Investigation of Measles During 2022-Outbreak in Pakistan

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2023

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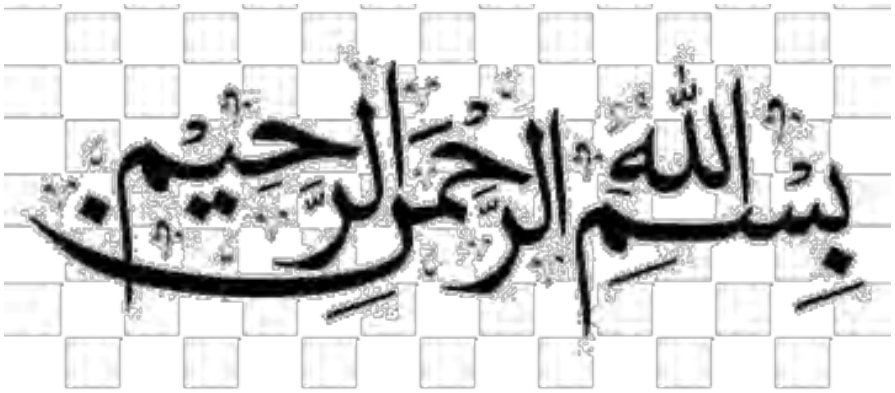
A thesis submitted in the partial fulfillment of the requirements for the degree of

MASTER OF PHILOSOPHY

In

BIOTECHNOLOGY

Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.



In the name of Allah, The Most Gracious, The Most Merciful

DECLARATION OF ORIGINALITY

I hereby declare that the work **"Investigation of Measles During 2022-Outbreak in Pakistan"** accomplished in this thesis is the result of my own research carried out in ***Infectious Diseases and Molecular Pathology laboratory***, Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan. This research study has not been published previously nor does it contain any material from the published resources that can be considered as a violation of international copyright law. Furthermore, I also declare that I am aware of the term "copy right" and plagiarism. If, any copyright violation is found in this research work, I will be responsible for the consequence of any such violation.

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DEDICATION

I dedicate this dissertation, wholeheartedly, to my beloved parents, siblings, and my respected supervisor. Without their prayers, support and guidance, accomplishment of this work would not have been possible for me.

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ACKNOWLEDGEMENT

I am overwhelmed in all the humbleness and beholden to Allah Almighty who is The Omniscient and guided me through this research to put the ideas into something concrete. Countless mercy on the Holy Prophet Mohammad (S.A.W).

First and the foremost would like to extend my deepest gratitude to my respected supervisor Dr. Muhammad Ali for his unwavering support and belief in me during my research work. His constructive feedback and mentorship have always been a source of encouragement for me. I also want to acknowledge Dr. Nighat Haider from Pakistan Institute of Medical sciences (PIMS) Islamabad, for her guidance and cooperation for the specimen collection during my research work and Higher Education Commission and Alliance of International Science Organizations (ANSO) for their funding for the project.

I want to acknowledge the help and support of my lab mates Hanbal Ahmad Khan, Asim Saeed, Iffat Irshad, Adnan Zeb my juniors Naveera Khan, Hassan Ayaz, and Ahsan Saeed.

I wish to express my deepest gratitude for my seniors Sidra Rahman, Syed Ahsan Shahid, Tazeen Naik and Sajid Ali Shah who have always been there to guide me in difficult situations and always provided me with their valuable suggestion.

How can I forget the most beautiful people in my life Dr. Naima Iram, Ms. Tehmina Shehzadi, Maryam Nawaz and my brothers who have always got my back in my darkest moments and lifted my spirit to not give up in face of any challenging situation.

Lastly, I am indebted to my beloved parents whose love and prayers are the guiding light for me and have provided me the strength and resilience to achieve my goals.

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LIST OF ABBREVIATIONS

MeV	Measles virus
RPV	Rinder pest virus
PPRV	Peste des petitis ruminant's virus
CDV	Canine distemper virus
PDV	Phocine distemper virus
CeMV	Cetacean morbillivirus
SSPE	Subacute sclerosing panencephalitis
MIBE	Measles inclusion-body encephalitis
RNP	Ribonucleoprotein
ADEM	Acute disseminated encephalomyelitis
CNS	Central nervous system
SLAMF1	Signaling lymphocytic activation molecule family 1
DCs	Dendritic cells
EBV	Epstein-Barr virus
URT	Upper respiratory tact
RT-PCR	Reverse Transcriptase Polymerase Chain reaction
MMR	Measles Mumps Rubella
MCV1	Measles containing vaccine first dose
MCV2	Measles containing vaccine second dose
CP	Complete parameters
DLC	Differential leucocytes
BSR	Blood sugar random
ALT	Alanine transaminase
SGPT	Serum glutamic pyruvic transaminase
TLC	Total Leucocyte count
MHC	Mean Corpuscular Hemoglobin
MCV	Mean Corpuscular volume
MCHC	Mean Corpuscular hemoglobin Concentration
RBC	Red blood cells
PCV	Packed cell volume
RDW-CV	Red cell distribution width-cell volume
NRBC	Nucleated red blood cell count
ANOVA	Analysis of variance
cDNA	Complementary DNA
TAE	Tris-acetate-EDTA
ALP	Alkaline phosphatase

Abstract

Measles is a contagious disease caused by an RNA virus. Resurgence of measles after Covid-19 and its severity among children has led to many speculations about the Measles vaccination coverage and its efficacy. In this study the clinical data of children <9 years (n=19) admitted at Pakistan Institute of Medical Sciences (PIMS) in measles ward was analyzed and the biological specimens were used for the molecular detection of measles virus. The blood samples (n=19) were processed for hematology and routine biochemistry tests. Five samples of blood serum and nasal swabs were used for the RNA Extraction to carry out Reverse Transcriptase-PCR. The results obtained from blood profile were statistically analyzed using SPSS-21 software for One-Way ANOVA (for Complete Parameters (CP), Kruskal Wallis and Mann-Whitney test for Differential leucocyte count (DLC) and Biochemical parameters). A $p<0.05$ was considered significant. The results suggest no significant difference of Complete blood parameters (CP) among non-vaccinated, partially vaccinated and fully vaccinated patients. Among DLC Basophils level was significantly different ($p=0.024$), being lower in partially vaccinated than non-vaccinated patients. Biochemical parameters showed that serum urea level was significantly different ($p=0.013$), showing a decline in fully vaccinated patients as compared to non-vaccinated patients. Moreover, a significantly higher level of Alkaline phosphatase as compared to normal range was observed in fully vaccinated patients. However, lower levels of MCH, MCV, MCHC, RBC, Hb, eosinophils and a higher level of RDW-CV was observed overall as compared to the normal range (healthy individuals). Two samples gave positive results for PCR as the bands were visualized for ~ 523 nucleotide region of measles virus H gene (partial). The results suggest that blood profile of patients may have certain parameters that could vary among the three studied groups (i.e., fully vaccinated, partially vaccinated and non-vaccinated individuals) and further research on measles virus (MeV) mutations, genotyping, phylogenetic analysis, and vaccine optimization could be helpful for deeper understanding of measles virus circulating in Pakistan.

1. Introduction

Measles virus was an inevitable infection that had been frequently experienced by most of the people during their early childhood. It has caused a great number of deaths across the world despite the availability of an effective live attenuated vaccine (Naim, et al., 2015). Measles virus is single stranded negative sense RNA virus belonging to family *Paramyxoviridae* and genus *Morbillivirus* (Rota et al., 2016). There are other five genera belonging to this family including Rinder pest virus (RPV), Peste des petitis ruminants virus (PPRV), Canine distemper virus (CDV), Phocine distemper virus (PDV), cetacean morbillivirus (CeMV) that cause similar infectious diseases in Cattle, sheeps and goats, Carnivores, seals and dolphins respectively (Rima and Duprex, 2006).

The only reservoir of measles virus is human being. The transmission of MeV takes place via respiratory aerosols as it is an airborne virus moreover it can also be transmitted via direct contact to the surface containing respiratory secretions from infected person (Rota et al., 2016). Measles virus has high infectivity rate as one infected person can infect more than 12 persons on average (Plotkin, 2019). As virus makes its way to human body it takes 7-10 days to manifest the symptoms in the form of cough, coryza, fever and following them the rash on face and other parts of body. These symptoms disappear usually after that and develop a lifetime immunity but in children with under developed immune system it can take severe form developing pneumonia, otitis media, encephalitis, blindness and other secondary infections caused due to bacteria and viruses (Griffin, 2013). Sometimes if virus persists in nervous system, it can cause severe neurodegenerative diseases including subacute sclerosing panencephalitis (SSPE) measles inclusion-body encephalitis (MIBE). These usually occur due to mutations in the F protein (Watanabe et al., 2019).

Measles has caused a huge mortality globally before the availability of vaccine in 1963. Approximately 30 million cases were reported and greater than 2 million deaths annually. But with the advent of industrialization, improved lifestyle, nutrition, and introduction of one dose of vaccine during the 1st year of life has declined the mortality rate. Also better health care facilities and antibiotic therapies for measles associated infections contributed toward decreased death rates (Rota et al., 2016).

One character that is common to all of the *Morbilliviruses* is the lack of neuraminidase activity (Griffin, 2013). Neuraminidases are the tetramers that express on the viral surface and serve

for the purpose of release of viral progeny and penetration of virus into host via mucous through desialylation of viral and cellular surface glycans (Chun et al., 2019). Measles virus is relatively stable as compared to other viruses of this family as it is monotypic having a single serotype. It has been divided into eight clades from A to H on the basis of different variable regions of MeV genome and 24 genotypes (Phan et al., 2018).

A cost effective live attenuated vaccine of measles is available and is usually given in two doses first dose at 9th month and second dose in the second year of life. Often measles vaccine is incorporated in mumps and rubella vaccine, and it is equally effective (WHO. 2019). Most of the countries have met WHO targets of measles complete immunization and have successfully eradicated measles but it still prevails in some African and Asian countries. Pakistan is also among those countries where there are still significant number of measles cases.(Mere et al., 2019) B3 genotype has been reported in Pakistan previously from different areas including Sindh, Punjab Islamabad and Khyber Pakhtunkhwa (Zaidi, Hameed, Suleman Rana, et al., 2018; Zaidi et al., 2017; Ilyas et al., 2020).

There is a resurgence of measles cases in Islamabad after covid-19 pandemic in 2022. Several factors have been speculated for this re-emergence of measles. Despite complete vaccination in some patients, they still have developed symptoms of measles and deaths have occurred simultaneously because of severity of disease.

2.1 Aims and Objectives

- To investigate the hematological and biochemical parameters of measles patients in Islamabad.
- To optimize conditions for amplification of measles virus H gene (partial genome).

2. Literature review

2.1 Measles Virus (MeV) structure

Measles virus Belongs to *Paramyxoviridae* family and genus *Morbillivirus*. It is single stranded, enveloped, and has non segmented negative sense 50S RNA genome (Rota et al., 2016). Under electron microscope the measles virus is pleomorphic with a diameter range of 300-1000nm. It has a helical nucleocapsid (60kDa) and a lipoprotein bilayer having glycoprotein spikes projected outside. Norrby et. al and St. Geme at. al., were the first to report the presence of RNA in measles virion (Norrby, et al., 1964; St. Geme, et al., 1964).

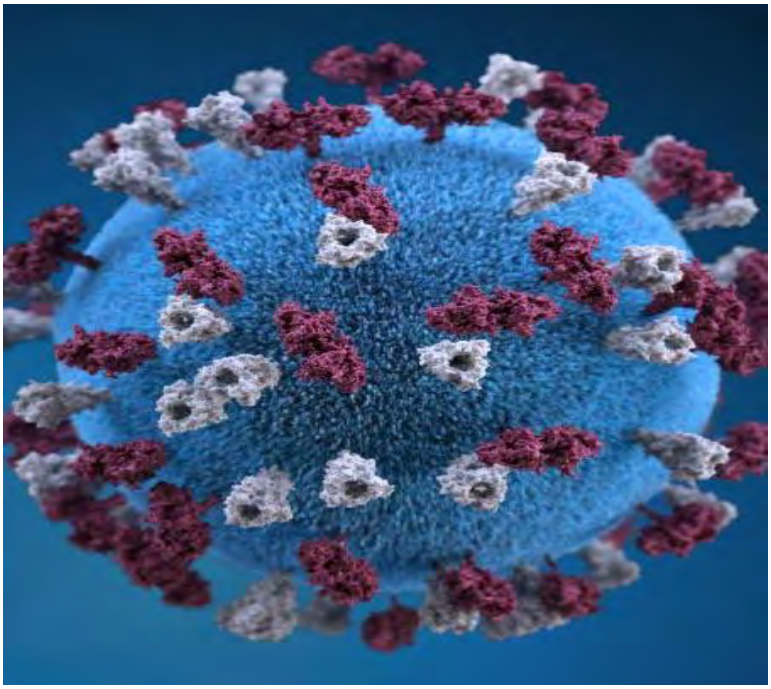


Figure 1. Morphology of measles virus under microscope (CDC., 2020).

2.1.1 Genome structure

The length of MeV genome is 15,894 nucleotides having 56 nucleotide 3' extracistronic region known as leader and then six genes N-P-M-F-H-L that code for 8 proteins including six structural proteins and two accessory proteins and ending with 40 nucleotides extracistronic region known as trailer. These extracistronic regions are essential for control of gene replication, transcription and flanking of genes (Griffin, 2013).

