's own genetic material and, if present, the virus's RNA.
The RNA is reverse transcribed to DNA using a specific enzyme. Scientists then add additional short fragments of DNA that are complementary to specific parts of the transcribed viral DNA. If the virus is present in a sample, these fragments attach themselves to target sections of the viral DNA. Some of the added genetic fragments are used for building DNA strands during amplification, while the others are used for building the DNA and adding marker labels to the strands, which are then used to detect the virus.

The mixture is then placed in an RT–PCR machine. The machine cycles through temperatures that heat and cool the mixture to trigger specific chemical reactions that create new, identical copies of the target sections of viral DNA. The cycle is repeated over and over to continue copying the target sections of viral DNA. Each cycle doubles the previous number: two copies become four, four copies become eight, and so on. A standard real time RT–PCR set-up usually

goes through 35 cycles, which means that, by the end of the process, around 35 billion new copies of the sections of viral DNA are created from each strand of the virus present in the sample. As new copies of the viral DNA sections are built, the marker labels attach to the DNA strands and then release a fluorescent dye, which is measured by the machine's computer and presented in real time on the screen. The computer tracks the amount of fluorescence in the sample after each cycle. When a certain level of fluorescence is surpassed, this confirms that

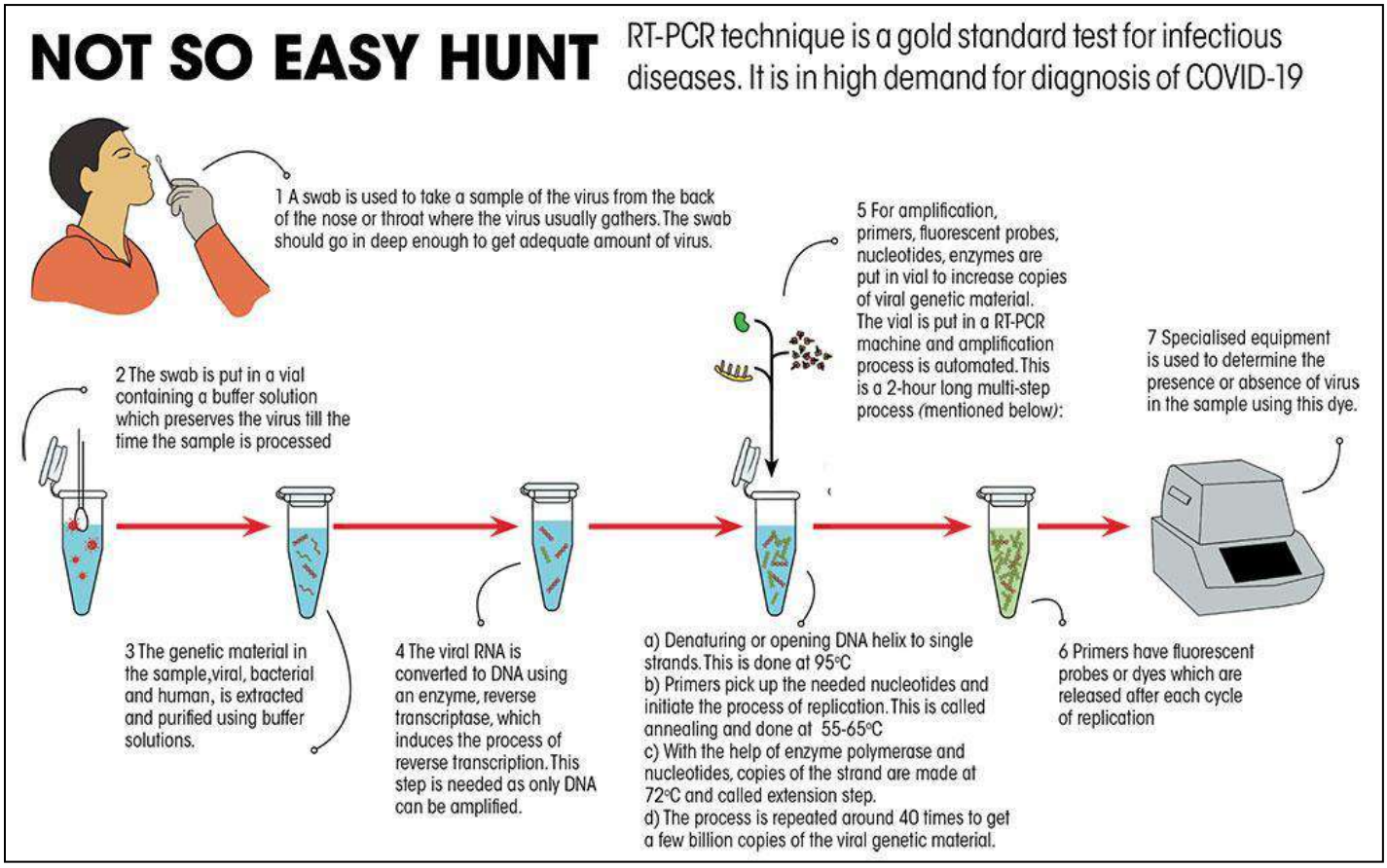


Figure 2 : Schematic representation of RT-PCR test for Covid-19

the virus is present. Scientists also monitor how many cycles it takes to reach this level in order to estimate the severity of the infection: the fewer the cycles, the more severe the viral infection is.

Scopes & Application

The exponential amplification via reverse transcription polymerase chain reaction provides for a highly sensitive technique in which a very low copy number of RNA molecules can be detected. RT-PCR is widely used in the diagnosis of genetic diseases and, semi-quantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression.

Research methods

Real time RT-PCR is commonly used in research methods to measure gene expression. For example, Lin et al. used qRT-PCR to measure expression of Gal genes in yeast cells. First, Lin et al. engineered a mutation of a protein suspected to participate in the regulation of Gal genes. This mutation was hypothesized to selectively abolish Gal expression. To confirm this, gene expression levels of yeast cells containing this mutation were analyzed using qRT-PCR. The researchers were able to conclusively determine that the mutation of this regulatory protein reduced Gal expression.[7] Northern blot analysis is used to study the RNA's gene expression further.

Gene insertion

RT-PCR can also be very useful in the insertion of eukaryotic genes into prokaryotes. Because most eukaryotic genes contain introns, which are present in the genome but not in the mature mRNA, the cDNA generated from a RT-PCR reaction is the exact (without regard to the error-prone nature of reverse transcriptases) DNA sequence that would be directly translated into protein after transcription. When these genes are expressed in prokaryotic cells for the sake of protein production or purification, the RNA produced directly from transcription need not undergo splicing as the transcript contains only exons. (Prokaryotes, such as E. coli, lack the mRNA splicing mechanism of eukaryotes).

Genetic disease diagnosis

RT-PCR can be used to diagnose genetic disease such as Lesch–Nyhan syndrome. This genetic disease is caused by a malfunction in the HPRT1 gene, which clinically leads to the fatal uric acid urinary stone and symptoms similar to gout. Analyzing a pregnant mother and a fetus for mRNA expression levels of HPRT1 will reveal if the mother is a carrier and if the fetus will likely to develop Lesch–Nyhan syndrome.

Cancer detection

Scientists are working on ways to use RT-PCR in cancer detection to help improve prognosis, and monitor response to therapy. Circulating tumor cells produce unique mRNA transcripts depending on the type of cancer. The goal is to determine which mRNA transcripts serve as the best biomarkers for a particular cancer cell type and then analyze its expression levels with RT-PCR.

RT-PCR is commonly used in studying the genomes of viruses whose genomes are composed of RNA, such as Influenzavirus A, retroviruses like HIV and SARS-CoV-2.

Discussion

In response to the SARS outbreak, we developed a real-time RT-PCR assay based on multiple primer and probe sets designed to different genomic targets to facilitate sensitive and specific detection of SARS-CoV in clinical specimens. A potential detection limit of <10 transcript copies was achieved with greater relative sensitivity than cell culture isolation or conventional RT-PCR. The potential for quantitation over a wide dynamic range (at least 6 logs) was demonstrated with low intra- and interassay variability and limited inhibition from exogenous nucleic acid extract from respiratory secretions. The increased sensitivity of the real-time RT-PCR assay over cell culture and conventional RT-PCR methods may aid detection of the virus at earlier stages of infection, when the virus is present at low titer in respiratory secretions (12). In addition, by eliminating the need for postamplification product processing, the real-time RT-PCR format permitted shortened turnaround time for reporting results, which proved critical during the SARS outbreak.

Although real-time RT-PCR offers clear advantages over more conventional RT-PCR formats, assay results must still be interpreted with caution. Asymptomatic spread of COVID-19 as reported by some research groups, made it crucial to develop multiplex and Point-of-Care techniques like isothermal amplification, CRISPR-based techniques and microfluidic techniques, so that they can be used to test the majority of the population and isolate infected persons mostly in remote areas, quarantine centers, in developing countries which lack enough resources and skills

Conclusion

In conclusion, real-time RT-PCR assay permitted rapid, sensitive, and specific detection of SARS-CoV in clinical specimens and provided needed diagnostic support during the recent SARS outbreak. Widely deploying this assay will enhance the ability to provide a rapid response in the event of the possible return of SARS. False-negative results could also potentially arise from mutations occurring in the primer and probe target regions in the SARS-CoV genome. This can be addressed by including multiple genetic targets in the assay and by carefully comparing the primer and probe sequences against published sequences of SARS-CoV as they become available. To avoid false-positive results, meticulous care has to be taken to prevent introduction of contaminating viral RNA or previously amplified DNA during preparation of the nucleic acid extracts and amplification reactions. In addition, all RT-PCR–positive specimens are to be retested from a second, unopened sample aliquot and confirmed in a second laboratory by using a real-time assay based on different genetic targets.

References

1. ^ Jump up to: *"ICTV Taxonomy history: Orthocoronavirinae". International Committee on Taxonomy of Viruses (ICTV). Retrieved 2020-01-24.*
2. ^ Jump up to: *Fan Y, Zhao K, Shi ZL, Zhou P (March 2019). "Bat Coronaviruses in China". Viruses. 11 (3): 210. doi:10.3390/v11030210. PMC 6466186. PMID 30832341.*
3. ^ *Cherry J, Demmler-Harrison GJ, Kaplan SL, Steinbach WJ, Hotez PJ (2017). Feigin and Cherry's Textbook of Pediatric Infectious Diseases. Elsevier Health Sciences. p. PT6615. ISBN 978-0-323-39281-5.*
4. ^ Jump up to: *Woo PC, Huang Y, Lau SK, Yuen KY (August 2010). "Coronavirus genomics and bioinformatics analysis". Viruses. 2 (8): 1804–20. doi:10.3390/v2081803. PMC 3185738. PMID 21994708. Coronaviruses possess the largest genomes [26.4 kb (ThCoV HKU12) to 31.7 kb (SW1)] among all known RNA viruses (Figure 1) [2,13,16].*
5. ^ Jump up to: *Almeida JD, Berry DM, Cunningham CH, Hamre D, Hofstad MS, Mallucci L, McIntosh K, Tyrrell DA (November 1968). "Virology: Coronaviruses". Nature. 220 (5168): 650. Bibcode:1968Natur.220..650.. doi:10.1038/220650b0. PMC 7086490. [T]here is also a characteristic "fringe" of projections 200 A long, which are rounded or petal shaped ... This appearance, recalling the solar corona, is shared by mouse hepatitis virus and*

several viruses recently recovered from man, namely strain B814, 229E and several others.

6. Infectious Diseases and How Nuclear Science Can Help,Nicole Jawerth, volume-61-2, jun 2020
7. *Lin L, Chamberlain L, Zhu LJ, Green MR (February 2012). "Analysis of Gal4-directed transcription activation using Tra1 mutants selectively defective for interaction with Gal4". Proc. Natl. Acad. Sci. U.S.A. 109 (6): 1997-2002. Bibcode:2012PNAS..109.1997L. doi:10.1073/pnas.1116340109. PMC 3277556. PMID 22308403.*
8. *Varkonyi-Gasic E, Hellens RP (2010). "qRT-PCR of Small RNAs". Plant Epigenetics. Methods in Molecular Biology. 631. pp. 109–22. doi:10.1007/978-1-60761-646-7_10. ISBN 978-1-60761-645-0. PMID 20204872.*
9. ^ *Torres RJ, Garcia MG, Puig JG (December 2012). "Carrier and prenatal diagnosis of Lesch-Nyhan disease due to a defect in HPRT gene expression regulation". Gene. 511 (2): 306–7. doi:10.1016/j.gene.2012.09.121. PMID 23046577.*
10. .^ *Xi L, Nicastri DG, El-Hefnawy T, Hughes SJ, Luketich JD, Godfrey TE (July 2007). "Optimal markers for real-time quantitative reverse transcription PCR detection of circulating tumor cells from melanoma, breast, colon, esophageal, head and neck, and lung cancers". Clin. Chem. 53 (7): 1206–15. doi:10.1373/clinchem.2006.081828. PMID 17525108.*
11. 4.^ *"Coronavirus: il viaggio dei test". Istituto Superiore di Sanità.*
12. Peiris JS, Chu CM, Cheng VC, Chan KS, Hung IFN, Poon LLM, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet*. 2003;361:1767–72 10.1016/S0140-6736(03)13412-5 [PMC free article] [PubMed] [CrossRef] [Google Scholar]
13. Emerg Infect Dis. 2004 Feb; 10(2): 311–316.doi: 10.3201/eid1002.030759 PMCID: PMC3322901. PMID: 15030703

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE,RAHARA

MCBA-DSE-II PROJECT

TOPIC:-MICROFLUIDIC DEVICES FOR THE DETECTION OF VIRUSES : ASPECTS OF EMERGENCY FABRICATION DURING THE COVID-19 PANDEMIC AND OTHER OUTBREAKS

SUBMITTED BY:-SUDIP DAS (623)

5TH SEMESTER

MICROBIOLOGY DEPARTMENT

GUIDED BY:-BIBHABASU HAZRA

Introduction

The preparation of this manuscript started amid one of the most severe virus outbreaks of our recent history. Since the identification of the new coronavirus (SARS-CoV-2) reported in December 2019, it rapidly spread worldwide .Between 30 January and 11 March, the World Health Organization (WHO) changed the COVID-19 outbreak status from 'Public Health Emergency of International Concern' to 'pandemic'. In a couple of months, the epicentre of this disease moved from Wuhan in the Province of Hubei, China, to Lombardy in Italy with about 800 deaths/day and a total of 10 000 fatalities as of 27 March 2020 .A few days later, the USA became the epicentre of the new coronavirus when around 103 000 cases and 1668 deaths were reported at the time. By 12 September 2020, there were more 6 300 000 cases, and about 191 000 deaths reported in the USA related to this disease .In a different direction, countries such as Singapore and South Korea did not experience the same trend in fatality rate numbers. The rapid and wide use of screening tests has been considered key to their achievements. In these countries, large efforts were made to increase the availability of test kits and to screen the population and isolate the infected citizens. The current good performance of these countries has been attributed to the lessons learned from past outbreaks (e.g. SARS, MERS).

Given the recent world pandemic of COVID-19, the need for the field- and clinic-ready diagnostic methods and devices is in high demand. The low-cost devices for rapid virus detections with high sensitivity must be available to minimize infection spreading in society. Virus detection from human specimens, including nasopharyngeal swabs, blood withdrawals, urine, or feces, is specifically challenging – particularly true for COVID-19 (SARS-CoV-2 detection from nasopharyngeal swabs) (Heesterbeek H et al, 2020, Hollingsworth TD et al, 2020). At present, the specimens are quite complex for further processing and subsequent assays. The current approach based on cell culturing to determine virus infection is difficult to process in the field. Moreover, some viruses are not culturable at all. Recently, a series of emerging microfluidic strategies have been developed to offer a range of advanced bioassay to rapidly determine virus infection progress. Both antibody- and nucleic acid amplification-based methods can be implemented on microfluidic platforms. Nucleic acid amplification methods, most notably polymerase chain reaction (PCR), are preferred for virus detection from human specimens (typically with reverse transcription), due to its high specificity and sensitivity. Beside the success of lab-based virus detections, the implementation of microfluidic platforms towards field- and clinic-ready detection has been considered challenging. For example, how can gene extraction and purification as well as thermal cycling

be implemented on a microfluidic device? Antibody-based methods, including lateral flow immune chromatographic assays or dip-stick assays, are simple and rapid, while the detection sensitivity is limited to determine low abundant virus in a clinical sample. Indeed, the challenge to bring lab-based microfluidic devices to the level of clinical diagnostics while accommodating their low cost, rapid assay time, and use in the clinics remains.

Brief Discussion

Microfluidic devices have attracted interest in the medical and diagnostic fields as they have the potential to perform many current large scale applications at a much smaller scale so as to reduce sample consumption and instrument size. Some applications, especially electrophoretic separations, have demonstrated excellent performance, such as rapid separations on the order of seconds and efficient separations of diagnostically relevant species (Li and Kricka, 2006; Reyes *et al.*, 2002; Verpoorte, 2002). For example, rapid DNA sequencing has been demonstrated in a high throughput format (Paegel *et al.*, 2002), and microfluidic-based protein and DNA separations, similar in working principle to gel electrophoresis techniques, have now been commercialized for over a decade (Panaro *et al.*, 2000). A further advantage of swift analysis is also appreciated in time-critical situations, such as during surgery or for analytes that change composition over time. Another intriguing advantage of microfluidic devices is their portability and potential for point-of-care diagnostics (Yager *et al.*, 2006), which has been demonstrated via a variety of marketed applications (Chin *et al.*, 2012). Additionally, as most microfluidic devices only require sample volumes in the nanoliter range or below for analyses, they are further suited for situations when sample amount is limited, such as in minimally invasive diagnosis. As medical and diagnostic applications of microfluidic devices focus on qualitative or quantitative determination of biomolecules, the biocompatibility of these devices becomes critical. The latter refers to several requirements for microfluidic devices. First, microchannel surfaces should resist non-specific adsorption of biomolecules (and provide a stable and non-altering composition over the course of an analysis. Due to the high surface-to-volume ratio apparent in microchannels, there is a high potential for surface adsorption and deterioration, especially in combination with diagnostic samples such as body fluids. Consequently, microfluidic applications strongly depend on the tailoring and control of surface properties in such devices. Second, for cell-based assays, the microfluidic environment has to be adapted so that cells can adhere to surfaces if required, and that intra- and inter-cellular processes proceed regularly, which poses an important requirement for the matrices used to embed or hold specific cells. Third, several other specific surface conditions arising from the

particular application at hand have to be considered, such as temperature compatibility, stability under flow and applied electric fields, and solvent compatibility. Considering these guidelines and requirements, most microfluidic applications require some sort of surface pretreatment prior to analysis. Treatments can involve passivation strategies to prevent non-specific adsorption or unwanted changes in surface properties during the course of an analysis. Furthermore, if the sensing element in a microfluidic application is a biomolecule, immobilization strategies rendering a high yield of active biomolecules on a surface are required. The control of specific biomolecule immobilization is thus another important requirement in many microfluidic applications.

Microfluidic devices can be created with a variety of materials, thus surface treatment strategies strongly depend on the properties of the material. Covalent immobilization schemes require specific active surface groups, which vary from material to material deeming such strategies specific for a given microfluidic device. Bifunctional linker molecules may also be used, which allow for specific linkage to a surface reactive group but also react specifically with functional groups on biomolecules. In contrast to covalent immobilization schemes, adsorptive coatings may present an alternative, less complex route to coat microfluidic surfaces for various purposes. Knowledge of the non-covalent interactions driving an adsorptive coating is important for the quality of the coating. In general, such interactions are determined by electrostatic, van der Waals, and/or hydrophobic interactions. The functionality of the coating can be tuned similarly to covalent strategies, and non-specific adsorption can be suppressed in most cases. Functional groups of the adsorptive coating material can also be used for further specific biomolecule immobilization (Huang C et al.2020). The ease of use of adsorptive strategies, and often diverse applicability to various materials, has led to their widespread recognition in microfluidics

An alternative to the time-consuming bench assays can be found in the field of microfluidics. Within a few decades, microelectronics and micro-electromechanical systems (MEMS) technologies have enabled the emergence of microfluidic devices capable of manipulating minute amounts of fluids and extracting information from it This approach also offers the potential to rapidly obtain information from the small sample volumes. It has increasingly been used for bedside or point-of-care testing (PoCT). Many microfluidic devices have been developed for early diagnosis of diseases or other health-related conditions (e.g. pregnancy test, glucose levels, pneumonia) by the detection of target elements in the circulation In recent years, microdevices have been developed to also detect smaller pathogens like viruses In this

work, we review the state-of-the-art in microfluidics devices for the manipulation and detection of infectious agents such as the coronavirus, the human immunodeficiency virus (HIV), influenza viruses and the Zika virus. (Sohrabi C et al 2019, Alsafi Z et al 2019) We discuss the detection approaches and the signalling methods used for identifying specific targets and informing the user about the detection. Moreover, we analyse similarities to the benchtop assays, and we end this work presenting a perspective on how such devices could be used in emergency fabrication situations.

Early diagnosis of viral diseases can lead to better and more accurate treatment. Cell culture-based techniques are the gold standard for viral detection. Rapid molecular techniques with high sensitivity involve the amplification of viral genomic material and may detect several viruses simultaneously. The two most important types of nucleic acid-based amplification tests (NATs) are nucleic acid sequence-based amplification (NASBA) and real-time polymerase chain reaction (real-time PCR) (Khan M et al 2019, Kerwan A et al 2019, Al-Jabir A et al 2019). NASBA is an isothermal and continuous amplification reaction in which three different enzymes are applied: RNase-H, AMV-RT, and T7-RNA polymerase. Real-time PCR involves the amplification of complementary DNA (cDNA) prepared from viral RNA in a real-time manner and is appropriate for the detection of minute amounts of nucleic acids.Another method is a biosensor that has high sensitivity and specificity, and most of the biosensors are based on electrochemical transduction.

Covid-19 can be diagnosed in different ways, including CT-Scan and RT-PCR. CT-Scan results indicate bilateral ground-glass and consolidative pulmonary opacities.qRT-PCR is currently used for the detection of SARS-CoV-2, but it is expensive, time-consuming, and not as sensitive as it should be. The shortage of equipment in healthcare centers and the need for better disease management require the development of more convenient and more reliable methods of diagnosis. This review aims to provide an overview of the microfluidic systems as a diagnostic method for RNA viruses with a focus on SARS-CoV-2, with the help of CRISPR technology. (Qin P et al 2019, Park M et al 2019, , Alfson KJ et al 2019).

Applications

PCR: A technique widely used to accurately and rapidly generate a large number of copies (amplification) of a DNA target sequence. The amplified DNA product can be used in a variety of ways, including for the diagnosis of infectious diseases. PCR reactions need a DNA template, primers that bind to the target DNA through complementary base pairing, an enzyme called DNA polymerase which synthesizes new complementary DNA strands, and nucleotides that will compose the new strands. All these reagents are combined and submitted to repeating heating and cooling cycles (Fernández-Carballo BL et al.2018). Each cycle doubles the amount of DNA, resulting in an exponential DNA increase. The number of cycles can vary between 25 and 40 until achieving a reasonable number of copies.

Real-time PCR: also known as quantitative PCR (qPCR), this technique allows for quantification of the target DNA while the reaction is happening. Real-time detection is possible because of the use of fluorescent reporter molecules that bind to double-stranded DNA during the PCR cycles. Levels of fluorescence increase with an increasing amount of PCR product. Fluorescence readings are performed with specialized thermal cyclers and the data needs to be analysed by instrument-specific software.

Reverse transcription PCR (RT-PCR): uses the same amplification principle as the traditional PCR, but differs in the sample preparation stage. While the PCR amplifies the fragments of DNA, RT-PCR is used for the amplification of RNA targets. The RNA templates are first converted into complementary DNA (cDNA) and then used as templates in PCR reactions. The amplification of RNA strands is relevant for the study of transcribed gene sequences and RNA-based viruses.

RT-LAMP integrated microfluidic:RT-LAMP versus commonly-used PCR does not require thermal cycles and is performed at a constant temperature between 60 and 65°C. Safavieh et al. designed cellulose-based paper microchips and amplified the target RNA using the RT-LAMP technique and detected the HIV-1 virus through the electrical sensing of LAMP amplicons. They developed an RT-LAMP paper microchip assay, which could be used as a simple and affordable method for the detection of HIV-1. Two other studies have shown that microfluidic-based RT-LAMP assay can affordably detect the Zika virus and Bacteriophage MS2 virus.

Nested PCR integrated microfluidic: Nested PCR is a modification of PCR, which involves the use of two primer sets and two successive PCR reactions. Therefore, it profits from higher sensitivity and specificity compared to conventional PCR. (Oshiki et al 2017). used a microfluidic nested-PCR device and next-generation sequencer to develop high-throughput detection and genotyping tool for 11 human RNA viruses including Aichi virus, astrovirus, enterovirus, norovirus (genogroups I, II, and IV), hepatitis A virus, hepatitis E virus, rotavirus, sapovirus, and human parechovirus. The results of this study showed that microfluidic nested PCR followed by MiSeq sequencing enabled efficient tracking of the fate of multiple RNA viruses in various environments like feces, sewage, and oysters.

