MOLECULAR CHARACTERIZATION OF MEASLES VIRUS IN CHILDREN WITH FEVER AND MACULOPAPULAR RASH IN SOUTHWESTERN NIGERIA

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B.Sc. MICROBIOLOGY, M.Sc. VIROLOGY (IBADAN)

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ABSTRACT

The use of live attenuated vaccines for measles has considerably reduced the incidence of the disease worldwide. However, measles remains endemic and a major cause of under-five morbidity and mortality in many developing countries. Molecular characterization of wild-type measles virus is a key component to laboratory surveillance activities in all phases of measles control. This study was therefore designed to carry out molecular characterization of measles virus strains circulating in south-western Nigeria.

Blood and throat swab samples were collected over a period of five years (2007-2011) from 1200 children (639 females and 561 males) aged six months to five years presenting with fever and maculopapular rash. The participants were consecutively recruited from Ibadan (Adeoyo General and Oni Memorial Children Hospitals) and Lagos (Isolo General and Massey Street Children Hospitals). All the samples were tested for the presence of IgM antibodies against measles virus, rubella virus and parvovirus B19 using Enzyme-Linked Immunosorbent Assay. Viral RNA were extracted from 286 throat swabs and transcribed to cDNA by reverse transcription using random hexamers. The cDNA was then amplified using a nested PCR and the amplicons directly sequenced in both forward and reverse directions. The virus sequences were edited and analysed for phylogenetic relationships by clustalW and maximum likelihood methods using specialised software based on their nucleotide and amino acid sequences. Data were analysed using descriptive statistics and ANOVA at P=0.05

One hundred and twenty eight (10.7%) children tested positive for measles IgM antibodies. The prevalence of measles infection was higher in female (11.58%) than in male (9.62%) participants though the difference was not significant (p=0.08). The rate of detection of measles virus was relatively constant (10.3%-11.9%) over the five year period. Rubella virus IgM antibodies were detected in 267 (24.7%) children, (151 females and 116 males) while

parvovirus B19 IgM antibodies were found in 96 (11.9%) children (51 females and 45 males). Twenty three (8.1%) of the 285 swabs processed were positive for measles by PCR out of which the target region was successfully sequenced from 11 of them. Ten of the sequenced strains were classified as genotype B3 cluster 1 while one showed high sequence similarity with measles virus of clade A.

Detection and isolation of measles virus of clade A in Nigeria was described for the first time and showed wider circulation of the clade outside of its previous regions of detection in Europe and Americas. Measles virus of genotype B3 cluster 1 is the predominant circulating strain in Southwestern Nigeria. There was uninterrupted transmission of endemic virus in the study areas.

Keywords: Measles virus, Circulating genotypes, Molecular characterization, IgM antibodies.

Word count: 425

DEDICATION

This thesis is dedicated first to

God almighty

And to my love and best friend

Olumide Omololu Faneye

And also to my lovely babes

Fiolabomi Ayomide Faneye and Fikunolami Iremide Faneye

for their endurance and unconditional love for me.

Finally to my beloved parents

Mr and Mrs Kolapo Ayoola Raji

You have been the best Dad and Mum to me, providing me with all the support.

CERTIFICATION

I certify that this work was carried out by Mrs Adedayo Omotayo Faneye in the Department of Virology, College of Medicine, University of Ibadan, Ibadan, Nigeria.

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Date----------

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CHAPTER ONE

1.0. INTRODUCTION

Measles is an acute infection characterised by a prodromal illness of fever, coryza, cough, conjunctivitis and presence of Koplik spots followed by the appearance of a generalised maculopapular rash (Griffin, 2007). It is caused by measles virus which is one of the most contagious viruses with >90% secondary attack rates among susceptible individuals (WHO, 2009). The virus can be transmitted through aerosols in respiratory droplets or by direct or indirect contact with nasal and throat secretions of infected persons. People with measles virus are considered infectious from four days before to four days after the onset of rash. The incubation period of measles after exposure before the onset of the symptoms is usually 10 to 12 days (Griffin, 2007).

An estimated 30% of reported cases of measles involve one or more complications (WHO, 2009). Complications of measles are more common among children younger than five years of age and adults 20 years of age and older. In developing countries where malnutrition and vitamin A deficiency is common, measles has been known to kill as many as one out of four infected people and it is a major cause of blindness in African children (CDC, 2012). Other complications of measles include diarrhoea (8%), pneumonia which majorly causes death in children with measles (6%), otitis media (7%) and post infection encephalitis (one per 1000 cases) (CDC, 2012).

Despite the availability of safe and effective vaccine for over 40 years, measles remains a leading cause of death globally among young children (0-5years). The World Health Organization estimated that 535,300 children died from measles in year 2000 (Wkly Epid Rec, 2006). Most of the deaths were recorded from developing countries in Africa and Asia region (Wkly Epid Rec, 2006). The World Health Assembly (WHA) and The United Nations

Children Organization set a goal of 90% reduction in measles mortality between 2000 and 2010 levels primarily by targeting children in WHO regions with the highest number of measles deaths.

In areas where measles remains endemic, transmission of the virus shows a seasonal trend (WHO, 2009). In temperate areas, the most intense virus transmission usually occurs in late winter and spring while in tropical areas like Nigeria the most intense virus transmission occurs in the dry season (WHO, 2009). Before vaccination programmes, childhood infection of measles was almost universal. Measles epidemics occurred in approximately four-year cycles with very high incidence alternating with low incidence, inter-epidemic periods. With the introduction and increase in coverage of measles vaccination, the incidence of the disease during epidemic periods has fallen and the intervals between epidemics have lengthened. Very high levels of population immunity have led to the elimination of the disease in many countries, but if this level of population immunity is not maintained, the cyclical pattern of measles outbreaks will reappear (WHO, 2009).

The widespread use of live attenuated vaccines for measles has dramatically reduced the worldwide incidence of measles. The disease has been successfully eliminated in the Western hemisphere (regions of the Americas) by effective vaccination programmes (UNICEF, 2006). Despite this success, measles remains endemic in developing countries. It remains a major cause of childhood morbidity and mortality accounting for an estimated 345, 000 deaths in 2005, 87% of which were in African and South Asian Regions (Wolfson *et al.,* 2007). Maintaining measles elimination requires achieving and maintaining very high levels of population immunity and good laboratory-based surveillance to rapidly detect and control outbreaks.

In 2005, the WHO identified some key strategies for the elimination of measles. These include:

- Maintainance of very high coverage (≥95%) with two doses of measles vaccine through high-quality routine immunization.
- Ensuring provision of measles vaccination opportunities through supplementary immunization activities in populations susceptible to measles.
- Strengthening of surveillance systems that include rigorous case investigation and laboratory confirmation of clinical cases

Surveillance for Measles

An essential component of the measles control program is surveillance. The purpose of disease surveillance is to provide information for public health actions and when needed to guide the planning, implementation and evaluation of public health interventions and systems (Rota *et al.*, 2009).

Surveillance for control and elimination of measles has two main objectives, which are:

1. Detection, investigation and characterization of measles sporadic cases and clusters in order to:
    a. Ensure proper management of cases and contacts;
    b. Understand the reason for occurrence and transmission of the disease (e.g. importation, failure to vaccinate or failure of the vaccine);
    c. Assess the sustainability of transmission (size, duration of clusters);
    d. Identify populations at risk of transmission; and
    e. Ensure a rapid and appropriate public health response to the event.
2. Monitoring of disease incidence and circulation of the virus in order to:

a. Provide information for priority-setting, planning, implementation and resource allocation for preventive programmes and for evaluating control measures;
b. Assess and document progress in disease control towards elimination
c. Identify changes in risk groups and disease epidemiology;
d. Assess the circulation of virus genotypes at national, regional and global levels.

Laboratory-Based Surveillance of measles

Routine laboratory confirmation of suspected cases of measles is based on detection of measles-specific Immunoglobulin M (IgM) in a single blood sample taken as soon as possible after the onset of rash. In some cases, molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) to detect viral RNA are used to complement serologic testing. Another important aspect of laboratory surveillance for measles is the genetic characterization of circulating wild-type viruses to support molecular epidemiologic studies (WHO, 2005b).

The combination of molecular epidemiologic techniques and standard case classification and reporting provides a very sensitive means to describe the transmission pathways of measles. In particular, sequence data can help confirm the sources of virus or suggest a source for unknown-source cases as well as establish links, or lack thereof, between various cases and outbreaks. Virologic surveillance is especially beneficial when it is possible to observe the change in viral genotypes over time in a particular country or region because this information, when analysed in conjunction with standard epidemiologic data, has helped to document the interruption of transmission of endemic measles. Thus, molecular characterization of measles viruses has provided a valuable tool for measuring the

effectiveness of measles control programs (Mulders *et al.*, 2001; Riddell *et al.*, 2005; Rota *et al.*,,. 1996, 2002; Rota and Bellini 2003).

Standard Methods for Molecular Epidemiology of Measles

There was no uniform nomenclature or analysis protocol to describe the genetic characteristics of wild-type measles viruses until the WHO made recommendations for a standard nomenclature for naming strains, describing genotypes, and conducting sequence analysis so that genetic data would be directly comparable between laboratories. These recommendations have been updated periodically since then (WHO, 1998, 2001a, 2001b, 2003, 2005b, 2005c, 2006, 2007a). The WHO recommends that the 450 nucleotides coding for the carboxyl terminal 150 amino acids of nucleocapsid gene are the minimum amount of sequence data required for genotyping a measles virus isolate or clinical specimen.

For molecular epidemiologic purposes, the genotype designations are considered the operational taxonomic unit, while related genotypes are grouped by clades. The WHO currently recognizes eight clades designated A, B, C, D, E, F, G, and H. Within these clades, there are 23 recognized genotypes, designated A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, E, F, G1, G2, G3, H1, and H2. Some clades contain only one genotype and, in such cases, the genotype designation is the same as the clade name. Other clades contain multiple genotypes and are designated by using the clade letter (in uppercase) and genotype number. All of the genotypes have an assigned reference strain chosen to represent the earliest isolation of virus from each genotype. Sequences from recent viral isolates are then compared to the set of WHO reference sequences, which are available from GenBank (World Health Organization 2005b) and the WHO Strain Banks, to determine the genotype.

MEASLES IN NIGERIA

Despite the fact that Nigeria integrated measles vaccination into the Expanded Programme on Immunization since 1989, measles still ravages Nigerian children each year. Suspected measles cases were about 32,000, 98,000 and 93490 in 2004, 2005 and 2006-2009, respectively while deaths attributed to measles were about 600 and 2,500 for 2004 and 2005 respectively. The north-western region of Nigeria with the lowest routine immunization coverage had the highest number of measles cases and mortality within 2006-2009 (Mohamed *et al.,* 2010). Fatiregun *et al.* (2014) also found 1631 cases of confirmed measles among children presenting with fever and rash between 2007 and 2012 in south-western Nigeria.

Presently, Nigeria's strategy for measles control includes case-based surveillance for measles and supplemental immunization activities. Nigeria adopted the case-based surveillance of measles in 2006 in which every reported case of fever and rash is tested for measles and rubella IgM antibodies. Nigeria is yet to integrate molecular epidemiology into her measles control programme. Thus, limited information is available on the genetic characteristics of endemic measles virus strains and those responsible for different epidemics in the country.

1.1 JUSTIFICATION FOR THE STUDY

Measles is still very active in Nigeria despite the availability of vaccine with over 13,518 confirmed cases between 2006 and 2009 (Mohamed *et al.,* 2010). The WHO (WHO Measles Lab Manual 2009) recommended that in addition to vaccination, laboratory surveillance should be included in each country's control programme. Molecular characterization of wild-type measles virus is a key component to laboratory surveillance activities in all phases of measles control**.** It also provides a means to differentiate vaccine-associated cases of measles from cases caused by infection with wild-type virus. Molecular characterization of measles

viruses provides a valuable tool for measuring the effectiveness of measles control programmes. Thus, there is the need for an update on the information on molecular characteristics of circulating strains of measles virus in Nigeria.

1.3 AIM OF THE STUDY

The aim of this study is to provide information on molecular characteristics of current circulating strains of measles in Lagos and Ibadan towards the control and final elimination of measles in Nigeria.