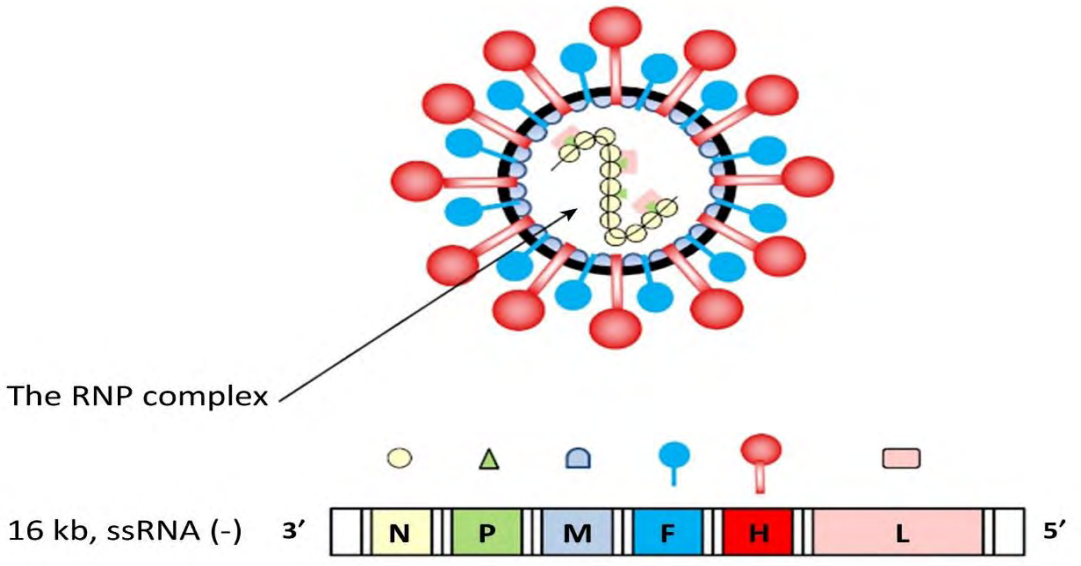


Figure 2. measles virus structure and genomic arrangement (Watanabe et al., 2019).

Table 1. Overview of measles viral genomic composition. (https://www.ncbi.nlm.nih.gov/nuccore/NC_001498.1?report=graph)

Sr no.	Gene name	Genomic length (nt)	Genomic location	Encoded proteins
1	N	1689	56-1744	Viral nucleocapsid protein
2	P	1655	1748-3402	Phosphoprotein /V/C protein
3	M	1467	3406-4872	Matrix protein
4	F	2372	4876-7247	Fusion protein
5	H	1958	7251-9208	Hemagglutinin protein
6	L	6643	9212-15854	Large Polymerase protein

2.1.2 Measles virus proteins

2.1.2.1 Structural proteins

i. Haemagglutinin (H) protein

Haemagglutinin is an integral surface membrane glycoprotein that is encoded by H gene containing 617 amino acids that form disulphide linked dimer. It is present in the form of spikes on viral surface. It contains Receptor binding sites (RBS) and initiates

the measles virus infection by binding with the cellular receptors (CD46, CD150 or SLAM) present on the host surface (Miyoshi et al., 2018). Several epitopes including, *I, II, iv, v, vi* have been reported to be present on H protein which are potential targets for the neutralizing antibodies. Another vital epitope present on the H protein is *vii* and has been found to be binding with several neutralizing antibodies. However, any mutations in this region can lead to MeV escape from neutralization (Tahara et al., 2013).

ii. Fusion (F) protein

Fusion protein is another premier protein encoded by F gene and consists of 550 amino acids. It has different conformational states mainly pre-fusion native state, that changes to pre-hairpin intermediate state leading to post hairpin state. It helps in the fusion of viral membrane with the host membrane which ultimately proceeds to the delivery of nucleocapsid into the cytoplasm of the host. This process is independent of pH and occurs directly at viral surface by mutual interaction of H and F proteins. In the later phases of infection cycle when fusion protein expresses on the surface of infected cells it promotes the fusion of coinciding cells and leads to syncytia formation. Syncytia formation is a cytopathic condition which leads to tissue necrosis. Mutations in F protein have been reported to cause central nervous system infection including measles inclusion body encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE) *etc* (Plattet et al., 2016).

iii. Nucleocapsid (N) protein

Nucleocapsid protein is a vital protein that plays central role in the MeV genomic RNA replication. It contains 535 amino acids and interacts with other components and with itself to encapsidate the viral genome. N protein is highly stable as it has shown resistance against high salinity and nuclease digestion. In the absence of other proteins in many other negative stranded viruses N protein self assembles into nucleocapsid formation. Bankamp et al demonstrated that N protein contains three functional domains one for its binding with P protein in order to form N_P complex, the second for Its self-interaction N-N in order to form nucleocapsid and the third one for binding of RNA in order to start initiation and elongation for the packaging of viral genome (Bankamp et al., 1996). As this protein is stable so any mutation in the carboxy terminus of N protein does not affect the product or the function contrary to other envelope proteins (Cattaneo et al., 1989).

iv. Phosphoprotein (P)

Phosphoprotein is a helical structure containing 507 amino acid that are encoded by P/V gene. Phosphoprotein plays key role in viral RNA synthesis and is an integral part of RNA polymerase and nascent chain assembly complex. The molecular activities of P protein include the condensation of other proteins and facilitating scaffolding, protein folding, RNA binding and RNA dependent RNA polymerase activity. P protein binds with unassembled N protein at their carboxy terminal as well as with large L protein. Moreover, a common feature of P protein is that it is poorly conserved and is phosphorylated by cellular kinases (Schneider et al., 1997).

v. Matrix protein (M)

Matrix protein is a 335 amino acid protein encoded by M gene. M protein plays critical role in viral assembly and interaction with RNP complex and viral membrane. It forms association with the inner surface of plasma membrane and interacts with the cytoplasmic tails of haemagglutinin and fusion proteins. M protein also have been reported to play regulatory role in the RNA synthesis by interacting with the specific residues of N protein at the carboxy terminus. This interaction also promote the efficient synthesis of measles virus infectious particles (Iwasaki et al., 2009).

vi. Large polymerase protein (L)

L protein is a large protein encoded by L gene containing 2183 amino acids. It mainly plays catalytic role by acting as RNA dependent RNA polymerase and catalyzing the replication of viral genomic RNA. For the transcription of viral mRNA, RNP tightly encapsidated with N protein serves as a template and polymerase binds with the viral mRNA at 3' leading to initiating the process of transcription with a declining efficiency from first gene to last one. The P protein acts as a processivity factor here (Bariˇ et al., 2005).

2.1.3 Nonstructural proteins.

V/C proteins

V and C proteins are comparatively smaller nonstructural proteins having a size of 299 and 186 amino acids respectively. Both are encoded by overlapping open reading frame of P gene via a mechanism known as RNA editing a process that can be described as the reiterated co-transcriptional insertion of non-templated G residues at a specific run of Cs in the template by the viral polymerase. V protein plays its role in host immune

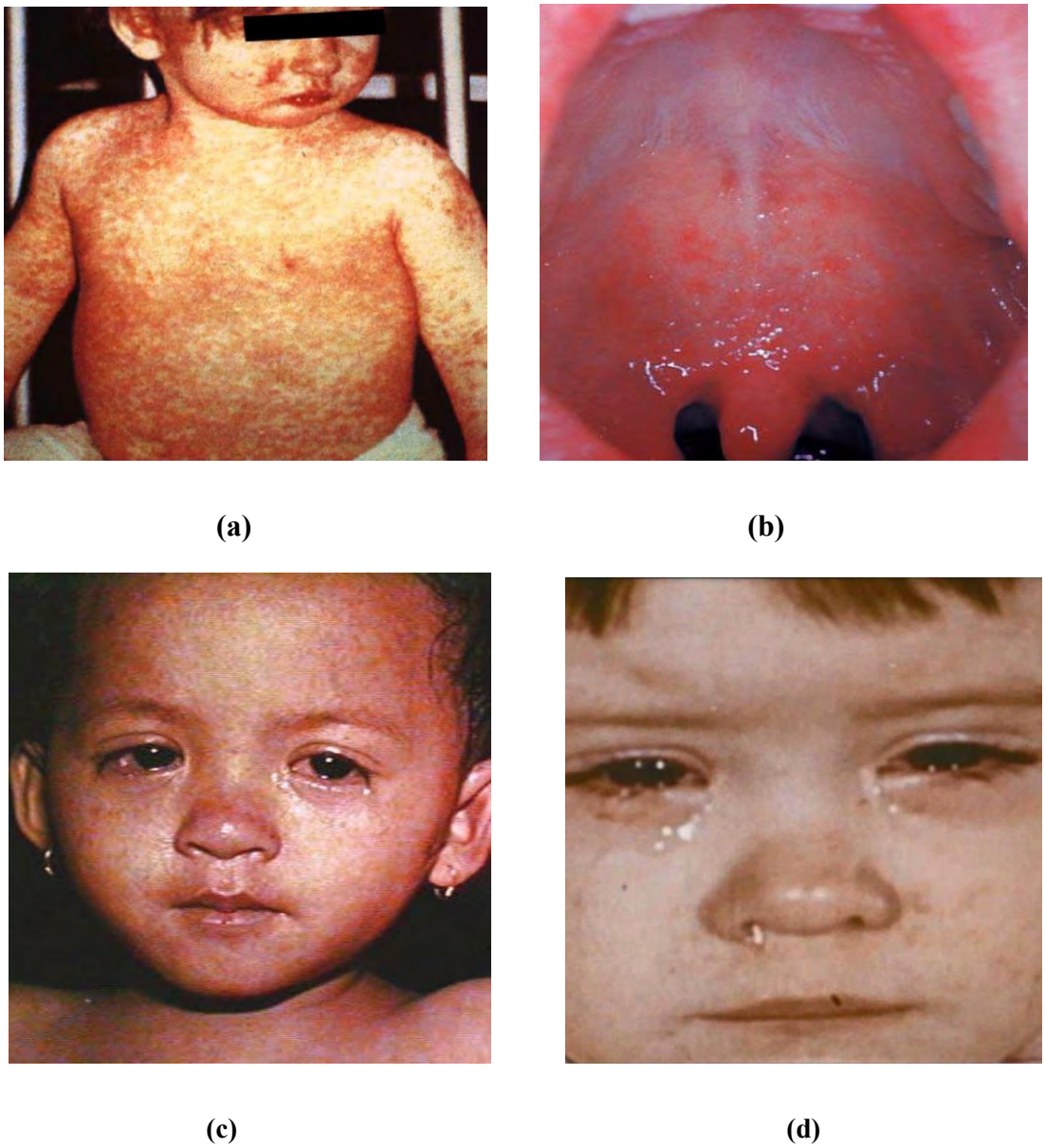
response suppression by blocking interferon alpha beta IFN α/β and signaling pathway resultantly preventing the establishment of antiviral state (Patterson et al., 2000).

The C protein have been reported to play inhibitory role and as a virulence factor. It is not important for the viral growth but the blocks induction of IFN α/β and IFNγ signaling (Shaffer et al., 2003).

2.2 Clinical manifestations and complications

Measles viruses manifest itself in several different forms including, skin, respiratory and brain infections etc. Generally, a maculopapular rash appears on skin at the onset of measles with other infections including dry cough, coryza, fever, conjunctivitis, and photophobia. Koplik spots are another characteristic feature of measles that appear on the mucosal surfaces of mouth. Delayed type hypersensitivity response also occurs against preexisting antigens including tuberculin but it is disappeared during acute measles and recovery stage (Rima and Duprex, 2006)

All these symptoms appear at different stages of acute measles infection. Measles infection can be divided into four stages including incubation period, prodromal phase, exanthem and recovery phase followed by immunity development. Cough, coryza and conjunctivitis are the characteristic features of prodromal phase that starts right after viral incubation. These symptoms are accompanied by fever, malaise, anorexia, and Kolpik spots. 1-2 days before the exanthem, oral lesions also appear. Exanthem phase is characterized by a characteristic pattern that gradually evolves as the phase proceeds. It starts from face and behind the ears 3-4 days following the onset of fever. It spreads to peripheral parts of body gradually and becomes more confluent. This phase spans over 3-7 days and then the rash starts fading in the same pattern as it spreads. Fever persists during this phase. In non-complicated measles cases the recovery phase is characterized by a persistent cough skin desquamation that is exaggerated in children facing malnutrition (Alves Graber et al., 2020).



(a) **(b)**

(c) **(d)**

Figure 3. Measles clinical manifestations. (a) Maculopapular rash (b) Koplik spots, (c) Conjunctivitis (d) Watery eyes and runny nose (Alves Graber et al., 2020).

- **Neurological complications:** MeV causes immunological amnesia by depleting B cell pools. Neuropathogenicity have also been attributed to MeV as it causes severe infections of central nervous system including measles inclusion body encephalitis (MIBE), Acute disseminated encephalomyelitis (ADEM) and subacute sclerosing panencephalitis (SSPE). The viral isolates from CNS of such infected patients have shown mutation in M protein and Fusion protein that led to hyperfusogenic phenotype (Ikegame et al., 2021). In SSPE cytoplasmic inclusion bodies are formed in the glia and neurons, but usually viral budding is not seen from the surface of infected cells. The associated manifestations are personality changes in patient, myoclonus and dementia that gradually leads to coma accompanied by

autonomic failure (Watanabe et al., 2019). The risk factor for MIBE is greater for young infants and immunocompromised patients as they are not fully capable of complete viral clearance. MIBE can lead to focal seizures, mental status changes, and sometimes auditory and visual impairment within one year of infection or the administration of live virus vaccination (Freeman et. al., 2004). ADEM is more severe as compared to other neurological complications, the mechanism behind ADEM is not clearly understood but one proposed reason can be the molecular mimicry of MeV proteins with the myelin protein. This disease can be characterized by demyelination, ataxia, motor and sensory loss, psychological changes and sometimes death (Buchanan et al.,2012). For such neurological disorders the infected lymphocytes might act as a carries for the MeV by crossing blood-brain barrier via receptor independent mechanism, as brain also contains lymphatic vessels (Laksono et al., 2016).

- **Respiratory complications:** *Otitis media* is the epithelial inflammation of eustachian tube that leads to different secondary bacterial infections and obstruction. It happens usually in children younger than 5 years and is less frequent in adults. *Laryngotracheobronchoitis* is a secondary bacterial infection of trachea that has been reported in children younger than 2 years and hospitalized for measles. *Pneumonia* is a respiratory complication that happens in almost all the measles patients and is a major cause of death in measles patients. Coinfection with several other viruses and bacteria have been reported in measles patients (Naim, 2015).
- **Gastrointestinal complications:** *Diarrhea* is a gastrointestinal complication that has been seen in children lesser than 5 years and adults above 30 years. It leads to dehydration. Other complications include hypocalcemia, hepatitis and an elevated level of creatinine phosphokinase (Naim, 2015).