Nucleic acid hybridization: Nucleic acid hybridization on a microfluidic chip integrated with the controllable micro-magnetic field has been reported as a rapid method for simultaneously detecting and subtyping multiple influenza viruses. The subtypes H1N1, H3N2, and H9N2 could be simultaneously detected in 80 min with detection limits about 0.21, 0.16, 0.12 nM, respectively. Therefore, this method can be a reliable technology platform with the ability of rapid diagnosis and subtyping of influenza viruses.

ELISA: The enzyme-linked immunosorbent assay (ELISA) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies, and hormones. Recently, it has been widely used with microfluidic devices resulting in a fast and affordable method of diagnosing RNA viruses.The commonly used ELISA and fluorescence-based Luminex assay typically consists of three steps and takes several hours to complete, but combining this method with the microfluidic system has led to efficient and rapid diagnosis.

Rolling circle amplification: Rolling circle replication is a process of rapid unidirectional replication of circular molecules of DNA and RNA, such as plasmids and the RNA genome of viroids. When mixed with microfluidic systems, some benefits like rapidity and cheapness are present. Rolling circle amplification combined with on-chip size-selective trapping of amplicons on silica beads showed that this system could be applied to diagnosing Ebola and influenza viruses(Phillips EA et al 2019,_Moehling TJ et al 2019,_Ejendal KF, et al 2019). In another study. The traditional approaches like virus isolation, serology, immunoassays, and RT-PCR are difficult and limited in terms of specificity and sensitivity for detecting RNA viruses. However, rolling circle amplification, in combination with padlock probes, had a higher specificity for detecting RNA viruses like Newcastle disease virus, avian coronavirus, and avian influenza virus

The microfluidic device integrated with porous silicon nanowire forest: The nanoscale features in silicon nanowires (SiNWs) can suppress phonon propagation, which is referred to when phonons propagate through a lattice, and sharply reduce their thermal conductivities compared to the bulk value. (Xia et al 2016). developed a microfluidic device embedded with porous silicon nanowire (pSiNW) forest for label-free size-based point-of-care virus capture in a continuous curved flow design. They worked on Influenza virus (H5N1) and demonstrated that this method could have high potentials for virus discovery, isolation, and culture.

Silica sol-gel coating/bonding method: Polydimethylsiloxane (PDMS) hybrid microchip using a simple epoxy silica sol-gel coating/bonding method. They showed that infectious reference viruses and nasopharyngeal swab patient specimens could be successfully tested using microchip Europium nanoparticle immunoassay (μENIA) on hybrid microchip platforms. The potential of this unique microchip nanoparticle assay was demonstrated in the clinical diagnosis of influenza viruses.

Aptamers: Aptamers are single-stranded artificial oligonucleotides (DNA or RNA) with a high affinity for binding to specific targets. They are of short length from 20 to 100 nucleotides and can bind to a variety of small (amino acids, antibiotics, and nucleotides) and large molecules (proteins, viruses, and bacteria.)

Future aspects

Microfluidic diagnostics is promising for early detection of a number of diseases, including cancer, diabetes, and cardiovascular diseases, in addition to serving for monitoring health conditions. To be efficient and cost-effective, portable PoC devices are made with microfluidic technologies, with which laboratory analysis can be made with small-volume samples. Recent years have witnessed considerable progress in this area with “epidermal electronics”, including miniaturized wearable diagnosis devices. These wearable devices allow for continuous real-time transmission of biological data to the Internet for further processing and transformation into clinical knowledge. Other approaches include bluetooth and WiFi technology for data transmission from portable (non-wearable) diagnosis devices to cellphones or computers, and then to the Internet for communication with centralized healthcare structures. There are, however, considerable challenges to be faced before PoC devices become routine in the clinical practice. For instance, the implementation of this technology requires integration of detection components with other fluid regulatory elements at the microscale, where fluid-flow properties become increasingly controlled by viscous forces rather than inertial forces. Another challenge

is to develop new materials for environmentally friendly, cheap, and portable microfluidic devices. In this review paper, we first revisit the progress made in the last few years and discuss trends and strategies for the fabrication of microfluidic devices. Then, we discuss the challenges in lab-on-a-chip biosensing devices, including colorimetric sensors coupled to smartphones, plasmonic sensors, and electronic tongues. The latter ones use statistical and big data analysis for proper classification. The increasing use of big data and artificial intelligence methods is then commented upon in the context of wearable and handled biosensing platforms for the Internet of things and futuristic healthcare systems.

Microfluidics is the name given to the technology for the fabrication of microminiaturized devices containing channels and chambers, with scale dimensions in the order of 1 mm or less, to control the flow behavior of small volumes of fluids. At these levels, the fluid-flow is laminar, i.e., without chaotic turbulence, allowing the control of transport and mixing of molecules to enable separation and detection of analytes with high accuracy and sensitivity.Several applications of microfluidics can be envisaged, then, in biotechnology, chemical synthesis, and analytical chemistry. In particular, such precise manipulation of molecular interactions allows for miniaturization and rapid processing of samples, with experimental techniques in lab-on-a-chip analytical platforms These point-of-care (PoC) devices are applied to detect analytes of biological interest and may provide rapid diagnostics even in remote regions (near a patient) with limited-resource or non-existing healthcare settings This can be made possible with synergistic integration of PoC devices with mobile communications, e.g. the fifth-generation wireless communications (5G), and the Internet of things (IoT) technologies (Mauk M et al 2017,Song J et al 2017, Bau HH et al 2017) . With fast Internet connectivity with low-latency, high bandwidth, cloud-based storage, and real-time extensive computing capabilities for billions of connected devices, one may expect the next-generation healthcare programs to be more patient-friendly and cost-efficient.

Conventional fabrication methods of microfluidic devices, such as lithography, soft-lithography , plasma treatment, and chemical vapor deposition, require cleanroom facilities and trained personnel. This makes them inefficient in terms of costs and fabrication speed to satisfy the increasing array of applications. Moreover, materials for microfluidics need to be optically transparent, mechanically strong, thermally stable, easily modifiable, and amenable for mass production .There is not a single material capable of satisfying all these requirements simultaneously. Hence, in general, a material is selected according to the application of interest. Plastics are cheap, flexible, and easy to control, seemingly suitable for general purposes.

However, their photolithography process is expensive and strong solvent materials cannot be used, which limits Sensors 2020, 20, 1951 3 of 19 their applicability. Silicon, on the other hand, exhibits good chemical and thermal compatibility, but it can be expensive, fragile, and opaque to visible and ultraviolet light, thus limiting its use in optical-based applications. Although optical transparency, chemical inertness, rigidity, and high temperature resistance turn glass into a good material for microfluidics, it requires slow and high-cost deposition techniques making it unsuitable for mass production. Some approaches, such as soda-lime glass , were proposed to address these limitations, but the need of rigorous chemical cleaning and thermal treatment for proper activation constitutes a major hurdle. Paper-based microfluidic devices appear to be advantageous for their cost effectiveness, capillary fluid-flow (power-free), high surface-area-to-volume ratio, and the ability to store reagents in active form within the fiber network .Microfluidic channels have been produced through an automated laser printer deposition of hydrophobic ink on paper for PoC applications as illustrated in. Permanent fixation of the hydrophobic barriers on the cellulose's capillaries of the paper is reached upon heating at 165 ◦C for 15 min, as depicted in the cross-section view at the bottom of Figure 1A. These laser printed microfluidic paper-based analytical devices (LP-µPADs) were used to determine the nitrite content in aqueous solutions and artificial urine samples and for detecting Escherichia coli (ATCC 25922) through the Griess colorimetric assay. To detect biomarkers at ultra-low concentrations for early diagnosis in asymptomatic patients of diseases such as Parkinson's disease, Alzheimer's disease, cancer, and malaria, new strategies need to be combined with microfluidic PoC devices. One such strategy includes magnetic nanoparticles for signal amplification and capture of analytes ,which also opens up the possibility for multiplex biomarker detection

Fabrication methods: Generally, there are two different kinds of microfluidic devices; channel-based and paper-based. The paper-based tool is made of a series of hydrophilic cellulose or nitrocellulose fibers that guide liquid in a paper by absorption. The channel-based one could be fabricated using four main methods, including laminate, molding, 3D-printing, and nanofabrication. Channel-based microfluidic devices need channels to create a bed for the integration of reagents.

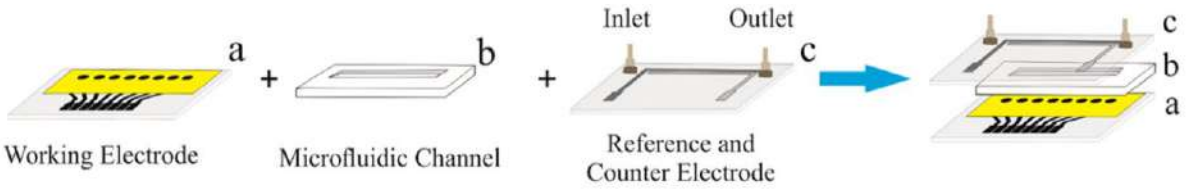
Laminate: Layers cut separately by using a knife or laser are fused to form the channel. Although it is an easy method, it is impossible to achieve sub-micrometer levels.

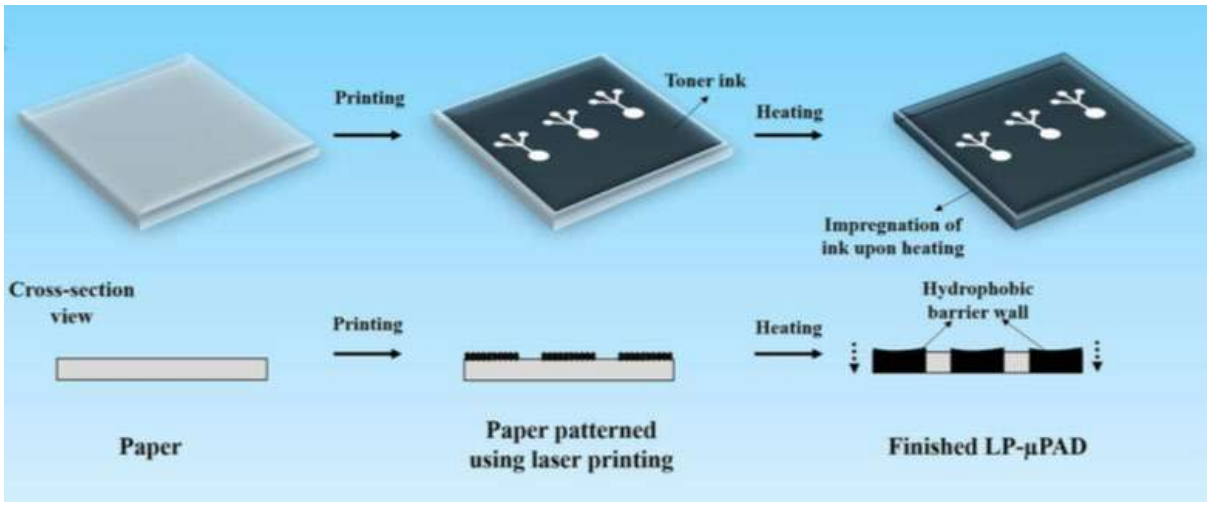
Moulding: Moulding is a technique that consists of four main steps, including shaping the mould, choosing the appropriate polymer, curing the polymer, and releasing the material from the mould. It includes three different methods, including replica moulding, injection moulding, and hot embossing.

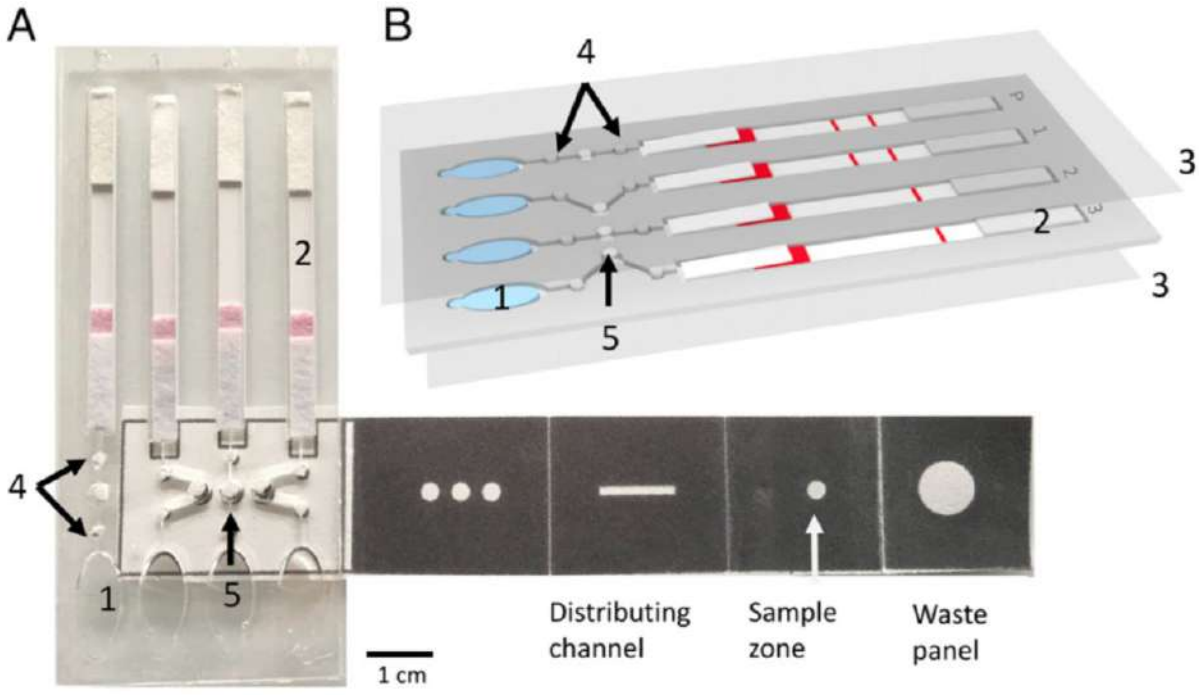
3D-printing: This is a method of fabricating layer by layer. It has two main parts. One is computer aided design. The second part is a 3D-printer that uses the computer format of stereolithography (STL), building up in 2D layers based on its resolution. There are different methods of 3D-printing, such as fused deposition modeling (FDM), STL and digital micromirror device-based projection printing (DMD-PP), multi-jet modeling, and two-photon polymerization.

Nanofabrication: In the top-down approach, the model size is reduced to the nanoscale until the desired shape and dimensions are achieved. In contrast, the bottom-up approach starts from atomic and molecular levels to finally shape the model.Extreme ultraviolet lithography (EUL), electron beam lithography (EBL), and nanoimprint lithography (NIL) are three different methods used in nanofabrication. EUL and EBL are not common in microfluidic fabrication, and the main reason is high costs.However, NIL, which is a special kind of replica moulding with the resolution of sub 15 nm, is affordable and has many applications in microfluidic fabrication.

Useful strategies for RNA virus detection: In recent years, portable microfluidic devices have reduced global cost per analysis and reagent consumption and also led to faster analyses due to shorter reactions. Among conventional methods for detecting RNA viruses, traditional cultural methods, serological methods, and molecular biology techniques can be mentioned. According to different studies, so much time and money can be spared when these methods are integrated into a microfluidic-based device.


a
+
b
+
Inlet
Outlet
c
c
b
a
Working Electrode
Microfluidic Channel
Reference and
Counter Electrode


Printing
Toner ink
Heating
Impregnation of
ink upon heating
Cross-section
view
Printing
Heating
Hydrophobic
barrier wall
Paper
Paper patterned
using laser printing
Finished LP-µPAD


A
B
4
3
2
1
5
3
2
4
1
5
1 cm
Distributing
channel
Sample
zone
Waste
panel

Conclusion

Within about half a year, COVID-19 has spread globally, causing hundreds of thousands of fatalities. The current scenario has put immense pressure on healthcare and economic systems worldwide. The sanitary situation is unparalleled in recent years. It has called for far-reaching restrictions in people's lives, such as social distancing requirements, work, school and travel restrictions, and local shutdowns. It has also led to a spike in demand for hygiene items such as 70% alcohol, personal protective equipment (PPE) such as masks and face shields, mechanical respirators and screen test kits. At the same time, we have witnessed an extraordinary effort from small and large companies, universities and common citizens to find ways to help. Despite of, or even because of, all these efforts, a critical bottleneck in this pandemic became evident: around the globe, there is a lack of reliable screen test kits for a new virus to prevent the unrestrained spreading of a disease such as COVID-19. Therefore, the ability to rapidly produce test kits for quick diagnosis seems to have become instrumental to avoid further social and economic disruptions while facing new varieties of contagious diseases. In the present work, we have discussed some of the most recent advancements in microfluidic-based detectors for viruses. The devices reviewed are based on a variety of approaches and methods but are all capable of detecting and discriminating viral targets in real test samples from medical patients. Many highly efficient microfluidic devices have taken advantage of well-established benchtop assays (i.e. implemented in macroscopic analysis tools rather than miniaturized, portable devices) and adapted them to miniaturized lab-on-a-chip versions. This is possible because recent concentrated research efforts on the SARS-CoV-2 virus have unveiled its genetic material, the proteins and other molecules that form the virus, and the memory antibodies for the disease These discoveries allowed for the development of new microfluidic detectors based on biological ligands. One such category of detectors uses *primers* as biological ligands when applying protocols involving the amplification of *genetic material* (e.g. DNA, RNA), such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). Another such category uses *antibodies* as biological ligands to bind target particles for the detection of viruses based on their *viral proteins* .Besides, other innovative solutions to detecting viruses exploit fabrication capabilities of micro- and nanotechnology to detect viruses with fewer or even no biological ligands but rather based on their geometry or electrical properties. Resistive pulse detectors are an example of this category of devices. The different detection approaches are compatible with different visualization methods to indicate detection events. Some common methods are based

on fluorescent markers, on colorimetric approaches, on electrochemically induced shifts in the spectroscopic response, on modified transfer functions of involved transistors, or on modified current signals. The combination of the various detection and visualization/interface methods allows for a range of sensitivities and resolution limits, but also strongly varies the requirements for device complexity and supporting biochemical supplies. A common guideline for the commercial success of micro- and nanotechnology products claims that 'simpler is better'. This attitude likely bears additional value when rapid development of new detector devices and fast fabrication are needed, such as under emergency fabrication requirements as in the situation imposed by a pandemic such as COVID-19. In this context, paper-based devices offer unique characteristics of the ubiquitous availability of material and relatively easy fabrication and disposal processes. This might be the simplest kind of a microfluidic device that can be rapidly adapted, and mass-produced for home testing populations. However, even those paper-based devices still require primers or antibodies for detection, which might constitute a problematic obstacle for developing, emerging or low gross domestic product countries .The same dependency on biotechnological material is required for electrochemistry-based detectors, which may additionally rely on large benchtop equipment such as spectrometers. An electrochemistry-based detection method that does not require much additional equipment or supplies is based on FET (immunoFET devices). The fabrication of sophisticated FET-based detectors, however, can be quite challenging and may not be feasible in many regions of the globe. New protocols to create micro and nanopores for RPS-based devices fall into the same category as the FET devices mentioned above – elimination of the need for biological ligands at the expense of much-increased fabrication challenges. The sensitivity of RPS detectors to the size and shape of the target particle eliminates the need for primers or antibodies, but there is still the need for highly complex fabrication capabilities and very sensitive amperemeters. In summary, a large variety of methods and approaches can be applied for the detection of viruses using microfluidic devices. They much vary in the complexity of the fabrication process, in the need for biochemical supplies and ancillary instruments, and detection properties. Therefore, no single approach stands out as a best-fit solution. Instead, a selection from the presented alternatives, or additional new ones potentially getting developed, should be made based on the complexity of the detection and discrimination of the specific virus of interest and based on local community needs and, cost considerations, and delivery timelines to find an adequate solution for producing screening tests and making them widely available.

REFERENCES:-

(Li and Kricka, 2006; Reyes *et al.*, 2002; Verpoorte, 2002)(Paegel *et al.*, 2002),(Panaro *et a*, 2000),(Yager *et al.*, 2006),(Chin *et al.*, 2012)

Weiss SR, Navas-Martin S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiol Mol Biol Rev*. 2005; **69**(4): 635- 664.

Ji W, Wang W, Zhao X, Zai J, Li X. Homologous recombination within the spike glycoprotein of the newly identified coronavirus may boost cross-species transmission from snake to human. *J Med Virol*. 2020; **92**(4).

Mauk M, Song J, Bau HH, et al. Miniaturized devices for point of care molecular detection of HIV. *Lab Chip*. 2017; **17**(3): 382- 394.

Ciftci S, Neumann F, Abdurahman S, et al. Digital rolling circle amplification–based detection of Ebola and other tropical viruses. *J Mol Diagn*. 2020; **22**(2): 272- 283.

Haleyur Giri Setty MK, Liu J, Mahtani P, et al. Novel time-resolved fluorescence europium nanoparticle immunoassay for detection of human immunodeficiency virus-1 group O viruses using microplate and microchip platforms. *AIDS Res Hum Retroviruses*. 2016; **32**(6):

Phillips EA, Moehling TJ, Ejendal KF, et al. Microfluidic rapid and autonomous analytical device (microRAAD) to detect HIV from whole blood samples. *Lab Chip*. 2019; **19**(20): 3375- 3386

Qin P, Park M, Alfson KJ, et al. Rapid and fully microfluidic Ebola virus detection with CRISPR-Cas13a. *ACS Sensors*. 2019; **4**(4): 1048- 1054.

Huang Cet al.2020Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. ***Lancet* 395**, 497–506.

Sohrabi C, Alsafi Z, O'Neill N, Khan M, Kerwan A, Al-Jabir A, Iosifidis C, Agha R. 2020World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19). ***Int. J. Surg.* 76**, 71–7.

WHO, 2020Coronavirus disease 2019 (COVID-19) Situation Report –67. Geneva, Switzerland: WHO.

Fernández-Carballo BLet al.2018Continuous-flow, microfluidic, qRT-PCR system for RNA virus detection. Oshiki et al 2017 ***Anal. Bioanal. Chem.* 410**, 33–43.

Subbaraman N. 2020Coronavirus tests: researchers chase new diagnostics to fight the pandemic.

Anderson RM, Heesterbeek H, Klinkenberg D, Hollingsworth TD. 2020How will country-based mitigation measures influence the course of the COVID-19 epidemic?***Lancet* 395**, 931–934.

A Comprehensive review of the Structure of SARS-CoV-2 and its Variants

SUBMITTED BY: SOHAMDEEP CHAKRABORTY

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE

DEPARTMENT OF MICROBIOLOGY

REGISTRATION NO: A01-1112-116-007-2019

ROLL NO: 608

SEMESTER: V

GUIDED BY: MR. AVIJIT CHAKRABORTY

A Comprehensive review of the Structure of SARS-CoV-2 and its Variants

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative pathogen of the coronavirus disease 2019 (COVID-19), has caused more than 179 million infections and 3.8 million deaths worldwide. Throughout the past year, multiple vaccines have already been developed and used, while some others are in the process of being developed. However, the emergence of new mutant strains of SARS-CoV-2 that have demonstrated immune-evading characteristics and an increase in infective capabilities leads to potential ineffectiveness of the vaccines against these variants. Rigorous determination of SARS-CoV-2 infectivity is very difficult owing to the continuous evolution of the virus, with its single nucleotide polymorphism (SNP) variants and many lineages. However, it is urgently necessary to study the virus in depth, to understand the mechanism of its pathogenicity and virulence, and to develop effective therapeutic strategies.