1.4 OBJECTIVES OF THE STUDY

This study on molecular characterization of measles virus in Lagos and Oyo state seeks to:-

1. Determine the prevalence of measles virus antibodies in children presenting with fever and maculopapular rash.
2. Determine the prevalence of antibodies to rubella virus and parvovirus B19 in children presenting with fever and maculopapular rash who tested negative for measles virus IgM antibody.
3. Isolate and characterize the circulating strains of measles virus in Lagos and Ibadan.
4. Determine any changes in the circulating genotypes in this region over time.

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORICAL REVIEW OF MEASLES VIRUS INFECTION

Measles a highly contagious disease is characterized by fever cough conjunctivitis and generalised maculoparpular rash. It was first distinguished from smallpox in the 9th century by an Arab physician called Rhazes of Baghdad (Brindley, 1748). Genetically and antigenically, measles virus is closely related to Rinderpest virus which is a pathogen of cattle (Barrett *et al.,* 1999, Sheshberadaran *et al*., 1986). Measles virus was believed to evolve in an environment where cattle and humans lived in close proximity (McNeil, 1976). The modern history of measles begins in 1670 with Thomas Sydenham's description of his son's attack and he was the first to clearly define measles as well as identifying some of its complications such as cancrum oris and encephalitis.

Many of the basic principles of measles epidemiology and infection were elucidated by studies of Peter Panum, a Danish physician who was sent to Faroe Islands in 1846 by his government to investigate an epidemic of measles on the Islands (Panum, 1938). He was able to establish four main facts which are:

A) Measles rash appears twelve to fourteen days after contact.

B) Its greatest infectivity is during the late prodrome (3-4 days before the rash appears).

C) The disease is highly contagious and it is spread by droplets; that is respiratory route of transmission.

D) The protection from an attack is life-long

Complications of measles were first described in 1790, by James Lucas who described post measles encephalitis (Lucas, 1790). Sub-acute sclerosing pan-encephalitis was also described

in 1933 by Dawson in a 16-year-old boy with progressive neurologic deterioration (Tellez-Nagel and Harter, 1966).

Measles virus was first isolated by Hektoen in 1910, the virus was first grown and subsequently attenuated by Enders in 1963 (Enders, 1963)

2.2 CLASSIFICATION OF MEASLES VIRUS

2.2.1 THE FAMILY PARAMYXOVIRIDAE

The family Paramyxoviridae include some common important disease-causing viruses of humans and animals, including measles virus.

The Paramyxoviridae are enveloped negative-stranded RNA viruses. They are related to two other families of negative-strand RNA viruses, namely the Orthomyxoviridae, and Rhabdoviridae for their envelope glycoproteins and organization and expression of its genome, respectively. A distinctive feature of the family Paramyxoviridae is the (F) Protein which causes viral cell membrane fusion at neutral pH (Lamb and Griffith, 2007)

The Paramyxoviruses contains non-segmented single-stranded RNA genomes of negative polarity and replicate entirely in the cytoplasm. Their genomes are about 15 to 19 KB in size containing six to ten closely linked genes.

They all have a lipid envelope that contain two surface glycoproteins (F and a second glycoproteins variously called HN, or H, or G) surrounding the virions. Inside the envelope, is a helical nucleocapsid core that contains the RNA genome as well as the necleocapsid (N), Phospho- (P) and large (L) proteins which initiate the intracellular virus replication. The viruses also have the matrix (M) protein which is important in virion architecture.

The family Paramyxoviridae is classified into two subfamilies, namely;

- Paramyxovirinae
- Pneumovirinae

The Paramyxovirinae contains five genera, which are;

- Respirovirus
- Rubulavirus
- Avulavirus
- Morbillivirus
- Henipavirus

While the Pneumovirinae has two general and this includes;

- Pneumovirus
- Metapneumovirus

This classification is based on morphologic criteria, the organization of the genome, the biological activities of the proteins, and the sequence relationship of the encoded proteins. The Pneumoviruses can be distinguished from Paramyxovirinae morphologically because they contain narrower nucleocapsids.

Table 2.1: Classification of the Family Paramyxoviridae

Family Paramyxoviridae		
Sub Family	**Genus**	**Species**
Paramyxovirinae	**Avulvavirus**	**Newcastle disease virus (Avian Paramyxovirus)**
Paramyxovirinae	**Henipaviruses**	**Hendra virus (HeV), Nipah virus (NiV)**
Paramyxovirinae	**Mobilivirus**	**Measles virus, Canine distemper virus (CDV), Rinderpest virus**
Paramyxovirinae	**Respirovirus**	**Human parainfluenza type 1 and 3**
Paramyxovirinae	**Rubulavirus**	**Mumps virus, Parainfluenza virus type 4, 4a, and 4b**
Pneumovirinae	**Pneumovirus**	**Human respiratory syncytial virus A1, B1, S2**
Pneumovirinae	**Metapneumovirus**	**Human metapneumovirus (hMPV) Avian metapneumovirus**
Unclassified paramyxoviruses		**Tupaia paramyxoviruse, Beilong virus, Manangle virus**

2.3 MEASLES VIRUS

Measles virus is a member of the **Mononegavirales** which comprises the **Rhabdoviridae, Filoviridae, Henipaviridae, Bornaviridae** and **Paramyxoviridae**. As a paramyxovirus, Measles virus has structural and biochemical features associated with this group. Measles virus is grouped into a separate genus **Morbillivirus** because it lacks a virion-associated neuraminidase activity. Other members include: Peste-des-petits-ruminants virus (PPRV), which infects sheep and goats, Rinderpest virus (RPV), which infects cattle, and Canine distemper virus (CDV), which infects dogs. All these viruses exhibit antigenic similarities, and all produce similar diseases in their host species.

2.3.1 Morphology of Measles Virus

Measles virus particles is made up of a lipid envelope surrounding a viral ribonucleoprotein complex, which is composed of genomic RNA associated with proteins. Measles virus has a trans-membrane fusion (F) and haemagglutinin (H) proteins projects from the envelope surface of the particle, and extends through the lipid bilayer into the cytosol. The N-terminus of the H protein protrudes through the cytoplasmic and viral membranes (type II glycoprotein), while the F protein is anchored near the C-terminus (type I glycoprotein). The virus also has a Matrix (M) protein which links the envelope to the Ribonucleoprotein core structure. The viral genomic RNA is fully condensed with N (nucleocapsid) protein to form the RNase-resistant Ribonucloprotein core structure. The virus has a helical nucleocapsid of about 1.2Aμm in length which is packed within the envelope in the form of a symmetric coil with the phosphoprotein (P) and large polymerase (L) proteins attached to it.

The virions are highly pleomorphic, with an average size of 120–250 nm. In an electron micrograph the virion is bounded by a lipid envelope which bears a fringe of spike-like projections (peplomers) 5–8 nm long. The membrane below the spikes is 10–20 nm thick and encloses the helical viral RNP core which has a diameter of 17 nm and a regular pitch of

5nm. Immediately below the membrane M proteins appear as a shell of electron-dense material (Bhella *et al.*, 2004)

2.3.2 Physical and Chemical Properties of Measles Virus

The structure of measles virus explains most of its physical properties. The stability and infectivity of the virus depends on the integrity of the envelope. Hence the virus is sensitive to any substance that disrupts its envelope, thus substances such as detergents or other lipid solvents including acetone and ether are capable of destroying the lipid envelop (Sibylle and Volker, 2009).

Measles virus particles are acid labile and are inactivated below pH 4.5, although they remain infective in the range pH 5-9. Measles virus is also thermo-labile. It may remain infective for two weeks at 4^0C, but it is completely inactivated after 30 minutes at 56^0C. At 37^0C it has a half-life of 2 hours. The virus can be stored for prolonged period at -70^0C and can also be freeze dried. These properties have important implications in the transport and storage of measles vaccine.

2.3.3 Genome Organization of Measles Virus

Measles virus is a non-segmented RNA genome of negative polarity, about 16 kb in length. It is flanked by non-coding 3'leader and 5'trailer sequences that contain specific encapsidation signals and the viral promoters used for viral transcription and/or replication (Parks *et al.*, 2001).

The genome encodes six structural genes for which the reading frame is arranged linearly and without overlap in the following order;

Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Fusion proein (F), Haemaglutination protein (H) and the Large protein (L)

The coding region of the measles virus genome is separated by intragenic regions which consist of a polyadenylation signal at the 3' end of each gene and a re-initiation signal for the distal gene. From the P gene, three non-structural proteins C, V, and R, are expressed.

2.3.4 Structural Proteins of Measles Virus

2.3.4.1 Nucleocapsid Protein (N)

The Nucleocapsid protein (N) is the first transcribed protein from the genome and it is the most abundant of the viral proteins. It binds to both RNA and P and is required for transcription and replication. N proteins serves to condense the viral genomic RNAs into a smaller more stable and more readily packaged form which gives the nucleocapsid its helical form (Bhella *et al.*, 2004). N proteins appear as a 60kD band on polyacrylamide gels but are often cleaved by cellular proteases during extraction. N can be transported into the nucleus of the infected cell but is usually retained in the cytoplasm by binding to the phosphoprotein (Huber *et al.*, 1991) the N- terminal portion of the protein is conserved and is required for self-assembly into nucleocapsids and for RNA binding. The C- terminal is more variable, intrinsically disordered, and acidic and belongs to a family of natively unfolded proteins that are structurally similar to the acidic activation domains of cellular transcription factors (Baczko *et al.*, 1992, Cattaneo *et al.*, 1989, Karlin *et al.*, 2003).

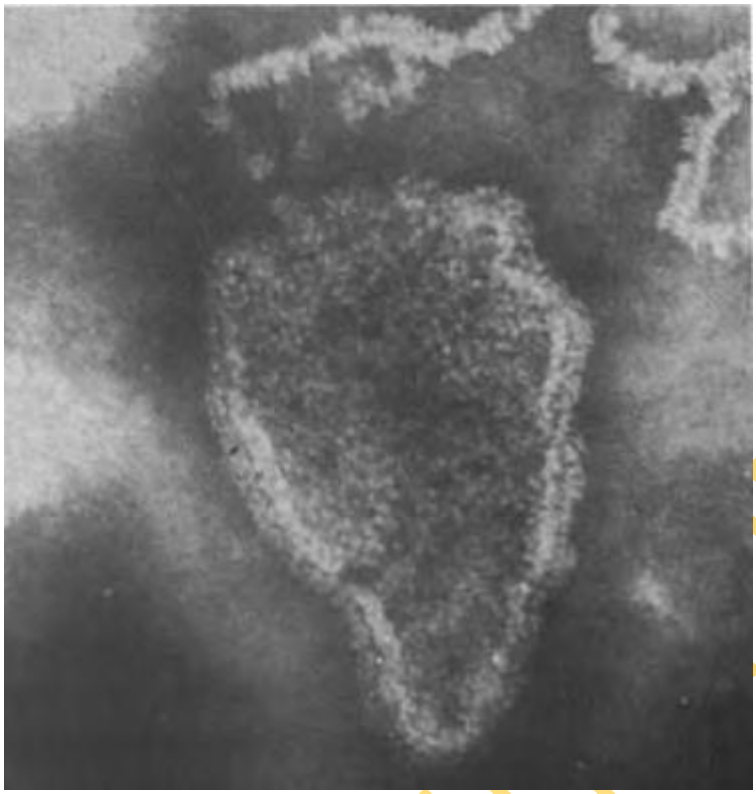


Figure 2.1: Electron micrograph of the measles virion (bar = 50 nm).
Source: Principles and Practice of Clinical Virology. Edited by A.J. Zuckerman, J.E.Banatvala, B.D. Schoub, P.D. Griffiths and P. Mortimer
 ISBN: 978-0-470-51799-4. Sixth Edition.