2.3 Viral transmission and immunology of measles of infection

2.3.1 Entry of MeV into the Host.

Measles virus like other morbillivirus transmits through respiratory route, and then causes systemic infection. It spreads from the infected subject to other hosts i.e. human and non-human primates via respiratory aerosols and droplets (Rima and Duprex, 2006). Two receptors have been identified for binding with measles virus envelop protein H i.e. Signaling lymphocytic

activation molecule family 1 (SLAMF1) or $CD150^+$ and the other is nectin-4 (Fukuhara et al., 2020).

2.3.2 Dissemination

As the measles virus enters a susceptible host via respiratory aerosols into respiratory tract. Early target cells for measles virus include $CD11c^+$ myeloid cells, alveolar macrophages and Dendritic cells that are present in submucosa of respiratory react and lungs. Two possible mechanisms have been proposed by studies. The **first one** includes the targeting of $CD150^+$ which are present in alveolar spaces or the binding of the virus with the dendrites of DC-$SIGN^+$ submucosal DCs that are present in the lumen of respiratory tract. From here the virus migrates to the tertiary lymphoid tissues, including bronchus associated lymphoid tissues, and into the draining lymph nodes. Here the virus replicates massively and is amplified due to abundant presence of $CD150^+$ B- and T-cells. (Fig. 4) (Laksono et al., 2016).

In the **second possible mechanism** the MeV infects the myeloid and lymphoid cells that are present in the conjunctiva. Langerhans cells, Dendritic Cells (DCs), macrophages, B-cells and $CD4^+$, $CD8^+$. T cells are present abundantly in lamina propria of conjunctiva which provides a very suitable site where viruses can replicate massively. Cell to cell transmission of MeV occurs in primary, secondary and tertiary lymphoid tissues that are connected to each other via dendrites. This infection progresses to lymphocytes and DCs present in skin and the epithelial submucosa, and these cells transmit the infection to the neighboring epithelial cells containing nectin-4^+or to the keratinocytes. The infection spreads to the liver, kidney, skin systemically and stimulates the leucocyte integrins lymphocyte function associated antigen-1 and later antigen-4 activation and expression. Viremia occurs as a result of circulating virus in blood which ultimately leads to immune suppression and other associated symptoms (Fig. 5) (Laksono et al., 2016).

Another mechanism has been proposed for the receptor independent entry of virus into the body which is known as in-cell infection. This mechanism was tested for Epstein-Barr virus (EBV) where the EBV infected B cells were internalized by carcinoma cells leading to in vivo viral dissemination, (Lee and overholtzer., 2015) the same can be speculated for measles virus (MeV) where the infected lymphocytes can be carrier for MeV into receptor negative cells and can cause infection dissemination independently for receptors (Laksono et al., 2016).

Following the prodromal phase MeV infection manifest itself by Kolpik spots that appear on buccal mucosa and lead to maculopapular skin rash. The infection of dermal endothelia cells and keratinocytes might be a possible reason behind such manifestations (Griffin DE., 2013).

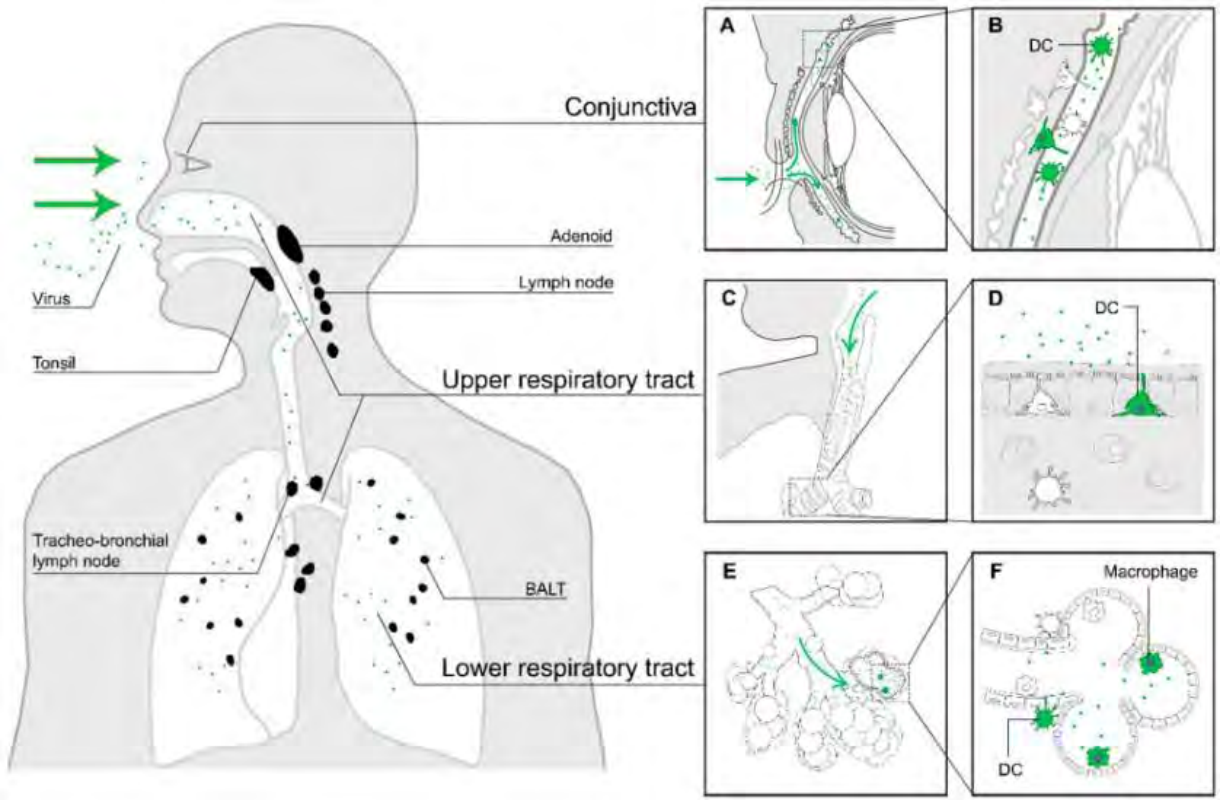


Figure 4. The first stage of viral dissemination into the body.

Right panel show the amplified version of primary targets of MeV and green arrows show the route of transmission (Laksono et al., 2016).

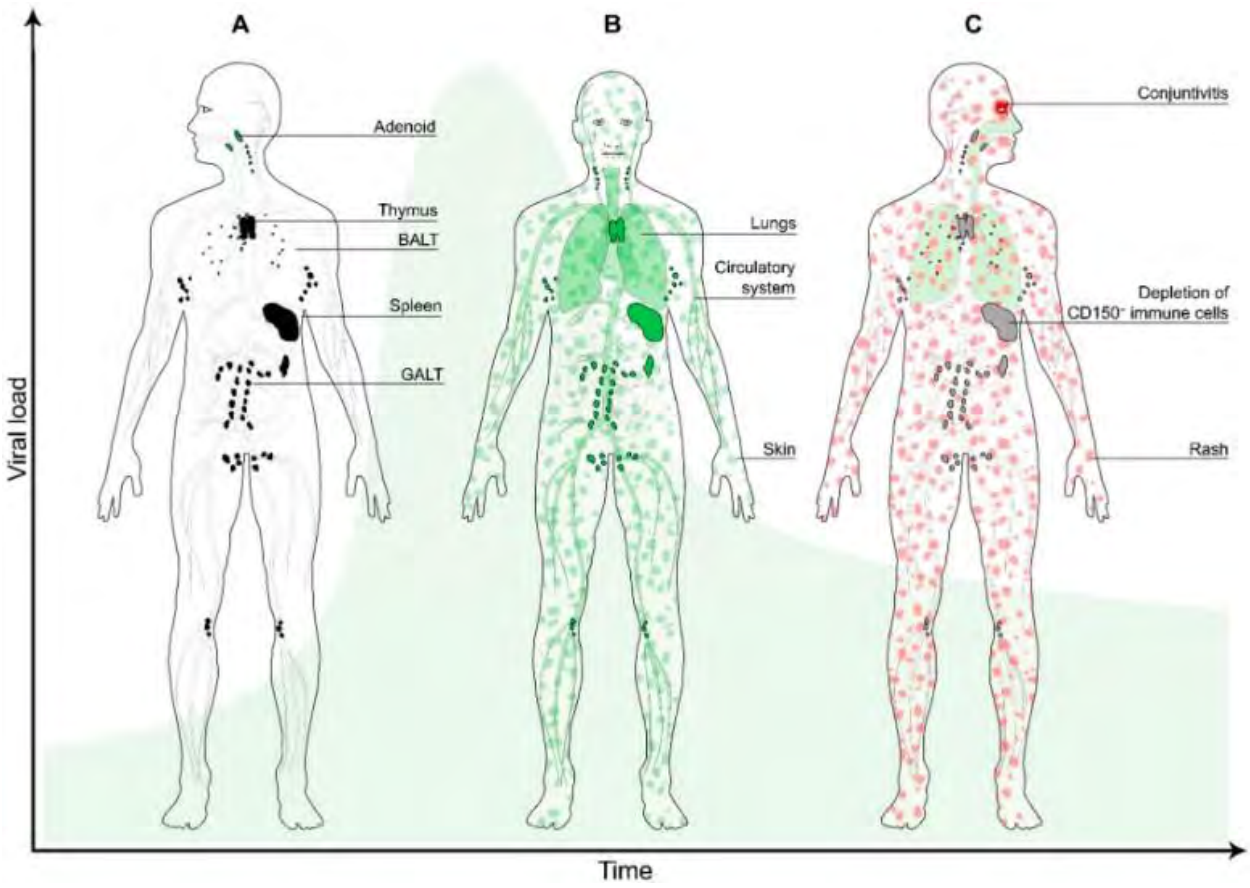


Figure 5. Systemic dissemination of measles infection.

A show the transmission of MeV to the lymphoid masses, B is the viremia stage and peak viral load that spreads systemically, and C shows the immune suppression, and initiation of clinical manifestation. The green curve in the background is representing the viral load with the progression of infection (Laksono et al., 2016)**.**

2.3.3 Transmission

Infectivity rate of measles is very high as one infected patient can transmit infection to approximately 12-18 susceptible people. MeV is released in the air by respiratory aerosols mainly via coughing (Noyce et al.2012). Cases have been reported where a single infected patient has spread infection to over 200 healthy people, (Christensen et al.,1953) this phenomenon can be referred to as “superspreading”.

Certain properties can increase the infectivity rate including sufficient shedding of MeV from the infected person and damaged epithelia, bronchi or brochioles are much more susceptible to MeV. Basolateral surface of epithelia cells is infected and the viral budding occures form the apical surface due to the presence of sorting signals in viral glycoprotein. CD150+ cells are abundantly present in the lymphoid tissues and the MeV that are produced here bind to these neighbouring cells. Measles virus that are produced in the epithelium of respiratory tract are

shed into the mucous present there and due to the scarcity of MeV receptors here they remain their as cell-free particles and with the help of mucocilliary escalator they travel to the upper respiratory tact (URT) where they are transmitted into the environment by coughing (Fig.6) (Ludlow et al., 2013). MeV is transmitte either in the form of of larger respiratory droplets for shorted distance or via touch or the small droplets can transmit the MeV over longer distance through air (Bloch et al., 1985).

Second impotrtanat prerequisite for the transmission of Mv is the viability of virus , as it should remain infectious till reaching to the new host. Direct contact of susceptible person's nose mouth or eyes with larger respiratory droplet containing infectious MeV virion increases the stability of MeV to cause infection. Smallar droplets can survive in the air for almost an hour. Humidity, air temperature and the speed also significantly affects the viral transmission rate (de jong, J.G. 1965).

The third important factor for viral transmission is viral infectious dose. In non human primates NHPs a single 50% tissue culture infectious dose was proved to be sufficient for the establishment of infection leading to systemic dissemination (Van Binnendijk, R.S. et al., 1994).

2.3.4 Immune suppression

Measles virus leads to a transient and immense immune suppression which renders the MeV patient prone to many oppertunistic infections, hence causing increased mortality in children. MeV excessively replicates in lymphoid tissues including tertiary lymphoid tissues Broncus associated lymphoid tissues (BALT) and Gut associated lyphoid tissues (GALT). Where the other viral or bacterial oppertunistic infections are occurred and lead to the accuulation of lymphocytes, hence forming germinal centers. CD11c+ , DCs and folicular DCs are abundantly present in these sited for the maintenance of these tissues. Such environment where there is abundant presence of CD150+ lymphocytes , DC-SIGN^{+} DCs becomes a perfect place for the profileration of MeV infection (De Swart, R.L., et al., 2007).

Invasion of MeV leads to lymphopenia when the infection is in its acute phase, this sharply decreases the level of T and B lymphocytes that are circulating in lymphoid tissues (Fig. 5c). This happens when there is viremia at its peak. MeV-infected cells are cleared by the cytotoxic T cells. The number of lymphocytes that had been reduced by lymphopenia are recovered to their normal range within a week, but the immune suppression caused by lymphopenia may

persist ranging from two weeks up to two years (Mina, M.J. et al., 2015). Many mechansims for the immune suppression by MeV have been proposed but none of them explains the paradox of measles where it induces a strong immune response , meanwhile renders lifelong

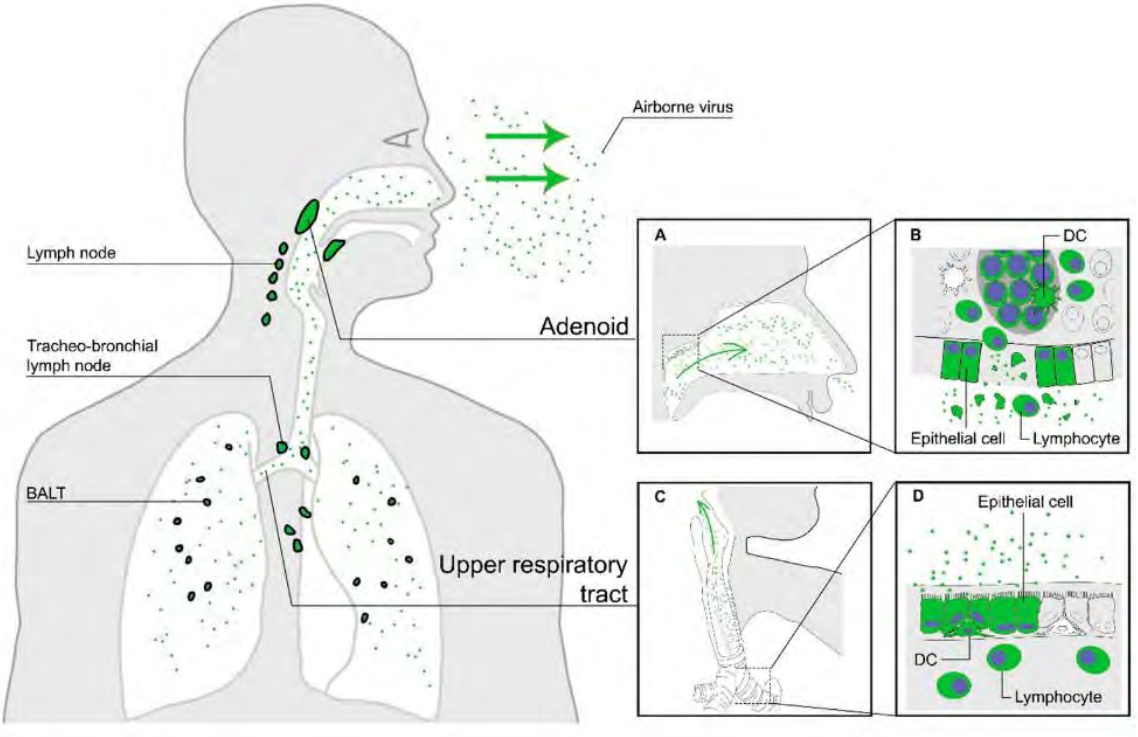


Figure 6. Transmission of M*e*V from the host body into the environment.