Key words: severe acute respiratory syndrome coronavirus 2, coronavirus disease 2019, vaccines, variant strains, antiviral therapy, SARS-CoV-2, PandemicMutation, Evolution pattern

Introduction

The SARS-CoV-2, which is responsible for the current Coronavirus disease (COVID-19), also belongs to the genus Beta-CoV and it is considered the third major coronavirus outbreak in the last 20 years, after Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). On March 11th, 2020, the World Health Organization (WHO) having established the spread (and severity) of the SARS-CoV-2 infection, declared that the COVID-19 outbreak recorded in the preceding months was a pandemic. It has currently affected >200 countries. As on October 2020, about 35.6 million people have been infected, with more than 1.04 million deaths. More than 24.8 million people have recovered completely, but a large

number of the infected people end up in critical condition that require respiratory assistance. Like other RNA viruses, SARS-CoV-2, while adapting to their new human hosts, is prone to genetic evolution with the development of mutations over time, resulting in mutant variants that may have different characteristics than its ancestral strains. Several variants of SARS-CoV-2 have been described during the course of this pandemic, among which only a few are considered variants of concern (VOCs) by the WHO, given their impact on global public health. Based on the recent epidemiological update by the WHO, as of December 11, 2021, five SARS-CoV-2 VOCs have been identified since the beginning of the pandemic:

- **Alpha (B.1.1.7):** first variant of concern described in the United Kingdom (UK) in late December 2020
- **Beta (B.1.351)**: first reported in South Africa in December 2020
- **Gamma(P.1)**: first reported in Brazil in early January 2021
- **Delta (B.1.617.2):** first reported in India in December 2020
- **Omicron (B.1.1.529):** first reported in South Africa in November 2021

Coronavirus classification and structure

- *Classification of Coronaviruses*

Coronaviruses (CoVs) are spherical and approximately 125 nm in diameter,21,22 with club-shape spikes projecting from the surface of the virus giving the appearance of a solar corona, prompting the name, coronaviruses. Within the envelope is the helically symmetrical nucleocapsids, which is actually uncommon among positive-sense RNA viruses. CoVs are classified under the order Nidovirales, family Coronaviridae, and subfamily Orthocoronavirinae (Fig. 1). With genome sizes ranging from 26 to 32 kilobases (kb) in length, CoVs have the largest genome for RNA viruses. Based on

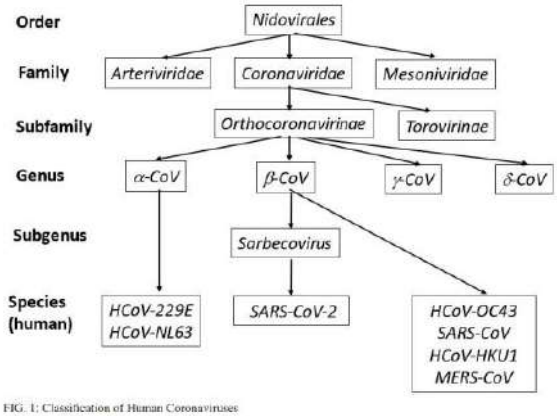


FIG. 1: Classification of Human Coronaviruses

genetic and antigenic criteria, CoVs have been organised into four groups: alphacoronavirus (α-CoV), betacoronavirus (β-CoV, gammacoronavirus (γ-CoV) and deltacoronavirus (d-CoV). For SARS-Cov-2, next-generation sequencing also shows 79% homology to SARS-CoV and 50% to MERS-CoV. Phylogenetic analysis has placed SARS-CoV-2 under the subgenus Sarbecovirus of the genus Betacoronavirus

- ***Genomic Structure and Function of Coronaviruses***

The organization of the coronavirus genome is 5′-leader-UTR- replicase-S (Spike)–E (Envelope)-M (Membrane)-N (Nucleocapsid)- 3′UTR-poly (A) tail with accessory genes interspersed within the structural genes at the 3′ end of the genome (Fig. 2). The four structural proteins are required by most CoVs to produce a structurally complete viral particle suggesting that some CoVs may encode additional proteins with overlapping compensatory functions. While each of the major protein plays a primary role in the structure of the virus particle, they are also involved in other aspects of the replication cycle. The S protein (~150 kDa) mediates attachment of the virus to the host cell surface receptors resulting in fusion and subsequent viral entry. In some CoVs, the S protein also mediate cell-cell fusion between infected and adjacent, uninfected cells resulting in formation of multinucleated giant cells, a strategy that allows direct viral spread between cells while avoiding virusneutralising antibodies.32,33 The S protein utilizes an N-terminal signal sequence to gain access into the endoplasmic reticulum (ER), and is heavily N-linked glycosylated. Homotrimers of the virus-encoded S protein make up the distinctive spike-like structure. This trimeric S glycoprotein is a class I fusion protein36 that mediates attachment to the host receptor. In most coronaviruses, S is cleaved by a host cell furin-like protease into two separate polypeptides, namely S1 and S2. S1 makes up the large receptor-binding domain of the S protein and S2 forms the stalk of the spike. The M protein (~25–30 kDa) with three transmembrane domains is the most abundant structural protein and defines the shape of the viral envelope. It has a small N-terminal glycosylated ectodomain and a much larger

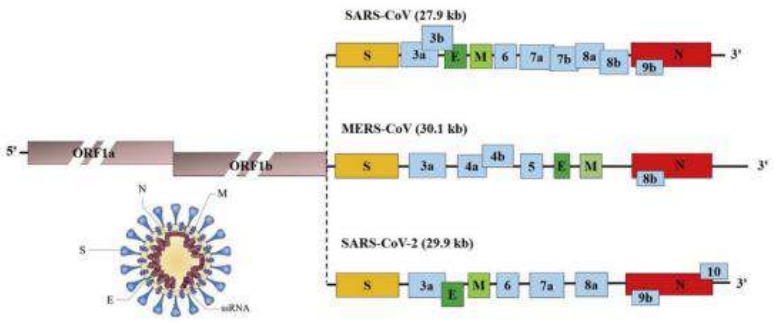


FIG. 2: Genomes of SARS-CoV, MERS-CoV and SARS-CoV-2 (Li *et al*. 2020)[71]
Available from https://doi.org/10.1016/j.jpha.2020.03.001

C-terminal endodomain that extends 6–8 nm into the viral particle. Studies have shown that the M protein exists as a dimer, and may adopt two different conformations allowing it to promote membrane curvature as well as bind to the nucleocapsid. Interaction of S with M protein is necessary for retention of S in the ER-Golgi intermediate compartment (ERGIC)/ Golgi complex and its incorporation into new virions, but is not required for the assembly process. Binding of M to N protein stabilises the nucleocapsid (N protein-RNA complex), as well as the internal core of virions, and, ultimately, helps complete the viral assembly. Together, M and E proteins make up the viral envelope and their interaction is sufficient for the production and release of virus-like particles (VLPs). The E protein (~8–12 kDa) is the smallest of the major structural proteins. This transmembrane protein has a N-terminal ectodomain and a C-terminal endodomain with ion channel activity. During the replication cycle, E is abundantly expressed inside the infected cell, but only a small portion is incorporated into the virus envelope. The majority of the protein participates in viral assembly and budding. Recombinant CoVs without E have been shown to exhibit significantly reduced viral titres, crippled viral maturation, or yield incompetent progeny, thereby demonstrating the importance of E protein in virus production and maturation. The N protein is the only one that binds to the RNA genome. The protein is composed of two separate domains, an N-terminal domain (NTD) and a C-terminal domain (CTD). It has been suggested that optimal RNA binding requires contribution from both these domains. It is also involved in viral assembly and budding, resulting in complete virion formation.

- ***Genomic structure of SARS-CoV-2***

The SARS-CoV-2 genome is similar to that of typical CoVs and contains at least ten open reading frames (ORFs). The 5'-terminal twothirds of the genome ORF1a/b encodes two large polyproteins, which form the viral replicase transcriptase complex. The other ORFs of SARSCoV-2 on the one-third of the genome encode the same four main structural proteins: spike (S), envelope (E), nucleocapsid (N) and membrane (M) proteins, as well as several accessory proteins with unknown functions which do not participate in viral replication (Fig. 2).

SARS-CoV-2 Variants

SARS-CoV-2 is prone to genetic evolution resulting in multiple variants that may have different characteristics compared to its ancestral strains. Periodic genomic sequencing of viral samples is of fundamental importance, especially in a global pandemic setting, as it helps detect any new genetic variants of SARS-CoV-2. Notably, the genetic evolution was minimal initially with the emergence of the globally dominant D614G variant, which was associated with increased transmissibility but without the ability to cause severe illness. Another variant was identified in humans, attributed to transmission from infected farmed mink in Denmark, which was not associated with increased transmissibility. Since then, multiple variants of SARS-CoV-2 have been described, of which a few are considered **variants of concern (VOCs)** due to their potential to cause enhanced transmissibility or virulence, reduction in neutralization by antibodies obtained through natural infection or vaccination, the ability to evade detection, or a decrease in therapeutics or vaccination effectiveness. With the continued emergence of multiple variants, the CDC and the WHO have independently established a classification system for distinguishing the emerging variants of SARS-CoV-2 into variants of concern(VOCs) and **variants of interest(VOIs)**.

SARS-CoV-2 Variants of Concern (VOCs)

- ***Alpha (B.1.1.7 lineage)***
    - In late December 2020, a new SARS-CoV-2 variant of concern, **B.1.1.7 lineage**, also referred to as **Alpha variant** or **GRY**(formerly GR/501Y.V1), was reported in the UK based on whole-genome sequencing of samples from patients who tested positive for SARS-CoV-2.
    - In addition to being detected by genomic sequencing, **the B.1.1.7** variant was identified in a frequently used commercial assay characterized by the absence of the S gene (S-gene target failure, SGTF) PCR samples. The B.1.1.7 variant includes 17 mutations in the viral genome. Of these, eight mutations (Δ69-70 deletion, Δ144 deletion, N501Y, A570D, P681H, T716I, S982A, D1118H) are

in the spike (S) protein. N501Y shows an increased affinity of the spike protein to ACE 2 receptors, enhancing the viral attachment and subsequent entry into host cells.

- This variant of concern was circulating in the UK as early as September 2020 and was based on various model projections. It was reported to be 43% to 82% more transmissible, surpassing preexisting variants of SARS-CoV-2 to emerge as the dominant SARS-CoV-2 variant in the UK. The B.1.1.7 variant was reported in the United States (US) at the end of December 2020.
- An initial matched case-control study reported no significant difference in the risk of hospitalization or associated mortality with the B.1.1.7 lineage variant compared to other existing variants. However, subsequent studies have since reported that people infected with B.1.1.7 lineage variant had increased severity of disease compared to people infected with other circulating forms of virus variants.
- A large matched cohort study performed in the UK reported that the mortality hazard ratio of patients infected with B.1.1.7 lineage variant was 1.64 (95% confidence interval 1.32 to 2.04, P<0.0001) patients with previously circulating strains. Another study reported that the B 1.1.7 variant was associated with increased mortality compared to other SARS-CoV-2 variants (HR= 1.61, 95% CI 1.42-1.82). The risk of death was reportedly greater (adjusted hazard ratio 1.67, 95% CI 1.34-2.09) among individuals with confirmed B.1.1.7 variant of concern compared with individuals with non-1.1.7 SARS-CoV-2.
- The B.1.1.7 variant emerged as one of the most dominant SARS-CoV-2 strains circulating in the US.

• *Beta (B.1.351 lineage)*

- Another variant of SARS-CoV-2, **B.1.351** also referred to as **Beta variant** or **GH501Y.V2** with multiple spike mutations, resulted in the second wave of COVID-19 infections, was first detected in South Africa in October 2020.

- The B.1.351 variant includes nine mutations (L18F, D80A, D215G, R246I, K417N, E484K, N501Y, D614G, and A701V) in the spike protein, of which three mutations (K417N, E484K, and N501Y) are located in the RBD and increase the binding affinity for the ACE receptors. SARS-CoV-2 501Y.V2(B.1.351 lineage) was reported in the US at the end of January 2021.
- This variant is reported to have an increased risk of transmission and reduced neutralization by monoclonal antibody therapy, convalescent sera, and post-vaccination sera.

Gamma(P.1 lineage)

- The third variant of concern, the **P.1 variant** also known as **Gamma variant** or **GR/501Y.V3**, was identified in December 2020 in Brazil and was first detected in the US in January 2021.
- The B.1.1.28 variant harbors ten mutations in the spike protein (L18F, T20N, P26S, D138Y, R190S, H655Y, T1027I V1176, K417T, E484K, and N501Y). Three mutations (L18F, K417N, E484K) are located in the RBD, similar to the B.1.351 variant.
- Notably, this variant may have reduced neutralization by monoclonal antibody therapies, convalescent sera, and post-vaccination sera.

Delta (B.1.617.2 lineage)

- The fourth variant of concern, B.1.617.2 also referred to as the **Delta variant** was initially identified in December 2020 in India and was responsible for the deadly second wave of COVID-19 infections in April 2021 in India. In the United States, this variant was first detected in March 2021
- The Delta variant was initially considered a variant of interest. However, this variant rapidly spread around the world prompting the WHO to classify it as a VOC in May 2021
- The B.1.617.2 variant harbors ten mutations (T19R, (G142D*), 156del, 157del, R158G, L452R, T478K, D614G, P681R, D950N) in the spike protein

- Researchers have predicted the B.1.617.2 variant to be the most dominant SARS-CoV-2 strain in the US in the coming weeks

• *Omicron (B.1.1.529 lineage)*

- The fifth variant of concern **B.1.1.529**, also designated as the **Omicron variant** by the WHO was first identified in South Africa on 23 November 2021 after an uptick in the number of cases of COVID-19.
- Omicron was quickly recognized as a VOC due to more than 30 changes to the spike protein of the virus along with the sharp rise in thenumber of cases observed in South Africa. The reported mutations include T91 in the envelope, P13L, E31del, R32del, S33del, R203K, G204R in the nucleocapsid protein, D3G, Q19E, A63T in the matrix, N211del/L212I, Y145del, Y144del, Y143del, G142D, T95I, V70del, H69del, A67V in the N-terminal domain of the spike, Y505H, N501Y, Q498R, G496S, Q493R, E484A, T478K, S477N, G446S, N440K, K417N, S375F, S373P, S371L, G339D in the receptor-binding domain of the spike, D796Y in the fusion peptide of the spike, L981F, N969K, Q954H in the heptad repeat 1 of the spike as well as multiple other mutations in the non-structural proteins and spike protein.
- Initial modeling suggests that Omicron shows a 13-fold increase in viral infectivity, and is 2.8 times more infectious than the Delta variant. Early reports also suggest that monoclonal antibodies including Bamlanivimab and the Rockefeller University antibody C144 are likely to have reduced efficacy against the Omicron variant, however, REGN-COV2 (Casirivimab and Imdevimab), as well as the Rockefeller University antibody C135 are predicted to still be effective against Omicron based on early modeling studies.
- The Spike mutation K417N (also seen in the Beta variant) along with E484A is predicted to have an overwhelmingly disruptive effect, making Omicron more likely to have vaccine breakthroughs

Effect of SARS-CoV-2

COVID-19 is primarily considered a viral respiratory and vascular illness as its causative agent, SARS-CoV-2, predominantly targets the respiratory and vascular systems.

The pathogenesis of SARS-CoV-2 induced pneumonia is best explained by two stages, an early and a late phase. The early phase is characterized by viral replication resulting in direct virus-mediated tissue damage, which is followed by a late phase when the infected host cells trigger an immune response with the recruitment of T lymphocytes, monocytes, and neutrophil recruitment which releases cytokines such as tumor necrosis factor-α (TNF α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), interleukin-6 (IL-6),), IL-1β, IL-8, IL-12 and interferon (IFN)-γ. In severe COVID-19, the immune system's overactivation results in a 'cytokine storm' characterized by the release of high levels of cytokines, especially IL-6 and TNF-α, into the circulation, causing a local and systemic inflammatory response. The increased vascular permeability and subsequent development of pulmonary edema in patients with severe COVID-19 are explained by multiple mechanisms, which includes a) endotheliitis as a result of direct viral injury and perivascular inflammation leading to microvascular and microthrombi deposition b) dysregulation of the RAAS due to increased binding of the virus to the ACE2 receptors and c)activation of the kallikrein-bradykinin pathway, the activation of which enhances vascular permeability, d)enhanced epithelial cell contraction causing swelling of cells and disturbance of intercellular junctions. Besides IL-6 and TNF-α, the binding of SARS-CoV-2 to the Toll-Like Receptor (TLR) induces the release of pro-IL-1β, which is cleaved into the active mature IL-1β that mediates lung inflammation, until fibrosis.

It can affect other major organ systems such as the gastrointestinal tract (GI), hepatobiliary, cardiovascular, renal, and central nervous system. SARS-CoV-2–induced organ dysfunction, in general, is possibly explained by either one or a combination of the proposed mechanisms such as direct viral toxicity, ischemic injury caused by vasculitis, thrombosis, or thrombo-inflammation, immune dysregulation, and renin-angiotensin-aldosterone system (RAAS) dysregulation.

Conclusion

Besides the importance of imposing public health and infection control measures to prevent or decrease the transmission of SARS-CoV-2, the most crucial step to contain this global pandemic is by vaccination to prevent SARS-CoV-2 infection in communities across the world. Extraordinary efforts by clinical researchers worldwide during this pandemic have resulted in the development of novel vaccines against SARS-CoV-2 at an unprecedented speed to contain this viral illness that has devastated communities worldwide. Vaccination triggers the immune system leading to the production of neutralizing antibodies against SARS-CoV-2. As per the WHO Coronavirus (COVID-19) Dashboard, more than 2.4 billion doses of vaccine doses have been administered as of 22 June 2021 with approximately 22% of the world's population receiving at least one dose of the vaccine.

The four novel vaccines, BNT162b2 vaccine, mRNA-1273 vaccine, Ad26.COV2.S vaccine and ChAdOx1 nCoV-19 were developed to target the SARS-CoV-2 spike protein main site where these variants have developed mutations, raising concerns regarding the efficacy of these vaccines against the new variants.

Refferences

- Evolution patterns of SARS-CoV-2: Snapshot on its genome variants
  Marta Giovanetti , Francesca Benedetti, Giovanni Campisi , Alessandra Ciccozzi , Silvia Fabris, Giancarlo Ceccarelli, Vittoradolfo Tambone, Arnaldo Caruso, Silvia Angeletti, Davide Zella , Massimo Ciccozzi
- A Comprehensive Review of COVID-19 Virology, Vaccines, Variants, and Therapeutics
  Lauren Forchette, William Sebastian, and Tuoen Liu

- Features, Evaluation, and Treatment of Coronavirus (COVID-19)
  Marco Cascella; Michael Rajnik; Abdul Aleem; Scott C. Dulebohn; Raffaela Di Napoli.

- Properties of Coronavirus and SARS-CoV-2 Yasmin A. MALIK Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

- Structure-based design of prefusion-stabilized SARS-CoV-2 spikes

  Ching-Lin Hsieh, Jory A Goldsmith, Jeffrey M Schaub, Andrea M DiVenere, Hung-Che Kuo, Kamyab Javanmardi, Kevin C Le, Daniel Wrapp, Alison G Lee, Yutong Liu, Chia-Wei Chou, Patrick O Byrne , Christy K Hjorth , Nicole V Johnson , John Ludes-Meyers, Annalee W Nguyen, Juyeon Park, Nianshuang Wang , Dzifa Amengor , Jason J Lavinder , Gregory C Ippolito, Jennifer A Maynard, Ilya J Finkelstein , Jason S McLellan

- SARS-CoV-2 variants of concern are emerging in India.
  Jasdeep Singh, Syed Asad Rahman, Nasreen Z. Ehtesham, Subhash Hira & Seyed E. Hasnain
- A new coronavirus associated with human respiratory disease in China
  Fan Wu, Su Zhao, Bin Yu, Yan-Mei Chen, Wen Wang, Zhi-Gang Song, Yi Hu, Zhao-Wu Tao, Jun-Hua Tian, Yuan-Yuan Pei, Ming-Li Yuan, Yu-Ling Zhang, Fa-Hui Dai, Yi Liu, Qi-Min Wang, Jiao-Jiao Zheng, Lin Xu, Edward C. Holmes & Yong-Zhen Zhang

Phage therapy: An alternative to antibiotics

By : Santanu Kuilya

Reg. No. : A01-1112-116-025-2019

Examination Roll No. :2022151206

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Guided by : Dr. Bibhabasu Hazra

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

ACKNOWLEDGEMENT

I would like to acknowledge and give my warmest thanks to my supervisor Dr. Bibhabasu Hazra who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator in charge; revered Bhrahamachari Parachaitanya Maharaj for allowing me to participate DSE project, which help me to venture relevant meaningful topics.

I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

Phage therapy: An alternative to antibiotics

Abstract:

The exercise of phage remedy, which makes use of bacterial viruses (phages) to deal with bacterial infections, has been round for nearly a century. The widespread decline with inside the effectiveness of antibiotics has generated renewed hobby in revisiting this exercise. Conventionally, phage remedy is based on using naturally-taking place phages to contaminate and lyse microorganism on the web website online of infection. Biotechnological advances have in addition multiplied the repertoire of ability phage therapeutics to consist of novel techniques the use of bioengineered phages and purified phage lytic proteins. Current studies on using phages and their lytic proteins, especially towards multidrug-resistant bacterial infections, indicate phage remedy has the ability for use as both an opportunity and a complement to antibiotic treatments. Antibacterial therapies, whether or not phage- or antibiotic-based, every have relative blessings and disadvantages; accordingly, many concerns ought to be taken into consideration while designing novel healing strategies for stopping and treating bacterial infections. Although a whole lot continues to be unknown approximately the interactions among phage, microorganism, and human host, the time to take phage remedy severely appears to be unexpectedly approaching.

Keywords: Bacteriophage, Bacteriophage therapy, Phage, Phage therapy.

INTRODUCTION:

Almost a decade before the discovery of penicillin, the controversial practice of phage therapy was being developed as a treatment for bacterial infections. Phages, short for bacteriophages, are bacteria-specific viruses that have been used as a treatment against pathogens such as Shigella dysenteriae as early as 1919. With an estimated 1031-1032 phages in the world at any given time, they make up the most abundant biological entity on Earth and play a crucial role in regulating bacterial populations; phages are responsible for the death of approximately 20%-40% of all marine surface bacteria every 24 h. Much of the controversy surrounding phage therapy was due to poor documentation of use and variable success. The complications in implementing phage therapy stemmed from how little was known about phages at the time of their discovery. In fact, the nature of their existence was a topic of contention until they were visualized in the 1940's after the invention of electron microscopy. A number of logistical and technical obstacles in developing phage therapy led to its widespread abandonment after the discovery of antibiotics.

The advent of pharmaceutical antibiotics in the mid-20th century, along with a better understanding of disease and sanitation, revolutionized healthcare and drastically improved both quality of life and life expectancy in the industrialized world. In 1900, life expectancy for men and women in the United States was 46 and 48, respectively, and the major causes of death were infectious diseases, many of which were bacterial (e.g., cholera, diphtheria,

typhoid fever, plague, tuberculosis, typhus, scarlet fever, pertussis, and syphilis). Antibiotics helped usher in a new era in medicine, rapidly becoming an indispensable medical tool with 262.5 million treatment courses prescribed in the United States in 2011 alone (842 prescriptions per 1000 persons) and an estimated 100000-200000 tons of antibiotics used globally between medicine, agriculture, and horticulture each year. Antibiotic resistance genes encoding for bacterial resistance to common antibiotics, including β-lactams, aminoglycosides, chloramphenicols, and tetracycline, are posing a major threat to current medical treatment of common diseases, and these genes now appear to be abundant in the environment. The spread of antibiotic resistance genes carries a unique danger in that many antibiotics have diminishing efficacy against common infections, particularly the difficult-to-treat nosocomial infections caused by the ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.).

Admonitions of a return to “the pre-antibiotic era” have become increasingly common and regulatory organizations such as the Centers for Disease Control (CDC) and WHO have declared antibiotic resistance a threat to global health. The CDC estimates antibiotic-resistant infections result in 2 million illnesses and at least 23000 deaths a year, with many more dying from conditions complicated by antibiotic resistant infections, costing the United States $55 billion annually. According to the United Kingdom government’s 2016 Review on Antimicrobial Resistance, an estimated 700000 people die each year globally from resistant infections with a projected cost of $100 trillion and a death toll of 10 million by 2050. In the United States, methicillin-resistant *S. aureus* (MRSA) infections alone account for more deaths than HIV/AIDS and tuberculosis combined. Since the discovery of antibiotics, there has been a steady stream of novel antibacterial pharmaceuticals in what has been dubbed the “antibiotic pipeline”. However, due to the rate at which bacteria evolve resistance to antibiotics, there has been less commercial interest in the research and development of novel compounds. In the years of 1983-1987, there were 16 new pharmaceutical antibiotics approved by the Food and Drug Administration (FDA) for use in the United States, this number has steadily trended downwards and between 2010-2016 only 6 new antibiotics were approved[12]. At the end of the antibiotic pipeline is the carbapenem class of antibiotics, often reserved as the “last resort” due to their adverse effects on health. Beginning in 2000, the incidence of carbapenem-resistant, hospital-acquired *K. pneumoniae* infections began to increase in the United States; due to the lack of treatment options, these infections are associated with a 40%-50% mortality rate. Reaching the end of the antibiotic pipeline could signal a shift in the global culture of infectious disease treatment and some claim is the imminent return to a pre-antibiotic era of medicine.

On September 21, 2016, the United Nations General Assembly convened to discuss the problem of antibiotic resistance and deemed it “the greatest and most urgent global risk”. In the hunt for alternative strategies for prophylaxis and control of bacterial infection, one of the

more popular suggestions involves revisiting the practice of phage therapy. Proponents of phage therapy tout several major advantages that phages have over antibiotics such as host-specificity, self-amplification, biofilm degradation, and low toxicity to humans.Owing to the development of analytical tools capable of studying these small biological entities (approximately 25-200 nm in length), such as next-generation sequencing and electron microscopy, the field of phage biology is only now reaching maturity. These technological advancements have ushered in a renaissance of phage therapy research as indicated by a wave of recent human clinical trials and animal research. To fully evaluate the viability of phage therapy, one must also consider the role of the indigenous gut phageome in human health and disease. However, this complex story is only beginning to unfold and will not be included in this review (for current literature review see Wahida, Ritter and Horz in 2016). This review aims at discussing historical use of phage therapy and current research on the feasibility of phage-based infection control with a focus on multidrug-resistant infections.