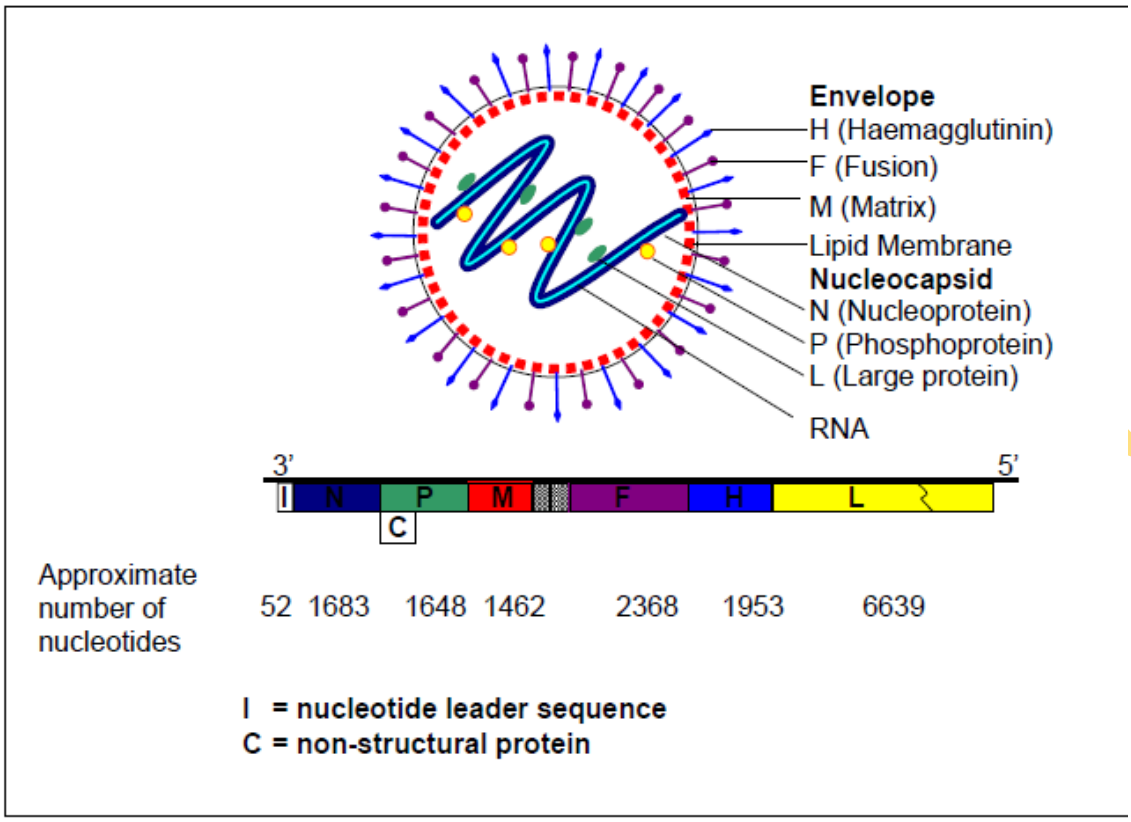


Figure 2.2: Schematic diagram of measles virus

Source: WHO manual for the laboratory diagnosis of measles and rubella virus infection. Second edition: WHO/IVB/0701

2.3.4.2 Phosphoprotein (P)

The Phosphoprotein (P) is a polymerase co-factor that is activated by phosphorylation, forms trimers, and links L to N to form the replicase complex (Curran *et al.,* 1995). It appears as a 72kD band on polyacrylamide gels and it is abundant in the infected cell, but only small amount are present in the packaged virus. P has a modular organization.

The Phospho-protein N-terminus (PNT) is poorly conserved, intrinsically unstructured, acidic, phosphorylated, and required for replication. Association of PNT with N proteins prevents it from binding to cellular RNA and initiates encapsidation of genomic viral RNA by binding in a sequence specific fashion to the leader (Huber *et al.*, 1991)

The Phosphoprotein C-Terminal (PCT) is well conserved and contains all domains required for transcription (Griffin, 2007)

2.3.4.3 Matrix Protein (M)

The Matrix (M) protein is found in the envelope of the measles virus. M protein is associated with nucleocapsids and with the inner layer of the plasma membrane in infected cells (Hirano *et al.,* 1992). M proteins interact with the intracytoplasmic regions of one or both transmembrane glycoproteins, modulates the targeting and fusogenic capacity of the envelope glycoproteins, and directs release of virus from the apical surface of epithelial cells (Blau *et al.,* 1995, Katz *et al.,* 1960).When M protein is deleted from the virus there will be an increase in cell to cell fusion thus lowering the production of infectious virus (Cathomen *et al.*, 1998)

2.3.4.4 Fusion (F) Protein

The F protein is a highly conserved type I transmembrane glycoprotein. The F protein causes fusion of viral and cell membrane at neutral pH. It is usually first synthesized as an inactive precursor (F_0) of about 60kD which after translation and glycosylation, is cleaved to yield two disulfide-linked subunits 41kD F_1 and 18kD F_2.

Glycosylation of the F_0 precursor is an essential prerequisite for cleavage, and all the potential N-glycosylation sites reside within the F_2 subunit. Mutations of any of these sites affect cell surface transport, proteolytic cleavage, stability and fusogenic activity of the F protein (Plemper *et al.,* 2003). Restricted processing of F is associated with persistent infection (Meertens *et al.*, 2003).

Fusion contains a highly conserved stretch of hydrophobic amino acid residues at the new N-terminus (amino acid 113 to 145) and this is postulated to play an important role in the fusion process (Richardson *et al.*, 1983). Fusion requires the expression of H and binding of H to a cell surface receptor, as well as expression of F (Zhang *et al.,* 2005) and the expression of these two proteins is important for syncytial formation and increases cell-to-cell spread of measles virus in vivo and in vitro (Moll *et al.,* 2001).

2.3.4.5 Hemagglutinin (H) Protein

The H protein is the receptor binding and hemagglutinatin protein and is an important determinant of morbillivirus cellular tropism. It is a type II transmembrane glycoprotein and it is found on the surfaces of infected cells and virions as disulfide-linked homodimers that associate to form tetramers (Blau *et al.,* 1995)

The mature H protein has a cytoplasmic tail of 34 amino acids preceding a single hydrophobic transmembrane region and a large C-terminal ectodomain with 13 strongly conserved cysteines. The cytoplasmic tail is essential for efficient transport to the cell surface

(Moll *et al.*, 2002, Moll *et al.*, 2004) H protein acts in conjunction with F protein during fusion and entry.

2.3.4.6 Large (L) Protein

The large (L) protein is a multi-domain protein with several highly conserved regions. L protein is present in small quantities in the infected cell, interacts with and functions in association with P, and is part of the viral nucleocapsid in the cell and in the virion. The N-terminal of the L protein binds to the C-terminal of the P protein that links to the nucleocapsid for transcription and replication (Horikami *et al.*, 1994, Kingston *et al.*, 2004).

2.3.5 Non Structural Proteins of Measles Virus

The P gene of measles virus, as with many members of the family Paramyxoviridae, encodes several proteins in addition to P protein and this includes the C protein and the V protein. C and V protein interact with cellular proteins and play a role in the regulation of transcription and replication (Liston *et al.*, 1995)

The C protein interferes with innate immune responses by inhibition of interferon (IFN) signaling, modulates viral polymerase activity, and has been implicated in prevention of cell death (Escoffier *et al.*, 1999, 31). Deletion of C decreases MV replication in peripheral blood mononuclear cells, thymic epithelial cells and infected monkeys, and decreases neuro-virulence for CD46 transgenic mice and this suggests an important in vivo role for C (Escoffier *et al.*, 1999, Tober *et al.*, 1998)

The V protein is phosphorylated, diffusely distributed in the cytoplasm of infected cells and affects N-P interaction (Tober *et al.*, 1998). V can interfere with IFN signalling in MV-infected cells in a strain-specific fusion (Ohno *et al.*, 2004). Deletion of V decreases the amount of virus released from glioblastoma cells, delays and prolongs MV replication in human thymic epithelial cells, and decreases MV replication in the lungs of cotton rats. Over

expression of V is associated with more rapid replication, but decreased cytopathic effect in Vero cells (Valsamakis *et al.,* 1998).

2.3.6 Biological Properties of Measles Virus

2.3.6.1 Hemagglutination and Hemadsoption

Measles virus binds to the erythrocytes of African green monkeys, Patas, and rhesus macaques and most efficiently agglutinates them. This is due to the presence of measles virus major receptor component CD46 on these cells. Measles virus does not agglutinate human erythrocyte because it lacks this receptor.

Hemagglutination (HA) occurs optimally at physiologic pH and 37^0C and cells productively infected with some strains of MV can be identified by their ability to adsorb monkey erythrocytes (hemadsorption).

HA and hemadsorption are properties of the H glycoprotein. HA of monkey erythrocytes is indicative of H protein binding to CD46, is improved by adaptation to growth in vero cells, and is dependent on the C-terminal 18 amino acid of H. Strains of measles virus with varying activities have been described. Many wild-type measles isolates have low hemagglutinatin activities (Saito *et al.*, 1992).

2.4 Cytopathic Effect of Measles Virus in Cell Lines

Measles virus replication in cell culture monolayer results in cytopathic changes of three varieties, which are;

i. Multinucleated giant cells (syncytia)
ii. Altered cell shape,
iii. Inclusion bodies

Infected cells can change from a normal polygonal shape to a stellate (a dendritic or spindle-shaped appearance with increased refractivity to light (Griffin, 2007).

Spindled-shaped cells and syncytia cells can contain intra-cytoplasmic and intra-nuclear inclusion bodies. Cytoplasmic inclusions are generally larger than nuclear inclusions and contain N-encapsidated RNA decorated with P and perhaps cellular proteins producing fuzzy or granular nucleocapsids (Bohn *et al.,* 1990).

2.4.1 Syncytia Formation

The formation of multinucleated giant (syncytia) cells results from fusion of infected cells with uninfected cells to produce syncytia with 50 or more nuclei bounded by a single cytoplasmic membrane. Nuclei in the centre of syncytia have marginated chromatin and are undergoing apoptotic cell deaths, leading to plaque formation in-vitro (Enders *et al.,* 1954). Syncytia formation is facilitated by basolateral expression of H and F protein.

2.5 Antigenic Composition and Strain Variation

Measles virus has long been considered an antigenically stable monotypic virus. Antisera from individuals infected decades ago retain the ability to neutralize more current wild-type strains of MV and vice versa, although with varying efficiency (Klingele *et al.,* 2000) Although measles is a monotypic virus, genetic and antigenic variation has been detected in wild-type viruses (Rota *et al.,* 1992). Sequence analysis of the Hemagglutinin (H) and Nucleoprotein (N) genes has shown that distinct lineages of wild-type viruses co-circulate.

Of the 6 genes comprising the viral genome, the H and N genes are the most variable with approximately 7% nucleotide variability between the most distantly related viruses. The single most variable part of the measles genome is the 450 nucleotides that codes for the carboxyl terminus of the N protein. Nucleotide diversity in this region can exceed 12%

between viruses from different genotypes, although the biological significance of this variation is not clear. Most molecular epidemiological studies of measles virus have used sequences derived from the N and/or H genes (WHO, 2001).

For molecular epidemiologic purposes, the genotype designations are considered the operational taxonomic unit for measles virus, while related genotypes are grouped by clades. WHO has identified eight clades designated A, B, C, D, E, F, G, and H. Within these clades, there are 23 recognized genotypes, designated A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, E, F, G1, G2, G3, H1, and H2 (Rota *et al.*, 2009).

Some genotypes are found in one geographic region, others are co-circulating, whereas others are inactive and may be extinct (Riddell *et al.*, 2005).