After the production of MV from Nectin-4+ epithelia cells of respiratory tract M*e*V *are* released into the lumen via mucous lining. (Green arrows in A and C). B is showing the destruction in upper respiratory tract caused by M*e*V released via infected lymphocytes. D is showing the destruction in lower respiratory tract leading to coughing (Laksono et al., 2016).

susceptibility of the infected person to other secondary infections. Experimental observations in NHPs explain this mechanism of immune suppression that is caused by depletion in $CD150^{+}$, T and B cells which is masked by the immense expansion of MeV specific lymphocytes. It explains that the short term lymphopenia leads to long term measles-associated immune suppression (Laksono et al., 2016).

2.4 Epidemiology of MeV

Despite being vaccine preventable measles has been a global problem in the form of regional endemics and imported cases. It has not been eliminated from all over the world and every year measles cases are reported from around the world (Fig. 7).

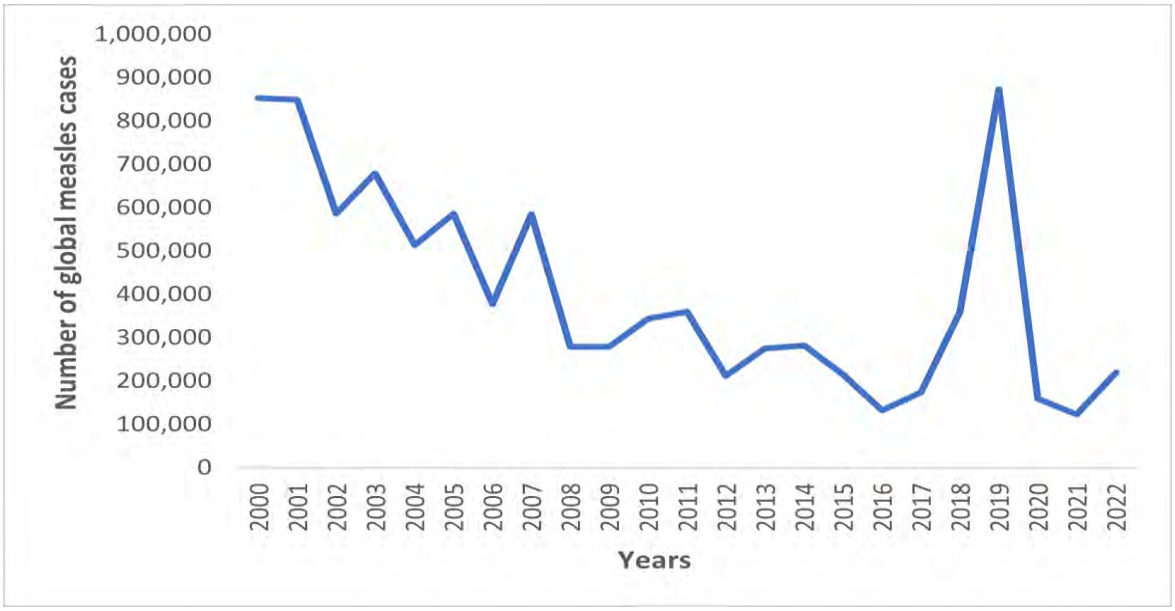


Figure 7. An overview of global measles cases during 2000-2022.

Global surveillance of measles is very important to keep a record of viral transmission and risk assessment regarding any imminent outbreak in any region. WHO is working efficiently for that sake by the establishment of global Measles and Rubella laboratory network (LabNet). LabNet is working on the surveillance, testing and reporting of measles in laboratories that are operational in 183 countries worldwide (Rota et al., 2011). This network supports the molecular characterization of MeV strains circulating in a specific region and then ensures the dissemination and sharing of data to strengthen the measles control program. WHO has recognized eight measles clades from A-H. These clades are further divided into 23 genotypes that are designated as A, B1-B3, C1, C2, D1- D10 E, F, G1, G2, H1 and H2 including one provisional genotype known as d11. Some genotypes having similar sequences are put into similar clusters such as B3 and H1. This process of measles virologic surveillance started in late 1980's after a resurgence of measles and then this process was expanded and optimized (Rota, et al., 1992).

Molecular characterization and surveillance of MeV helps to know the type of circulating virus and in the determination of strain whether it is wild type, imported from some other region or vaccine based. All the currently available measles vaccines have been derived from A genotype (Rota, et. al., 1994).

There are some countries that have effectively eliminated measles, imported measles cases are reported sporadically in such countries and multiple genotypes are reported that can be imported from other countries where measles is yet endemic (Fig.8).

2.4.1 Epidemiology of Measles in African Region

According to WHO report of 2007-2009 the most widely circulating measles genotype across the African region was B3 that occupies a major ratio (89%) of overall reported genotypes. B3 genotype has been reported to be citrculating in Malawi, Liberia, Mauritania and most of the African region excluding North africa. B3 has been divided into two clusters and the cluster 1 has been reported from Tanzania, Kenya, Nigeria, Cameroon and Ghana while Cluster 2 has been circulating in the western Africa mostly. Other genotypes include D2, D4, D10 that have been found in the Southern and Eastern Africa. B2 and D8 have also been reported in sporadic outbreaks from different parts of Africa (Rota et al., 2011). Currently B3 and D8 are found to be causing outbreks iin Africa during 2022 D8 have been involved in importation from African region to others (WHO., 2022).

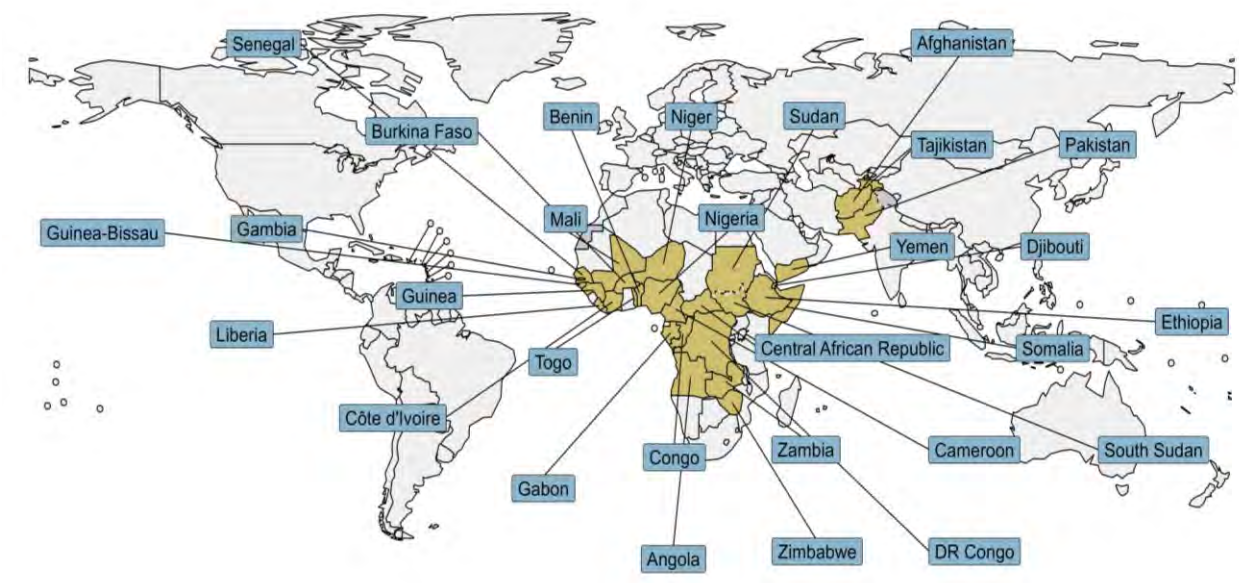


Figure 8. Largest measles outbreaks during 2022 (WHO. 2022).

Despite the monotypic nature of measles its genotypes among the eight clades have been increasing over the years. A, E and F clades have single genotype while B, C, D, G and H

clades have multiple genotypes that have been reported from different regions of the world (Riddell MA., 2005).

2.4.2 Epidemiology of Measles in American Region

Measles have been eliminated from most of the American region. No more endemic transmission of any measles genotype is being reported but the virologic surveillance of MeV strains involved in some outbreaks have been imported from other endemic areas. There are five different genotypes that have been detected in measles cases and endemic during 2007-2009. These include B3, D4, D5, D8 and H1 from Canada, Peru, United States, Chile, Argentina, Jamaica and Venezuela etc. (Rota et al., 2011). According to WHO 2022 reports measles B3 from United States and D8 from South America have been involved in some measles cases (WHO., 2022).

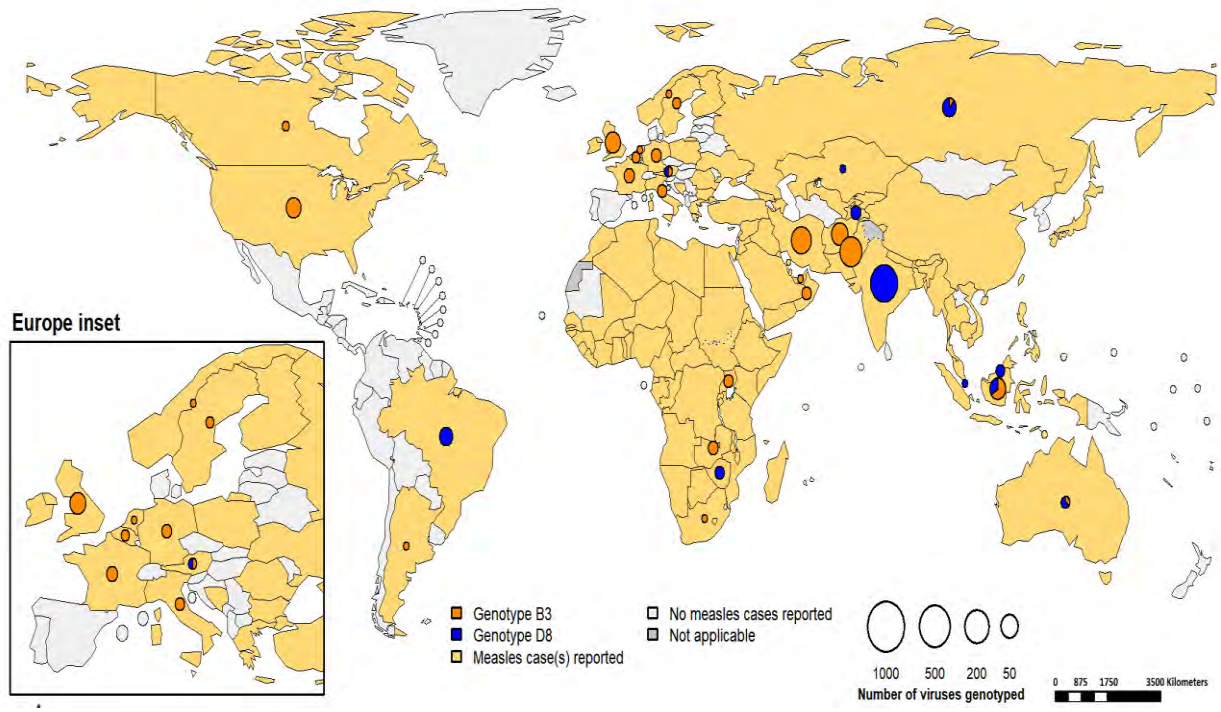


Figure 9. Overview of Circulating Genotypes in different WHO regions during 2022 (WHO, 2022).

2.4.3 Epidemiology of Measles in European region

Europe had adopted the target to eliminate the measles in 2015 completely. They adopted several effective strategies to attain this status. Europe has very heterogenous approach for

measles elimination. Out of 53 countries in this region 52 have either well established National Measles and rubella laboratories or they have access to laboratories in other countries. Before the elimination during 2007-2009 47 out of all the 53 countries reported measles outbreaks. Six countries reported no measles case and there was no reporting from one country. Different genotypes were reported from 26 by the analysis of 200 provided sequences. The reported genotypes were B3, D4, D5, D6, D8, D9, H1 and A (Vaccine associated). D4 and D5 were the most prevalent on involved in endemic transmission and causing a huge number of measles cases (Rota et al., 2011). During 2022 sporadic cases and outbreaks were being reported that were mainly caused by B3 genotype and some from D8 (WHO.,2022).

2.4.4 Epidemiology of Measles in Western Pacific region.

Western pacific region had targeted to eliminate measles by 2012. Out of 1127 sequences submitted from this region during 2007-2009, 990 sequences came out to be from H1 genotype. H1 has been the indigenous strain in China, having three clusters i.e., H1*a*, H1*b* and H1*c*. This genotype has also been involved in outbreaks at Viet Nam. In western pacific region H1, D9, G3 and D5 have been involved in endemic transmissions and D4, D5, D9, H1 and B3 have been found to be involved in imported cases in the region. D3, genotype has caused several endemic transmissions in Philippines. D3 and G9 have also caused some endemic transmissions in this region (Rota et al., 2011). Recently during 2022 D8 and B3 genotype has been detected in imported cases in western pacific region (Fig. 9) (WHO.,2022).