PHAGE BIOLOGY BASICS:

Phages are simple, but distinctly diverse, non-dwelling organic entities together with DNA or RNA enclosed inside a protein capsid. As naturally-going on bacterial parasites, phages are incapable of reproducing independently (i.e., non-dwelling) and are in the end depending on a bacterial host for survival. Phages usually bind to particular receptors at the bacterial mobileular surface, inject their genetic fabric into the host mobileular, after which both combine this fabric into the bacterial genome (so-called “temperate” phages) and reproduce vertically from mom to daughter mobileular, or hijack the bacterial replication equipment to supply the following technology of phage progeny and lyse the mobileular (so-called “lytic” phages). Upon achieving a important mass of phage progeny, which may be everywhere from some to over one thousand viral particles, relying on environmental factors, the lytic proteins emerge as lively and hydrolyze the peptidoglycan mobileular wall, freeing novel phage to reinitiate the lytic cycle .Most phages are infectious best to the microorganism that convey their complementary receptor, which successfully determines lytic phage host range[20]. Host specificity varies amongst phages, a number of that are pressure-particular, while others have established the functionality of contamination throughout a number of bacterial traces or even genera .Bacteria have developed severe mechanisms to withstand contamination via way of means of lytic phages, and phages have an similarly magnificent range of mechanisms for breaking this resistance. For microorganism, this may encompass alteration or lack of receptors and integration of phage DNA into the clustered often interspaced palindromic repeats/CRISPR related system (CRISPR/Cas) system, at the same time as for phage this may encompass reputation of recent or altered receptors and anti-CRISPR genes. The maximum not unusual place lytic phages related to human pathogens and the intestine microbiota are with inside the orders *Caudovirale*s, usually regarded as “tailed phages” which incorporate double-stranded DNA genomes, and *Microviridae*, that are tailless, single-stranded DNA viruses . In comparison to lytic phages, lysogenic phages combine their genetic fabric into the bacterial chromosome with inside the shape of an endogenous prophage (much less usually phage DNA can stay separate as a plasmid however nevertheless be stably transmitted throughout bacterial generations). The bacterial lysogenic then propagates the prophage with

every mobileular division. Environmental stressors at the bacterial host are able to inducing the lysogenic phage from the latent prophage shape, triggering a transition to the lytic cycle and the discharge of phage progeny into the environment. When incorporating their genetic fabric into the bacterial genome, prophage-encoded genes emerge as to be had for transcription via way of means of the host. Up to 18 prophages had been located in a single bacterial genome, as with inside the meals pathogen Escherichia coli *(E. coli)* O157:H7 pressure Sakai, with prophage-encoded genes comprising as much as 20% of bacterial chromosomal content. Prophage genes may be useful to the bacterial host and may encode for virulence factors (e.g., diphtheria toxin, shiga toxin, and botulinum toxin), metabolic genes, and antibiotic resistance genes (e.g., β-lactamases)[29-32]. Phage biologists now understand that phage lifecycles can fall on a spectrum among lytic and lysogenic with pseudolysogenic, chronic, and cryptic lifecycles as examples of new classifications. Conventional phage remedy is predicated on strictly lytic phages, which obligately kill their bacterial host. For treatment, lytic phages are compiled into arrangements called “phage cocktails” which encompass more than one phages tested to have in vitro efficacy towards the goal pathogen.

PHAGE THERAPY VS ANTIBIOTIC THERAPY:

Both antibiotics and phages function as antibacterials that disrupt bacterial colonies through lysis or inhibition, yet several key differences make each antibacterial more or less appropriate depending on the situation.

Safety:

Adverse reactions to antibiotics are properly documented and consist of times of anaphylaxis, nephrotoxicity, cardiotoxicity, hepatotoxicity, and neurotoxicity, in addition to some of gastrointestinal and hematological headaches. The majority of damaging reactions; in those uncommon times the anaphylaxis is related to unique training of antibiotics or is the made from excessive tissue concentrations . In evaluation to the complete literature on antibiotic protection, phage remedy has best lately received interest via way of means of western remedy and, as a result, a great deal of the to be had statistics on phage protection is new. Although oral phage management is normally taken into consideration to be safe, a chief attention for phage remedy is the translocation of phage throughout the intestinal epithelium in which they ultimately flow into withinside the blood .Some facts display that phage translocation can also additionally advantage the host via way of means of downregulating the immune reaction to indigenous intestine microbe antigens thru the inhibition of interleukin-2, tumor necrosis factor, and interferon gamma production. Other research observed a bunch innate immune reaction geared toward getting rid of phage after management in mice. While the professionals of phage remedy probably outweigh the cons in non-immunocompromised patients, the immunological reaction to phage can be indicative of the capability for an damaging response in immunocompromised patients, that can hypothetically get worse a patient's condition. There is presently no consensus in this opportunity as different researchers argue it's miles not likely phage remedy will elicit such an damaging response in immunocompromised patients. Additional headaches consist of the

opportunity that phage cocktails set off a kingdom of intestinal barrier disorder, in any other case acknowledged as “leaky intestine”. Tetz and Tetz used a mouse version to illustrate that oral management of a business Russian phage cocktail changed into able to growing intestinal permeability and raising serum degrees of inflammatory circulating immune complexes withinside the blood, which might be related to some of pathological conditions .However, any other take a look at discovered no extensive boom in cytokine degrees in reaction to phage treatment .The capability for phage remedy to disrupt ordinary intestinal barrier feature could have critical implications for numerous problems lately connected to intestinal barrier disorder consisting of Crohn’s disease, inflammatory bowel disease, and kind 1 diabetes .Pincus et al, observed that the inflammatory reaction to phage changed into depending on web website online of infection. Clearly, most of the protection worries with phage remedy nevertheless want to be addressed. It is probably that the physiological reaction to phages additionally differs among people and is depending on the unique phage traces used. To decide the protection of phage remedies with reference to human health, destiny investigations will want to cognizance on human scientific trials as a great deal of the modern studies at the immunological reaction to phage is restricted to animal models.

Specificity:

In stark comparison to antibiotics, phages have a tendency to be precise toward each species and stress. In sure conditions this may be a chief benefit, thinking about the well-documented, collateral consequences of broad-spectrum antibiotics on commensal intestine microbes, which might be infamous for secondary results along with antibiotic-related diarrhea and *C. difficile* infection. Other results of antibiotic perturbations withinside the intestine microbial network encompass danger of asthma, obesity, and diabetes .The modern information of collateral harm because of phage remedy is constrained, but, as compared to antibiotics, phage remedy has been said to bring about much less perturbation of the intestine microbiome even as nevertheless successfully decreasing intestine carriage of pathogens along with *Shigella sonnei* and uropathogenic *E. coli*. While stress and species specificity of antibacterial compounds gives many advantages, it comes with some of inherent constraints. By concentrated on a unmarried pathogen, phage remedy can be much less powerful in opposition to infections along with inflamed burn wounds, which might be regularly colonized through multiple stress of microorganism .This may be accounted for through growing phage cocktails infective in opposition to a number of regarded pathogens, however the fulfillment of this method relies upon on expertise of which pathogens are being treated. Logistically, host specificity substantially affects remedy improvement and testing, and additionally limits the opportunity of large-scale manufacturing and distribution, a wonderful benefit of broad-spectrum antibiotics. Bourdin et al, cross-inoculated phages from 2 wonderful geographic areas (Mexico and Bangladesh) in opposition to diarrhea-related E. coli from the identical areas and determined that phage confirmed excessive stress specificity to the E. coli in their indigenous region. In a randomized scientific trial, administered a normally used Russian E. coli phage cocktail to a cohort of a hundred and twenty Bangladeshi kids with microbiologically-validated enterotoxogenic E. coli diarrhea. No development of scientific final results changed into located in sufferers receiving the phage

cocktail as compared to placebo. These findings are in step with the in vitro paintings that shows phage cocktails are higher tailored to nearby bacterial populations, and bacterial host variety may be limited each spatially and temporally. In an in vitro cross-inoculation of a phage cocktail in opposition to shiga toxin-generating *E. coli* O157:H7, lysis took place in isolates of each human and bovine origin, which shows a capacity for nearby phage cocktails for each scientific and agricultural settings . Determined that phages concentrated on antibiotic-resistant microorganism are much more likely to be determined withinside the surroundings of the inflamed patient, which, on this case, changed into the sanatorium effluent wherein the antibiotic-resistant microorganism have been isolated. Regional specificity might also additionally consequently be high quality while searching out phages that focus on precise bacterial strains. Regional specificity can be useful in locating phages with the finest infectivity toward the goal pathogen, this will particularly gain areas with constrained get entry to to antibiotics. Together, the mounting proof for the nearby adaptivity of phage shows that regulatory pipelines have to additionally be unexpectedly adaptable (i.e., taking into consideration the alternative or addition of phages into cocktails with out requiring in addition scientific trials) for phage remedy to paintings on a worldwide scale.

Biofilm penetration:

Antibiotic remedy is especially powerful with planktonic microorganism, inclusive of V. cholerae and Yersinia pestis, but is constrained in treating biofilm-primarily based totally bacterial infection. Phages, however, are ready with enzymes (e.g., EPS depolymerase) at the outdoors of the capsid that degrade the extracellular polymeric substances (EPS) and disperse bacterial biofilms, permitting the phage to get right of entry to microorganism embedded in the EPS matrix .The phage progeny launched upon of entirety of the lytic cycle propagate the dispersal of the biofilm via the elimination of biofilm-embedded microorganism in next layers .In order to penetrate dense biofilms, excessive doses of antibiotics are usually required to have a look at any inhibition of bacterial growth, but whole eradication is uncommon and regrowth of colonies starts after the cease of antibiotic remedies .Although low concentrations of many antibiotics are usually taken into consideration non-toxic, excessive concentrations can bring about tissue toxicity , on the Eliava Institute of Bacteriophages in Tbilisi, Georgia discovered that the utility of phages on in vitro colonies of the pathogen *P. aeruginosa* now no longer best averted extra biofilm formation via way of means of the pathogen however additionally degraded current biofilm. Phage remedies have removed biofilms fashioned via way of means of *L. monocytogenes*, *P. aeruginosa*, and Staphylococcus epidermidis at the floor of scientific gadgets .These findings are especially applicable to the hassle of continual infections due to implanted scientific gadgets inclusive of catheters, lenses, and prostheses in which biofilm formation is common.

Phage cocktails:

Due to the big variety of environmental phages, designing a phage cocktail is considerably extra complex than designing a routine for mixture antibiotic remedy. Composition of the phage cocktail is important for the achievement of phage remedy. Factors withinside the creation of a phage cocktail are past the scope of this overview and were very well mentioned elsewhere, however one of the essential logistical demanding situations is whether or not to method phage remedy with a standardized or a custom designed cocktail. Customizing phage cocktails to every contamination is time ingesting and luxurious however, on the opposite stop of the spectrum, a "one-size-fits-all" method might not offer the stress specificity required for favorable scientific outcomes. Other concerns are the collateral consequences of phages at the indigenous microbiota, a subject that has now no longer but been completely explored[88,94,95]. In cocktail layout, one ought to additionally bear in mind phage lifecycle. Lysogenic phages appear like very not unusualplace withinside the indigenous intestine microbiota, with prophages comprising the bulk of the intestine virome .Some therapeutically promising lysogenic phages correctly silence virulence genes in pathogenic microorganism or offer genes for brief chain fatty acid metabolism, while different lysogenic phages complement genes for virulence and antibiotic resistance .Antibiotic resistance genes were accrued from the phage fraction of DNA in wastewater and were pronounced to persist longer in phages than in microorganism. Antibiotic resistance genes also are gift withinside the phage fraction of human fecal samples and antibiotic remedy in mice enriches the abundance of phage-encoded antibiotic resistance genes, indicating a probable function for phages as a reservoir for antibiotic resistance genes .The hypothetical ability for lysogenic phages to complicate current infections thru the horizontal switch of antibiotic resistance genes to infectious microorganism in large part excludes them from attention for maximum phage cocktails. Yet, Regeimbal et al , proven the opportunity for an modern software of lysogenic phages via way of means of designing an "clever" five phage cocktail that removed *A. baumannii* pores and skin wound contamination in a mouse model. This clever phage cocktail changed into composed of four phages that had been incapable of lysing the *A. baumannii* host and 1 phage that best inhibited boom in vitro. The boom-inhibiting phage focused capsulated *A. baumannii*, deciding on for the lack of the capsule. The elimination of the capsule, a recognized virulence factor, reduced the virulence of the bacterium and made it liable to lysis from the four extra phages. This kind of cocktail layout represents the start of novel remedy alternatives for getting rid of bacterial infections which can be resistant to traditional remedy. Lysogenic phages have many fascinating homes that can be beneficial for this kind of in situ manipulation of character bacterium, and probably the human intestine microbiome metagenome , however first lots extra desires to be recognized approximately the function of lysogenic phages withinside the human intestine phageome for this to be completed correctly and correctly.

CONCLUSION:

The to be had literature on using phages and phage-derived proteins for fighting bacterial infections, particularly the ones of multidrug-resistant bacteria, an increasing number of

indicates promise for the possibility of phage remedy as both an opportunity or a complement to antibiotics. However, discrepancies in current findings at the immunomodulatory effects, the host range, and the capacity for horizontal gene switch make it abundantly clean that we want a higher knowledge of the interplay among phage, microbiome, and human host earlier than enforcing phage remedy on a huge scale. Phage lysins might also additionally consequently be a far greater sensible healing device for his or her reduced immunological capacity, amongst different motives consisting of ease of production, purification, and storage. Despite the promising initial findings on phage and phage-derived lytic proteins, it's miles greater than possibly that no panacea for antibiotic-resistant infections will arise. The elevated efficacy of antibacterial marketers while utilized in conjunction means that remedy the usage of a few mixture of phage, phage-derived lytic proteins, bioengineered phage, and/or antibiotics could be essential for addressing the developing trouble of antibiotic-resistant infections.

REFERENCES:

1. Chanishvili N. Phage therapy--history from Twort and d'Herelle through Soviet experience to current approaches. Adv Virus Res. 2012;83:3–40. [PubMed] [Google Scholar]

2. Suttle CA. Marine viruses--major players in the global ecosystem. Nat Rev Microbiol. 2007;5:801–812. [PubMed] [Google Scholar]

3. Wittebole X, De Roock S, Opal SM. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. Virulence. 2014;5:226–235. [PMC free article] [PubMed] [Google Scholar]

4. Ackermann HW. The first phage electron micrographs. Bacteriophage. 2011;1:225–227. [PMC free article] [PubMed] [Google Scholar]

5. Yoshikawa TT. Antimicrobial resistance and aging: beginning of the end of the antibiotic era? J Am Geriatr Soc. 2002;50:S226–S229. [PubMed] [Google Scholar]

6. Hicks LA, Bartoces MG, Roberts RM, Suda KJ, Hunkler RJ, Taylor TH, Schrag SJ. US outpatient antibiotic prescribing variation according to geography, patient population, and provider specialty in 2011. Clin Infect Dis. 2015;60:1308–1316. [PubMed] [Google Scholar]

7. Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, Vlieghe E, Hara GL, Gould IM, Goossens H, et al. Antibiotic resistance-the need for global solutions. Lancet Infect Dis. 2013;13:1057–1098. [PubMed] [Google Scholar]

8. Zhang XX, Zhang T, Fang HH. Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol. 2009;82:397–414. [PubMed] [Google Scholar]

9. Centers for Disease Control. 2017. Antibiotic Resistance: The Global Threat. 2015; Accessed Mar 29, 2017. Available from: https://www.cdc.gov/drugresistance/pdf/antibiotic_resistant_fs.pdf. [Google Scholar]

10. World Health Organization. 2017. Antibiotic resistance - a threat to global health security. 2013. Accessed Mar 29. Available from: http://www.who.int/drugresistance/activities/wha66_side_event/en/ [Google Scholar]

11. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009;48:1–12. [PubMed] [Google Scholar]

12. Luepke KH, Suda KJ, Boucher H, Russo RL, Bonney MW, Hunt TD, Mohr JF. Past, Present, and Future of Antibacterial Economics: Increasing Bacterial Resistance, Limited Antibiotic Pipeline, and Societal Implications. Pharmacotherapy. 2017;37:71–84. [PubMed] [Google Scholar]

13. Centers for Disease Control and Prevention. Vital signs: carbapenem-resistant Enterobacteriaceae. MMWR Morb Mortal Wkly Rep. 2013;62:165–170. [PMC free article] [PubMed] [Google Scholar]

14. United Nations. 2017. PRESS RELEASE: High-Level Meeting on Antimicrobial Resistance. 2016; Accessed Mar 29, 2017. Available from: http://www.un.org/pga/71/2016/09/21/press-release-hlmeeting-on-antimicrobial-resistance/ [Google Scholar]

15. Bourdin G, Navarro A, Sarker SA, Pittet AC, Qadri F, Sultana S, Cravioto A, Talukder KA, Reuteler G, Brüssow H. Coverage of diarrhoea-associated Escherichia coli isolates from different origins with two types of phage cocktails. Microb Biotechnol. 2014;7:165–176. [PMC free article] [PubMed] [Google Scholar]

16. Donlan RM. Preventing biofilms of clinically relevant organisms using bacteriophage. Trends Microbiol. 2009;17:66–72. [PubMed] [Google Scholar]

17. Wahida A, Ritter K, Horz HP. The Janus-Face of Bacteriophages across Human Body Habitats. PLoS Pathog. 2016;12:e1005634. [PMC free article] [PubMed] [Google Scholar]

18. Delbrück M. The growth of bacteriophage and lysis of the host. J Gen Physiol. 1940;23:643–660. [PMC free article] [PubMed] [Google Scholar]

19. Weinbauer MG. Ecology of prokaryotic viruses. FEMS Microbiol Rev. 2004;28:127–181. [PubMed] [Google Scholar]

20. Rakhuba DV, Kolomiets EI, Dey ES, Novik GI. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. Pol J Microbiol. 2010;59:145–155. [PubMed] [Google Scholar]

21. Koskella B, Meaden S. Understanding bacteriophage specificity in natural microbial communities. Viruses. 2013;5:806–823. [PMC free article] [PubMed] [Google Scholar]

22. Motlagh AM, Bhattacharjee AS, Goel R. Biofilm control with natural and genetically-modified phages. World J Microbiol Biotechnol. 2016;32:67. [PubMed] [Google Scholar]

23. Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. Nat Rev Microbiol. 2010;8:317–327. [PubMed] [Google Scholar]

24. Koskella B, Brockhurst MA. Bacteria-phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS Microbiol Rev. 2014;38:916–931. [PMC free article] [PubMed] [Google Scholar]

25. Minot S, Sinha R, Chen J, Li H, Keilbaugh SA, Wu GD, Lewis JD, Bushman FD. The human gut virome: inter-individual variation and dynamic response to diet. Genome Res. 2011;21:1616–1625. [PMC free article] [PubMed] [Google Scholar]

26. Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, Gordon JI. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature. 2010;466:334–338. [PMC free article] [PubMed] [Google Scholar]

27. Ohnishi M, Kurokawa K, Hayashi T. Diversification of Escherichia coli genomes: are bacteriophages the major contributors? Trends Microbiol. 2001;9:481–485. [PubMed] [Google Scholar]

28. Hatfull GF, Hendrix RW. Bacteriophages and their genomes. Curr Opin Virol. 2011;1:298–303. [PMC free article] [PubMed] [Google Scholar]

29. Penadés JR, Chen J, Quiles-Puchalt N, Carpena N, Novick RP. Bacteriophage-mediated spread of bacterial virulence genes. Curr Opin Microbiol. 2015;23:171–178. [PubMed] [Google Scholar]

30. Modi SR, Lee HH, Spina CS, Collins JJ. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. Nature. 2013;499:219–222. [PMC free article] [PubMed] [Google Scholar]

31. Quirós P, Colomer-Lluch M, Martínez-Castillo A, Miró E, Argente M, Jofre J, Navarro F, Muniesa M. Antibiotic resistance genes in the bacteriophage DNA fraction of human fecal samples. Antimicrob Agents Chemother. 2014;58:606–609. [PMC free article] [PubMed] [Google Scholar]

32. Colomer-Lluch M, Jofre J, Muniesa M. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. PLoS One. 2011;6:e17549. [PMC free article] [PubMed] [Google Scholar]

33. Wang X, Wood TK. Cryptic prophages as targets for drug development. Drug Resist Updat. 2016;27:30–38. [PubMed] [Google Scholar]

34. Sulakvelidze A, Alavidze Z, Morris JG. Bacteriophage therapy. Antimicrob Agents Chemother. 2001;45:649–659. [PMC free article] [PubMed] [Google Scholar]

35. Hodyra-Stefaniak K, Miernikiewicz P, Drapała J, Drab M, Jończyk-Matysiak E, Lecion D, Kaźmierczak Z, Beta W, Majewska J, Harhala M, et al. Mammalian Host-Versus-Phage immune response determines phage fate in vivo. Sci Rep. 2015;5:14802. [PMC free article] [PubMed] [Google Scholar]

36. Carlton RM. Phage therapy: past history and future prospects. Arch Immunol Ther Exp (Warsz) 1999;47:267–274. [PubMed] [Google Scholar]

37. Weber-Dabrowska B, Mulczyk M, Górski A. Bacteriophage therapy of bacterial infections: an update of our institute's experience. Arch Immunol Ther Exp (Warsz) 2000;48:547–551. [PubMed] [Google Scholar]

38. Watanabe R, Matsumoto T, Sano G, Ishii Y, Tateda K, Sumiyama Y, Uchiyama J, Sakurai S, Matsuzaki S, Imai S, et al. Efficacy of bacteriophage therapy against gut-derived sepsis caused by Pseudomonas aeruginosa in mice. Antimicrob Agents Chemother. 2007;51:446–452. [PMC free article] [PubMed] [Google Scholar]

39. Ramesh V, Fralick JA, Rolfe RD. Prevention of Clostridium difficile-induced ileocecitis with bacteriophage. Anaerobe. 1999;5:69–78. [Google Scholar]

40. Nale JY, Spencer J, Hargreaves KR, Buckley AM, Trzepiński P, Douce GR, Clokie MR. Bacteriophage Combinations Significantly Reduce Clostridium difficile Growth In Vitro and Proliferation In Vivo. Antimicrob Agents Chemother. 2016;60:968–981. [PMC free article] [PubMed] [Google Scholar]

41. Biswas B, Adhya S, Washart P, Paul B, Trostel AN, Powell B, Carlton R, Merril CR. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant Enterococcus faecium. Infect Immun. 2002;70:204–210. [PMC free article] [PubMed] [Google Scholar]

42. Wang J, Hu B, Xu M, Yan Q, Liu S, Zhu X, Sun Z, Tao D, Ding L, Reed E, et al. Therapeutic effectiveness of bacteriophages in the rescue of mice with extended spectrum beta-lactamase-producing Escherichia coli bacteremia. Int J Mol Med. 2006;17:347–355. [PubMed] [Google Scholar]

43. Wang J, Hu B, Xu M, Yan Q, Liu S, Zhu X, Sun Z, Reed E, Ding L, Gong J, et al. Use of bacteriophage in the treatment of experimental animal bacteremia from imipenem-resistant Pseudomonas aeruginosa. Int J Mol Med. 2006;17:309–317. [PubMed] [Google Scholar]

44. Soothill JS. Treatment of experimental infections of mice with bacteriophages. J Med Microbiol. 1992;37:258–261. [PubMed] [Google Scholar]

45. Pouillot F, Chomton M, Blois H, Courroux C, Noelig J, Bidet P, Bingen E, Bonacorsi S. Efficacy of bacteriophage therapy in experimental sepsis and meningitis caused by a clone O25b: H4-ST131 Escherichia coli strain producing CTX-M-15. Antimicrob Agents Chemother. 2012;56:3568–3575. [PMC free article] [PubMed] [Google Scholar]

46. Jun JW, Shin TH, Kim JH, Shin SP, Han JE, Heo GJ, De Zoysa M, Shin GW, Chai JY, Park SC. Bacteriophage therapy of a Vibrio parahaemolyticus infection caused by a multiple-antibiotic-resistant O3: K6 pandemic clinical strain. J Infect Dis. 2014;210:72–78. [PMC free article] [PubMed] [Google Scholar]

47. Wills QF, Kerrigan C, Soothill JS. Experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model. Antimicrob Agents Chemother. 2005;49:1220–1221. [PMC free article] [PubMed] [Google Scholar]

48. Chan BK, Sistrom M, Wertz JE, Kortright KE, Narayan D, Turner PE. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. Sci Rep. 2016;6:26717. [PMC free article] [PubMed] [Google Scholar]

49. Kutateladze M, Adamia R. Phage therapy experience at the Eliava Institute. Med Mal Infect. 2008;38:426–430. [PubMed] [Google Scholar]

50. Fish R, Kutter E, Wheat G, Blasdel B, Kutateladze M, Kuhl S. Bacteriophage treatment of intransigent diabetic toe ulcers: a case series. J Wound Care. 2016;25 Suppl 7:S27–S33. [Google Scholar]

51. Chanishvili N, Sharp R. Bacteriophage therapy: experience from the Eliava Institute, Georgia. Microbiol Australia. 2008;29:96–101. [Google Scholar]

52. Kutateladze M, Adamia R. Bacteriophages as potential new therapeutics to replace or supplement antibiotics. Trends Biotechnol. 2010;28:591–595. [PubMed] [Google Scholar]

53. Monk AB, Rees CD, Barrow P, Hagens S, Harper DR. Bacteriophage applications: where are we now? Lett Appl Microbiol. 2010;51:363–369. [PubMed] [Google Scholar]

54. Nannapaneni R, Soni KA. USA: John Wiley & Sons, Ltd; 2015. Use of Bacteriophages to Remove Biofilms of Listeria monocytogenes and other Foodborne Bacterial Pathogens in the Food Environment. Biofilms in the Food Environment, Second Edition; pp. 131–144. [Google Scholar]

55. Endersen L, O'Mahony J, Hill C, Ross RP, McAuliffe O, Coffey A. Phage therapy in the food industry. Annu Rev Food Sci Technol. 2014;5:327–349. [PubMed] [Google Scholar]

56. Schofield DA, Sharp NJ, Vandamm J, Molineux IJ, Spreng KA, Rajanna C, Westwater C, Stewart GC. Bacillus anthracis diagnostic detection and rapid antibiotic susceptibility determination using 'bioluminescent' reporter phage. J Microbiol Methods. 2013;95:156–161. [PubMed] [Google Scholar]

Project report on :

Cholera Disease

Submitted by : **Raju Mondal**

Reg.no. : **A01-1112-116-023-2019**

Roll no. : **624** semester : **v**

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Guided by: Debasish Malik
Department of Microbiology

ABSTRACT

Cholera is an acute diarrhoeal disease that can kill within hours if left untreated. Researchers have estimated that each year there are 1.3 to 4.0 million cases of cholera, and 21 000 to 143 000 deaths worldwide due to cholera (1) Most of those infected will have no or mild symptoms and can be successfully treated with oral re-hydration solution.Severe cases will need rapid treatment with intravenous fluids and antibiotics.Provision of safe water and sanitation is critical to prevent and control the transmission of cholera and other waterborne diseases.Oral cholera vaccines should be used in conjunction with improvements in water and sanitation to control cholera outbreaks and for prevention in areas known to be high risk for cholera.A global strategy on cholera control, Ending Cholera: a global roadmap to 2030, with a target to reduce cholera deaths by 90% was launched in 2017

INTRODUCTION

Cholera is an acute secretory diarrheal illness caused by the bacteria Vibrio cholerae. It is estimated to cause upwards of four million cases per year, worldwide. High-volume fluid loss with electrolyte derangements that can progress to hypovolemic shock and ultimately death characterizes this gastrointestinal disease.[1][2][3] The infection is transmitted via the fecal-oral route and can vary in severity. The key is replacing the fluid and electrolytes lost as soon as possible.