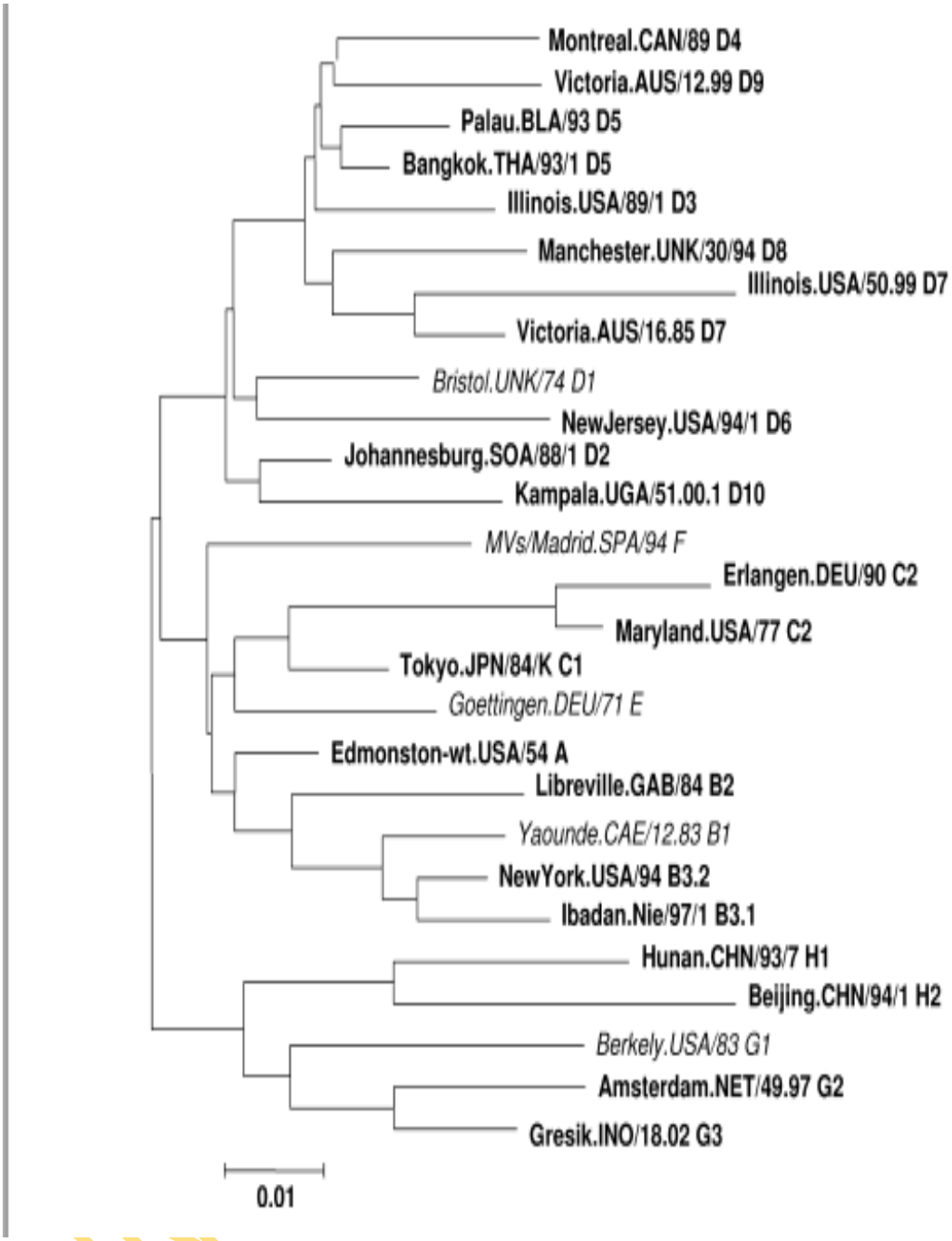


Figure 2.3: Phylogenetic tree showing evolutionary relationships of wild type and vaccine strains of measles virus. (Riddell *et al.*, 2005)

Source: D.E. Griffin and M.B.A. Oldstone (eds.) Measles – Pathogenesis and Control. 129 © Springer-Verlag Berlin Heidelberg 2009

Table 2.2: WHO List of Reference Strains to be Used Genetic Analysis of Wild Type Measles Virus

Table 7.1 Reference strains to be used for genetic analysis of wild-type measles viruses: 2008

Genotype	Status[a]	Reference strains (MVi)[b]	H gene accession[c]	N gene accession
A	Active	Edmonston-wt.USA/54	U03669	U01987
B1	Inactive	Yaounde.CAE/12.83 "Y-14"	AF079552	U01998
B2	Active	Libreville.GAB/84 "R-96"	AF079551	U01994
B3	Active	New York.USA/94	L46752	L46753
		Ibadan.NIE/97/1	AJ239133	AJ232203
C1	Active	Tokyo.JPN/84/K	AY047365	AY043459
C2	Active	Maryland.USA/77 "JM"	M81898	M89921
		Erlangen.DEU/90 "WTF"	Z80808	X84872
D1	Inactive	Bristol.UNK/74 (MVP)	Z80805	D01005
D2	Active	Johannesburg.SOA/88/1	AF085198	U64582
D3	Active	Illinois.USA/89/1 "Chicago-1"	M81895	U01977
D4	Active	Montreal.CAN/89	AF079554	U01976
D5	Active	Palau.BLA/93	L46757	L46758
		Bangkok.THA/93/1	AF009575	AF079555
D6	Active	New Jersey.USA/94/1	L46749	L46750
D7	Active	Victoria.AUS/16.85	AF247202	AF243450
		Illinois.USA/50.99	AY043461	AY037020
D8	Active	Manchester.UNK/30.94	U29285	AF280803
D9	Active	Victoria.AUS/12.99	AY127853	AF481485
D10	Active	Kampala.UGA/51.00/1	AY923213	AY923185
E	Inactive	Goettingen.DEU/71 "Braxator"	Z80797	X84879
F	Inactive	MVs/Madrid.SPA/94 SSPE	Z80830	X84865
G1	Inactive	Berkeley.USA/83	AF079553	U01974
G2	Active	Amsterdam.NET/49.97	AF171231	AF171232
G3	Active	Gresik.INO/17.02	AY184218	AY184217
H1	Active	Hunan.CHN/93/7	AF045201	AF045212
H2	Active	Beijing.CHN/94/1	AF045203	AF045217

[a] Active genotypes that have been isolated within the past 15 years
[b] WHO name; other names that have been used in the literature appear in quotation marks
[c] Sequences available at GenBank (http://www.ncbi.nlm.nih.gov) or from WHO strain banks
Reproduced from World Health Organization 2005b

Source: D.E. Griffin and M.B.A. Oldstone (eds.) Measles – Pathogenesis and Control. 129 © Springer-Verlag Berlin Heidelberg 2009

2.6 Replication Cycle of Measles Virus

2.6.1 Cellular Receptor

The cellular receptor for wild-type measles virus has been shown to be a glycoprotein that interacts with the H protein present on human and monkey cells (Blau *et al.,* 1995). Two receptors have been identified for measles virus and they are membrane cofactor protein or CD46 (Dorig *et al.,* 1993) and SLAM or CD150 (Tatsuo *et al.,* 2000).

CD46 is a widely distributed human complement regulatory protein expressed on all nucleated cells and preferentially on the apical surface of polarized epithelial cells. CD46 homolog is found expressed on monkey erythrocytes. Several isoforms of CD46 are expressed in a tissue-specific manner and all of them support measles virus uptake. CD46 is involved in protecting uninfected cells from complement-mediated lysis by recruiting theC3b/C4b components, thereby rendering them in accessible to degradation by serum proteases.

CD150 (also referred to as Signalling Lymphocyte Activation Molecule, SLAM), is a CD2-like molecule of the Ig super family. It is expressed on cells of the immune system including activated and memory T and B cells, activated monocytes and mature dendritic cells. It is an important co-stimulatory molecule with two highly glycosylated immunoglobulin like domains (V and C2) and structural features of the CD2 family. Measles virus H protein binds to the V domain of human SLAM. Interaction with H proteins intracellularly and extracellularly results in down-regulation of SLAM expression on the surface of infected cells (Tatsuo *et al.,* 2001). CD150 supports entry of all known measles virus strains

Some studies have suggested measles virus may use additional receptor because the distributions of SLAM and CD46 in tissues do not account for the tropism and sites of

measles virus replication in acute natural infection where epithelial and endothelial cells, as well as cells of the immune system, are infected or in chronic infection where cells of the central nervous system (CNS) are important targets (Moench *et al.*, 1988). Likewise several in vitro studies have shown that infection can occur with or without either of CD46 or SLAM (Hashimoto *et al.*, 2002).

2.6.2 Intracellular Replication

The time taken for measles virus replication in a host cell varies and becomes shorter as the virus adapts to growth in vitro. The origin and activation stage of the host cell influences the efficiency of measles virus replication as well as their ability to produce type 1 interferon (IFN) or the ability of the virus strain to prevent IFN production and /or signalling.

After the virus ribonucleoprotein complex has been delivered into the cytoplasm of the infected cell viral transcription is initiated by specific attachment of the polymerase complex to the promoter located within the 3'end of the genome and progresses to the 5' end by transcribing mono and bicistronic mRNAs. Replication of measles virus is dependent on protein synthesis.

The synthesis of viral proteins is directed by the viral mRNAs. The proteins are then translocated and modified through the Golgi apparatus and finally inserted into the plasma membrane. Nascent RNA genomes condense with N protein to form the nucleocapsid, and P and L proteins bind to these structures in the perinuclear area. M protein combines with both cytoplasmic nucleocapsids and plasma membrane resident virus glycoproteins, and possibly interacts with cytoskeletal components during intracellular transport of viral ribonucleoproteins. Progeny nucleocapsid structures assemble beneath the modified areas of the membrane and are pinched off in the budding process. The ability of M protein to aggregate in a crystalline array most likely enables distortion of the membrane into an

outward-facing bulge, and ultimately budding of the nucleocapsid inside a small vesicular structure –the new virion. During the replication process the large amount of glycoprotein inserted in to the cell membrane causes it to develop the capacity to attach to adjacent cells, while the F protein promotes fusion with adjacent cells and this leads to the formation of multinucleated giant cells which are characteristic for measles infection. Host cells are rapidly killed by this fusion.

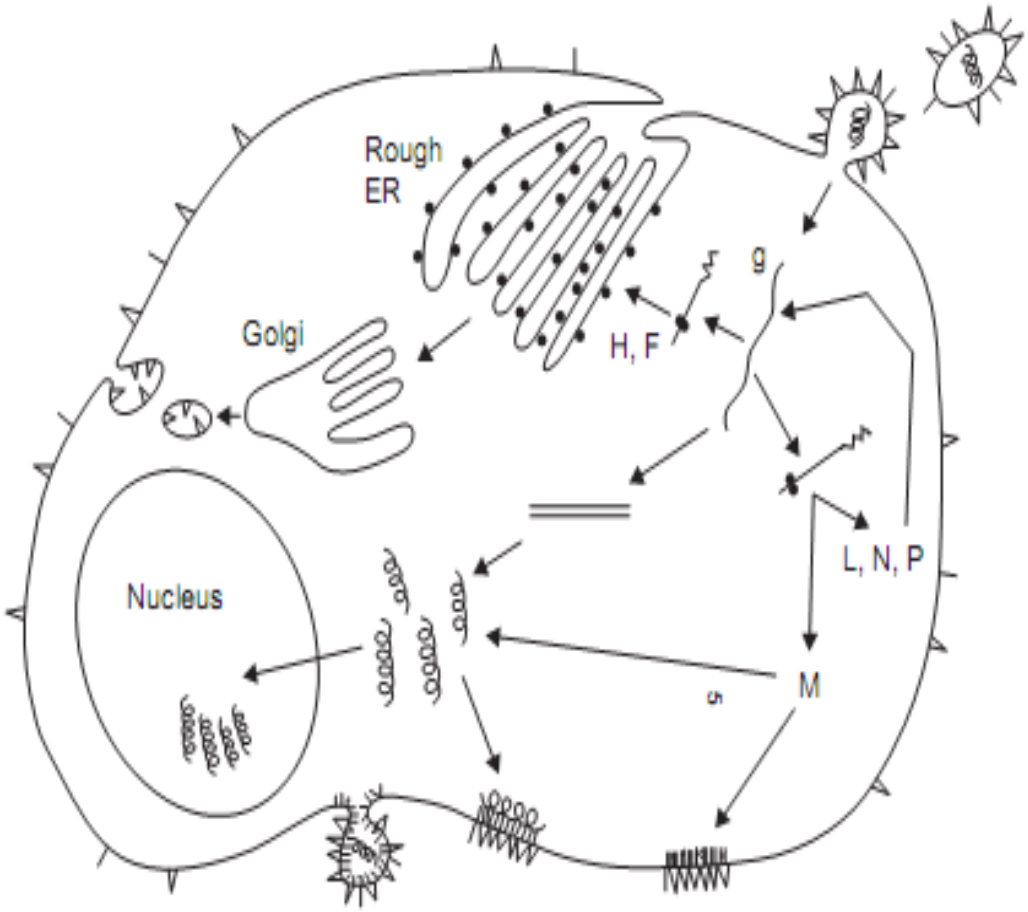


Figure 2.4: Schematic representation of the events occurring in measles virus replication

Source: Principles and Practice of Clinical Virology. Edited by A.J. Zuckerman, J.E. Banatvala, B.D. Schoub, P.D. Griffiths and P. Mortimer. © 2009 John Wiley & Sons Ltd. ISBN: 978-0-470-51799-4. Sixth Edition

2.7 Pathogenesis and Pathology of Measles Virus

2.7.1 Measles Disease

Measles begins by a latent period of 10 to14 days and a two to three day prodromal fever, coryza, cough, and conjunctivitis followed by the appearance of a characteristic maculopapular rash. The onset of the rash coincides with the appearance of the immune response and initiation of virus clearance (Fig 4). Recovery from the infection is accompanied by lifelong immunity to re-infection. Monkeys exposed to infected humans or experimentally infected with wild-type strains of MV develop a similar disease (Van Binnendijk *et al.*, 1995) and much of our more detailed understanding of pathogenesis and sites of virus replication comes from studies of nonhuman primates.