2.4.5 Epidemiology of Measles in Southeast Asian region

Most measles cases in this region have been reported form India. Out of total 98 reported genotypes during 2007-2009 a majority were sent to WHO from India. D4 and D8 genotypes had been detected from India and Nepal previously. Moreover, they had been involved in importation from India to United States. D5 and D9 had been isolated from Thailand and H1 from Democratic People's Republic of Korea. G2, G3, D5 and D9 have been found to be involved in small scale outbreaks in Indonesia, East-Timor, Maldives, and Myanmar *etc.* (Rota et al., 2011).

D8 genotype was being detected from utterpardesh in India during 2012. Similar strain was detected in several other outbreaks in other regions of India. All of these strains that were detecetd from different regions of India shoewed a significant diversity (3.4-0.2%) from each other. This genotype differed from the WHO reference sequence at 20 positions with 11

substutions that led to change in amino acids and 9 other caused silent mutations (Shakya et al., 2012).

2.4.6 Epidemiology of measles in Eastern Mediterranean Region

There are 22 countries in this region and WHO has established measles surveillance laboratories in all these regions that are working effectively for timely reporting of measles situation. Out of all these 22 countries 17 reported 166 measles sequences that belonged to B3, D4, D5, D8, and H1 genotypes. D4 genotype had been found to be involved in 53% of outbreaks in this region. It has been involved in regional endemics in Syria, Egypt, Iraq, and Iran while in other countries such as Morocco, Oman, and Bahrain D4 was involved in some measles cases that were imported from endemic areas. During 2009 B3 genotype caused outbreaks in Libya and Yemen and these genotypes were imported to Tunisia and Oman. Genotype B3 had been the second major genotype involved in outbreaks in Eastern Mediterranean Region occupying 29% of all the cases (Rota et al., 2011). Currently during the 2022 B3 genotype has been prevalent in some measles cases in this region (WHO.,2022).

- **Measles Epidemiology in Pakistan.**

In 1997 WHO Regional Committee for Eastern Mediterranean had adopted the resolution to eliminate the measles from this region by 2010. In 2011 the overall progress was reviewed which indicated the delay in the fulfillment of this goal and then the date for this target was extended to 2015. Different strategies were being adopted and implemented by the member state to meet the target. In 2017 there was a significant upsurgence of measles indicating 33,943 cases in the region and two countries' contributed to 90% of all these cases including Somalia (80%) and Pakistan (20%). (World Health Organization Eastern Mediterranean Regional Office, 2019) Pakistan has been struggling with measles since a long time and has witnessed outbreaks every year in a mild to severe form (Fig. 10). A sharp rise in measles cases was seen during 2018 where more than 30000 cases were reported in the country. During all these years

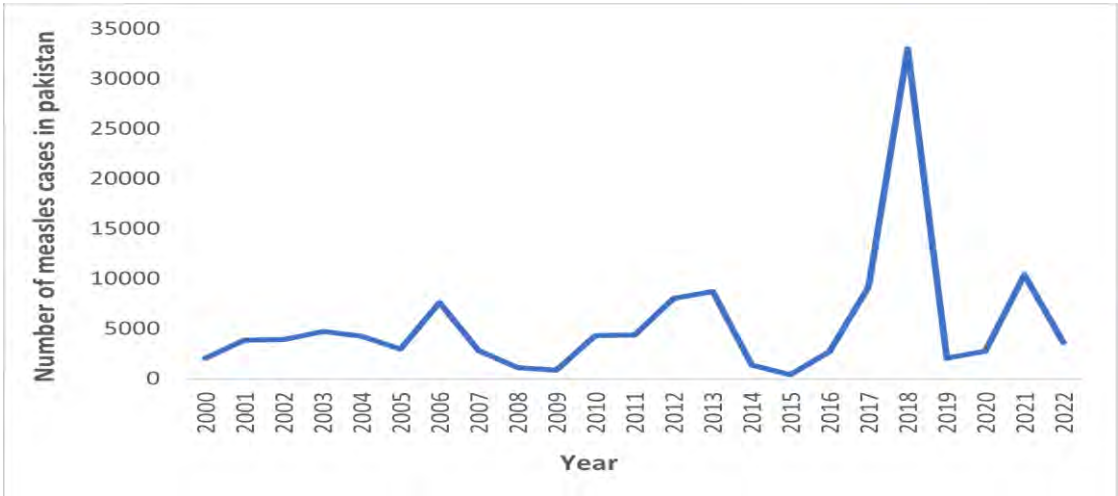


Figure 10. An overview of measles cases in Pakistan during past 22 years.

the most prevalent genotype has been B3 although D4 and H1 have also been reported from Pakistan. Zaidi et. al reported measles B3 genotype from different districts of Sindh province during 2013 that was further clustered into B3.1 sub group (Zaidi et al., 2017). Similar genotype had been found to be circulating in Islamabad during 2013-2015 (Zaidi, Hameed, Suleman Rana, et al., 2018). During the same period the similar genotype (B3) had been the most prevalent one. However, 2 cases of D4 genotypes were also being reported (Zaidi, Hameed, Ali, et al., 2018). Currently WHO report during 2022 has shown the prevalence of same genotype involved in measles endemic (WHO., 2022).

2.5 Diagnosis and treatment of MeV

2.5.1 Diagnosis of Measles

Diagnostic virology has emerged as an integral part of infectious disease surveillance and treatment in the face of ongoing epidemics, small scale endemics and pandemics. A variety of diagnostic methods are being utilized for the detection of diseases at different stages and several biological specimens are used according to the compatibility and efficacy of diagnostic test. Viruses are a grave concern for the modern world where they are mutating rapidly, taking new forms, and rendering existing therapies and treatments ineffective. An early-stage diagnosis can help greatly to minimize the overall impact of disease (Woo et al., 2012). MeV can be typical or atypical. Inexperienced medical practitioners cannot always correctly diagnose measles on the basis of clinical manifestations (Dunn et al., 2020). Serological and Molecular assays are the part of mainstream medical diagnosis. Both assays are used for the

diagnosis of diseases on different principles. Serological assay includes enzyme linked immune reactions where the quantification of viral antibodies present in the blood serum provides the basis for the severity of disease or the presence of viral infection. In case of measles virus IgM antibodies are produced as immune response and serological assays detect their presence. These immunological assays are useful when the immune system has already produced enough antibodies that could be detected by the assay. (Ilyas et al., 2020).

Several rapid diagnostic tests (RDT) are also available for quick diagnosis of measles that work on this method. Molecular based detection of viruses has revolutionized virologic diagnosis. They are rapid accurate and highly sensitive. Recent covid-19 pandemic has highlighted the importance of rapid diagnosis at the early stages of viral infection so that the disease could be cured timely and effectively. Moreover RT-PCR based detection can be effective for the detection of cases at the early stage of outbreak and it optimizes the process of disease surveillance and identification of viral strains and genetic characterization of viral isolates. The sensitivity of RT-PCR varies with respect to the biological specimen that can be blood serum, nasopharyngeal, throat swab or nasal swab. The optimum results are obtained when the sample is collected in the prodromal stage of measles when the viral load is maximum (Brown et al., 2020a). RT-PCR based detection have proved to be highly efficient as it has shown more than 90% sensitivity for the detection of different viral diseases including Covid-19, Rubella and Measles *etc*. (Dhamad and Rhida, 2020; Jin and Thomas, 2007; Lassel et al., 2013). Global measles and Rubella laboratory Network (GMRLN) has recommended two sequences for the determination of measles virus genotype. The primary target is 450 nucleotide sequence that encodes the 150 carboxy terminal amino acids of N protein (N450) and the secondary target is the whole coding region of haemagglutinin (H) gene (Song et al., 2022).

2.5.2 Measles treatment

There is no specific antiviral treatment for measles. Though some preventive measures are suggested such as good nutrition and excessive intake of liquids and hydration because water is lost due to vomiting and diarrhea. Antibiotics are suggested for eye and ear infections and pneumonia. The supplementation of vitamin A is suggested for measles patients as it reduces the risk of eye damage and blindness (WHO.,2019).

2.5.3 Measles vaccination

Live attenuated vaccine for measles is available that is administered in combination with Mumps and Rubella vaccine (MMR). It is given in two doses the first dose in the 12-15 months and the second dose for 4-6 years. MMR can be administered in children between 6-11 months if the disease is endemic or the child must travel to some other place. The MMR vaccine has shown 93% effectiveness for its MCV1 and that is increased up to 97% after the administration of MCV2. If the child gets these two doses at early stage, they are not exempted from getting the booster doses in later year of age i.e., 4-6 year. The vaccine should be administered carefully for the immunocompromised people because even the attenuated viral particles can cause the disease in such patients, so they are forced to rely on herd immunity (Gupta et al., 2020).

3. MATERIAL AND METHODS

3.1 Hematological and Biochemical Assessment of Children Infected with Measles Virus: 2022 Outbreak in Pakistan

3.1.1 Inclusion/Exclusion Criteria

Measles patients included in this study were 19 below 9 years admitted at Pakistan Institute of Medical Sciences (PIMS) children measles ward. Only the patients whose vaccination status was known were included in this study and the rest of them were excluded. Written informed consent was obtained from the parents/Guardian of young Children for the collection of clinical samples.

3.1.2 Clinical Specimens Collection

Children aged 1 month to 9 years having laboratory confirmed measles and showing mild to severe symptoms of measles were enrolled in the current study. The data set of measles patients were divided into three subgroups based on their vaccination status including three Not vaccinated, eight partially vaccinated and eight fully vaccinated. Patients who did not received any dose of MMR vaccine till the time of admission at PIMS were considered as not vaccinated, those who received one dose of vaccine at the time of admission at hospital were assigned as partially vaccinated status and the patients who had received two doses of MMR vaccine were considered fully vaccinated. Gender was also recorded but not used for analysis due to the limited number of samples.

Venous blood sample was successfully collected from all the patients (n=19) in a 5 ml heparin vacutainer tube by the medical practitioner. Blood samples were processed for analyzing hematological parameters (CP, DLC) and routine biochemistry test (BSR, total bilirubin, serum creatinine, SGPT (ALT), alkaline phosphatase, serum urea, calcium, potassium, sodium).

3.1.3 Blood sample processing

3.1.3.1 For hematological parameters

Blood was taken intravenously (1.5 ml) from the measles patients into a 5ml EDTA tube. The tubes were placed on the rotor to maintain homogeneity of blood samples. The blood sample was fed to the probe of TOSOH automated hematology analyzer and the results of CP (TLC, Platelets count, MHC, MCV, MCHC, RBC, Hb, PCV, RDW-CV, Platelet distribution width,

mean platelet volume) and DLC (Neutrophils, Lymphocytes, Monocytes, Eosinophils, Basophils, NRBC_per 100WBCs, Immature granulocytes) were recorded.

3.1.3.2 For Biochemical parameters

Whole blood sample (2ml) was taken from measles patients intravenously into a yellow top 5ml glass tube. The blood was allowed to clot by setting it aside for 15-20 minutes. When the blood is fully clotted the tubes were centrifuged at 1000-2000g for 10 minutes (Thermo-scientific LABOFUGE 200, Sweden). After centrifugation the tubes were gently taken out and placed in a rack the blood cells settled down and serum separated out in the upper layer above which gel is present that was gently rimmed, and the sample was centrifuged again. The gel settled above the blood clot and the serum was separated into clear test tubes labeled with patients ID. The samples were placed in TOSOH G11 HPLC Analyzer, Japan and the report generated for the study parameters was recorded.

3.1.4 Statistical Analysis

Statistical analysis was performed on data sets obtained from laboratory tests. One-Way ANOVA was performed for comparing variations of TLC, Platelets count MCH, MCV, MCHC, RBC, Hb, PCV, RDW-CV, Platelets distribution width, Mean platelet vol. between three patients' groups. *P* value below 0.05 was considered significant. Non-parametric tests (Kruskal-Wallis, Mann-Whitney) were performed for DLC and Biochemical parameters.

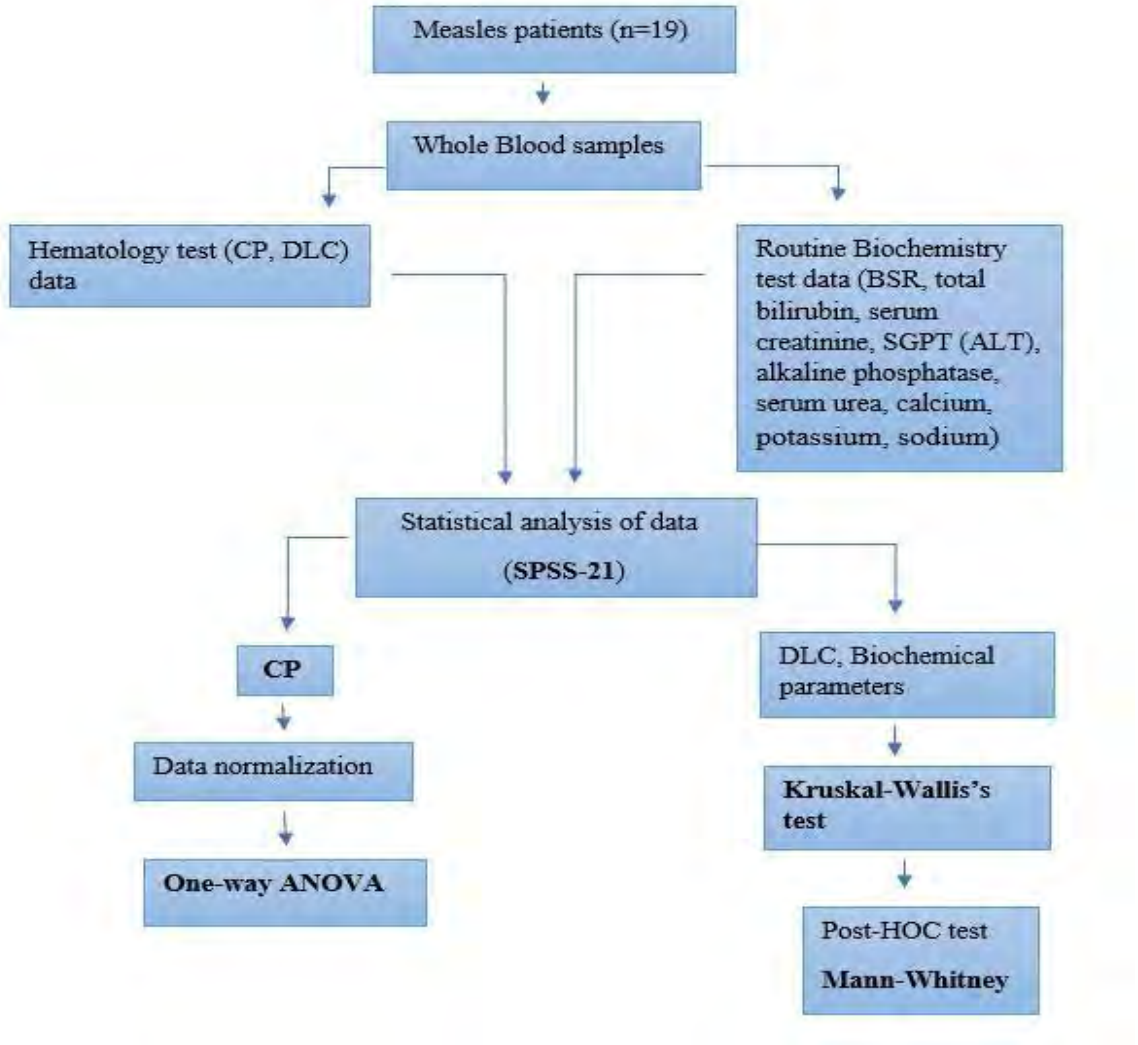


Figure 11. Consort Diagram.