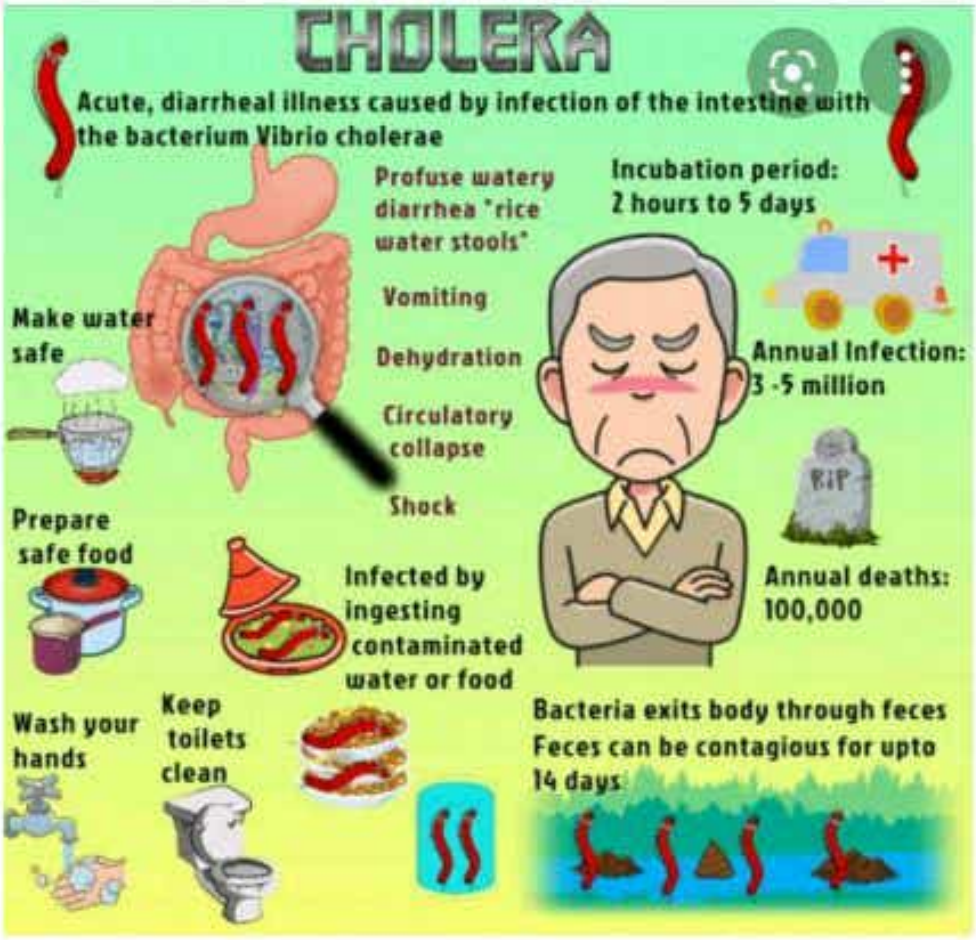


Fig: Diagram of Cholera disease...Source: histopathology-india.net

ETIOLOGY

TRANSMISSION AND THE ROUTE OF TRANSMISSION

Vibrio cholerae is a facultative, gram-negative, comma-shaped, oxidase-positive rod that is prevalent in developing countries. Two serotypes have been identified to cause outbreaks. O1 is responsible for all recent outbreaks, whereas O139 causes sporadic outbreaks, specifically in Asia. There is no etiologic difference between the two. V. cholerae is found in food (classically shellfish) and poorly sanitized water. The bacteria is known to spread via the fecal-oral route and is thus endemic to areas associated with inadequate food and water hygiene.[4][5]

The organism is acquired via the fecal-oral route and a large dose is required to develop infectivity. Factors that increase susceptibility include:

Use of proton-pump inhibitors (PPIs) and antihistamines

Poor sanitation

Overcrowding

Prior vagotomy

Helicobacter pylori infection

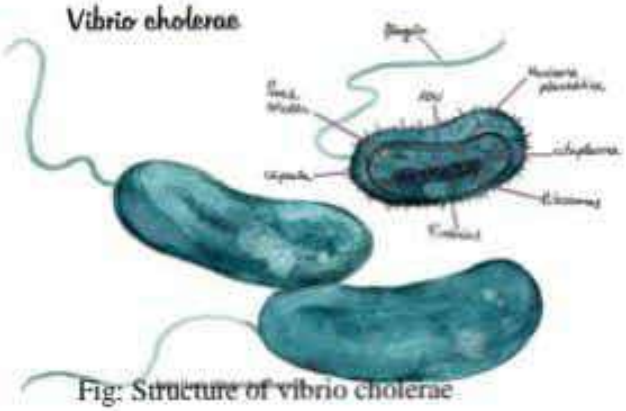


Fig: Structure of vibrio cholerae

Epidemiology

There are about four million cases of cholera worldwide annually, with over 140,000 deaths attributed to the disease. Nearly 1.8 million people worldwide obtain their drinking water from sources contaminated with human feces that may act as a reservoir for the cholera bacteria. Outbreaks are known to occur, specifically in the developing world where sanitation and water filtration standards may not exist. Currently, cholera is known to be endemic in approximately 50 nations, mostly throughout Asia and Africa. The incidence is tied to a seasonal distribution, depending on the timing of the region's rainy season. Epidemics can be more widespread, however, involving other parts of the world, including South and Central America. The introduction of the species to a new region with a collapse of hygiene and health services has been known to lead to the propagation of epidemics.

Transmission

Cholera disease is usually transmitted by ingestion of food or water contaminated directly or indirectly by the excretions of infected people, e.g. faeces or vomit (Tauxe and Blake, 1992; Rabbani and Greenough, 1999). In historical treaties on cholera, seaborne transport of cholera provides the fundamental theory of propagation. However, early records show an association with contaminated water, rivers or swampy areas, or marshes where the streams were much reduced (Colwell, 1996). Cholera infects only humans and always transmits by fecal oral route; there is no insect vector or animal reservoir hosts (WHO, 2010a), but V. cholerae is able to accumulate in zooplankton, which are fed-upon by oysters (Colwell, 2013

Two main transmission routes of V. cholerae have been documented. The first and primary route is via the aquatic environment, and the second route occurs when the infection is transmitted from infected to healthy people by water or food contamination (Ruiz-Moreno et al., 2010). Once the primary transmission has initiated an outbreak, the secondary transmission causes epidemics in the endemic areas.

Incubation period

The incubation period is usually very short and ranges from 2 h to 5 days. After a typical incubation period of 1to 2 days, there can be an extended period up to 5 days in some cases; the infected people experience a sudden onset of watery diarrhoea. Vomiting is common and often precedes the onset of diarrhoea with fluid loss often 500 to 1,000 mL/h, leading to severe dehydration. Over a 24 h period, up to 20 litres of watery diarrhoea can be excreted in adults. If left untreated, the mortality rate may reach 40 to 60% (Speck, 1993; Sack et al., 2004; WHO, 2009a). The stool of the infected people became highly infectious containing high counts of choleragenic V. cholerae up to 109 CFU/mL of stool (Dizon et al., 1967). Without antibiotic treatment, organisms are secreted in the stool for an average of 6 days
(Lindenbaum et al., 1967). The incubation period for non-cholera-genic Vibrio spp. infection is usually 12 h to 3 days, but can be extended for up to one week (Seas and Gotuzzo, 2009).

Reservoirs

Asymptomatically infected people having sub-clinical doses of V. cholerae are some of the main reservoirs of cholera. Once conditions are favourable, those people are able to spread the disease. The aquatic environments can also be a reservoir for Vibrio cholerae even in the absence of outbreaks (Akoachere and Mbuntcha, 2014). Since the nineteenth century, the physician Snow recognized that water acts as a reservoir for cholera diseases and that V. cholerae is able to spread by infected water (Shears, 1994). Recently many researchers have confirmed that the different water sources are able to serve as a reservoir for V. cholerae and responsible for spreading cholera disease (Colwell and Huq, 1994; Shapiro et al., 1999; Blokesh and Schoolnik, 2007). Akoachere and Mbuntcha (2014) investigated well-, tap- and stream-water samples in Bepanda, Cameroon, as reservoirs of Vibrio cholerae; they found that 33% of 96 isolates were confirmed as V. cholerae O1.

Symptoms

Cholera is an extremely virulent disease that can cause severe acute watery diarrhoea. It takes between 12 hours and 5 days for a person to show symptoms after ingesting contaminated food or water (2). Cholera affects both children and adults and can kill within hours if untreated.

Most people infected with V. cholerae do not develop any symptoms, although the bacteria are present in their faeces for 1-10 days after infection and are shed back into the environment, potentially infecting other people.

Among people who develop symptoms, the majority have mild or moderate symptoms, while a minority develop acute watery diarrhoea with severe dehydration. This can lead to death if left untreated.

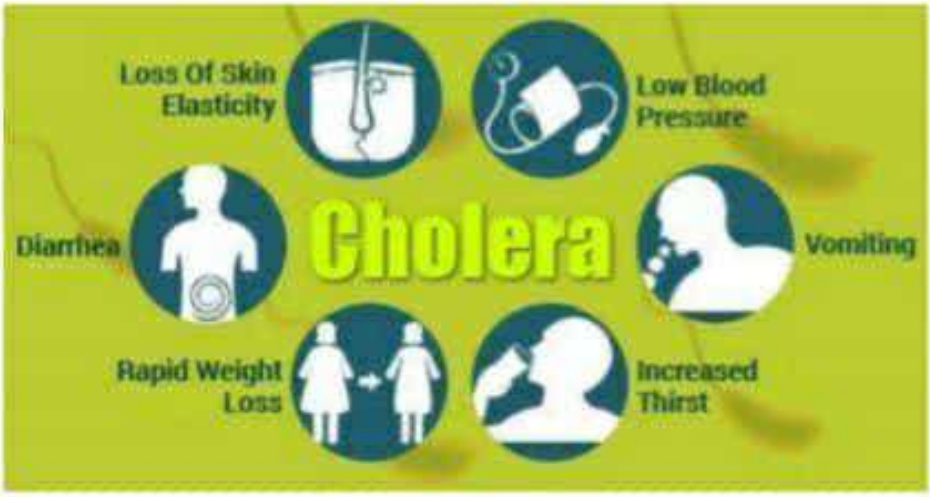


Fig: Schematic representation of Cholera

Diagnosis

Challenges still remain vis-à-vis the diagnosis and dection of Vibrio cholerae infections, due to limited resources especially in developing countries. The most commonly performed methods for serodiagnosis of Vibrio cholerae O1 infection comprise the measurement of antibacterial antibodies by agglutination or vibriocidal antibody essays (Barret et al., 1994). The vibriocidal antibody test has been used widely for serologic surveys as it is a friendly method based on the titration of large numbers of sera by microtiter procedure. According to Barret et al., (1994), the acquisition of antibody through natural exposure or vaccination has demonstrated obvious correlation to immunity on a population basis and in volunteers challenged with the organism.

More procedures such as the indirect hemagglutination test and enzyme-linked immunosorbent assays (ELISAs) and cell culture models for detecting cholera toxin (CT) have been also reported to provide marked improvement in the ability to measure antitoxic antibodies (Barret et al., 1994). Rapid diagnostic tests such as reverse passive latex agglutination test (RP LA) have also been proved effective for the detection of CT (Yamasaki et al., 2013). Recently, various tests have been developed and they mostly focus on the detection of the lipopolysaccharide of V. cholerae O1 and O139 by monoclonal antibodies, using the vertical-flow immunochromatography principle (Keddy et al, 2013). These are commercial membrane-based rapid diagnostic tests that have been used to detect the presence of cholera infection under laboratory and field conditions with variable sensitivity and specificity. The immunochromatographic Test Strip developed by Yamasaki et al., (2013) demonstrated the capability to detect the CT in culture supernatant of all 15 toxigenic *V. cholerae* isolates examined, whereas no falsepositive signal was detected in all 5 nontoxigenic V. cholerae isolates examined. A combination of different target analytes for example, immunoassay which detects the existence of CT can result in the surveillance of toxigenic *V. cholerae*.

Treatment

Cholera is an easily treatable disease. The majority of people can be treated successfully through prompt administration of oral rehydration solution (ORS). The WHO/UNICEF ORS standard sachet is dissolved in 1 litre (L) of clean water. Adult patients may require up to 6 L of ORS to treat moderate dehydration on the first day.

Severely dehydrated patients are at risk of shock and require the rapid administration of intravenous fluids. These patients are also given appropriate antibiotics to diminish the duration of diarrhoea, reduce the volume of re hydration fluids needed, and shorten the amount and duration of V. cholerae excretion in their stool.

Mass administration of antibiotics is not recommended, as it has no proven effect on the spread of cholera may contribute to antimicrobial resistance.

Rapid access to treatment is essential during a cholera outbreak. Oral rehydration should be available in communities, in addition to larger treatment centres that can provide intravenous fluids and 24 hour care. With early and proper treatment, the case fatality rate should remain below 1%.

Zinc is an important adjunctive therapy for children under 5, which also reduces the duration of diarrhoea and may prevent future episodes of other causes of acute watery diarrhoea.

Breastfeeding should also be promoted.

Reference

(1) Updated global burden of cholera in endemic countries.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4455997/

Ali M, Nelson AR, Lopez AL, Sack D. (2015). PLoS Negl Trop Dis 9(6): e0003832. doi:10.1371/journal.pntd.0003832.

(2) The incubation period of cholera: a systematic review.

https://www.ncbi.nlm.nih.gov/pubmed/23201968

Azman AS, Rudolph KE, Cummings DA, Lessler J. J Infect. 2013;66(5):432-8. doi: 10.1016/j.jinf.2012.11.013. PubMed PMID: 23201968; PubMed Central PMCID: PMC3677557.

(3) Cholera Annual Report 2019

Weekly Epidemiological Record 31 September 2020, Vol 95, 38 (pp 441-448).

(4) Cholera vaccines: WHO position paper – August 2017

http://apps.who.int/iris/bitstream/10665/258764/1/WER9234-477-498.pdf Weekly Epidemiological Record

25 August 2017, No 34, 2017, 92, 477–500.

[1] Weil AA,Ryan ET, Cholera: recent updates. Current opinion in infectious diseases. 2018 Oct

[PubMed PMID: 30048254]

[2] Ajayi A,Smith SI, Recurrent cholera epidemics in Africa: which way forward? A literature review. Infection. 2019 Jun [PubMed PMID: 30084058]

[3] Baker-Austin C,Oliver JD,Alam M,Ali A,Waldor MK,Qadri F,Martinez-Urtaza J, Vibrio spp. infections. Nature reviews. Disease primers. 2018 Jul 12[PubMed PMID: 30002421]

[4] Wolfe M,Kaur M,Yates T,Woodin M,Lantagne D, A Systematic Review and Meta-Analysis of the Association between Water, Sanitation, and Hygiene Exposures and Cholera in Case-Control Studies. The American journal of tropical medicine and hygiene. 2018 Aug [PubMed PMID: 29968551]

[5] Rabaan AA, Cholera: an overview with reference to the Yemen epidemic. Frontiers of medicine. 2018 Jun 22 [PubMed PMID: 29934743]

[6] Chatterjee P,Kanungo S,Dutta S, Challenges for programmatic implementation of killed whole cell oral cholera vaccines for prevention and control of cholera: a meta-opinion. Expert opinion on biological therapy. 2018 Sep [PubMed PMID: 30107757]

[7] Williams PCM,Berkley JA, Guidelines for the management of paediatric cholera infection: a systematic review of the evidence. Paediatrics and international child health. 2018 Nov [PubMed PMID: 29790841]

[8] Kirkeby S,Lynge Pedersen AM, Modifications of cholera toxin subunit B binding to human large intestinal epithelium. An immunohistochemical study. Microbial pathogenesis. 2018 Aug 23 [PubMed PMID: 30145256]

[9] Wu J,Yunus M,Ali M,Escamilla V,Emch M, Influences of heatwave, rainfall, and tree cover on cholera in

Bangladesh. Environment international. 2018 Aug 11[PubMed PMID: 30107291] [10] Richterman A,Sainvilien DR,Eberly L,Ivers LC, Individual and Household Risk Factors for Symptomatic Cholera

Infection: A Systematic Review and Meta-analysis. The Journal of infectious diseases. 2018 Oct 15 [PubMed PMID: 30137536]

[11] Lekshmi N,Joseph I,Ramamurthy T,Thomas S, Changing facades of {i}Vibrio cholerae{/i}: An enigma in the epidemiology of cholera. The Indian journal of medical research. 2018 Feb [PubMed PMID: 29806601]

[12] Curran KG,Wells E,Crowe SJ,Narra R,Oremo J,Boru W,Githuku J,Obonyo M,De Cock KM,Montgomery JM,Makayotto L,Langat D,Lowther SA,O'Reilly C,Gura Z,Kioko J, Systems, supplies, and staff: a mixed-methods study of health care workers' experiences and health facility preparedness during a large national cholera outbreak, Kenya 2015. BMC public health. 2018 Jun 11 [PubMed PMID: 29890963]

[13] Ngwa MC,Liang S,Mbam LM,Mouhaman A,Teboh A,Brekmo K,Mevoula O,Morris JG Jr, Cholera public health surveillance in the Republic of Cameroon-opportunities and challenges. The Pan African medical journal. 2016 [PubMed PMID: 27800077]

[14] Clements JD,Norton EB, The Mucosal Vaccine Adjuvant LT(R192G/L211A) or dmLT. mSphere. 2018 Jul 25 [PubMed PMID: 30045966]

[15] Brandt KG,Castro Antunes MM,Silva GA, Acute diarrhea: evidence-based management. Jornal de pediatria. 2015 Nov-Dec .

STEM CELL THERAPY

Abstract

Stem cells are a population of undifferentiated cells characterized by the ability to extensively proliferate (self-renewal), usually arise from a single cell (clonal), and differentiate into different types of cells and tissue (potent). There are several sources of stem cells with varying potencies. Pluripotent cells are embryonic stem cells derived from the inner cell mass of the embryo and induced pluripotent cells are formed following reprogramming of somatic cells. Pluripotent cells can differentiate into tissue from all 3 germ layers and multipotent stem cells may differentiate into tissue derived from a single germ layer such as mesenchymal stem cells. Stem cells can be used in cellular therapy to replace damaged cells or to regenerate organs. In addition, stem cells have expanded our understanding of development as well as the pathogenesis of disease. Disease-specific cell lines can also be propagated and used in drug development. Despite the significant advances in stem cell biology, issues such as ethical controversies with embryonic stem cells, tumor formation, and rejection limit their utility. However, many of these limitations are being bypassed and this could lead to major advances in the management of disease. This review is an introduction to the world of stem cells and discusses their

definition, origin, and classification, as well as applications of these cells in regenerative medicine.

Introduction

Stem cells have the ability to differentiate into specific cell types. The 2 defining characteristics of a stem cell are perpetual self-renewal and the ability to differentiate into a specialized adult cell type.

There are 2 major classes of stem cells: pluripotent cells, which can become any cell in the adult body, and multipotent cells, which are restricted to becoming a more limited population of cells. Stem cells have great potential in tissue regeneration and repair, but much still needs to be learned about their biology, manipulation, and safety before their full therapeutic potential can be achieved.

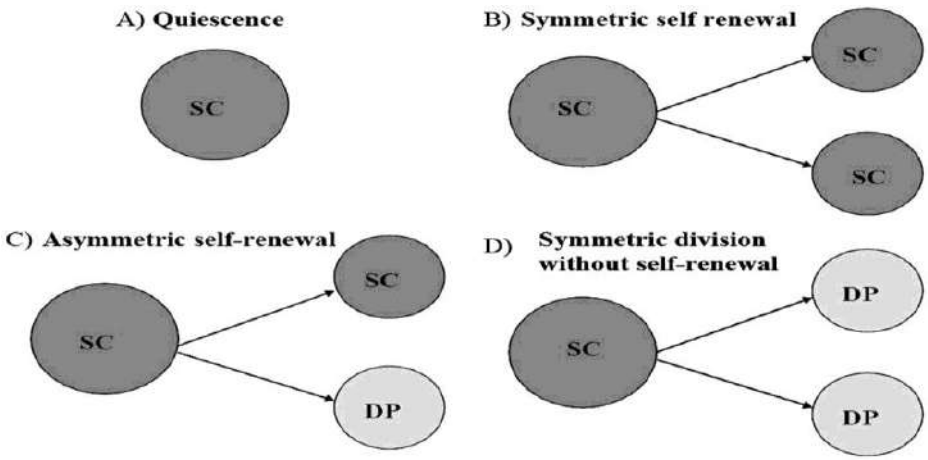


Figure-1: Derivation of stem cells, Introduction to Stem Cell Therapy. Picture adopted from Jesse K. Biehl and Brenda Russell work. https://www.ncbi.nlm.nih.gov › pmc

Introduction to Stem Cell Therapy – NCBI

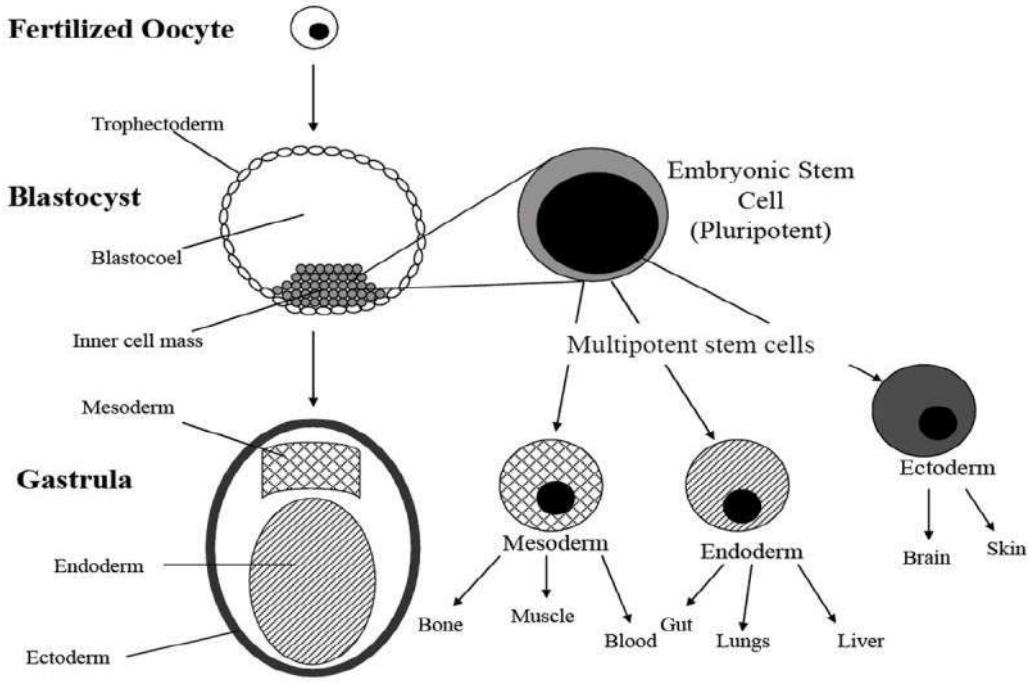


Figure-2: Stem cell fates, Introduction to Stem Cell Therapy. Picture taken from Jesse K. Biehl and Brenda Russell work. Picture adopted from Jesse K. Biehl and Brenda Russell work. https://www.ncbi.nlm.nih.gov › pmc

Introduction to Stem Cell Therapy – NCBI

Stem cell therapy promotes the repair response of diseased, dysfunctional or injured tissue using stem cells or their derivatives. It is the next chapter in organ transplantation and uses cells instead of donor organs,which are limited in supply. Diseases can be cured by Stem Cell Transplants are- Acute leukemia, lymphoma, anemia, Osteopetrosis etc.