2.7.2 Entry and Sites of Primary Replication

The virus is spread by aerosol or respiratory droplets and enters by the respiratory route. Initial infection is established in the respiratory tract with virus replication in tracheal and bronchial epithelial cells and pulmonary macrophages. This was demonstrated from studies of experimentally infected primates and human autopsy tissues taken during the incubation period of measles. (Sakaguchi *et al.*, 1986)

From the respiratory tract, the virus spread extends to local lymphatic tissues, perhaps carried by pulmonary macrophages or dendritic cells. The virus then replicates in the lymphoid tissues and this leads to the appearance of lymphoid or reticuloendothelial giant cells first described by Warthin (1931) and Finkeldey *et al.*, (1931).

These are large cells of up to 100 Âµm or more in diameter that may contain 100 nuclei aggregated near the center. Inclusion bodies are not generally present. Warthin-Finkeldey

cells tend to be located at the periphery of germinal centres and in sub-mucosal lymphoid tissue.

2.7.3 Spread

Virus amplification in regional lymph nodes results in the apparent viraemia and spread of virus through the blood to infect a variety of organs. Studies have documented a cell-associated viraemia present before and at the time of the rash (Forthal *et al.*, 1992) Plasma viraemia is rarely detected in measles infection and if at all only before the appearance of neutralizing antibodies, but viral RNA can be detected in plasma by RT-PCR.

Monocytes are the primary cells infected in the blood are (Esolen *et al.*, 1993). Infection of monocytes and macrophage cell lines by some strains of measles virus increases the expression of LFA-1, an adhesion molecule that promotes adherence to endothelial cells and may contribute to virus dissemination.

2.7.4 Target Cells and Tissues

Lymphoid organs and tissues such as thymus, spleen, lymph nodes, appendix and tonsils are prominent sites of virus replication (Sakaguchi *et al.*, 1986). This is shown by the increasing numbers of giant cells in lymphatic tissue before the appearance of the rash (Hathway, 1935).

In the spleen, measles virus replicates primarily in macrophage-rich areas. While in the thymus, infection of epithelial cells and thymocyte apoptosis lead to a prolonged decrease in the size of the thymic cortex, whereas other lymphoid tissues recover promptly.

Measles virus also spreads to numerous other organs, including the skin, conjunctivae, kidney, lung, gastrointestinal tract, respiratory mucosa, genital mucosa, and liver. In these various sites, measles virus replicates primarily in endothelial cells, epithelial cells, or monocytes and macrophages. Endothelial cell infection can be accompanied by vascular

dilatation, increased vascular permeability, mononuclear cell infiltration, and infection of surrounding tissue (Denton, 1925)

The histopathology of the measles rash suggests that the initial event is the infection of dermal endothelial cells. This is followed by spread of infection into the overlying epidermis with infection of keratinocytes in the stratum granulosum leading to focal keratosis and edema. Epithelial giant cells form and mononuclear cells accumulate around vessels.

Koplik's spots found on the oral mucosa are pathologically similar and involve the sub-mucous glands

Pathologic examination of other tissues shows multinucleated epithelial giant cells that resemble those formed in tissue culture. Epithelial giant cells are prominent at the onset of the rash and are readily demonstrated in nasal secretions and the conjunctivae during the first days of the rash. Measles virus infected epithelial cells are also shed into the urine (Gremillion *et al.*, 1981).

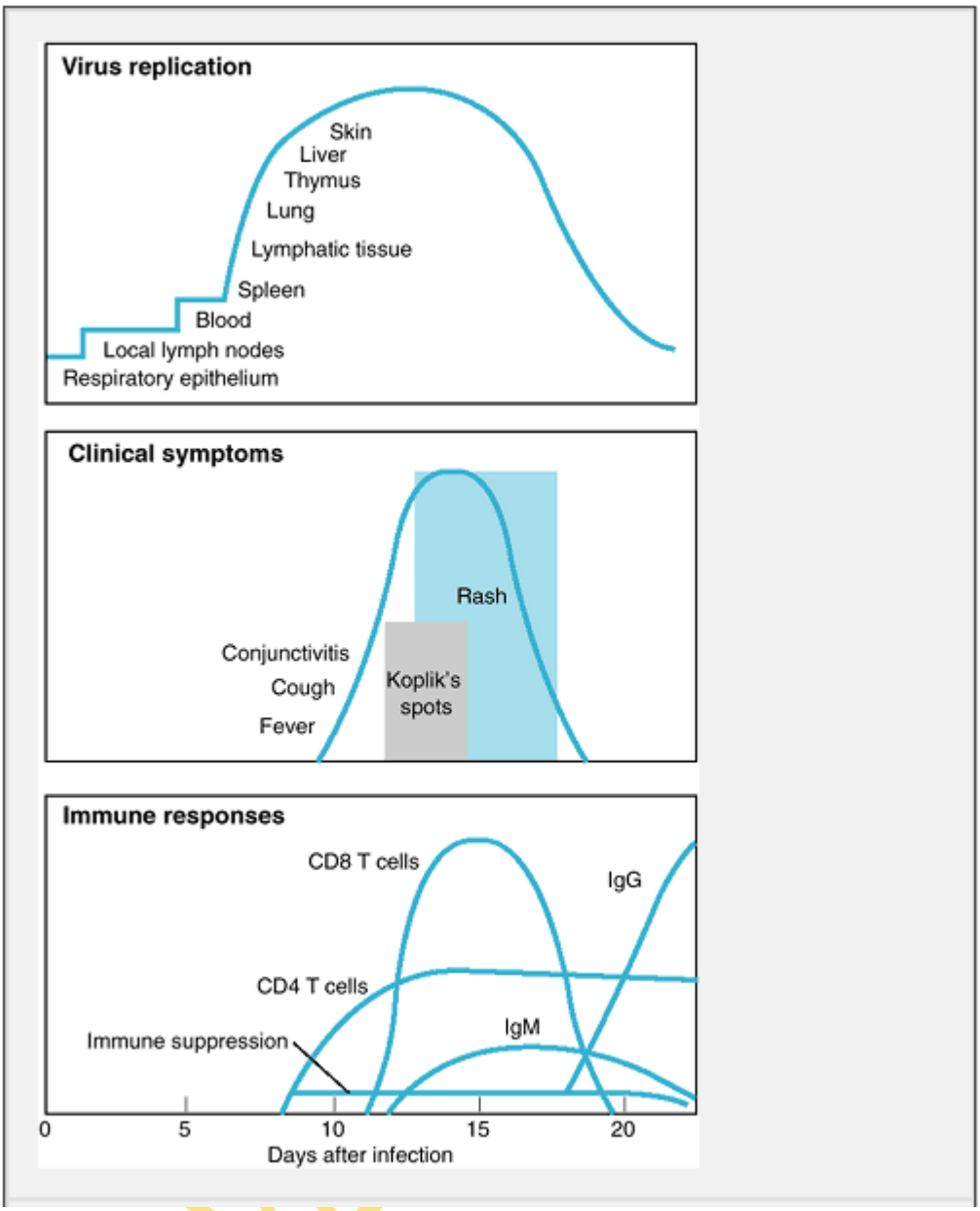


Figure 2.5: Characteristics of classic measles disease showing the typical immune responses and clinical symptoms. Source: Fields virology 5th EDITION.

2.8 Immune Responses to Measles Virus Infection

The immune responses to measles virus, which are important for clearance of virus and recovery from infection, are directly responsible for several of the clinical manifestations of measles. Innate responses can contribute to control of virus replication during the incubation period, but the onset of clinically apparent disease coincides with the appearance of measles virus specific adaptive immune responses (Griffin, 2007).

During measles infection there is marked activation of the immune system as shown by spontaneous proliferation of peripheral blood mononuclear cells, polyclonal activation of B cells, expression of activation antigens on T cells and increased plasma levels of cytokines and soluble cell surface proteins (Griffin *et al.,* 1989, Griffin *et al.,*1992, Griffin *et al.,*1996).

This activation coincides with the appearance of immune suppression. Immune suppression and immune activation continue for many weeks after apparent recovery from measles.

2.8.1 Early Non-Specific Responses to Measles Infection

The principal actors in the early non-specific immune response are alpha/beta interferon (IFN α/β) induction, complement activation, natural killer cell (NK) and macrophage activation, and interferon gamma (IFNγ) and interleukin-12 (IL-12) production.

Measles virus infection of cell lines in vitro has been shown to induce IFN (Volckaert-Vervliet *et al.*, 1977), but its induction by wild type strains are less efficient than by vaccine strains (Crespi *et al.*, 1988). The induction of IFN by measles virus inhibits the development of dendritic cells, stimulates the maturation of immature dendritic cells and terminal differentiation of cortical thymic epithelial cells (Hahm *et al.*, 2005). IFN decreases measles virus replication and increases expression of major histocompatibility complex (MHC) class 1 antigen and toll-like receptor type 3 (TLR-3) on infected cells (Leopardi *et al.*, 1992,

Tanabe *et al.*, 2003). Increased levels of IFN are present in serum 8 to 11 days after measles immunization, but elevated plasma levels of biologically active IFN have not been documented during natural infection (Petralli *et al.*, 1965).

Measles virus does not impede either complement activation in vitro or IFNγ in vivo (Griffin *et al.*, 1990). However, measles virus has been shown to depress IL-12 synthesis in vitro and studies of activity natural killer (NK) cells indicate that NK cell function is lower than normal during measles infection rather than as might have been predicted elevated (Griffin *et al.*, 1990).

2.8.2 Antibody Response to Measles Infection

Measles specific IgM antibodies are first delectable when rash appears. This is followed by a switch to IgG2 and IgG3 and then, in the memory phase to IgG1 and IgG4 (Bouche *et al.*, 2002). IgG is initially of low avidity and this increases steadily over several months (Tuokko *et al.*, 1995). IgA, IgM, and IgG antibodies to MV are found in secretions and sampling of saliva has provided a non-invasive method for determining immune status (Brown *et al.*, 1991).

Measles antibodies are usually produced against all measles proteins, but the most abundant and most rapidly produced is the antibody to N protein and the absence of this antibody is a reliable indicator of measles seronegativity (Graves *et al.*, 1984). Antibodies to measles H protein are measured by the inhibition of the agglutination of monkey erythrocytes by measles virus and are the primary antibodies measured by neutralization of virus infectivity (Giraudon *et al.*, 1981). Antibodies to F protein contribute to virus neutralization by preventing fusion of the virus membrane with the cell membrane at the time of virus entry (Malvosin *et al.*, 1990).

Antibody, which can protect from MV infection, may contribute to recovery from infection and may play a role in establishing persistent infection (Endo *et al.,* 2001). Transient depletion of B cells, however, does not affect virus clearance in infected monkeys (Permar *et al.,* 2004). Antibody binding to infected cells alters intracellular virus replication and may contribute to control of infection.

2.8.3 Cellular Immunity

Burnet F.M 1964 postulated that the ability to recover from measles is an indication of adequate T-lymphocyte mediated immune response. Evidence shows that CD8 T-cells are activated and expanded during measles (Ward *et al.,* 1990). Measles virus antigens that induce CD8 T cells include the N, P, H and F proteins (Van Binnendijik *et al.,* 1993, Hikman *et al.,* 1997).

CD4 T cells are also activated in response to measles virus infection and they proliferate during rash (Gerlier *et al.,* 1994). There is evidence of elevated soluble CD4 in plasma for several weeks after recovery from measles infection (Griffin *et al.,* 1993). Classic CD4 T-cell responses, such as measles virus specific proliferation and production of cytokines, are stimulated during measles infection as well (Van Binnendijik *et al.,* 1990). Lymphocyte proliferation can be induced by most measles virus proteins in immune individuals (Muller *et al.,* 1996).