Whole blood samples from nineteen patients were taken and processed for hematology (Complete blood test, CBC) and routine biochemistry tests. The data obtained from the test results was analyzed in SPSS-21 software. Complete parameter (CP) data was analyzed by One-way ANOVA. Differential leucocyte count (DLC) and biochemical parameters were analyzed by Kruskal-Walli's test and by Mann-Whitney test.

3.2 Molecular detection of MeV in Children admitted at PIMS measles ward during 2022 outbreak in Pakistan.

3.2.1 Sample collection.

In this study the serum and nasal swab samples were used. Samples were collected from the children under 9 years, admitted at Pakistan Institute of Medical sciences Islamabad (PIMS) measles isolation ward.

3.2.2 Inclusion/Exclusion criteria

Only the children under the age of 9 years and showing the clinical symptoms of measles including rash and fever were included in this study and rest of the patients were excluded.

3.2.3 Primer designing for the Measles Hemagglutinin (H) gene.

The primers for the H gene were designed by using primer3 software (https://bioinfo.ut.ee/primer3-0.4.0/) by using reference sequence NC_001498.1. Multiple Measles whole genome sequences were taken and aligned in Bioedit. The conserved region of H gene in all the sequences was selected for the primer synthesis. The primers were dually validated by using primer blast software and Snap-gene online tool (https://www.snapgene.com/) via *in-silico* PCR. A detail of primers used in the study is given in table below (Table 2).

Table 2. Overview of primers used for the Amplification of measles H gene.

Sr. no.	Primer sequence for H gene	Sequence region	Product size (nt)	Reference sequence
1.	**Forward primer.** 5' TTTCAGCAACTGCATGGTGG 3' **Reverse primer.** 5' TGGCGGGATAGTCAGCCAAT 3'	8119-8138 8644-8625	~526	NC_001498.1
2.				

3.2.4 RNA Extraction.

Viral RNA was extracted from serum and nasal swab samples of measles patients using WizPrep™ Total RNA Mini Kit by following standard manufacturer's protocol for the separation of serum form the blood samples. The blood collected in yellow top gel tubes was centrifuged at 8000 rpm for 1 minute The serum was separated in the upper layer of gel tube and it was transferred into an autoclaved 1.5 ml Eppendorf tube. The Eppendorf was labelled according to the patient ID.

For extraction of RNA 200µl serum and nasal swab sample (preserved in viral transfer media) was added into an autoclaved Eppendorf and by adding 50µl Proteinase K the sample was mixed thoroughly. From the RNA extraction kit 200 µl Viral lysis (VL) buffer and 15 µl carrier

RNA was added and the sample was mixed thoroughly by vertexing. The sample was incubated at 66˚C for 10 minutes. After incubation 280 µl absolute ethanol was added into the sample. The sample was mixed thoroughly and was loaded into the purification column. Column was centrifuged at 5000rpm for 1 minute and the flow through was discarded. Column washing was carried out by adding 500 µl wash buffer-1 and the sample was centrifuged at 5000rpm for 1 minute. Flow through was discarded. After first washing 500 µl wash buffer-2 was added into the column and again it was centrifuged at 5000 rpm for 1 minute. The flow through was discarded. Column was again washed with wash buffer-2 by adding 500 µl volume of wash buffer-2 and centrifuging it at 14000 g for 3 minutes. Now the column was transferred into a new autoclaved Eppendorf and 30-50 µl of elution buffer was added into the middle of the purification column, right above the filter membrane. The column along with Eppendorf was centrifuged at 5000 g for 1 minute. The flow through contains extracted RNA that was

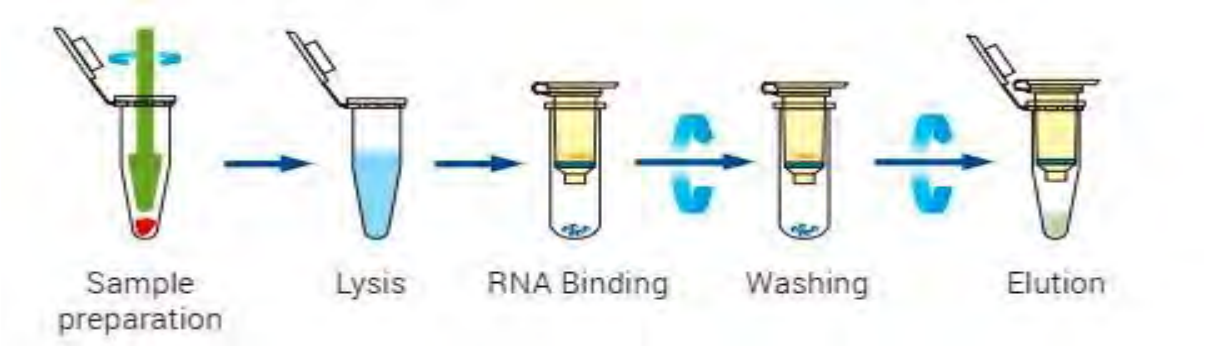


Figure 12. Schematic diagram for the RNA Extraction.

processed for cDNA synthesis.

3.2.5 Measles cDNA synthesis for PCR amplification.

First strand cDNA Synthesis Kit (Revert Aid, Thermo Scientific) was used for the synthesis of cDNA from the extracted RNA by using it as template. Composition of the reaction mixture (Table. 3) and conditions (Fig. 13) for the Reverse Transcriptase PCR are given below.

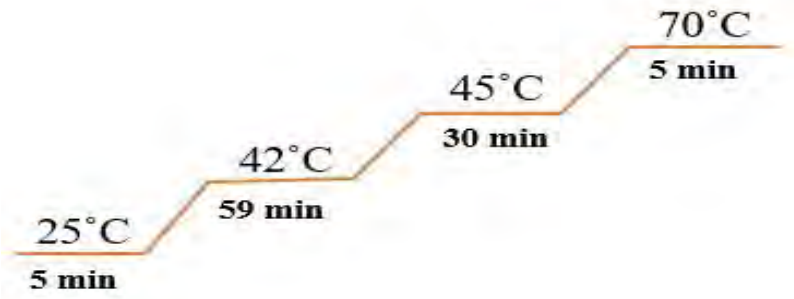


Figure 13. Reverse Transcription PCR Cycle parameters for cDNA Synthesis.

Table 3. Description of Reverse transcriptase PCR reaction mixture.

Sr no.	Reaction Components	Volume
1.	5x reaction buffer	8 µl
2.	10 mM dNTP Mix	4 µl
3.	Random hexamer primer/ R primers	4 µl
4.	RevertAid RT Enzyme (20U/µl)	2 µl
5.	Ribolock (20U/µl)	0.5 µl
6.	Template RNA	16 µl
7.	ddH_2O	5.5 µl
	Total volume	**40 µl**

3.2.6 Polymerase Chain Reaction (PCR) for the Amplification of H gene (partial).

The conditions for the designed primers were optimized by gradient PCR. For the amplification of measles virus Haemagglutinin gene polymerase chain reaction (PCR) was carried out using Master mix (2X Phusion High-Fidelity, Thermo Scientific). The amplified product of PCR was used as template for carrying out second round PCR. The volumes and PCR cycle conditions are given in Fig. 14. The amplified product was processed through gel electrophoresis for visualization.

Table 4. Overview of reaction mixture for amplification of MeV H gene.

Sr. no	Reaction components	Volume
1.	2X Phusion HF Master Mix	5µl
2.	Forward primer	0.75µl
3.	Reverse primer	0.75µl
4.	ddH_2O	0.5µl
5.	Template	3µl
	Total volume	**10 µl**

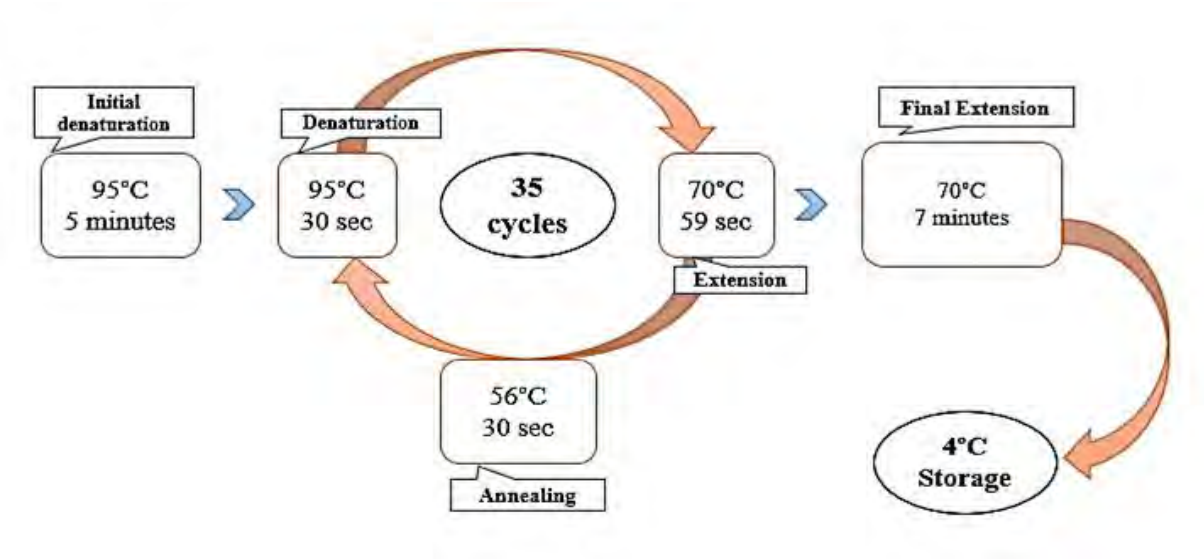


Figure 14. Overview of PCR parameters for the amplification of H gene (partial).

Table 5. Details of Reaction mixture for the second round PCR.

Sr. no	Reaction components	Volume
1.	Phusion Master Mix	10µl
2.	Forward primer	1µl
3.	Reverse primer	1µl
4.	ddH_2O	2µl
5.	Template	6µl
	Total volume	**20 µl**

3.2.7 Preparation of TAE buffer

10X TAE buffer was prepared by adding. 54g tris-base into 350 ml of deionized water. 27.5g of boric acid and 4.65g of EDTA were also added and mixed thoroughly to form solution. The final volume was raised to 500ml by adding 150ml of deionized water. For working solution 1X TAE buffer was prepared from 10X by taking 270ml of deionized water and adding 30 ml of 10X TAE.

3.2.8 Gel Electrophoresis

1.5% gel was prepared by taking 60ml of 1X TAE buffer in an autoclaved conical flask and adding 0.8g of Agarose (TopVision) into it. The mixture was heated in microwave oven for 30

seconds. The mixture was turned into a clear solution and after cooling it down a little, 4µl of Ethidium Bromide was added into the liquid solution to visualize the product under UV transilluminator. The gel was poured into the gel casting tray where the comb was already placed for the formation of wells. The gel was set aside for solidification. After the solidification the gel casting tray was placed into the electrophoretic tank and 1X TAE buffer was poured into the tank until the gel is completely immersed into the buffer. The comb was taken out. 7µl of PCR product was mixed into 3 µl of loading dye and the sample was loaded into the wells. 1kb gene ruler was used for the comparison of PCR product size. Electrophoresis was carried out at 90V for 30 minutes and the gel was visualized at under UV transilluminator.

Table 6. Composition of 1.5% Agarose gel solution.

Sr. no	Components of 1.5% Agarose gel	Volume
1.	1X TAE buffer	60ml
2.	Agarose	0.8g
3.	Ethidium Bromide	3µl

3.2.9 DNA Extraction/Elution from Agarose gel

The bands/amplified fragments from the first round PCR were cut from the Agarose gel and were eluted by using Gene JET Gel Extraction Kit (Thermo scientific). The visible DNA fragment was excised form the Agarose gel and was added into a pre-weighed autoclaved microcentrifuge tube. The weight of the gel was recorded and the binding buffer from the extraction kit was added into the gel slice with and equal (1:1) weight: volume ratio. The microcentrifuge tube was heated at 65 ºC for 10 minutes and the product was gently mixed after every three minutes so that it gets completely dissolved into the binding buffer. When the gel was completely dissolved it was transferred into the purification column (maximum 800 µl). The column was centrifuged at 12000rpm for 1 minute and the flow through was discarded. 700 µl of wash buffer was added followed by centrifugation at 12000 rpm. Flow through was discarded. Another centrifugation was carried out to completely remove the residual wash buffer. The column was transferred into Now another autoclaved microcentrifuge and 30 µl of elution buffer was added into the column followed by centrifugation at 12000 rpm for 1 minute. The column was removed, and the eluted product was used for the second round PCR (Fig. 15).