Brief Description

The pluripotency nature of embryonic stem cells is used with somatic cell nuclear transfer to create induced pluripotent stem cells (IPSC). As of now the only established therapy using stem cells is hematopoietic stem cell transplantation. This usually takes the form of a bone-marrow transplantation, but the cells can also be derived from umbilical cord blood.

Evaluation and pre-transplant testing

Identification of the appropriate type of transplantation— Autologous (from the patient), syngeneic (from an identical twin), or allogeneic(from a related, unrelated or cord blood donor).

Obtaining stem cells from the patient or a donor —

- Blood is taken from a vein and circulated through a machine that removes the stem cells and returns remaining blood and plasma back to the patient.
- Stem cells are collected by a needle placed in the soft center in marrow of the bone and harvested in an operating room.

Conditioning treatment — administration of chemotherapy and/or radiation to the patient in order to destroy all of the diseased cells in the body and to create space in the bone marrow for the transplanted stem cells to populate.

Infusion of healthy stem cells into the patient — a painless process in which stem cells are transplanted into the patient through intravenous infusion.

Engraftment and recovery — transplanted stem cells begin to grow and reproduce healthy blood cells. For bone marrow or blood stem cell transplant, engraftment takes between two and three weeks; for cord blood transplant takes three to five weeks.

Post-transplant care — including regular examinations to monitor allogeneic transplant patients for signs of graft vs. Host disease and to watch autologous and allogeneic patients for immune system recovery, complications related to chemotherapy or radiation and cancer recurrence a physician is referred.

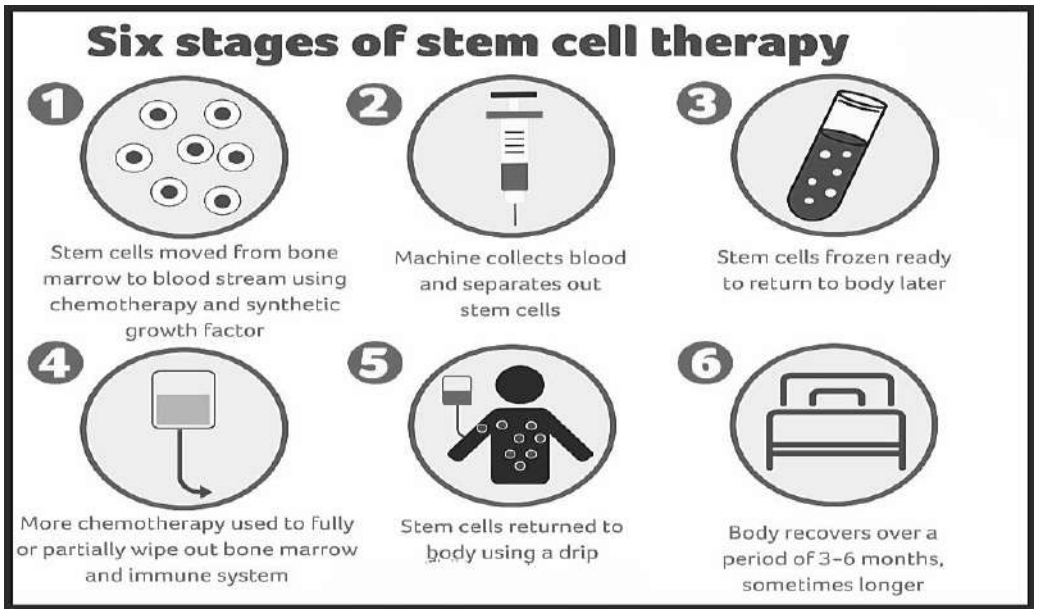


Figure-3:Procedure of stem cell therapy, picture adopted from mstrust.org.uk

Scope

Cancer Stem Cells

- Cellular origin of cancer stem cells
- Genetic constraints, including epigenetic modifications, that influence growth process.

Embryonic Stem Cells/Induced Pluripotent Stem (iPS) Cells

- Signaling pathways/factors regulating and/or initiating ESC/iPS cells pluripotency and differentiation.

Regenerative Medicine

- Potential applications for stem cell-based strategies in pathological conditions.
- Gene therapies for inherited diseases.

Tissue-Specific Stem Cells

- Regulation of and pathways that govern stem cell self-renewal and/or differentiation
- Cellular interactions and signaling pathways necessary for tissue specificity.

Translational and Clinical Research

- Studies describing new model systems with potential for drug development
- Translation into pharmaceutical, clinical or practical finding.

Application

Stem cells themselves do not serve any single purpose but are important for several reasons.

First, with the right stimulation, many stem cells can play role of any type of cell, and regenerate damaged tissue, under the right conditions. This potential could save lives or repair wounds and tissue damage in people after an illness or injury.

Tissue regeneration

It is probably the most important use of stem cells.Until now, a person who needed a new kidney, for example, had to wait for a donor and then undergo a transplant but by instructing stem cells to differentiate in a certain way,

scientists could use them to grow a specific tissue type or organ.

Cardiovascular disease treatment

In 2013, a team of researchers from Massachusetts General Hospital reported in PNAS Early Edition that they had created blood vessels in laboratory mice, using human stem cells.

Within 2 weeks of implanting the stem cells, networks of blood-perfused vessels had formed which are as good as the nearby natural ones and the authors hoped that this type of technique could eventually help to treat people with cardiovascular and vascular diseases.

Brain disease treatment

Doctors may one day be able to use replacement cells and tissues to treat brain diseases, such as Parkinson's and Alzheimer's.

In Parkinson's, for example, damage to brain cells leads to uncontrolled muscle movements. Scientists could use stem cells to replenish the damaged brain tissue and bring back the specialized brain cells that stop the uncontrolled muscle movements.

Cell deficiency therapy

Scientists hope one day to be able to develop healthy heart cells in a laboratory that they can transplant into

people with heart disease. Similarly, people with type I diabetes could receive pancreatic cells to replace the insulin-producing cells that their own immune systems have lost or destroyed.

Blood disease treatments

Doctors now routinely use adult hematopoietic stem cells to treat diseases, such like leukemia, sickle cell anemia, and other immunodeficiency problems.

Hematopoietic stem cells occur in blood and bone marrow and can produce all blood cell types, including red blood cells that carry oxygen and white blood cells that fight disease.

Donating or harvesting stem cells

People can donate. Trusted Source stem cells to help a loved one, or possibly for their own use in the future.

Donations can come from the following sources:

1. **Bone marrow:** These cells are taken under a general anesthetic, usually from the hip or pelvic bone. Technicians then isolate the stem cells from the bone marrow for storage or donation.
2. **Peripheral stem cells:** A person receives several injections for the bone marrow to release stem cells into the blood. Next, blood is removed from the body, a machine separates the stem cells, and doctors return the blood to the body.

3. **Umbilical cord blood:** Stem cells can be harvested from the umbilical cord after delivery, with no harm to the baby.

This harvesting of stem cells can be expensive, but the advantages for future needs include:

- The stem cells are easily accessible.
- less chance of transplanted tissue being rejected if it comes from the recipient's own body.

Future perspective

The progress in the field of stem cells research represents great scope of stem cells regenerative therapeutics. It can be estimated that by 2024 or so we will be able to produce wide array of tissue and organs from adult stem cells.

Inductions of pluripotent stem cell differentiation into adult cells have better therapeutic future due to least ethical constraints with adult cells. In the coming future, there might be new pharmaceutical compounds; those can activate tissue specific stem cells, promote stem cells to migrate to the site of tissue injury, and promote their differentiation to tissue specific cells.

The existing stem cell therapeutics advancements are more experimental and high in cost; due to that application on broad scale is not possible in current scenario. In the near future, the advancements of medical science presume using

stem cells to treat cancer, muscles damage, and spinal cord injuries etc. and It will bring benefits to the patients suffering from wide range of injuries and disease.

For advancement of application of stem cells, there is a need of clinical trials, which needs funding from both public and private organizations. Regulatory guidelines at each phase of clinical trial is a must to comprehend the success and efficacy in time frame.

Conclusion

The promises of cures for human ailments by stem cells have been much touted but many obstacles must still be overcome.

First, more human pluripotent and multipotent cell research is needed since stem cell biology differs in mice and men.

Second, the common feature of unlimited cell division shared by cancer cells and pluripotent stem cells must be better understood in order to avoid cancer formation.

Third, the ability to acquire large numbers of the right cells at the right stage of differentiation must be mastered.

Finally, clinical trials must be completed to assure safety and efficacy of the stem cell therapy. When it comes to stem cells, knowing they exist is a long way from using them therapeutically.

Reference

1. Mahla RS (2016). “Stem cells application in regenerative medicine and disease threpeutics”. International Journal of Cell Biology. 2016 (7): 1–24. Doi:10.1155/2016/6940283. PMC 4969512. PMID 27516776.
2. Müller, Albrecht M.; Huppertz, Sascha; Henschler, Reinhard (July 2016). “Hematopoietic Stem Cells in Regenerative Medicine: Astray or on the Path?”. Transfusion Medicine and Hemotherapy. 43 (4): 247–254. Doi:10.1159/000447748. ISSN 1660-3796. PMC 5040947. PMID 27721700.
3. Lyon, Louisa (1 October 2018). “Stem cell therapies in neurology: the good, the bad and the unknown”. Brain. 141 (10): e77. Doi:10.1093/brain/awy221. ISSN 0006-8950. PMID 30202947.
4. Ian Murnaghan for Explore Stem Cells. Updated: 16 December 2013 Why Perform a Stem Cell Transplant?

5. Bone Marrow Transplantation and Peripheral Blood Stem Cell Transplantation In National Cancer Institute Fact Sheet web site. Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services, 2010. Cited 24 August 2010
6. Karanes C, Nelson GO, Chitphakdithai P, Agura E, Ballen KK, Bolan CD, Porter DL, Uberti JP, King RJ, Confer DL (2008). "Twenty years of unrelated donor hematopoietic cell transplantation for adult recipients facilitated by the National Marrow Donor Program". Biology of Blood and Marrow Transplantation. 14 (9 Suppl): 8–15. Doi:10.1016/j.bbmt.2008.06.006. PMID 18721775.
7. Malard F, Mohty M (2014). "New Insight for the Diagnosis of Gastrointestinal Acute Graft-versus-Host Disease". Mediators Inflamm. 2014: 701013. Doi:10.1155/2014/701013. PMC 3964897. PMID 24733964.
8. "Prochymal – First Stem Cell Drug Approved". 22 May 2012.
9. "A Stem-Cell-Based Drug Gets Approval in Canada". 17 May 2012.
10. Rosemann A (December 2014). "Why regenerative stem cell medicine progresses slower than expected" (PDF). J Cell Biochem. 115 (12): 2073–76. Doi:10.1002/jcb.24894. PMID 25079695.

11. European Medicines Agency. “First stem-cell therapy recommended for approval in EU”. Retrieved 12 December 2014.

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE

PROJECT REPORT

Amogh Maitra

Regn. No. – A01-1112-116-020-2019

Roll No – 621

Semester V

Guided by - Debasish Malik

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

A highly contagious disease: Varicella zoster.

Abstract

The study on Varicella-zoster virus (VZV) a human alphaherpesvirus that causes varicella (chicken pox) which is a very common illness during childhood, takes place along with fever, viremia, and scattered vesicular lesions on the skin. Rarely, it is seen it reactivates again to cause shingles caused by herpes-zoster, very much same alike to chickenpox. The virion consists of a nucleocapsid surrounding a core that contains the linear, double-stranded DNA genome; a protein tegument separates the capsid from the lipid envelope, which incorporates the major viral glycoproteins. VZV is found in a worldwide geographic distribution but is more prevalent in temperate climates. Varicella is highly contagious which spreads from person to person by direct contact or inhalation of aerosols and enters through the nose and mouth and attacks mostly the cranial and spinal nerves. Primarily the virus draws out antibodies, which bind to many classes of viral proteins compromising the virus-specific cellular immunity Acyclovir, valaciclovir are some drugs used to treat varicella and herpes zoster. A live attenuated varicella vaccine was developed from the Oka strain and is recommended as a immunity booster for children.

Introduction

Varicella-zoster virus (VZV) is a ubiquitous human alphaherpesvirus. *Human alphaherpesvirus 3* (HHV-3) [1], is usually referred to as the Varicella-zoster virus (VZV), is one of nine herpes viruses known to infect humans. It causes chickenpox (varicella), a disease most commonly affecting children, teens, and young adults, and shingles (herpes zoster) in adults. VZV infections are species-specific to humans, but can survive in external environments for a few hours.[2] Several symptoms of rashes, high fever are seen. Therapeutic drugs are often prescribed or advised to take for reducing the symptoms of itchiness and discomfort.

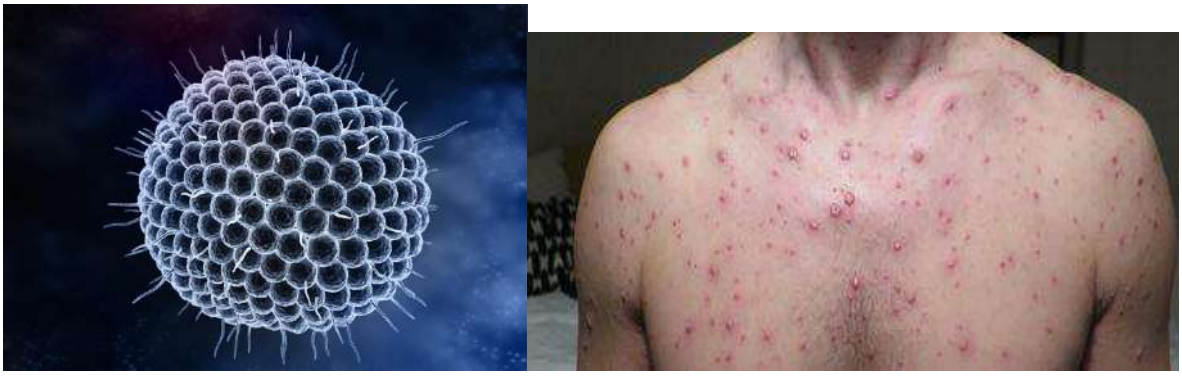


Figure 1. Varicella zoster virus the causative agent of the disease chickenpox. (Left) The rashes of chickenpox- the main symptom of the disease. (Right)

Images adopted from National Foundation for Infectious Diseases.

Epidemiology

The annual incidence of herpes zoster infection is estimated at a total of 315 cases per 100,000 inhabitants for all ages and 577 cases per 100,000 for people 50 years of age or older [4] VZV can also infect the central nervous system, with a 2013 article reporting an incidence rate of 1.02 cases per 100,000 inhabitants in Switzerland, and an annual incidence rate of 1.8 cases per 100,000 inhabitants in Sweden [5], according to a study from Sweden by Nilsson et al (2015).

In about one third cases, VZV reactivates later in life, producing a disease known as shingles or herpes zoster [6] as it is commonly called. The individual lifetime risk of developing herpes zoster is found to be between 20% and 30%, or approximately 1 in 4 people. However, for individuals of age 85 and above, this risk gets increased to 1 in 2 people.[7]

According to the evolution and phylogeography, there are at least five clades of this virus.[8] Clades 1 and 3 include European/North American strains; clade 2 are Asian strains, especially from Japan; and clade 5 appears to be based in India. Clade 4 includes some strains from Europe but its geographic origins need further clarification. There are also four genotypes that do not fit into these clades.[9] Allocation of VZV strains to clades required sequence of whole virus genome. Practically all molecular epidemiological data on global VZV strains distribution are obtained with targeted sequencing of selected regions.

Morphology and Genome

VZV is closely related to the herpes simplex viruses (HSV), sharing much genome homology. The known envelope glycoproteins (gB, gC, gE, gH, gI, gK, gL) correspond with those in HSV. VZV virions are spherical and 180–200 nm in diameter. Their lipid envelope encloses the 100 nm nucleocapsid of 162 hexameric and pentameric capsomeres arranged in an icosahedral form. Its DNA is a single, linear, double-stranded molecule, about 125,000 nt long. The capsid is surrounded by loosely associated proteins known collectively as the tegument; many of these proteins play critical roles in initiating the process of virus reproduction in the infected cell. The tegument is in turn covered by a lipid envelope studded with glycoproteins that are displayed on the exterior of the virion, each approximately 8 nm long.[11]

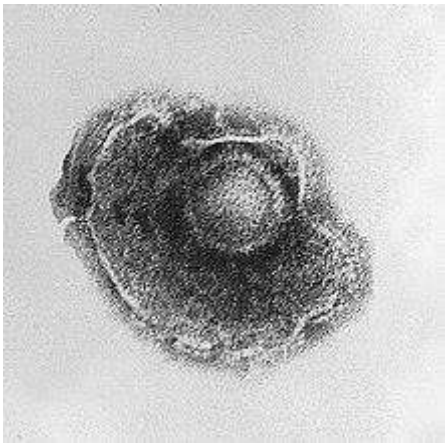


Figure 2. Electron Micrograph of Human alphaherpesvirus 3 virion

The genome was first sequenced in 1986,[12] having a linear duplex DNA molecule with 124,884 base pairs. The genome has 2 predominant isomers, depending on the orientation of the S segment, P (prototype) and IS (inverted S) which are present with equal frequency for a total frequency of 90–95%. The L segment can also be inverted resulting in a total of four linear isomers (IL and ILS). Their genome encodes for atleast 70 genes from the respective open reading frames.

According to (Grose et al., 1991) the glycoproteins of varicella-zoster virus (VZV) and their role as immunogens is of great significance when the relevance of studies of VZV to the selection of a glycoprotein subunit herpes simplex virus (HSV) vaccine was done. It was seen that HSV types 1 and 2 and VZV are alpha-herpesviruses, which are characterized by common biologic features such as a relatively short replication cycle and a latent state, in neurologic tissues. The three viruses also conserve several glycoprotein genes, including gB, gC, gE, gH, and gI. While the known properties of the VZV glycoproteins closely resemble those of their homologous HSV counterparts and may provide further insight into biologic functions of the immunogens. In particular, VZV glycoproteins gpII and gpIII closely resemble their HSV homologs gB and gH in that all four harbour complement-independent neutralization epitopes. [13]

Transmission and Pathogenesis

The virus spreads mainly through close contact with someone who has chickenpox (varicella virus). It is transmitted from person to person by directly touching the blisters, saliva or mucus of an infected person. The virus can also be transmitted through the air by coughing and sneezing (aerosols).] It enters the body through the mouth and nose after contact with an infected person. It then travels along the cranial and spinal nerves lying dormant in it . When the virus is reactivated, it travels to the area of the skin served by those nerves, where it causes a distinctive, stripe-like rash.

During primary infection, VZV hijacks T cells to disseminate to the skin and establishes latency in ganglia. The human skin, dorsal root ganglia or foetal thymus that contains T cells gets infected with the viruses in the presence of inhibitors of viral or cellular functions that initiates molecular mechanisms of VZV-host interactions. [14]

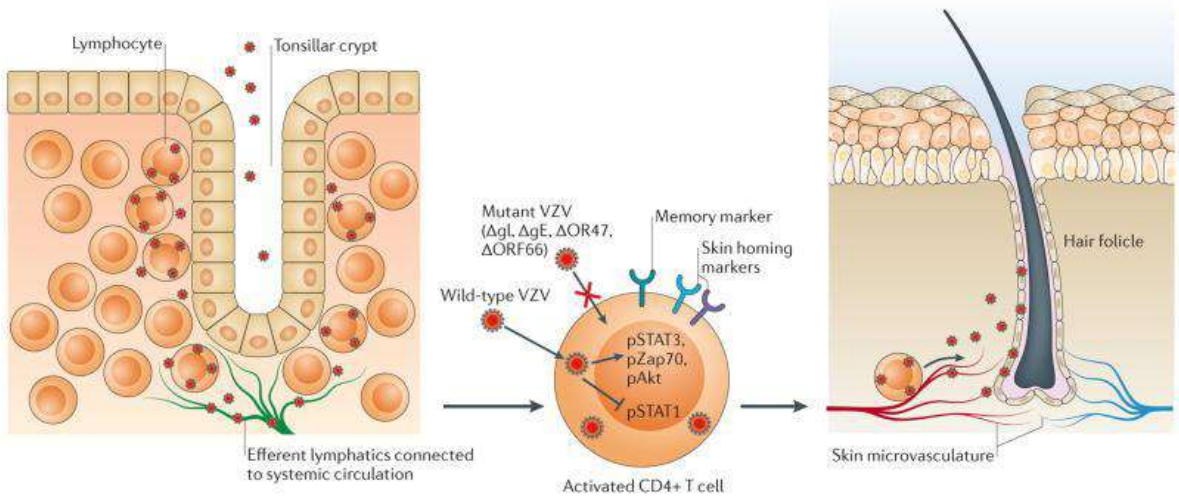


Figure 3. Molecular mechanisms of VZV on T cell tropism. Image adopted from "Molecular mechanisms of varicella zoster virus pathogenesis" Nature Reviews Microbiology.

Primary VZV infection elicits immunoglobulin G, M and A antibodies, which bind to many classes of viral proteins. Virus-specific cellular immunity is critical for controlling viral replication in healthy and immunocompromised patients with primary or recurrent VZV infections.

Incubation Period

The average incubation period for varicella is 14 to 16 days after exposure to varicella or a herpes zoster rash, with a range of 10 to 21 days. A mild prodrome of fever and malaise may occur 1 to 2 days before the onset of rash, particularly in adults. In children, the rash is often the first sign of disease.
A person with chickenpox is considered contagious in beginning 1 to 2 days before the rash onset until all the chickenpox lesions have crusted (scabbed).[15] The period of illness is not prolong, about 3 to 5 days but observation is required to check that no new rashes have popped up.

Shingles lesions are associated with pain and burning, sensations tend to occur on the skin. The skin lesions starts to grow within 3 to 4 days of incubation causing acute pain over course of several weeks.
Other serious complications like postherpetic neuralgia can also occur in which a chronic pain persists more than three months found in 10-15% of cases.

Symptoms

Primary varicella zoster virus (VZV) infection results in chickenpox which is characterized by fever, viremia, and scattered vesicular lesions of the skin. This is a common childhood illness.

As the characteristic of the alphaherpesviruses, VZV signs and symptoms includes painful vesicular rashes filled with pus that turns itchy ruptures and turn into scab, spreading over the entire body. The rashes may first show up on the face, throat, the back, lower back, the chest [15] and shoulders and then, including inside the mouth, on the eyelids, and in genital area. Often seen the rashes involves one or more adjacent dermatomes. The rash and the other symptoms start appearing when the virus is most contagious. Some other symptoms are high fever, malaise, nausea, vomiting and fatigue.

VZV establishes viral latency and remains dormant in the nervous system of the infected person in the trigeminal and dorsal root ganglia. [16] It can even result to complication sometimes with secondary infections including encephalitis, pneumonia or bronchitis.

VZV, later can again reactivate and produce a disease, shingles.[6] Symptoms to identify are painful red fluid-filled blisters or rash in linear distribution on the skin, start to begin few days after pain, burning sensation and itching, numbness and sensitivity to touch, fatigue.

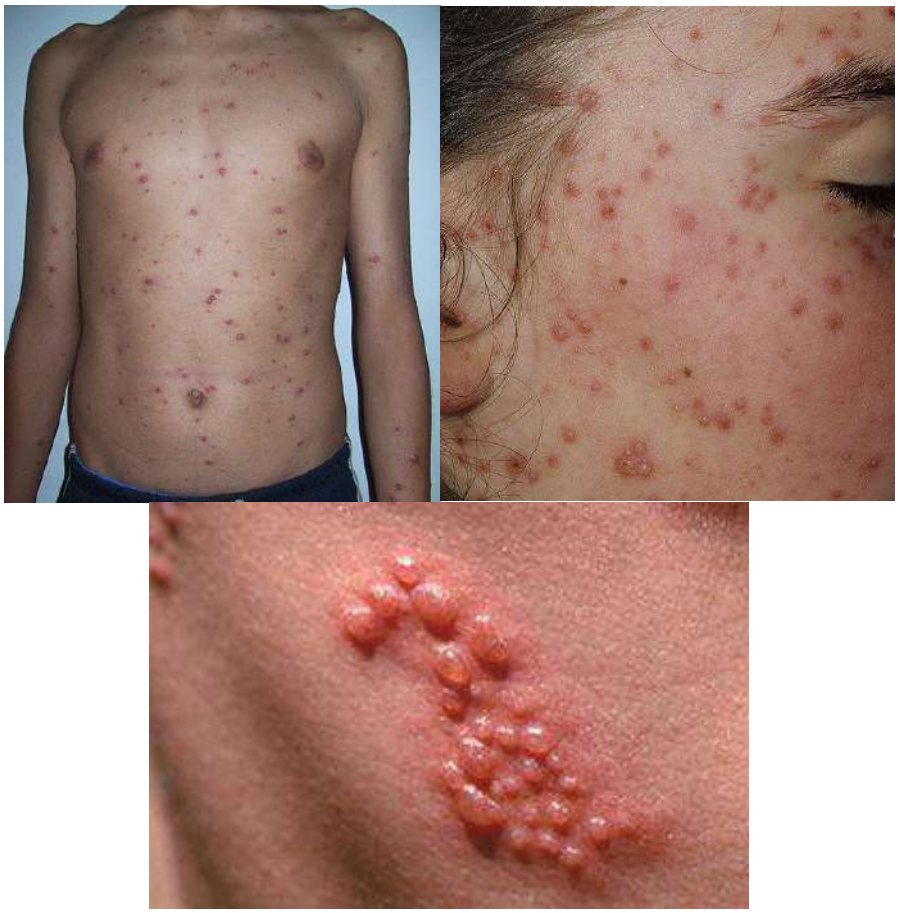


Figure 4. Rashes of chickenpox (varicella zoster) on face and body (above). Blisters of shingles (herpes zoster) on adjacent dermatomes on the skin (below). Images adopted from WebMD.