2.8.4 Duration of Measles virus Immune Response

Epidemiologic studies have documented that long-term protection from re-infection does not require re-exposure (Panum, 1938). Immunologic memory includes continued production of antibody and circulation of MV-specific T cells (Black *et al.,* 1962, Howe *et al.,* 2005). Extensive replication of measles virus in lymphoid tissue may maximize the interaction of

viral antigen with antigen-retaining follicular dendritic cells in germinal canters, leading to long-term antibody production.

2.8.5 Immune Suppression during Measles

There is in-vivo and in-vitro evidence of immune suppression during measles infection and is the trigger of the increased susceptibility to other infections that is a characteristic of measles. Production of antibody and cellular immune responses to new antigens is impaired during measles infection, and there is also evidence of in vitro suppressed lymphoproliferative responses to mitogens and abnormal production of lymphokine (Tamashiro *et al.,* 1987), (Coovadia *et al.,* 1974). All these abnormalities suggest defects in the responses of both monocytes and lymphocytes.

Monocytes are infected during measles (Esolen *et al.,* 1995) and monocyte function is abnormal with low levels of tumour necrosis factor (TNFα) production during both acute and convalescent phases of the disease (Griffin *et al.,* 1987). Circulating T cells are decreased in number during the acute phase of measles and CD4:CD8 ratios are often reduced (Griffin *et al.,* 1986, Dawson, 1944). Lymphocytopenia, which may result from death of infected cells or from changes in lymphocyte trafficking associated with the fever of acute disease, is more marked in girls and in malnourished children (Ryon *et al.,* 2002). A greater decrease in CD4 T cells has been associated with more severe disease (Kiepeila *et al.,* 1987). CD4:CD8 ratios are lower in boys than girls (Ryon *et al.,* 2002).

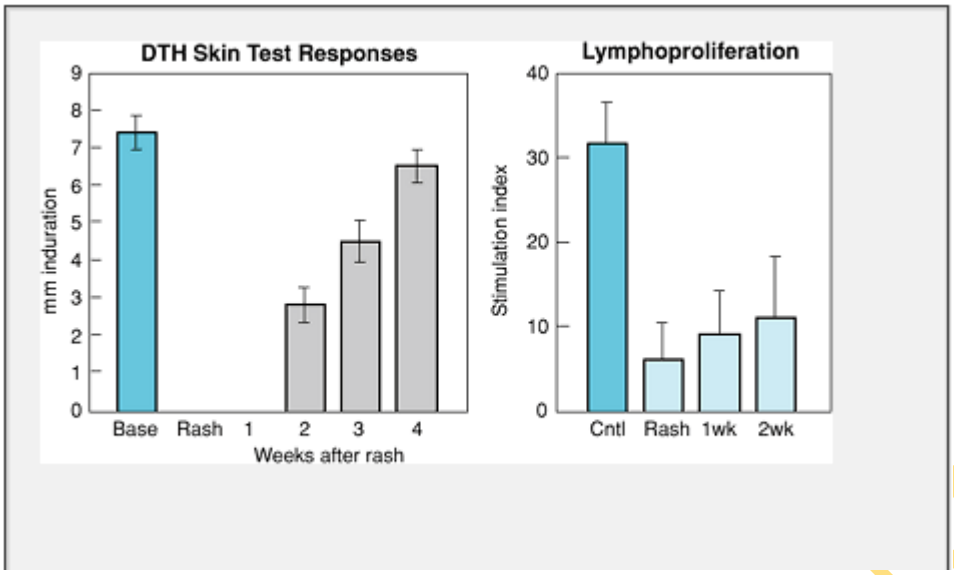


Figure 6: Immunosuppression during measles. Changes during measles in tuberculin-induced delayed type hypersensitivity (DHT) skin test responses in children with measles who had previously received immunization against tuberculosis and changes in proliferation of peripheral blood mononuclear cells to the mitogens phytohemagglutinin. (Source: Fields Virology

2.9 Clinical Manifestation of Measles

2.9.1 Acute Measles and its Complications

Measles has an incubation period of 10 to 14 days, spanning the time from exposure to the onset of clinical disease. The first prodromal symptoms of measles are fever, malaise, and anorexia followed by cough, coryza, and conjunctivitis. The prodromal period lasts 2 to 3 days and during this time small bright red spots with a bluish-white speck at the center, Koplik's spots, may become visible on the buccal mucosa, providing early diagnostic evidence of measles (Griffin *et al.*, 1996). They appear first opposite the molars and spread to cover the buccal and labial mucosa.

The prodromal period ends when the rash appears. The rash is characteristically maculopapular and appears first on the face and behind the ears and then spreads to the trunk and extremities. It begins to fade 3 to 4 days after it appears. Generalized involvement of lymphoid tissue can result in lymphadenopathy, mild splenomegaly, and appendicitis. In uncomplicated measles, clinical recovery begins soon after appearance of the rash.

2.9.2 Respiratory Disease

The most common respiratory symptoms seen in measles infection are manifestations of diffuse mucosal inflammation in response to widespread infection of epithelial cells. Interstitial pneumonitis caused by measles virus replication and inflammation in the lower respiratory tract is common in uncomplicated disease, but frequently is detectable only by an x-ray study or by measuring the alveolar-arterial oxygen gradient. Pneumonitis is more likely to be clinically severe during pregnancy. Symptomatic giant cell pneumonia is seen primarily in immunocompromised individuals. Most of the severe pneumonia that complicates measles and leads to chronic pulmonary disease is caused by secondary bacterial and viral infections

(Gremillion *et al.*, 1981). Other common respiratory complications caused by secondary infections are otitis media and laryngotracheobronchitis (Beckford *et al.*, 1985). In addition to increasing susceptibility to new infections, previously latent viral and bacterial infections may be reactivated (Suga *et al.*, 1992)

2.9.3 Gastrointestinal Disease

Diarrhoea is a common complication of measles, particularly in young patients requiring hospitalization. Many epithelial surfaces are infected with MV and this can lead directly to gastrointestinal symptoms. However, diarrhoea is frequently associated with secondary bacterial and protozoa infections (Greenberg *et al.*, 1991). This complication often compounds the borderline nutritional status of young children in developing countries.

2.9.4 Myocardial Disease

Symptomatic cardiac disease is uncommon, however, and is most often related to transient conduction abnormalities. In autopsy studies, myocardial and pericardial lesions are usually attributable to systemic bacterial infections (Grifiin, 2007)

2.9.5 Neurologic Disease

In immunologically competent individuals measles virus hardly replicates in the brain parenchyma during acute disease. However the occasional appearance of Measles Inclusion Body Encephalitis (MIBE) in immunocompromised individuals and the occurrence of Sub-acute Sclerosing Panencephalities (SSPE) in immunocompetent individuals at young age show the ability of measles virus to occasionally enter the brain and replicates in neurons and glial cells.

Acute disseminated encephalomyelitis complicates 1 in 1,000 cases of measles, mostly in individuals over the age of 2 years and usually occurs within 2 weeks after the onset of the rash (Miller, 1964)

2.9.6 Eye Disease

Measles is considered to be an important cause of childhood blindness associated with corneal lesions. In areas of vitamin A deficiency, the two problems are synergistic and corneal ulceration resembling keratomalacia is a frequent complication of measles. In many parts of Africa most childhood blindness from measles infection is due to corneal lesions (Oyedele 2002).

2.9.7 Atypical Measles

A more severe form of measles, with unusual clinical features, was seen in individuals who had previously received an inactivated measles vaccine used in the mid1960s. Atypical measles differs from typical measles by having higher and more prolonged fever, unusual skin lesions, and severe pneumonitis (Fulginiti *et al.*, 1967). The rash often is accompanied by evidence of hemorrhage or vesiculation and begins on the extremities and spreads to the trunk. Pneumonitis is associated with distinct nodular parenchymal lesions and hilar adenopathy (Young *et al.*, 1970). Abdominal pain, hepatic dysfunction, headache, eosinophilia, pleural effusions, and edema are also described in atypical measles infection.

2.9.8 Measles in the Immunocompromised Host

Children with hypogammaglobulinaemia appear to recover uneventfully from measles virus infection, whereas in those with deficiencies in cellular immune responses virus clearance is delayed and persistence infection is established. Two types of progressive diseases have been identified to complicate measles infection in these individuals. They are giant cell pneumonia and measles inclusion body encephalitis disease. These diseases usually occur in the absence

of typical rash and other characteristic features of measles (Markowitz *et al.*, 1988, Budka *et al.*, 1996,).

Giant cell pneumonia is characterized by increasing respiratory insufficiency beginning 2 to 3 weeks after a history of exposure to measles. Virus can be isolated from lung tissue and bronchial and nasopharyngeal washings (Markowitz *et al.*, 1988,). Pathology shows multinucleated epithelial alveolar and bronchiolar giant cells with intranuclear and intra-cytoplasmic inclusion bodies (Archibald *et al.*, 1974). Evidence is often seen of systemic disease, with giant cells visualized in multiple organs.

Measles inclusion bodies encephalitis sometimes occur with giant cell pneumonia, but most times it presents as an isolated primary manifestation of progressive measles virus infection in immunocompromised individuals. It presents as neurologic disease which usually becomes evident 1 to 6 months after exposure to measles and this is followed by behavioural abnormalities and progresses to myoclonus, focal seizures, confusion, and finally coma. Pathology shows gliosis with inclusions in glial cells and neurons.

2.9.9 Sub-acute Sclerosing Pan-encephalitis (SSPE)

This is a rare, fatal, slowly progressive degenerative disease of the brain. It is generally seen in children and young adults and follows measles after an interval of six to eight years. The course of SSPE usually starts with a generalized intellectual deterioration or psychological disturbance which may not be recognised as illness until more definitive signs appear. The signs include neurological or motor dysfunctions and may take the form of dyspraxia, generalised convulsions, aphasia, visual disturbances or mild repetitive simultaneous myoclonic jerks in 75% of cases the invasion of the retina by the virus leads to a chorioretinitis, often affecting the macular area followed by blindness. Finally the disease

proceeds to progressive cerebral degeneration leading to coma and death. Death usually occurs within months to years after onset of the disease (Freeman, 1969).

At the time that neurologic symptoms occur in cases of SSPE, neurons and glia contain nuclear and cytoplasmic viral inclusion bodies. The antibody responses to the virus are vigorous and evident both in serum and CSF, and an extensive mononuclear inflammatory reaction occurs in the CNS. Both white matter and grey matter are affected and virus replication is defective.

2.10 Epidemiology of Measles Virus

Measles is one of the most infectious of communicable diseases. It is estimated that 76% of household exposures of susceptible persons leads to measles (Hope-Simpson, 1952). The efficient spread of measles virus is mediated by aerosol droplets and respiratory secretions, which can remain infectious for several hours. Measles transmission is efficient through direct exposure to an infected individual, but the virus can survive for hours in respiratory droplets thus direct contact is not always required. Infected individuals are most infectious from 4 to 5 days before, through 4 days, after the appearance of the rash (Christensen *et al.*, 1953).

There are no animal reservoirs or evidence exists of latent or epidemiologically significant persistent infection. Therefore, maintenance of measles virus in a population requires a continuous supply of susceptible individuals. If the population is too small to establish endemic transmission; the virus cannot be maintained (Black, 1966). Mathematical calculations and studies of islands and cities with populations of different sizes have shown a requirement for a population of 250,000 to 500,000 to establish measles as an endemic disease (Keeling, 1997).

In large population centres, measles is endemic with occasional epidemics as the numbers of susceptible individuals increase. In temperate climates, measles is more frequent in the winter and early spring when lowered relative humidity would favour the form of measles transmission. In equatorial regions epidemics of measles occur in the hot dry season. The frequency of epidemics is determined by the number of susceptible individuals, the duration of infectiousness, and patterns of population mixing (Keeling, 1997).The size of the population is also a primary determinant of the age of sero-conversion. The average age of infection is earlier in urban than in rural areas in both developed and developing countries (Cutts *et al.,* 1994). In developing countries with large populations, high birth rates lead to infection at an early age. Very young infants are protected from measles (and from response to vaccine) by maternal antibody. The duration of protective antibody in the infant is dependent on the level of maternal antibody, a primary determinant of the level of antibody in the infant at birth (Griffin, 2007).