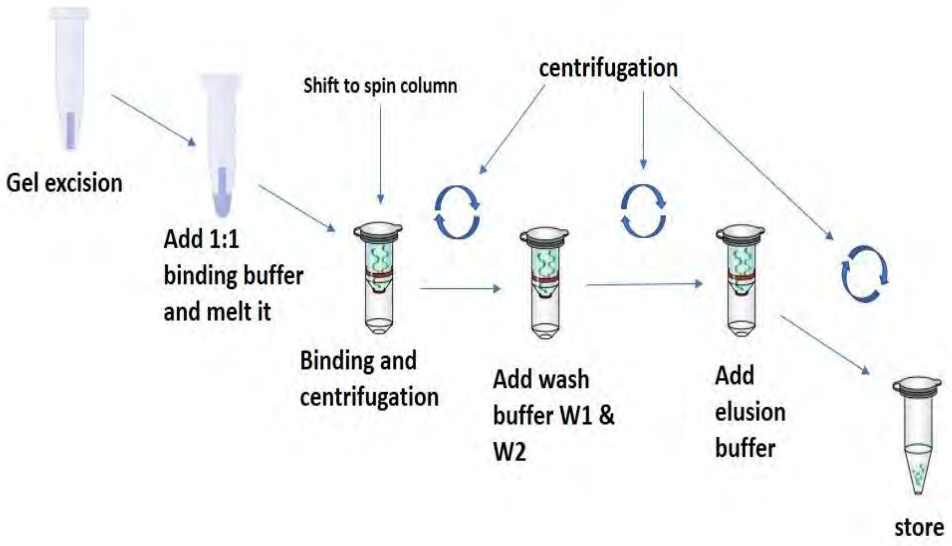


Figure 15. Schematic diagram for DNA extraction from Agarose gel.

4. Results

4.1 Hematological and Biochemical Assessment of Children Infected with Measles Virus: 2022 Outbreak in Pakistan

The total number of measles patients recruited in the current study are 19 including six females and thirteen male patients from one month to nine years old children. Their vaccination status has been shown in the figure below. (Fig16).

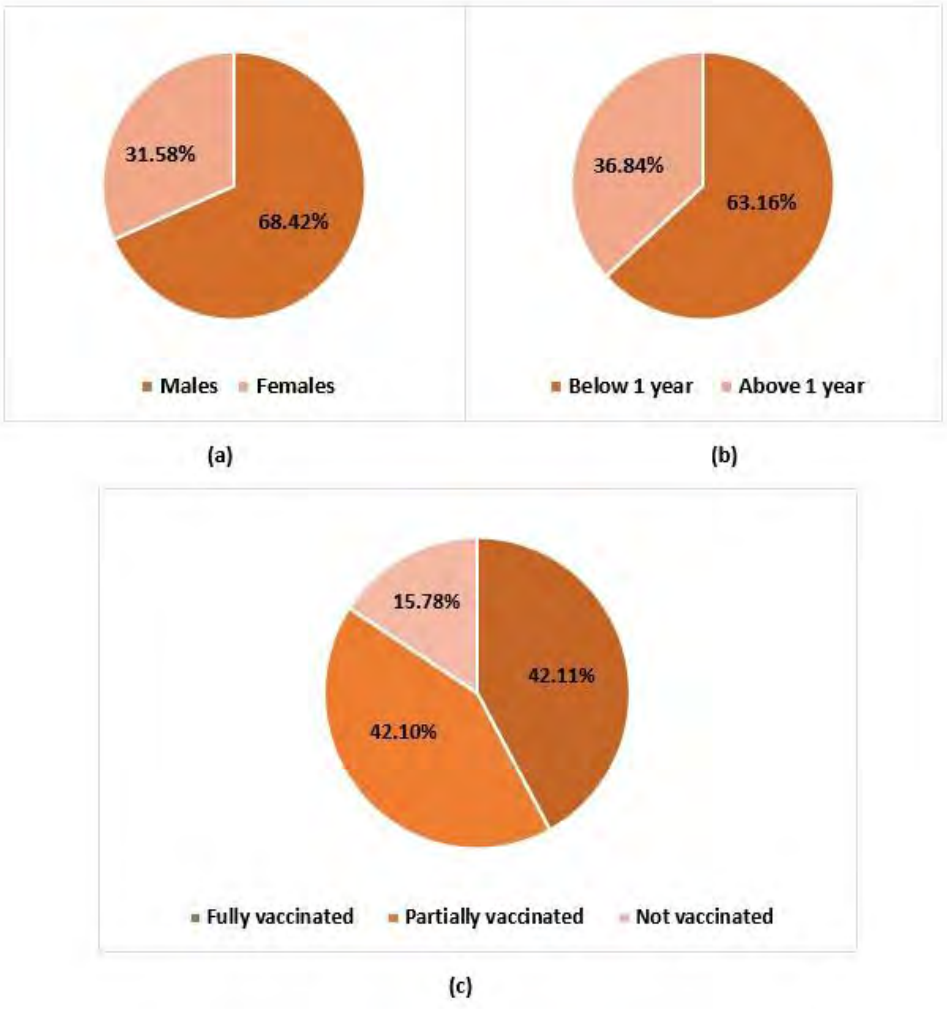


Figure 16. (a) gender ratio of measles patients, (b) the age ratio (upto-9 years), (c) the ratio of vaccination status of measles patient.

*(No dose= not vaccinated, 1 dose =partially vaccinated, 2 doses=fully vaccinated).

4.1.1 Hematological Parameters

4.1.1.1 Complete Blood Parameters (CP)

Our results showed that there was no significant difference between not vaccinated, partially vaccinated and fully vaccinated patients for TLC, [F $(_{2, 16})$ = 0.284, p =0.756] Platelets count [F $(_{2, 16})$ = 2.172, p = 0.146] MCH, [F$(_{2, 16})$ = 1.451, p = 0.264] MCV, [F$(_{2, 16})$ = 1.174, p = 0.335] MCHC, [F$(_{2, 16})$ = 1.575, p =0.237] RBC, [F$(_{2, 16})$ = 2.077, p = 0.158], Hb, [F$(_{2, 16})$ = 0.27, p = 0.974] PCV, [F$(_{2, 16})$ = 0.150, p =0.862] RDW-CV, [F$(_{2, 16})$ =3.257 , p =0.065] Platelets distribution width, [F$(_{2, 16})$ = 1.139, p =0.345] Mean platelet vol. [F$(_{2, 16})$ = 0.260, p = 0.774]. Lower levels of MCH, MCV, MCHC, RBC, Hb as compared to the normal range in healthy subjects were observed in all the three groups of measles patients. Moreover, there was a very high RDW-CV in all the groups as compared. (Table.7).

4.1.1.2 Differential Leucocyte Count

There was no significant difference (p>0.05) between neutrophils, lymphocytes, monocytes, eosinophils, NRBC_per100WBCs, immature granulocytes among not vaccinated, partially vaccinated, and fully vaccinated measles patients, except for Basophils (p=0.024) that was significantly different between non vaccinated and partially vaccinated patients (Fig.17) Moreover, a lower level of Eosinophils as compared to the normal range in healthy subjects prevailed in all the three patient groups. (Table. 8).

Table 7. Comparison of complete blood parameters (CP) of non-vaccinated, partially vaccinated, and fully vaccinated measles patients as compared to the normal range (Healthy individuals).

Parameters	Not vaccinated	Partially vaccinated	Fully vaccinated	Normal range
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	
TLC ($\times 10^9$/L)	12.8 ± 4.2	10.2 ± 1.9	9.4 ± 2.3	6-18
Platelet count (×1000/µl)	399 ± 73.5	442.3 ± 58.9	292.6 ± 44.7	200-550
MCH (pg)	18.3 ± 2.7	21.5 ± 1.3	22.1 ± 0.8	25-29
MCV (fl)	60 ± 8.3	68 ± 3.6	69.8 ± 2.3	72-84
MCHC (g/dL)	30.5 ± 0.5	31.5 ± 0.4	31.6 ± 0.3	32-36
RBC (million/ µl)	5.6 ± 0.3	4.9 ± 0.4	4.6 ± 0.1	6-18
Hb (g/dL)	10.2 ± 1.4	10.3 ± 0.4	10.1 ± 0.3	11-15.5
PCV	33.4 ± 4.1	32.6 ± 1.4	31.9 ± 0.9	30-38
RDW-CV (%)	20.8 ± 1.7	17.4 ± 0.8	16.7 ± 0.8	10-15
Platelets distribution width (%)	15.1 ± 0.3	15.5± 0.2	15.5 ± 0.12	15-17
Mean platelet vol.	8.6 ± 0.30	8.4 ± 0.4	8.2 ± 0.3	6.5-12

Table 8. Comparison of differential leucocyte count (DLC) in non-vaccinated, partially vaccinated, and fully vaccinated measles patients as compared to the normal range (Healthy individuals).

Differential Leucocyte Count (DLC) %	Non-vaccinated		Partially vaccinated		Fully vaccinated		Normal range
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**	
Neutrophils	56.6	11.6	54.9	6.3	48.8	7.9	30-60
Lymphocytes	34.1	12.2	38.5	6.0	43.1	6.8	25-55
Monocytes	8.5	0.9	6.4	1	7.2	2.5	2-10
Eosinophils	0.43	0.3	0.09	0.1	0.72	0.5	1-6
Basophils	0.3	0.1	0.1	0.03	0.2	0.03	0.0-2.0
NRBC_per100WBCs	0.6	0.6	0.07	0.05	0.00	0.0	0.00-2
Immature granulocytes	0.5	0.1	0.3	0.1	0.4	0.10	0.0-100

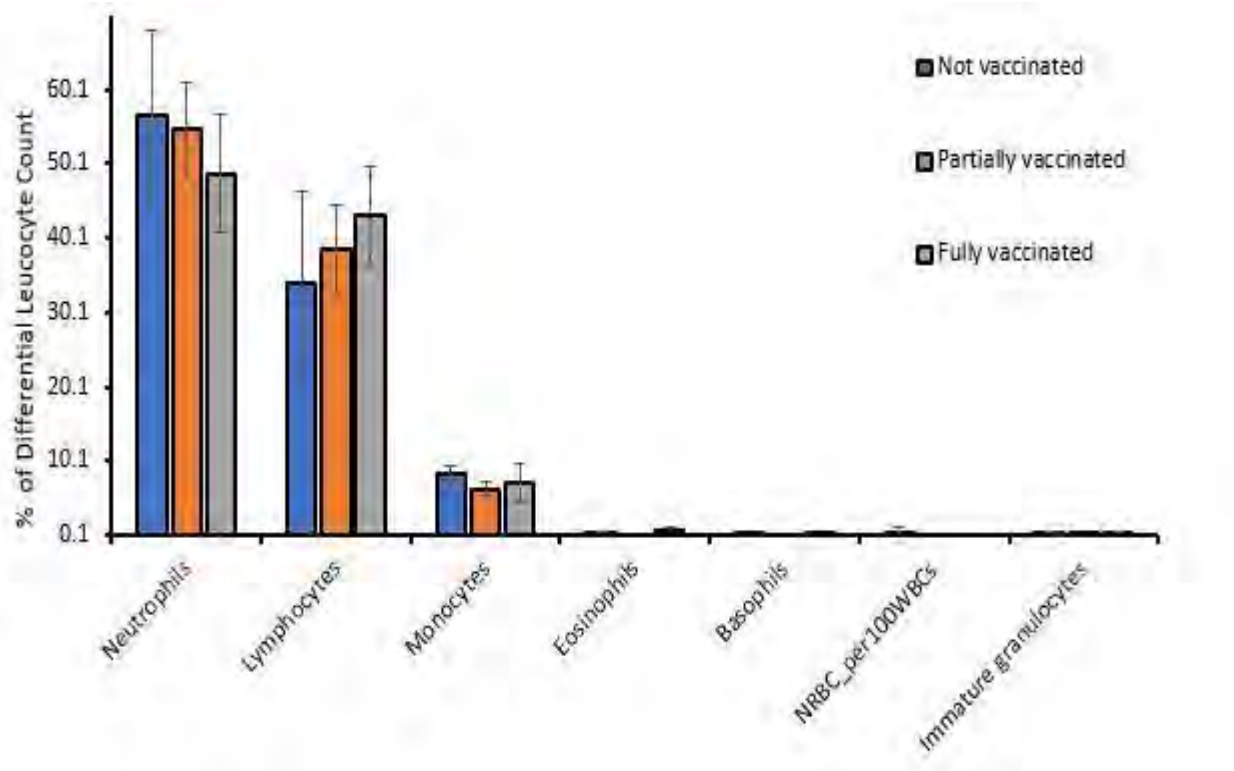


Figure 17. Comparison of differential leucocyte count in non-vaccinated, partially vaccinated, and fully vaccinated measles patients as compared to the normal range.

4.1.2 Biochemical Parameters

Statistical analysis of biochemical parameters including Blood Sugar Random (BSR), total bilirubin, serum creatinine, SGPT (ALT), alkaline phosphatase, serum urea, calcium, potassium, sodium of three groups of measles patients showed no significant difference (p>0.05) among all the parameters except serum urea that was significantly different (p=0.013) between not vaccinated and vaccinated group. Moreover, a significantly higher level of Alkaline phosphatase as compared to normal range was being observed in fully vaccinated patients (Table 9).

Table 9. Statistics for the comparison of biochemical parameters among three measles patients' group as compared to the normal range (Healthy individuals).