Diagnosis and Treatment

The general diagnosis of chickenpox is done based on the rash. If there is any doubt about the diagnosis, chickenpox can be confirmed with lab tests, including blood tests or a culture of lesion samples.

It can be treated by a number of drugs and therapeutic agents including acyclovir for the chicken pox, famciclovir, valaciclovir for the shingles. [17]

Acyclovir is a licensed antiviral drug frequently used as the drug of choice in primary VZV infections, which might lessen the severity of chickenpox when given within 24 hours after the rash first appears.

The severity of the disease might be controlled by famiciclovir and valaciclovir but are not approved as appropriate for everyone especially for children affected with chickenpox.

A live attenuated VZV Oka/Merck strain vaccine is available which is developed by Merck, Sharp & Dohme from the Oka strain virus isolated in the 1970s.

It is now recommended for routine childhood immunization and a 90% success in reductions in the incidence of varicella cases and hospitalizations and deaths due to VZV.

Clinical data has proved that the vaccine is effective for over ten years in preventing varicella infection in healthy individuals, and when breakthrough infections do occur, illness is typically mild.[18]

In 2006, the CDC's Advisory Committee on Immunization Practices (ACIP) recommended a second dose of vaccine before school entry to ensure the maintenance of high levels of varicella immunity.[19]

A subunit vaccine of *V. zoster* (glycoprotein E) named Shingrix was developed by GlaxoSmithKline which was approved in the United States by the FDA in October 2017.[20]

The ACIP recommended Shingrix for adults over the age of 50, mainly. Data says that the efficacy of is >90% against shingles across all age groups, as well as sustained efficacy over a 4-year follow-up. It is given as two intramuscular doses, two to six months apart.[21] This vaccine has shown to be immunogenic and safe in adults with human immunodeficiency virus. [22]

Figure 5. Live Attenuated Vaccine for Varicella Zoster Virus (VZV)

Image adopted from HealthCentral

References

1. *"ICTV Taxonomy history: Human alphaherpesvirus 3". International Committee on Taxonomy of Viruses (ICTV). Retrieved 9 January 2019.*
2. *"Pathogen Safety Data Sheets: Infectious Substances – Varicella-zoster virus". Canada.ca. Pathogen Regulation Directorate, Public Health Agency of Canada. 2012-04-30. Retrieved 2017-10-10*
3. *Sandri-Goldin RM, ed. (2006). Alpha Herpesviruses: Molecular and Cellular Biology. www.horizonpress.com. Caister Academic Press. ISBN 978-1-904455-09-7.*
4. *Nilsson, Jonas; Cassel, Tobias; Lindquist, Lars (22 May 2015). "Burden of herpes zoster and post-herpetic neuralgia in Sweden". BMC Infectious Diseases. 15: 215. doi:10.1186/s12879-015-0951-7. PMC 4493830. PMID 26002038.*
5. *Becerra JC, Sieber R, Martinetti G, Costa ST, Meylan P, Bernasconi E (July 2013). "Infection of the central nervous system caused by varicella zoster virus reactivation: a retrospective case series study". International Journal of Infectious Diseases. 17 (7): e529-34. doi:10.1016/j.ijid.2013.01.031. PMID 23566589.*
6. *Harpaz, R; Ortega-Sanchez, IR; Seward, JF (6 June 2008). "Prevention of herpes zoster: recommendations of the Advisory Committee on Immunization Practices (ACIP)". Morbidity and Mortality Weekly Report (MMWR). 57 (RR-5): 1–30, quiz CE2-4. PMID 18528318.*
7. *Pinchinat, Sybil; Cebrián-Cuenca, Ana M; Bricout, Hélène; Johnson, Robert W (December 2013). "Similar herpes zoster incidence across Europe: results from a systematic literature review". BMC Infectious Diseases. 13 (1): 170. doi:10.1186/1471-2334-13-170. PMC 3637114. PMID 23574765.*
8. *Chow VT, Tipples GA, Grose C (August 2013). "Bioinformatics of varicella-zoster virus: single nucleotide polymorphisms define clades and attenuated vaccine genotypes". Infection, Genetics and Evolution. 18: 351–6. doi:10.1016/j.meegid.2012.11.008. PMC 3594394. PMID 23183312.*
9. *Grose C (September 2012). "Pangaea and the Out-of-Africa Model of Varicella-Zoster Virus Evolution and Phylogeography". Journal of Virology. 86 (18): 9558–65. doi:10.1128/JVI.00357-12. PMC 3446551. PMID 22761371*
10. *Mettenleiter; et al. (2008). "Molecular Biology of Animal Herpesviruses". Animal Viruses: Molecular Biology. www.horizonpress.com. Caister Academic Press. ISBN 978-1-904455-22-6.*

11. Han DH (25 October 2017). "General Characteristics and Epidemiology of varicella-zoster virus (VZV)". The Lecturio Medical Concept Library. Medical Professionals Reference (MPR). Retrieved 2021-06-14.
12. Davison AJ, Scott JE (September 1986). "The complete DNA sequence of varicella-zoster virus". The Journal of General Virology. 67 (Pt 9) (9): 1759–816. doi:10.1099/0022-1317-67-9-1759. PMID 3018124
13. Grose C . "Glycoproteins of varicella-zoster virus and their herpes simplex virus homologs" (Nov-Dec 1991) PMID: 1664135 doi: 10.1093/clind/13.supplement_11.s960 PMID: 1664135
14. L Zerboni L, Sen N, Oliver SL, Arvin AM "Molecular mechanisms of varicella zoster virus pathogenesis" Nature Reviews Microbiology (February 2014) doi: 10.1038/nrmicro3215 PMID: 24509782
15. Tortora G. Microbiology: An Introduction. Pearson. pp. 601–602.
16. Steiner I, Kennedy PG, Pachner AR (November 2007). "The neurotropic herpes viruses: herpes simplex and varicella-zoster". The Lancet. Neurology. 6 (11): 1015–28. doi:10.1016/S1474-4422(07)70267-3. PMID 17945155. S2CID 6691444.
17. "Zovirax (acyclovir) dosing, indications, interactions, adverse effects, and more". Medscape Reference. WebMD. Archived from the original on 19 February 2014. Retrieved 5 February 2014.
18. "Prevention of varicella: Recommendations of the Advisory Committee on Immunization Practices (ACIP). Centers for Disease Control and Prevention". MMWR. Recommendations and Reports. 45 (RR-11): 1–36. July 1996. PMID 8668119.
19. Marin M, Güris D, Chaves SS, Schmid S, Seward JF (June 2007). "Prevention of varicella: recommendations of the Advisory Committee on Immunization Practices (ACIP)". MMWR. Recommendations and Reports. 56 (RR-4): 1–40. PMID 17585291
20. Gruber MF (20 October 2017). "Biologics License Application (BLA) for Zoster Vaccine Recombinant, Adjuvant" (PDF). Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, US Food and Drug Administration. Retrieved 16 November 2018.
21. Han HD (25 October 2017). "ACIP: New Vaccine Recommendations for Shingles Prevention". Medical Professionals Reference (MPR). Retrieved October 30, 2017.
22. Berkowitz EM, Moyle G, Stellbrink HJ, Schürmann D, Kegg S, Stoll M, et al. (April 2015). "Safety and immunogenicity of an adjuvanted herpes zoster subunit candidate vaccine in HIV-infected adults: a phase 1/2a randomized, placebo-controlled study". The Journal of Infectious Diseases. 211 (8): 1279–87. doi:10.1093/infdis/jiu606. PMC 4371767. PMID 25371534.

Ramakrishna Mission Vivekananda Centenary College

A Project report on

Infants Nutrition and it's Intestinal Microbiome

Submitted by : Biswayan Bera

Reg.no. : A01-1112-116-006-2019

Roll no. : 607 semester : v

Department of Microbiology

Guided by — Mr. Avijit Chakraborty

Department of Microbiology

Ramakrishna Mission Vivekananda Centenary College

Acknowledgement

I would like to acknowledge and give my warmest thanks to my supervisor Avijit Chakraborty who provided me the innovative ideas and guided me to make this paper possible. His guidance and advice carried me through all the stages of writing this project. I would also like to thank our departmental coordinator in charge; revered Bhrahamachari Parachaitanya Maharaj for giving me this opportunity, which help me to venture relevant meaningful topics.

Finally, I would like to acknowledge with gratitude, the support and love of my parents and friends. They all kept me going, and this paper would not have been possible without their cordial support.

Infants Nutrition and it's Intestinal Microbiome

ABSTRACT:

The microbiota has recently been recognized as a driver of health that affects the immune system ,nesvous and metabolic system. The gut microbiota begins developing early in life, and this initial colonization is remarkably important because it may influence long term micrtobiota composition and activity.breast milk particular are thought to play a major role in shaping the early life microbiota and promoting its development. 1000 different bacterial species cohabit the human intestinal tract . Here discuss about these microbes come from and its potential role.

Introduction:

The interaction between the microbial ecosystem and the host represents a long evolutionary symbiosis that is essential for optimal health throughout life. The resident microbiota, with its broad genetic and metabolic diversity, exerts an effect on host metabolism, physiology, and immune system development. For this reason, the microbiota is now recognized as a "virtual organ" . The microbiota encompasses 2 predominant bacterial phylotypes, bacteroidetes and firmicutes, with the phyla proteobacteria, actinobacteria, fusobacteria, and verrucomicrobia being present at a relatively lower abundance .

The whole microbial distribution varies along the gastrointestinal tract, with microbial densities and diversities increasing both from the proximal to the distal gut and along the tissue-lumen axis. Although newborns were initiallythought to be born sterile, it is now believed that the colonization of the gut starts during pregnancy and continues after birth until 2 years of age, when it reaches a relatively stable composition resembling that of an adult. In this case human milk acts as a ideal source of nutrition . Human milk is the gold standard for infant nutrition in the first 12 months of life for term and preterm newborn infants. Beyond nutritional components, HM contains important bioactive compounds such oligosaccharides, cytokines, immunoglobulins, microbes, and proteins among others that directly influence the developing infant and shape the intestinal microbiota colonization.Breastfeeding practices have been associated with a risk reduction of NEC and LOS in preterm infants.

In this review , I considered different aspects of the microbiota's influence on infants health. I summarize factors that contribute to the establishment of a protective microbiota early in life and describe the interactions between human milk oligosaccharides the microbiota. Besides, how whole intestinal microbiome develop in infant health and how it affects on the infant's nutrition and immune systems.

Infant-Feeding Patters :

The World Health Organization (WHO) recommends that infants be exclusively breastfed for the first six months, followed by breastfeeding along with complementary foods for up to two years of age or beyond. Multivariable linear regression models were used to examine the associations between child's weight, height and BMI at ages 1, 3 and 5 years respectively and the feeding patterns scores adjusted for parental and child (gestational age, sex and child care attendance) characteristics. The effect of the feeding patterns on the change in anthropometric measurements, from birth to 1 year, from 1 to 3

years and from 3 to 5 years, was estimated with the final value as the outcome, adjusted for the initial value and other potential confounders. Discussion Infant feeding patterns were not significantly related to anthropometric measurements at 1, 3 and 5 y, but they were related to height and weight growth both during the first year and from 1 to 3 years. High scores on infant feeding pattern characterized by long breastfeeding, later main meal food introduction and use of homemade foods were related to significant lower 01 years and higher 1-3 years or 3-5 years increase in weight and height. This specific growth pattern was explained by the long breastfeeding duration.

The most dramatic changes in the composition of the intestinal microbiome begin during the first year of life with the rapid microbial colonization of the newborn intestinal tract (Palmer, Bik, DiGiulio, Relman, & Brown, 2007). Research suggests that the microbial population that develops during the early months of a newborn's life varies highly from infant to infant (Palmer et al., 2007; Stark & Lee, 1982), and that the newborn diet represents an essential extrinsic factor related to the establishment of the gut microbiota (Fanaro et al., 2003).

Diet Composition:

Many studies suggest that the gut microbial profile of breastfed infants is dominated by Bifidobacterium with the addition of a few other anaerobes and small numbers of facultative anaerobic bacteria (Stark & Lee, 1982). It is thought that the colonization of Bifidobacterium and Bacteroides (another commonly found organism in the breastfed infant gut) is stimulated by the presence of human milk oligosaccharides (HMOs), the most abundant carbohydrate component inbreast milk. Infants lack the enzymes necessary to digest HMOs causing them to pass into the lower intestinal tract where they are thought to function as a prebiotic, stimulating the growth of Bifidobacterium and Bacteroides (Marcobal & Sonnenburg, 2012). In addition to Bifidobacterium and Bacteroides, Streptococcus, and Lactobacillus have been found in breastfed infants (Harmsen et al., 2000).

Diet continues to play a primary role in generating compositional change and diversity in the microbiome as dietary patterns progress over the first three years. Studies have shown that major shifts in the taxonomic groups of the microbiome have been observed with changes in diet such as weaning to solid foods (Koenig et al., 2011). The introduction of table food to the breastfed infant causes a rapid rise in the number of enterobacteria and enterococci, followed by progressive colonization by Bacteroides spp., Clostridium, and anaerobic Streptococcus. In formula-fed infants, however, the transition to solid food does not have as great an impact on gastrointestinal flora (Stark & Lee, 1982). As the amount of solid food in the diet increases, the bacterial flora of both breast and bottle-fed babies approach that of adults (Stark & Lee, 1982) with a sustained increase in the abundance of Bacteroidetes, elevated fecal short chain fatty acid levels, enrichment of genes associated with carbohydrate utilization, vitamin biosynthesis, xenobiotic degradation, and a more stable community composition characteristic of the adult microbiota (Koenig et al., 2011).

In summary, diet composition and patterns during the first three years of life may impact the diversity and functional capacity of the gut microbiome with potential downstream effects (as detailed below) on infant development and disease risk (Johnson & Versalovic, 2012). Understanding the colonization patterns of the gut microbiota during infancy and early childhood, the factors that influence colonization (see Figure 3), and the mechanisms through which the gut microbiota interact with immune regulation, the endocrine system, and

metabolism may help in the development of strategies to guide the formation of healthpromoting microbiotas that could then be maintained throughout the lifespan. In infant body diet composition is very effective as the whole microbiome include there and those function is regulate the. Infants development.

Clinical implementation of Infant:

Currently, the American Academy of Pediatrics Committee on Breastfeeding recommends that all preterm infants receive human milk . According to these recommendations, a mother's own milk, fresh or frozen, should be the primary diet and should be fortified appropriately for infants born weighing <1.5 kg. If the mother's own milk is unavailable despite significant lactation support, pasteurized donor milk should be used. Currently in level 3 neonatal intensive care units, preterm infants receive enteral feeds with mother's own milk, donor milk, or formula. A Cochrane review suggested that donor breast milk is associated with a lower risk of NEC but slowergrowth in the early postnatal period, but the quality of the evidence is limited (40). Further research is needed to confirm these findings and measure the effect of fortified or supplemented donor breast milk. Whether the lack of bacteria in donor milk may incur risks when compared with an infant's own mother's milk remains speculative. Antibiotics should be used judiciously across the lifespan, but perhaps especially so during the first 1,000 days. In cases where antibiotic treatment is necessary, practitioners might consider probiotic supplements as early evidence suggests this may lessen the deleterious impact of antibiotics on the infant gut microbiome

Finally, perinatal and pediatric healthcare providers should support the early initiation of breastfeeding for all newborns since breastfed infants are less likely to be colonized by potentially pathogenic organisms like C. difficile. The promotion of breastfeeding may support the proliferation of beneficial microbes, thus providing protection from those linked with atopy or NEC.

There is a broad consensus that the intestinal microbiota plays an important physiologic role for the host. This has significant implications for very low birth weight infants. The neonatal microbiome is likely to be influenced by the human-milk microbiome, and if we can optimize beneficial microbes this may lead to numerous benefits including decreased mortality, less antibiotic use and thus less antibiotic resistance, decreased rates of NEC and sepsis, and both direct and indirect cost savings.

Developing Microbiome:

From the human microbiome project we can analysis how much microbe reschedule in various regions of body and develop the hole system of our body specifically the region of intestinal microbiota. The total number of bacterial cells residing in the human intestine far exceeds the number of host cells. More than 3 million genes have been identified in this "microbiome," which is >100-fold of our ownhumangenes(1). Butinterestingly,

most of the microbiome in the human intestine is restricted to 4 dominant phyla: Firmicutes, Bacteroides, Actinobacteria and Proteobacteria. The intestinal microbes our council or in my own they have the proper mechanism for digest food material and help in the development of the immune system and post Natal maturation. Intestine.

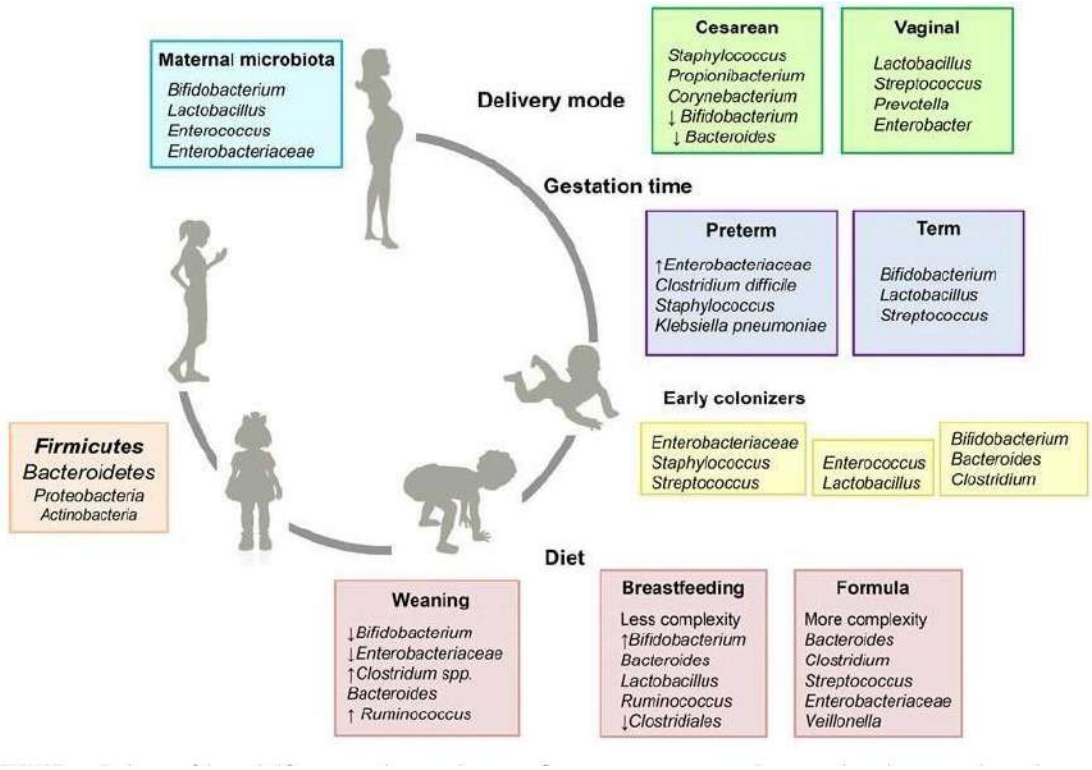


Figure 1: Evolution of early life gut microbiota and events influcing its composition.

The amniotic fluid (AF)3 from mothers of infants born prematurely is often colonized by microbes. The quantity of microbial DNA correlates inversely with gestational age, and directly with IL-6 and white blood cells in AF (2,3). Although evidence supports that the majority of microbes found in AF are of vaginal origin, seeding the AF via translocation through the chorioamniotic intracellular junctions (4), other origins such as maternal intestinal translocation or hematogenous spread from periodontal tissue are also possible. An injection of endotoxin or IL-1 into AF invokes a strong inflammatory response in theileumofthe125-dovinefetus. Fetal intestinal inflammation and fetal inflammatory response syndrome induced by ureaplasma is blunted with intraamniotic injection of IL-1b receptor antagonist (IL1RA) (10,11). Thus, the fetal intestine is a likely origin of fetal inflammatory response syndrome that is responsible for triggering preterm labor.

AF is difficult to obtain routinely for the purpose of research investigations. However, meconium and infant stools are readily available, and collection is noninvasive. The first stool in the newborn (meconium) reflects the in utero fetal intestinal environment. If microbes in AF enter the intestine of the fetus and cause an inflammatory response, then microbial remnants as well as some of the markers of inflammation would be expected to be present in the meconium of these infants.

Indeed, in our ongoing studies in premature infants at risk of necrotizing enterocolitis (NEC), we found significant quantities of microbial DNA in the meconium of these infants (12). We speculate that a relation exists between the fetal microbiome as evaluated in meconium and spontaneous preterm labor. The mechanisms of this relation remain poorly understood, but research in the next few years is likely to yield information that should lead to microbehost based interventions for prevention.

Human milk as a source of microbes:

In the year open 1900,And it's before there were only source of nutrition was human milk which act as a high nutrient food for infants. the composition of human make is identical for optimising infant health. Besides nutritional value off human milk,

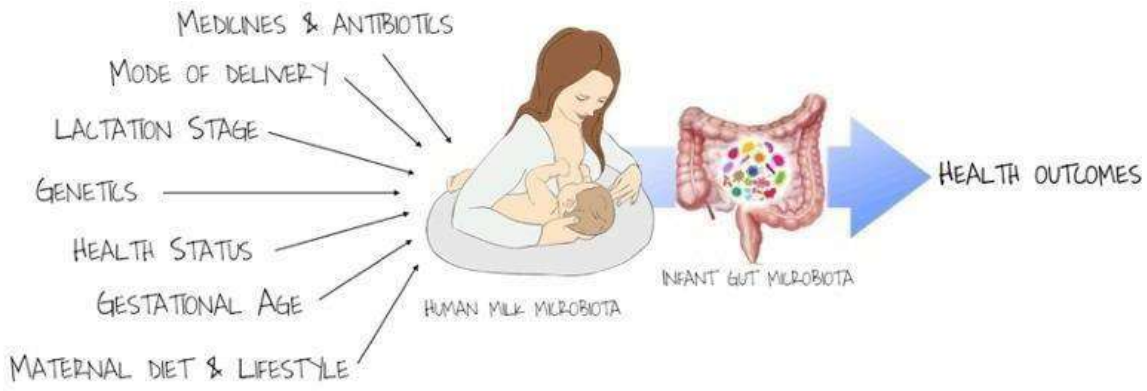


Figure 2: Modulation of infants gut microbiota via mother gut & human milk microbiota

it has also immunological role. The protective role of human milk seems to be the consequence of a synergistic action of the wide range of health-promoting components such as carbohydrates, nucleotides, FAs, immunoglobulins, cytokines, immune cells, lysozymes, lactoferrin, and other immunomodulatory factors (32). Formula and donor breast milk contain many ingredients similar to an infant'sownmother's breast milk, but most notably, because of heat treatment, they both lack live immune cells and microbes that provide the infant both direct and indirect protection against disease (33) .That is accepted that the microbiota of intestinal area of the breast feed infant's provide anti infective properties. That intestinal microbiota has the ability to regulate and increase the affinity of postnatal development of mew system.

Human milk also contains a group of bacteria called the human-milk microbiome (34). Human microbiome develop with the advent of culture independent techniques and the development of the "omics". Culture-dependent techniques can identify from 2 to 18 different bacterial species in an individual milk sample. Some of the most commonly cultured species include Staphylococcus epidermidis, Staphylococcus aureus, Lactobacillus salivarius, Lactobacillus fermentum, Bifidobacterium breve, and Bifidobacterium bifidum (34). Two of these bacterial groups, Lactobacillus and Bifidobacterium, are recognized to have health-promoting properties and are present in commercial foods such as yogurt and used in pharmaceutical probiotics to enhance protective gut microbiota, thus improving intestinal microbial balance. Hindi culture

independent techniques gram negative bacteria is not identified. the human milk microbiome is a very large and complex system with great diversity. The newer culture-independent techniques using 16S ribosomal RNA have allowed a more comprehensive assessment of the bacterial diversity of human milk (34). Some bacterial genera obviously present in basement of women that are Streptococcus, Staphylococcus, Serratia, Pseudomonas, Cornybacteria, Ralstonia, Proprionibacterium, Sphingomonas, and Bradyrhizobiaceae.