2.10.1 Molecular Epidemiology of Measles Virus

Measles virus is monotypic thus infection by any measles virus confers immunity to all serotype. Despite this fact, sequence analysis of the variable C-terminus of the N and H gene have been useful for identification of different genotypes and analysis of the molecular epidemiology of measles. Genotyping of a given measles strain is based on the C -terminal 151-amino-acid sequence of the N protein, where up to 10.6% divergence in the amino acid sequence between unrelated strains can occur. Several lineages of MV that have characteristic temporal and geographic distributions have been identified (Rima *et al.,* 1995, Rota *et al.,* 2003). Currently 8 distinct clades with at least 22 genotypes are recognized. (Fig 2.3) Assembly of this increasingly large database of MV genotypes has aided in the identification of global measles transmission pathways. Identification of the source of the

MV causing disease has become increasingly important as control programs are implemented and identification of cases as imported or indigenous is necessary (Rota *et al.,* 2001).

2.11 Diagnosis of Measles Infection

Measles is characterized by prodromal fever, conjunctivitis, coryza, cough and Koplik spots on the buccal mucosa. A characteristic red rash (maculopapular erythematous rash) appears on the third to seventh day of fever, beginning on the face, becoming generalized and lasting four to seven days. The clinical case definition for measles includes generalized maculopapular rash of 3 days or more, fever of 38°C (101°F), and at least one of the following: cough, coryza, or conjunctivitis (CDC, 1983). Not all of these signs and symptoms are present in measles, however, and many are shared with other diseases such as scarlet fever, rubella. Parvovirus B19, human herpesvirus-6 and 7, dengue fevers etc. Fever, rash, or both may also be absent during measles in very young infants, immunocompromised patients, malnourished children, and previously immunized individuals (Edmonson *et al.,* 1990).

The non-specific nature of the prodromal signs and the existence of mild cases make clinical signs unreliable as the sole diagnostic criteria of measles, thus the need for laboratory methods of distinguishing measles from other similar cases. Measles laboratory diagnostics procedure consist of isolation of virus, direct detection of the virus, viral RNA or viral antigens in secretion, detection of IgM or low avidity IgG antibody by enzyme immunoassay (EIA), or documentation of seroconversion using hemagglutination inhibition (HI), complement fixation (CF), virus neutralization or IgG-specific EIA on serum taken during the acute and convalescent phases of disease.

2.11.1 Microscopy

Production of multinucleate giant cells with inclusion bodies is pathognomonic for measles during the prodromal phase. Epithelial cells from nasopharynx, buccal mucosa, conjunctivae or urine can be used for direct cytologic examination for giant cells and inclusions and for antigen detection. These cells can be detected by fixing the smear with formalin and staining with haematoxylin and eosin. Direct and indirect immunofluorescence have also been widely used to stain cells shed in nasal secretions. The use of monoclonal antibodies has improved the sensitivity and reliability of measles virus detection (WHO 2007).

2.11.2 Serological Methods

The clinical diagnosis of measles is most often confirmed by serology. Diagnosis of measles is made if antibody titres rise by more than four- fold between the acute and the convalescent phase serum pairs or if measles-specific IgM is found in serum or saliva (Helfand *et al.*, 1997). IgM antibody appears at the time of the rash and can be detected by 3 days and can persist for up to 4 weeks after the onset of the rash in most individuals. Measles virus specific IgG peaks approximately 2 weeks later and gradually increases in avidity.

Serological tests such as Hemaggutination Inhibition (HAI), plaque reduction neutralization PRN), Haemolysin inhibition (HLI), complement fixation (CF) and enzyme linked immunoassay have been used for serological diagnosis of measles. EIA is the most commonly used of these tests because it allows the differential detection of IgG and IgM and because it is convenience. Different methods of ELISA that can be used to detect measles-specific IgM include IgM capture ELISA and Indirect ELISA. PRNT remains the standard against which other tests are measured. It is more sensitive than HI or EIA tests and provides the best correlate for protection from infection and, therefore, remains the best measure of response to vaccination (Chen *et al.*, 1990).

2.11.3 Virus Isolation

Virus can be cultured from peripheral blood mononuclear cells, respiratory secretions, conjunctival swabs, and urine collected during the prodrome and rash stages of the disease, but culture is rarely used as the means of diagnosing acute disease. Cell lines vary in their susceptibility to infection by wild-type isolates with human cord blood leukocytes, a marmoset B-cell line (B95-8 or B95a cells), and vero cells expressing SLAM being most sensitive (Kouomou, & Wild, 2002). Vero/SLAM has an advantage over B95a cells is that they are not persistently infected with virus and therefore, present less of a biological hazard. CPEs develop usually between 48 hours and 15 days, and consist either of a broad syncytium or of a stellate form, both of which can reveal nuclear and cytoplasmic inclusion bodies. Vero-SLAM cell line is now recommended for routine isolation of measles virus in WHO laboratory network. The current disadvantage of the Vero-SLAM cells is that they require culture medium containing Geneticin to retain SLAM expression. However, once cell stocks are prepared in the presence of Geneticin, SLAM expression will be retained for at least 15 subsequent passages without Geneticin, saving on the increased cost of the tissue culture medium containing Geneticin.

2.11.4 Detection of Viral RNA

Measles RNA can be detected by RT-PCR using primers targeted to highly conserved regions of measles virus N, M, and/or F gene. RNA can be extracted from a wide variety of specimen such as peripheral blood mononuclear cells, respiratory secretions, conjunctival swabs, and urine collected during the prodromal phase and rash stages of the disease.

2.11.4.1. Principle and Application of Reverse - Transcription (RT)

Reverse Transcription (RT reaction) is a process in which single stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total RNA or poly(A) RNA, a reverse transcriptase enzyme (a polymerase that synthesizes DNA from RNA), a primer, dNTPs and RNase inhibitor. The resulting cDNA can be used in RT-PCR reaction. Three types of primers can be used in RT reaction these are oligo (dT) primers, random (Hexamer) and gene specific primers. The RNA template used in RT reaction is usually 1-2µg in concentration. Other components used includes RT buffer which contains ions and cations such as $MgCl_2$ required by the enzyme.

2.11.4.2 Principle and Application of Polymerase Chain Reaction

PCR is a revolutionary method developed by Kary Mullis in the 1980s. It is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. It is an extremely sensitive technique and possible to achieve a sensitivity of down to 1 DNA molecule in a clinical specimen.

Component of PCR reaction includes the followings:-

DNA template: - the sample DNA that contains the target sequence.

DNA polymerase: - a type of enzyme that synthesizes new strands of DNA complementary to the target sequence.

Primers - short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.

Nucleotides (dNTPs or deoxynucleotide triphosphates): - single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands.

Buffer solution: - this provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.

Divalent Cations: -this could be Magnesium or Manganese ions

Monovalent Cations: - Potassium ions.

2.12 MANAGEMENT OF MEASLES VIRUS INFECTION

As an acute self-limiting disease, measles will run its course in the absence of complications without the need for specific intervention. Treatment is supportive and there is no standard antiviral treatment for measles. Administration of high doses of vitamin A during acute measles decreases morbidity and mortality even in the absence of clinical evidence of vitamin A deficiency. In areas of vitamin A deficiency and xerophthalmia, supplementation prevents blindness caused by measles-induced corneal destruction. The WHO recommends high-dose vitamin A supplementation for all children with measles in countries where the fatality rate is 1% or greater

Infection of the undernourished, the immunocompromised or children suffering from chronic debilitating diseases is more serious, these patients, children under one year of age and pregnant women after household contacts can be protected by the administration of human anti-measles gammaglobulin (0.25–0.5 ml/kg). If given within the first three days after exposure, it is usually effective, yet the effectiveness is partially or completely lost if the globulin is given four to six days, or later, respectively, after exposure.

Respiratory tract infections of measles virus causes considerable damage to the ciliated epithelium and this may lead to superimposed bacterial infection. For pneumonia, distinction between primary viral infections and the superimposed bacterial infection can sometimes be difficult, and thus, treatment with antibiotics is required.

2.13 Prevention and Control of Measles

Measles virus has no animal reservoir and is thus targeted for a controlled campaign aimed at eradication (Mitchell and Balfour, 1985). Attenuated measles vaccines based on adaptation of MV to growth in chick cells have been developed and are now widely used to prevent measles infection (Katz *et al.*, 1959). Attenuated live virus vaccine has dramatically decreased the incidence of measles in all countries in which it has been effectively delivered. Countries like United States and Canada where vaccination of all children is required at or before commencing school have achieved a significant reduction of case reports of measles infection falling by over 99% but without complete eradication. Other industrialised countries where measles vaccination is not mandatory still experience local epidemics. In developing countries, where the consequences of measles infection are most severe, considerable progress has been made in controlling acute measles and reducing related mortality by the implementation of mass immunization campaigns.

There are two types of measles vaccines; Live attenuated vaccines and the inactivated vaccines.

2.13.1 Inactivated Vaccines

Inactivated vaccine was intended for use in young children less than a year of age, who are most prone to serious complications, to avoid the use of a live vaccine. The alum-precipitated inactivated vaccine was used in a three-dose regimen to elicit a suitable antibody response. Recipients of the inactivated vaccine developed moderate levels of neutralizing and HI antibodies and low levels of CF antibody (Carter *et al.*, 1962). Studies in rhesus macaques have shown that the inactivated vaccine induces no CD8 T-cell response and that the avidity of the MV antibody induced does not mature (Norrby *et al.*, 1995). This left vaccinees open

to virus attack, and their partial immunity led to serious hypersensitivity reactions to infection and a more severe disease called Atypical measles

2.13.2 Attenuated Live Vaccines

The first attenuated live measles vaccine was developed by adaptation of the Edmonston strain of MV to chick embryos and subsequently to chick embryo fibroblasts after passage in primary renal and amnion cells to produce the Edmonston B virus (Enders *et al.*, 1962). When administered intramuscularly or subcutaneously 12–18 months after the disappearance of maternal anti bodies, this vaccine achieved seroconversion in 95% of recipients, but side effects of mild measles such as fever and rash were observed in 5–10% of the vaccinees. These reactions were reduced by giving measles virus gammaglobulin at doses appropriate to prevent side effects but not to promote vaccine failure at the same time as the vaccine.

The Edmonston B vaccine was further attenuated and thus rendered less reactogenic by passage in chick embryos at lowered temperature to yield the Schwartz and Moraten (Enders) strains which was licensed in 1965 (Swarz; 1962). The Schwarz vaccine currently serves as the standard measles vaccine in much of the world. The lyophilized vaccine is relatively stable, but the reconstituted vaccine rapidly loses infectivity at room temperature.

The recommended age of vaccination has varied from 6 to 15 months. The probability of seroconversion and the levels of antibody induced are determined by the level of persisting MV-specific maternal antibody and by the age of the infant. The recommended age of immunization is determined, therefore, by a balance between the optimal age for seroconversion and the probability of acquiring measles before that age (Cutts *et al.*, 1995). In areas where measles remains prevalent, measles vaccination is routinely performed at 9 months, whereas in areas with little measles, vaccination is often at 12 to 15 months of age. Respiratory routes of vaccination have been advocated for younger infants, but have proved

either unsuccessful or impractical, although they may hold promise for use in older children (Cutts *et al.*, 1997).

2.13.3 Side Effects of Live Vaccination, Adverse Reactions, Precautions and Contraindications

Majority of measles vaccine recipients remains asymptomatic but in rare occasions, a transient rash or low-grade fever sometimes associated with moderate febrile seizures is observed after 5-12 days in some vaccines.

Live measles vaccine should not be administered to women who are or are considering becoming pregnant with in the next three months because of the theoretical risk of foetal infection. The decision to administer or delay measles vaccination in case of current febrile illness depends on the cause of the illness and the severity of the symptoms. Hypersensitivity reactions following administration of live measles vaccine are rare and usually occur at the injection site. People with a history of anaphylactic reactions following egg ingestion should, however, be vaccinated with extreme caution, and individuals who have experienced anaphylactic reactions to neomycin should not be given the vaccine. Unlike with natural measles, exacerbation of tuberculosis has not been observed after measles vaccination.

2.13.4 Vaccination of Immunocompromised and HIV-infected Individuals

Replication of vaccine viruses can be enhanced in immunocompromised recipients, such as those with leukaemia, lymphoma, generalized malignancy or therapy with alkylating agents, anti-metabolites, radiation or large doses of corticosteroids. Thus, patients with such conditions or therapies (except f or HIV infection) should not be given live measles vaccine. Short-term corticosteroid therapy does not contraindicate live measles vaccination.