Biochemical parameters	Non-vaccinated	Partially vaccinated	Fully vaccinated	Reference range
	Mean± SD	**Mean± SD**	**Mean ± SD**	
BSR (mg/dL)	124 ± 19.7	102.9 ± 7.8	121.6 ± 17.5	80-160
Total bilirubin (mg/dL)	0.4 ± 0.03	0.3 ± 0.02	0.4 ± 0.02	Upto-1.0
Serum creatinine (mg/dL)	0.3 ± 0.01	0.3 ± 0.03	0.3 ± 0.6	Upto-1.2
SGPT (ALT) (U/L)	25.7 ± 6.8	43.1 ± 11.2	40.5 ± 10.4	Upto-42
Alkaline phosphatase (U/L)	119.3 ± 0.7	123.9 ± 8.1	137.8 ± 22.7	Upto-135
Serum urea (mg/dL)	23.7 ± 2.7	27.6 ± 2.9	15.4 ± 1.5	12-50
Calcium (mg/dL)	9.3 ± 0.4	9.1 ± 0.2	8.7 ± 0.2	8.5-10.5
Potassium (m.mol/L)	4.2 ± 0.1	4.5 ± 0.1	4.3 ± 0.2	3.5-5
Sodium (m.mol/L)	133.6 ± 2.8	133.4 ± 2.2	135.5 ± 0.8	135-145

***Abbreviations.** **BSR**=Blood sugar random, **SGPT**= Serum glutamic pyruvic transaminase, **ALT**= Alanine transaminase.

4.2 Molecular detection of MeV in Children admitted at PIMS measles ward during 2022 outbreak in Pakistan.

4.2.1 Results of PCR amplified product

PCR was conducted for six measles samples. Out of these six samples four were negative. And two MH3 and MH29 gave positive results as the bands were visualized in the range of ˜523 bp (The product size of H gene primers) (Fig. 18).

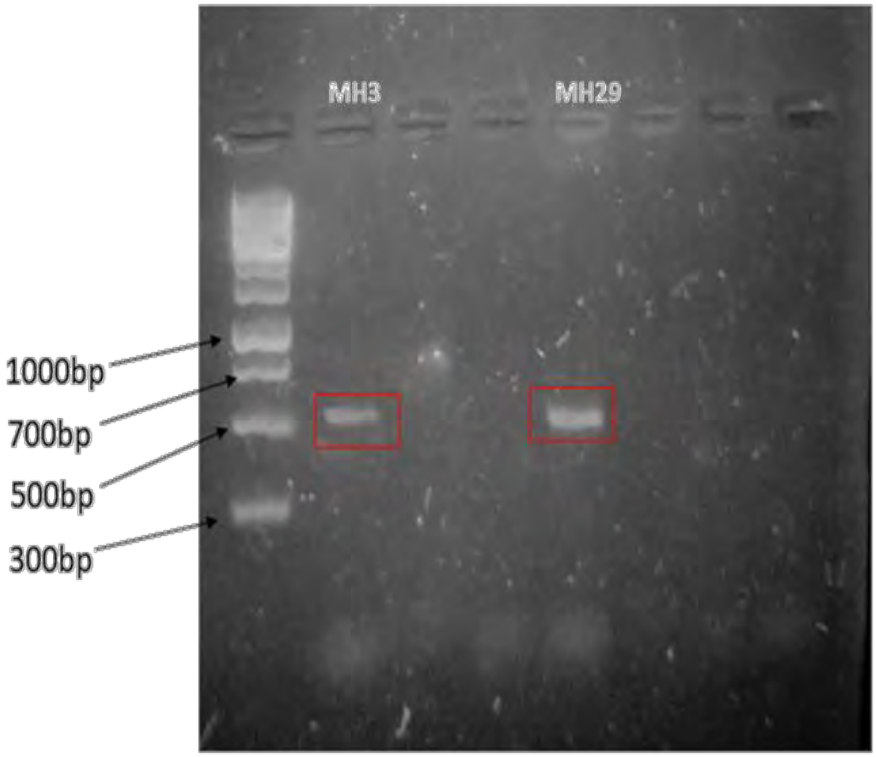


Figure 18. Visualization of agarose gel under UV transilluminator. Two samples gave positive result (MH3, MH29). Bands appeared in the range of ~ 523 bp (Target region in H gene).

Lane 1 represents 100 bp ladder (Thermo scientific), while 2 and 5 represent Amplified PCR product ~523 bp.

5. Discussion

The symptoms and clinical manifestations of measles can vary in different patients depending on their age, nutritional status and immunocompetency. The analysis of clinical data of measles patients (n=19) suggested that most of the measles patients were anemic with a lower level of MCH, MCV, MCHC, RBCs and hemoglobin and a very high ratio of RDW-CV than the normal range among the healthy subjects (Table. 7). The anemic condition (Hb<11) prevailed among all the patients irrespective of their vaccination status.

Our study is comparable with previous studies involving viral infections including dengue, hepatitis, HIV and Covid-19 that led to decrease in hemoglobin hence causing anemia in the infected patients (Mahardhika et al., 2020; McHutchison et al. 2006; Masaisa et al., 2011; Hariyanto, and Kurniawan., 2020). Hemoglobin contains iron that carries oxygen to different organs of the body and when the concentration of hemoglobin decreases it causes hypoxia which could ultimately leads to organ dysfunction specifically targeting respiratory organs. Anemic conditions might have aggravated the respiratory problems in hospitalized measles patients, as many of them (n=7) were put on ventilators for proper breathing (Hemaeur et al.,2017). The genetic factor of the infected host can possibly play a role in the viral induction of anemia (Morinet et al., 2011). The nutritional status and composition of diet also contributes towards anemia (Shah et al., 2020). Some studies also suggest that the antiviral therapy or administration of antiviral drugs during treatment might induce anemia, by hemolysis or other related mechanisms (McHutchison and Logo., 2006). Among all patient groups there was no significant difference of hemoglobin level and other indicators of anemia, so we might infer that the vaccination did not affect the hemoglobin level, there might be some other reason including the viral induction of hemolysis, the poor nutritional status of children or the genetic factor of infected patients.

Measles vaccine in combination with other vaccines could cause mild adverse effects following the immunization (AEFI). Studies have reported the incidence of fever, neurological symptoms, agitation, nervousness, gastrointestinal diseases, thrombocytopenia, serious, redness, swelling, local pain, lymphadenitis *etc.* (Bellavite and Donzelli.,2021). Our results showed a declined level of eosinophils in the patients (Table. 8) that were partially vaccinated (One dose of MMR vaccination). Some of these patients also showed the symptoms of

hyperpyrexia, skin rash, and excessive crying that is comparable with already reported studies (Bellavite and Donzelli.,2021).

The paradigm of eosinophils includes the destructive and inflammatory functions in cells. They are recruited because of T helper cells Th2 type reactions releasing cytotoxic granule proteins, different lipid mediators and cytokines that promote the parasite destruction, inflammation, and tissue damage. Under baseline conditions eosinophils perform homeostatic, protective and immunoregulatory functions in different organs of the body including gastrointestinal tract, lungs, thymus mammary glands, and adipose tissues (Bellavite and Donzelli.,2021). Eosinophils showed a decline (0.09%) in partially vaccinated patients as compared to non-vaccinated (0.43%) and then gradually increased (0.72%) in fully vaccinated patients. With this transition in eosinophil level, we can speculate that the previous history of administration of MMR vaccine 1st dose could be associated with the altered level of eosinophils might have affected the level of eosinophils in measles patients that gradually recovered in fully vaccinated patients. The manifestation of allergy inflammation and breathing issues in partially vaccinated patients could be possibly related to AEFI. As the vaccination contains live attenuated measles strain that might trigger the immune response in patients having an already compromised immune system possibly due to age, nutritional or genetic factors etc.

Some other significant variations among the clinical data of measles patients were observed in the level of Alkaline phosphatase that was slightly higher than the normal range in fully vaccinated patients, and the level of serum-urea that remarkably declined from non-vaccinated toward fully vaccinated measles patients. Although the later one was found to be in normal range in all the measles patients as compared to the healthy subjects. (Table. 9) There is no direct evidence for the influence of MMR vaccination history on biochemical parameters in measles patients. But viral infections including hepatitis B and C have been reported to cause an increase in the level of serum Alkaline phosphatase (ALP) (Alemam et al., 2020). Alkaline phosphatase level in not vaccinated and partially vaccinated patients lies under normal range but higher in fully vaccinated patients as compared to the normal subjects. We can infer that the vaccination has somehow played a role in increasing ALP level. ALP is a membrane bound glycoprotein that promotes the hydrolysis of several kinds of phosphate monoesters. It is reported to be increased in patients with bone and liver diseases, but the level of ALP is slightly higher in infants and adolescents as compared to the adults due to bone growth. An elevated level of ALP has been reported to be associated with respiratory infections (Sakurai and

Higashiguchi., 2021). In our case complete MMR vaccination increases the level of ALP so we can say that the vaccination might have contributed to the aggravation of respiratory problems in measles patients despite curing the diseases. Vaccination history affected the level of serum urea among measles patients by decreasing its level from non-vaccinated to completely vaccinated patients, but the fluctuation is under the normal range as compared to the healthy subjects, so we did not consider it under AEFI.

Our Hematological and Biochemical assessment of measles patients indicated that the MMR vaccination proved to be suboptimal in immunizing the measles patients. The results also indicated some mild adverse effects following the immunization. There can be several possible reasons behind that including the poor storage conditions of vaccines that rendered ineffectiveness. The other possible reasons could be the malnourishment of measles patients as all the participants included in this study were <9 years so they might be immunocompromised having not fully developed immune system which exacerbated the symptoms of disease and did not promote the immunization offered by the vaccine. Another possible reason behind it can be the variation in the measles virus structure as it is an RNA virus which has a very high rate of mutations.

In our Molecular detection of MeV both serum and nasal swabs have been used for the conventional PCR. Haemagglutinin (H) gene has been targeted for MeV detection as it is a vital gene for the detection and genotyping of the measles virus. Global measles and Rubella Laboratory Network has suggested the whole coding region of H gene for the detection and genotyping of measles virus (Song et al., 2022).The primers designed for the starting coding region of H gene (partial) were being validated with multiple tools to have the optimum amplification of MeV. They produced bright bands on the gel which confirmed the presence of measles virus in the children. RT-PCR based assay needs more careful handling as a slight negligence can give false positive results. Now a days molecular diagnostic techniques have been incorporated into routine laboratory diagnosis. The repertoire of molecular based detection is vast as it plays role in diagnosis at early stage of disease outbreak and later the characterization of viral strains can help in the disease surveillance, preparedness. Molecular detection paves the way to case-based disease surveillance that can provide a better insight into different factors effecting the severity of disease. Moreover, the sequencing of the viral isolates can help in the differentiation of wild type virus from vaccine derived hence providing a guideline for the optimization of vaccination strategies and supplementary immunizations.

Conclusion

Despite the MMR vaccination administration/campaigns, still there are measles cases being reported in the country. Our study indicated toward differences in blood profiles of the patients with different vaccination status (non-vaccinated, fully vaccinated and partially vaccinated). However, this study includes limited sample size and not a broad range of biological specimens have been used for molecular detection. Further research could be conducted on a large set of measles patients with respect to their nutritional status, specific genetic factors, immunocompetency and efficacy of MMR vaccination. The investigation for the optimization of vaccine cold chain storage can be an area of further exploration. Also, the use of multiple biological samples such as throat swabs and urine samples can be included in the molecular diagnosis. Moreover, the purified PCR product can be sequenced for the phylogenetic and mutational analysis to highlight the unexplored aspects of measles prevalence.

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Research Publication

Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences 60(S): 55-63 (2023)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(60-sp1)791

Pakistan Academy of Sciences

Research Article

Hematological and Biochemical Assessment of Children Infected with Measles Virus: 2022 Outbreak in Pakistan

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Abstract: Measles is a contagious disease caused by an RNA virus. Resurgence of measles after Covid-19 and its severity among children has led to many speculations about the Measles vaccination coverage and its efficacy. In this study, the clinical data of children <9 years (n=19) admitted at the Pakistan Institute of Medical Sciences (PIMS) in the measles ward was analyzed. The blood samples were processed for hematology and routine biochemistry tests. The results obtained were statistically analyzed on SPSS-21 software by using One-Way ANOVA for Complete Parameters (CP), Kruskal Wallis, and Mann-Whitney test for Differential leucocyte count (DLC) and Biochemical parameters. A $p<0.05$ was considered significant. The results suggest no significant difference in Complete blood parameters (CP) among non-vaccinated, partially vaccinated and fully vaccinated patients. Among DLC Basophils level was significantly different ($p=0.024$), being lower in partially vaccinated than non-vaccinated patients. Biochemical parameters showed that serum urea level was significantly different ($p=0.013$), showing a decline in fully vaccinated patients as compared to non-vaccinated patients. Moreover, a significantly higher level of Alkaline phosphatase as compared to the normal range was observed in fully vaccinated patients. However, lower levels of MCH, MCV, MCHC, RBC, Hb, eosinophils, and a higher level of RDW-CV were observed overall as compared to the normal range (healthy individuals). The results suggest improvements are needed in vaccination strategies for effectively controlling the disease. Anemic conditions in overall measles patients indicate poor health conditions. This study contains a limited sample size, further research on measles virus (MeV) mutations, and vaccine optimization could be helpful for the complete eradication of measles from Pakistan.

Keywords: Measles, Hematology, Biochemical parameters, Measles vaccination, Measles in children.

1. INTRODUCTION

Measles virus (MeV) is a very contagious virus that had been a cause of high fatality rates throughout the world before the advent of the Measles vaccine [1]. Measles virus is a single-stranded negative-sense RNA virus belonging to the family Paramyxoviridae and genus Morbillivirus [2]. There are other five genera belonging to this family including Rinderpest virus (RPV), Peste des Petitis Ruminant's Virus (PPRV), Canine Distemper Virus (CDV), Phocine Distemper Virus (PDV), Cetacean Morbillivirus (CeMV) that cause similar infectious diseases in Cattle, sheep and goats, Carnivores, seals and dolphins respectively [3].

The only reservoir of the measles virus is human beings. The transmission of MeV takes place via respiratory aerosols as it is an airborne virus. Moreover, it can also be transmitted via direct contact with the surface containing respiratory secretions from the infected person [2]. Measles virus has a high infectivity rate as one infected person can infect more than 12 persons on average [4]. As the virus makes its way to the human body the prodromal phase spans over 7-10 days and then MeV manifests itself in the form of cough, coryza, and fever followed by a rash on the face and the other parts of the body. These symptoms disappear usually with a decrease in viral load conferring lifetime immunity. However, in children with an underdeveloped immune system, it can take severe form developing pneumonia, otitis

Received: December 2022; Accepted: January 2023
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nvestigation of Measles During 2022-Outbreak in Pakistan

14/02/23

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