Human milk has a microbiome that tends to stable overtime. There are three evidence of human milk microbiome. The first hypothesis is that hormonal changes during pregnancy cause the gastrointestinal tract of the mother to be more permeable, and this facilitates bacterial uptake by the bloodstream, which transports the bacteria to the mammary gland. The second hypothesis is knownasthe contamination theory. This postulates that human-milk microbes come from direct contamination of the mother's skin and oral secretions from the infant. The third hypothesis involves active migration of bacteria with the aid of dendritic cells. This final hypothesis is thought to be the most likely mechanism based on the current research and is important for future research that may lead to ways to manipulate the transport of certain favorable bacterial strains from the maternal gut to improve maternal and infant health.

Gut microbiota reduce obesity and metabolic disease:

A recent meta-analysis , which included seven studies conducted in different locations (>1825 gut samples obtained from 684 infants), reported that exclusive breastfeeding practices shape the gut microbiota by promoting higher relative abundances of Bacteroidetes and Firmicutes. This change in bacterial composition is accompanied by increases in predicted microbial pathways related to carbohydrate, lipid and vitamin metabolism, as well as detoxification pathways, when compared to non-exclusively breastfed neonates. The identified differences in the predicted microbial pathways were higher in the non-breastfed neonates delivered by C-section when compared to the vaginally delivered neonates. Recently, it has been demonstrated that the shifts induced by C-section delivery on infant gut microbiome may be partially reestablished to a similar-vaginal birth microbiota by exclusive breastfeeding. Furthermore, exclusive breastfeeding practices of a longer duration were associated with ower gut microbiota dysbiosis related to diarrhea. The gut microbiota differences between the exclusively and non-exclusively breastfed neonates persisted until six months of age, suggesting the short- and long-term benefits of exclusive breastfeeding in terms of the gut microbiota across different populations. In this way infant regulate its metabolic and protective mechanism of it's own body.

Conclusion:

Breast milk represents a complex and dynamic system that allows mother-infant communication and signaling. The components of the "mother-breast milk-infant" triad are closely connected to each other, and every single variation could affect the trajectory of infant development or maternal health [8]. Taking care of women's health, in terms of diet and lifestyle, during the preconception period, pregnancy, and breastfeeding could represent a prevention strategy in terms of improving the offspring health. Nowadays, the increased percentage of women who follow elimination diets by choice or by necessity underlines the importance of providing specialist care in order to prevent malnutrition and the adverse associations with maternal health and infant's growth and development.

Breast milk is one of the main factors driving the proliferation of a protective gut bacterial community enriched in bifidobacteria. Bioactive factors in human milk may promote the growth of beneficial bacteria and therefore ameliorate infant health. Although breast milk is considered the ideal nutrition for newborns, formulas represent an alternative for those who are unable or choose not to breastfeed. Formula composition has greatly improved within the past decade. The addition of prebiotics to infant formula has shown beneficial effects on the gut microbiota. Novel ingredients may further contribute to minimize differences between breastfed and formula-fed infants. The maternal metabolic status both before and during gestation exerts a significant influence on the infant microbiota at the beginning of life. Although the maternal diet has an influence on the infant microbiota, it appears that early-life nutritional patterns have a stronger effect on the microbiota.

Reference:

1. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons gene catalog.

2. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature 2012;489:242–9. .

3.EvansJM,MorrisLS,MarchesiJR.Thegutmicrobiome:theroleofavirtual organ in the endocrinology of the host. J Endocrinol 2013;218:R37–47.

4. Fischbach MA, Sonnenburg JL. Eating for two: how metabolism establishes interspecies interactions in the gut. Cell Host Microbe 2011;10:336–47.

5. Arrieta MC, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B. The intestinal microbiome in early life: health and disease. Front Immunol 2014;5:427.

6. Chassaing B, Etienne-Mesmin L, Gewirtz AT. Microbiota-liver axis in hepatic disease. Hepatology 2014;59:328–39.

7. Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 2012;10:735–42.

8. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 2012;13:701–12

9. Clarke G, O'Mahony S, Dinan T, Cryan J. Priming for health: gut microbiota acquired in early life regulates physiology, brain and behaviour. Acta Paediatr 2014;103:812–9.

10. Patterson E, Cryan JF, Fitzgerald GF, Ross RP, Dinan TG, Stanton C. Gut microbiota, the pharmabiotics they produce and host health. Proc Nutr Soc 2014;73:477–89.

11. Macfarlane GT, Macfarlane S. Bacteria, colonic fermentation, and gastrointestinal health. J AOAC Int 2012;95:50–60.

12. Jacobi SK, Odle J. Nutritional factors influencing intestinal health of the neonate. Adv Nutr 2012;3:687–96.

13. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, W, Pettersson S. Host-gut microbiota metabolic interactions. Science 2012;336:1262–7.

14. De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, Backhed F, Mithieux G. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell 2014;156:84–96.

15. Selkrig J, Wong P, Zhang X, Pettersson S. Metabolic tinkering by the gut microbiome: implications for brain development and function. Gut Microbes 2014;5:369–80.

16. O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res 2015;277:32–48.

17. Clarke G, Stilling RM, Kennedy PJ, Stanton C, Cryan JF, Dinan TG. Minireview: gut microbiota: The neglected endocrine organ. Mol Endocrinol 2014;28:1221–38.

18. LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol 2013;24:160–8.

19. Lyte M. Microbial endocrinology: host-microbiota neuroendocrine interactions influencing brain and behavior. Gut Microbes 2014;5:381–9.

20. Rautava S, Luoto R, Salminen S, Isolauri E. Microbial contact during pregnancy, intestinalcolonization and human disease. Nat Rev Gastroenterol Hepatol 2012;9:565–76.

21. Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt PA, Stobberingh EE. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 2006;118:511–21.

22. ScholtensPA,OozeerR,MartinR,AmorKB,KnolJ.Theearlysettlers:intestinal microbiology in early life. Annu Rev Food Sci Technol 2012;3:425–47.

23. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci USA 2010;107:11971–5.

24. Arboleya S, Binetti A, Salazar N, Fernandez N, Solis G, HernandezBarranco A, Margolles A, de Los Reyes-Gavilan CG, Gueimonde M. Establishment and development of intestinal microbiota in preterm neonates. FEMS Microbiol Ecol 2012;79:763–72.

25. Berrington JE, Stewart CJ, Embleton ND, Cummings SP. Gut microbiota in preterm infants: assessment and relevance to health and disease. Arch Dis Child Fetal Neonatal Ed 2013;98:F286– 90.

26. Hunt KM, Foster JA, Forney LJ, Schutte UM, Beck DL, Abdo Z, Fox LK, Williams JE, McGuire MK, McGuire MA. Characterization of the diversity and temporal stability of bacterial communities in human milk. PLoS One 2011;6:e21313.

27. Fernández L, Langa S, Martin V, Maldonado A, Jimenez E, Martin R, Rodriguez JM. The human milk microbiota: origin and potential roles in health and disease. Pharmacol Res 2013;69:1– 10.

28. Jost T, Lacroix C, Braegger CP, Rochat F, Chassard C. Vertical motherneonate transfer of maternal gut bacteria via breastfeeding. Environ Microbiol 2014;16:2891–904.

29. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, Welling GW. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 2000;30: 61–7

30. Adlerberth I, Wold AE. Establishment of the gut microbiota in western infants. Acta Paediatr 2009;98:229–38.

31. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol 2007;5:e177.

32. Veereman-Wauters G, Staelens S, Van de Broek H, Plaskie K, Wesling F, Roger LC, McCartney AL, Assam P. Physiological and bifidogenic effects of prebiotic supplements in infant formulae. J Pediatr Gastroenterol Nutr 2011;52:763–71.

33. Sierra C, Bernal MJ, Blasco J, Martinez R, Dalmau J, Ortuno I, Espin B, Vasallo MI, Gil D, Vidal ML, et al. Prebiotic effect during the first year of life in healthy infants fed formula containing gos as the only prebiotic: a multicentre, randomised, double-blind and placebo-controlled trial. Eur J Nutr 2015;54:89–99.

34. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci USA 2011;108 Suppl 1: 4578–85.

35. Schulfer A, Blaser MJ. Risks of antibiotic exposures early in life on the developing microbiome. PLoS Pathog 2015;11:e1004903.
36.KorenO,GoodrichJK,CullenderTC,SporA,LaitinenK,BackhedHK,Gonzalez A, Werner JJ, Angenent LT, KnightR,etal.Hostremodelingofthegut microbiome and metabolic changes during pregnancy. Cell 2012;150:470–80.

37. Kerr CA, Grice DM, Tran CD, Bauer DC, Li D, Hendry P, Hannan GN. Early life events influence whole-of-life metabolic health via gut microflora and gut permeability. Crit Rev Microbiol 2015;41:326–4

38. Le Huërou-Luron I, Blat S, Boudry G. Breast-v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. Nutr Res Rev 2010;23:23–36.

39. Maga EA, Weimer BC, Murray JD. Dissecting the role of milk components on gut microbiota composition. Gut Microbes 2013;4:136–9.

40. Singhal A, Macfarlane G, Macfarlane S, Lanigan J, Kennedy K, EliasJonesA,StephensonT,DudekP,LucasA.Dietarynucleotides and fecal microbiota in formula-fed infants: a randomized controlled trial. Am J Clin Nutr 2008;87:1785–92.

41. Kunz C, Rudloff S, Baier W, Klein N, Strobel S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu Rev Nutr 2000;20:699–722.

42. Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O'Ryan ML, Ruiz- Palacios G, Hilty MD, Pickering LK, Prieto PA. Variability of human milk neutral oligosaccharides in a diverse population. J Pediatr Gastroenterol Nutr 2000;30:181–92

43. Rudloff S, Kunz C. Milk oligosaccharides and metabolism in infants. Adv Nutr 2012;3:398S– 405S.

44. Marcobal A, Sonnenburg JL. Human milk oligosaccharide consumption by intestinal microbiota. Clin Microbiol Infect 2012;18 Suppl 4:12–5.

45. Goehring KC, Kennedy AD, Prieto PA, Buck RH. Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PLoS One 2014;9:e101692.

46. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 2012;22:1147–62.

47. Duska-McEwen G, Senft AP, Ruetschilling TL, Barrett EG, Buck RH. Human milk oligosaccharides enhance innate immunity to respiratory syncytial virus and influenza in vitro. Food Nutr Sci 2014;5:1387–98.

48. Wang B. Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. Adv Nutr 2012;3:465S–72

49. Bienenstock J, Buck RH, Linker H, Forsythe P, Stanisz AM, Kunze WA. Fucosylated but notsialylated milk oligosaccharides diminish colon motor contractions. PLoS One 2013;8:e76236.

50. Vázquez E, Barranco A, Ramirez M, Gruart A, Delgado-Garcia JM, Martinez-Lara E, Blanco S, Martin MJ, Castanys E, Buck R, et al. Effects of a human milk oligosaccharide, 29-fucosyllactose, on hippocampal long-term potentiation and learning capabilities in rodents. J Nutr Biochem 2015;26:455–65.

51. Pacheco AR, Barile D, Underwood MA, Mills DA. The impact of the milk glycobiome on the neonate gut microbiota. Annu Rev Anim Biosci 2015;3:419–45.

52. Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA. Infants fed a lower calorie formula with 29-fucosyllactose (29-fl) show growth and 29-fl uptake like breast-fed infants. J Pediatr Gastroenterol Nutr 2015 July 6 (Epub ahead of print).

53. Garrido D, Barile D, Mills DA. A molecular basis for bifidobacterial enrichment in the infant gastrointestinal tract. Adv Nutr 2012;3:415S–21S.

PROJECT REPORT ON :**EMPHASIS OF MYCORRHIZA IN ENVIROMENT**

SUBMITTED BY:ARINDAM GANGULY

RAMAKRISHNA MISSION VIVEKANANDA CENTENARY COLLEGE

DEPARTMENT OF MICROBIOLOGY

REG. NO:A01:1112-116-006-2018

ROLL NO:1606

SEMESTER:V

GUIDED BY:Dr. SUJOY PAL

INTRODUCTION- Mycorrhizae literally translates to “fungus-root.” Mycorrhiza defines a (generally) mutually beneficial relationship between the root of a plant and a fungus that colonizes the plant root. In many plants, mycorrhiza are fungi that grow inside the plant’s roots, or on the surfaces of the roots. The plant and the fungus have a mutually beneficial relationship, where the fungus facilitates water and nutrient uptake in the plant, and the plant provides food and nutrients created by photosynthesis to the fungus. This exchange is a significant factor in nutrient cycles and the ecology, evolution, and physiology of plants.In some cases, the relationship is not mutually beneficial. Sometimes, the fungus is mildly harmful to the plant, and at other times, the plant feeds from the fungus.Not all plants will have mycorrhizal associations. In environments in which water and nutrients are abundant in the soil, plants do not require the assistance of mycorrhizal fungi, nor might mycorrhizal fungi germinate and grow in such environments.Plants associate with other life forms (animals, bacteria or fungi) to complete their life cycle, to fight against pathogens or to thrive in adverse environments. The plant root and its associated living organisms are together called ‘rhizosphere’, the region of mycorrhizal association. Mycorrhiza is one of the best examples of symbiotic1 association between plants and fungi. The term ‘mycorrhiza’ comes from Greek – mycos meaning fungus and rhiza meaning roots. In nature, more than eighty percent of angiosperms, and almost all gymnosperms are known to have mycorrhizal associations. There are mainly two types of mycorrhizal associations found in nature (Box 1) namely, endomycorrhizae or arbuscular-mycorrhizae (AM), eg., Endogone, Rhizophagus, etc. and ectomycorrhizae (EM), eg., Laccaria bicolor, Amanita mascaria, etc. Mycorrhizal associations help the host plants to thrive in adverse soil conditions and drought situations by increasing the root surface and mineral uptake efficiency. Environmental threats like increased temperature, changing climate and associated drought, soil infertility, etc., are some of the major challenges in agriculture and have to be mitigated to ensure global food security. In this context, mycorrhiza-based crop production is one of the key components of sustainable agriculture practice

- TOPIC DESCRIPTION:- There are two predominant types of mycorrhizae: ectomycorrhizae, and endomycorrhizae. They are classified by where the fungi colonize on the plants.

- ECTOMYCORRHIZAE:- Ectomycorrhiza tend to form mutual symbiotic relationships with woody plants, including birch, beech, willow, pine, oak, spruce, and fir. Ectomycorrhizal relationships are characterized by an intercellular surface known as the Hartig Net. The Hartig Net consists of highly branched hyphae connecting the epidermal and cortical root cells. Additionally, ectomycorrhiza can be identified by the formation of a dense hyphal sheath surrounding the root's surface. This is known as the mantle. In other words, ectomycorrhiza live only on the outside of the root. Overall, only 5-10% of terrestrial plant species have ectomycorrh

- ENDOMYCORRHIZAE:- On the other hand, endomycorrhizae are found in over 80% of extant plant species -including crops and greenhouse plants such as most vegetables, grasses, flowers, and fruit trees. Endomycorrhizal relationships are characterized by a penetration of the cortical cells by the fungi and the formation of arbuscules and vesicles by the fungi. In other words, endomycorrhizahave an exchange mechanism on the inside of the root, with the fungi's hyphae extending outside of the root. It is a more invasive relationship compared to that of the ectomycorrhiza.Endomycorrhiza are further subdivided into specific types: Arbuscular Mycorrhizae, Ericaceous Mycorrhizae, Arbutoid Mycorrhizae, and Orchidaceous Mycorrhizae.

❖ EXAMPLES OF MYCORRHIZA:-

❖ Orchid Mycorrhiza--As mentioned above, some orchids cannot photosynthesize prior to the seedling stage. Other orchids are entirely non-photosynthetic. All orchids, however, depend on the sugars provided by their fungal partner for at least some part of their lives. Orchid seeds require fungal invasion in order to germinate because, independently, the seedlings

cannot acquire enough nutrients to grow. In this relationship, the orchid parasitizes the fungus that invades its roots. Once the seed coat ruptures and roots begin to emerge, the hyphae of orchidaceous mycorrhiza penetrate the root's cells and create hyphal coils, or pelotons, which are sites of nutrient exchange.

- Arbuscular Mycorrhiza--Arbuscular mycorrhizae are the most widespread of the micorrhizae species and are well known for their notably high affinity for phosphorus and ability for nutrient uptake. They form arbuscules, which are the sites of exchange for nutrients such as phosphorus, carbon, and water. The fungi involved in this mycorrhizal association are members of the zygomycota family and appear to be obligate symbionts. In other words, the fungi cannot grow in the absence of their plant host.

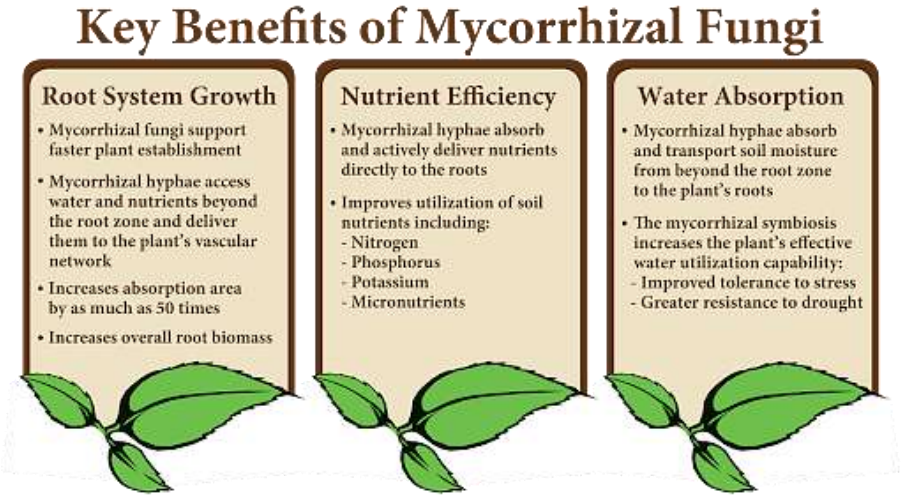
- Ericaceous Mycorrhiza--Ericaceous mycorrhizae is generally found on plants of the order Ericales and in inhospitable, acidic environments. While they do penetrate and invaginate the root cells, ericoid mycorrhiza do not create arbuscules. They do, however, help regulate the plant's acquisition of minerals including iron, manganese, and aluminum. Additionally, mycorrhizal fungi form hyphal coils outside of the root cells, significantly increasing root volume.

- Arbutoid Mycorrhiza--Arbutoid mycorrhiza are a type of endomycorrhizal fungi that look similar to ectomycorrhizal fungi. They form a fungal sheath that encompasses the roots of the plant; however, the hyphae of the arbutoid mycorrhiza penetrate the cortical cells of plant roots, differentiating it from ectomycorrhizal fungi.

- Ectotrophic Mycorrhiza--The fungi involved in this mycorrhizal association are from the Ascomyota and Basidiomyota families. They are found in many trees in cooler environments. Unlike their wood-rotting family members, these fungi are not adapted to degrade cellulose and other plant materials; instead, they derive their nutrients and sugars from the roots of their living plant host.

❖ BENEFITS OF MYCORRHIZAE--

- It enhances the water and nutrient intake.
- The association reduces irrigation requirements.
- Need for artificial fertilizers reduces.
- Plant health thrives and becomes stress tolerant.
- Higher transplanting success.
- Makes the plants better at surviving.
- It makes the plants more resistant to diseases and droughts.
- Mycorrhizal plants release chemicals which keep the insects away from it.
- It makes the plants more resistant to toxins present in the environment.



❖ **ROLE OF MTCORRHIZAE IN AGRICULTUIRE AND FORESTY:**

- Role in Agriculture**:**
    a. The mycorrhizal association helps in the formation of dichotomous branching and profuse root growth, thus enhances plant growth.

    b. Ectotrophic mycorrhiza helps in uptake of mineral ions and also acts as reservoir.

    c. They also help in absorption of nutrients.

d. In nutrient deficient soil, the mycelial association helps in the absorption of N, Ca, P, Zn, Fe, Na and others.

e. Mycorrhizal association is obligatory for the germination of orchid seeds.

f. Mycorrhizal growth in orchids (Rhizoctonia repens with Orchis militaris tuber tissues) causes the synthesis of phytoalexins — orchinol and hirsinol. Both the compounds act as a barrier to protect infection by other pathogens.

g. Inoculation of VAM as biofertiliser provides a distinct possibility for the uptake of P in phosphorus-deficient soil.

- Role in Foresty:
    a. Mycorrhiza plays an important role to establish forest in unfavourable location, barren land, waste lands etc.

    b. Trees with facultative endomycorrhiza act as first invader in waste lands as pioneer in plant succession.

    c. The application of mycorrhizal fungi in forest bed enhances the formation of mycorrhizal association that prevents the entry of fungal root pathogens. This method is very much effective in the root of Pinus clausa against Phytophthora cinnamoni infection.

    d. Mycorrhiza mixed nitrogenous compounds such as nitrate; ammonia etc. is available to the plants. Thus it helps in plant growth, especially in acid soil.

❖ THINGS TO REMEMBER:--

- It is a mutual beneficial association between host plant and fungi
- The association is crucial in maintaining nutrient cycles and balance in ecology.

- The plant receives essential nutrients and moisture from the fungi.
- The fungi gets its carbohydrate requirements from the plant.
- There are mainly 2 types of mycorrhizae - ectomycorrhiza and endomycorrhiza.
- Sometimes mycorrhizal association can be harmful to the plant at high colonization sites of fungi.
- The association helps the plants to keep the insects away and improve the plant health.
- This association also regulates the environment around the plant to make it suitable for its growth.

❖ CONCLUSION—

Though the study of mycorrhizal fungi has been a neglected field, yet in India works on mycorrhizal fungi was started by B.K. Bakshi at Forest Research Institute, Dehra Dun. His project report Mycorrhiza and its role in forestry (1974) published by F.R.I, has served as a mile stone for the beginner.

Mishra and Sharma (1981) reported the association with Pinus kesiya of Amanita muscaria, Boletus edulis, Cenococcus geophilus, Inocybe rimosa, Russula roseipes, Scleroderma aurantium, Suillus bovinus from Meghalaya of North East Himayala. Moreover, association of ectomycorrhizal fungi with some other gymnosperm

(Cedrus, Cryptomeria and Pinus) has been explored by R.R. Mishra and his research group. The work on this aspect done by K.S. Thind and his research group at Chandigarh cannot be overlooked. Lakhanpal (1987) has reported 72 fungal species forming mycorrhiza in several forest trees in the Western Himalaya.At Forest Research Institute, Dehra Dun, in addition to significant research work done by B.K. Bakshi and coworkers, mycorrhizal researches are being strengthened. Amanita muscaria, L. laccata and Scleroderma citrinus were recorded from Pinus patula, and A. muscaria, and L. chinensis were identified from Eucalyptus globulus.

Variations in genotypes of the host, biotic disturbances in the forest, tree density, soil temperature, soil pH, moisture and the presence of antagonistic microorganisms were the factors that governed the occurrence of ectomycorrhizal fungi in different oak forests.

The western and eastern Ghats of South India have been extensively surveyed by A. Mahadevan and his research group of Madras

University. Dubey (1998) have reviewed the influence of nutrients on formation and growth of ectomycorrhiza.In India, B.K. Bakshi (1974) was the first to publish an account of 14 spore types of 5 genera of Endogonaceae (VAM Fungi) such as Glomus, Gigaspora, Acaulospora, Endogone and Sclerocystis from forest soils.

Sharma (1986, 1987) recorded the species of Acaulospora, Gigaspora, Glomus and Sclerocystis from rhizosphere soils of different forest trees of Meghalaya. Negi (1993) recorded Endogone sp. in soils of Cupressus torulosa and VA-mycorrhizal fungi in root tissues from Nainital. Vesicles of varying colours and numbers were observed on C. torulosa roots throughout the year.

The maximum infection was recorded in winter and minimum in summer seasons. In Himachal Pradesh 10 districts were periodically surveyed over a wide altitudinal range of 500 to 2500 meters. A total of ten VAM fungi of four genera were recorded.

A significant work on VAM fungi has been done by Sudhir Chandra and Kehri of the University of Allahabad especially on management of waste land and introduction in crop field for high yield of crops. D.J. Bagyaraj and his research

group have done excellent work on VAM fungi as far as crop improvement is concerned. Reena and Bagyaraj (1990) screened several VAM fungi for their suitability to use as inoculants for two slow growing tree species. Acacia nilotica and Calliandra calothyrsus.

REFERENCES—

https://en.wikipedia.org/wiki/Ectomycorrhiza

http://www.growersgold.net/what-is-mycorrhizae

Zeng, R. (2006). Disease Resistance In Plants Through Mycorrhizal Fungi Induced Allelochemicals. Allelochemicals: Biological Control of Plant Pathogens and Diseases Disease Management of Fruits and Vegetables, 181-192. doi:10.1007/1-4020-4447-x_10

https://en.wikipedia.org/wiki/Mycorrhiza#Occurrence_of_mycorrhizal_association

http://www.mykepro.com/mycorrhizae-benefits-application-and-research.aspx

https://mycorrhizae.com

https://en.wikipedia.org/wiki/Occurrence_of_mycorrhizal_association

http://www.growersgold.net/about mycorrhizae