All HIV infected children, either symptomatic or not should receive measles vaccination including those with acquired Immune Deficiency Syndrome (AIDS), since measles in these children can be severe. Exposed HIV infected as well as other immunocompromised individuals should receive high dose of measles Ig regardless of their previous vaccination status.

2.13.5 Effectiveness of Vaccination in Measles Control

Immunization alters the epidemiology of measles by reducing the susceptible individuals in the population. In countries that have achieved high levels of vaccine coverage, the average age for measles is increased because herd immunity reduces transmission and indirectly protects children from infection. Also the interval between measles outbreaks increases. Sufficiently high levels of vaccination can as well interrupt endemic transmission (Cutts *et al.*, 1994).

A two-dose schedule is one of the strategies used to ensure high vaccine coverage in the quest for measles elimination, particularly in developing countries where malnutrition, crowding and intensity of exposure aggravate measles infection and secondary infections are common and take severe courses in young infant (Aaby, 2007).

The United Nations in 2002 launched a four part key strategies to reduce deaths due to measles by half, and these strategies involves;

1. Provision of one dose of measles vaccine to all infants by 12 months of age via routine health services.
2. A sound opportunity for immunization for all children from 9 months to 15 years through mass vaccination campaigns to cope with primary and secondary vaccine failures.

3. Establishment of effective surveillance and
4. Improvement of clinical management of complicated cases, including vitamin A supplementation, which can effectively reduce measles morbidity.

These measures increased vaccine coverage with the first routine dose from 71 to 77% globally between 1999 and 2005, and over 360 million children aged below 15 years received immunization through mass vaccination campaigns. Within this period, reduction of the estimated measles mortality was most effective in the Western Pacific region (81%), followed by Africa (75%) and the Eastern Mediterranean Region (62%). Overall, more than 7.5 million deaths from measles were efficiently prevented.

2.13.6 Surveillance of Measles

An essential component of measles control program is laboratory based surveillance. This provides confirmation of cases and genetic characterization of circulating wild-type viruses. Laboratory based surveillance involves routine confirmation of suspected cases based on detection of measles virus-specific IgM in a single blood or oral fluid sample obtained as soon as possible after rash onset and molecular techniques such as RT-PCR to detect viral RNA (CDC, 2005).

Another important phase of laboratory surveillance of measles is the genetic characterization of circulating wild-type viruses to support molecular epidemiologic studies (Rota *et al.*, 2009). Laboratory-based surveillance for measles and rubella is performed throughout the world by the WHO Measles and Rubella Laboratory Network (LabNet). This network provides for standardized testing and reporting in all WHO regions and supports genetic characterization of currently circulating viral strains. LabNet has been responsible for standardization of the nomenclature and laboratory procedures that are used to describe the

genetic characteristics of wild-type measles and rubella viruses which has allowed sharing of virologic surveillance data throughout the measles control programs (CDC, 2005)

The combination of good surveillance for suspected cases, good molecular epidemiologic techniques, and good standard case classification and reporting provides a sensitive means to describe the transmission pathways of measles virus. Molecular characterization of wild type viruses has provided a valuable tool for measuring the effectiveness of measles control programs

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical approval

Ethical approval for this study was obtained from the following authorities;

- Oyo State Hospital Management Board
- Central Public Health Laboratory, Yaba Lagos
- Lagos State Health management service

3.2 STUDY SITES

Two hospitals within Ibadan metropolis; Oni memorial Children Hospital and State Hospital Ade-Oyo and two hospitals in Lagos; Massey Street Children Hospital, and Isolo General Hospital as well as Central Public Health Laboratory, Yaba Lagos were used as sample collection sites in this study. These hospitals were selected because they are either a specialised paediatric hospital or have specialised paediatric department as well as having a high population of patients visiting the hospitals.

3.3 STUDY POPULATION

Children under five years of age presenting with the following clinical symptoms i.e. maculopapular rash not more than 3 days with a history of fever, cough and or coryza and/or conjunctivitis in the above mentioned hospitals and health centres between 2007 and 2011 were recruited for the study. Table 3 shows the annual, gender and age distribution of the children used for this study.

A total of 1200 children were recruited between 2007 and 2011. They were aged five months to five years with the mean age of 3.4 years. Five hundred and sixty one (46.75%) of these children were males and 643 (53.25%) were females.

Inclusion Criteria:

Children of five years of age and below presenting with fever and maculopapular rash whose parent or caregiver consented were included in the study.

Exclusion Criteria:

Children older than five years and those whose parents or caregiver did not consent were excluded from the study.

3.3 SAMPLE COLLECTION

3.3.1 Blood Sample

About 2ml of blood was collected from each child by venepuncture with needle and syringe into sterile EDTA bottles. Each sample was labelled appropriately with date and time of collection. They were transported to the laboratory maintaining cold chain. The blood samples were centrifuged at 1000g for 10 minutes to separate the plasma and stored at -80^0C until used for analysis. Table 3.1 shows the annual distribution of the blood samples collected.

Table 3.1: Annual and gender distribution of children recruited.

Year	Male	Female	Total
2007	120	181	301
2008	63	85	148
2009	137	114	251
2010	139	159	298
2011	98	104	202
Total	557	643	1200

3.3.2 Throat Swab Sample Collection

Throat or nasopharyngeal swab was collected from each child by firmly rubbing the back of the throat and nasopharyngeal passage with sterile cotton swabs to dislodge epithelial cells. The swabs were placed in 2ml of sterile viral transport medium in labelled screw-capped tubes. The specimens were transported to the laboratory on ice packs to maintain cold chain. In the lababoratory the samples were vortexed for two minutes to dislodge all the cells in to the medium and the medium was aliquoted in to two screw capped tubes and stored at -80^{0}C until analysed. Table 4 shows the annual distribution of the throat swab samples collected.

3.3.3 Urine Sample Collection

Early morning urine sample was collected from each child into a sterile 50ml centrifuge tube and capped immediately. The urine samples was placed on ice and transported to the lab while maintaining cold chain. In the laboratory the urine specimens were centrifuged at 2500 revolution per minute for 10minutes at 4^{0}C using a cold centrifuge. The supernatant was discarded and the pellet formed was re- suspended in 2ml of 2% MEM. The re-suspended specimen was aliquoted in to two cryovials and frozen at -70^{0}C until analyses. Table 3.2 shows the annual distribution of the urine samples collected.

Table 3.2: Annual distribution of Blood Throat swab and Urine samples collected from febrile children with maculopapular rash in Lagos and Ibadan between 2007 and 2011

Year	Blood			Throat Swab			Urine		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
2007	120	181	301	33	37	70	30	32	62
2008	63	85	148	14	19	33	10	14	24
2009	137	114	251	30	33	63	30	31	61
2010	139	159	298	36	32	68	32	35	67
2011	98	104	202	29	23	52	23	27	50
Total	557	643	1200	142	144	286	125	139	264

3.4 Serology

All the blood samples were screened for measles IgM antibody using Enzyme-linked Immunosorbent Assay (ELISA) test kit by DRG Diagnostics (Frauenbetgstr Marburg Germany) according to the manufacturer's instructions. All the samples that were negative for measles IgM antibody were screened for antibodies to rubella and parvovirus B19 using ELISA test by DGR Diagnostics (Frauenbetgstr, Margurg). Germany according to the manufacturer's instruction.

3.4.1 Measles Virus IgM ELISA

The DGR Diagnostics measles virus IgM ELISA Kit is a solid phase enzyme-linked immunosorbent assay. The microtiter plate is coated with measles virus antigen. The samples are diluted with sample diluent and additionally incubated with IgG-RF-Sorbent, containing hyper-immune anti-human IgG-class antibody to eliminate competitive inhibition from specific IgG and to remove rheumatoid factors. This pre-treatment prevents false negative and false positive results.

Sample Dilution

Prior to the ELISA each sample was first to be diluted with sample diluent. The pre-diluted samples was incubated with IgG-RF-Sorbent.

Procedure

Each sample was diluted 1:50 with sample diluent and mixed well. The pre-diluted samples were further diluted 1:1 with IgG-RF-Sorbent and mixed well. Afterwards the samples were incubated at room temperature for 15 minutes. 100µl of these diluted samples was used for the ELISA assay.

ELISA Procedure

Well A1 was left empty as the blank well. Negative control (100µl) was dispensed into well B1and 100µl of Cut-Off control was dispensed into well C1 and D. Also 100µl of positive control was dispensed into well E1, afterwards 100µl of the pre-treated samples were dispensed into all the remaining wells with new disposable tips for each sample. The ELISA plate was covered with foil and incubated at room temperature for 60 minutes. After the incubation the foil covering was removed and the wells were washed in the ELISA washer using the reconstituted wash solution six times. The plate was then gently tapped to an absorbent paper to remove residual droplets from the wells. One hundred microliter of conjugate was dispensed into each well except well A1. The plate was covered with foil and incubated for 30 minutes at room temperature. After the incubation period the foil was removed and the plate was washed and gently tapped on absorbent paper to remove residual droplets. One hundred microliter of Substrate solution was dispensed into all the wells. The plate was then covered with foil and incubated for 15 minutes at room temperature in the dark and 100µl of stop solution was dispensed into all the wells. The optical density was then read at 450nm with a microtiter plate reader within 30 minutes after adding the stop solution.

Validation of the Test Run

The test run is considered valid if:

Substrate in well A1 - Absorbance value of substrate blank in well A1 is lower than 0.100

Negative in well B1 - Absorbance value of negative control is lower than 0.200

Cut-Off in well C1 and D1 - Absorbance value of cut of control is between 0.300-0.600

Positive in well E1 - Absorbance value of positive control is greater than 0.600

Interpretation of Result

All samples with absorbance values more than 20% above that of the mean of the cut-off controls were considered positive

All samples with absorbance value more than 10% below the mean of the cut-off controls were considered negative.

Samples with absorbance value below 20% of the mean of the cut-off and above 10% below the mean of the cut-off were treated as equivocal and repeated. If afterwards still having absorbance value below that considered positive were regarded as negative.

3.4.2 Rubella Virus IgM ELISA

The DGR Diagnostics rubella virus IgM ELISA kit is a solid phase enzyme-linked immunosorbent assay. The microtiter plate is coated with rubella virus antigen. The samples were diluted with sample diluent and additionally incubated with IgG-RF-Sorbent, containing hyper-immune anti-human IgG-class antibody to eliminate competitive inhibition from specific IgG and to remove rheumatoid factors. This pre-treatment avoids false negative and false positive results.

Sample Dilution

Prior to the ELISA, each sample was first to be diluted with sample diluent. The pre-diluted samples was incubated with IgG-RF-Sorbent.

Procedure

Each sample was diluted 1:50 with sample diluent and mixed well. The pre-diluted samples were further diluted 1:1 with IgG-RF-Sorbent and mixed well. Afterwards the samples were

incubated at room temperature for 15 minutes and 100µl samples were used for the ELISA test.

ELISA Procedure

Well A1 was left empty as the blank well. One hundred microliter of negative control was dispensed into well B1and 100µl of Cut-Off control was dispensed into well C1 and D. Also 100µl of positive control was dispensed into well E1, afterwards 100µl of the pre-treated samples were dispensed into all the remaining wells with new disposable tips for each sample. The ELISA plate was covered with foil and incubated at room temperature for 60 minutes. After the incubation the foil covering was removed and the wells washed in the ELISA washer using the reconstituted wash solution six times. The plate was gently tapped to an absorbent paper to remove residual droplets from the wells. One hundred microliter of conjugate was dispensed into each well except well A1. Afterwards the plate was covered with foil and incubated for 30 minutes at room temperature. After the incubation period the foil was removed, the plate washed and gently tapped on absorbent paper to remove residual droplets. One hundred microliter of Substrate solution was dispensed into all the wells. The plate was covered with foil and incubated for 15 minutes at room temperature in the dark and 100µl of stop solution was dispensed into all the wells. The optical density was read at 450nm with a Microtiter plate reader within 30 minutes after adding the stop solution.

Validation of the Test Run

The test run is considered valid if:

Substrate in well A1 - Absorbance value of substrate blank in well A1 is lower than 0.100

Negative in well B1 - Absorbance value of negative control is lower than 0.200

Cut-Off in well C1 and D1 - Absorbance value of cut of control is between 0.300-0.600

Positive in well E1 - Absorbance value of positive control is greater than 0.600

Interpretation of Result

All samples with absorbance values more than 20% above that of the mean of the cut-off controls were considered positive

All samples with absorbance value more than 10% below the mean of the cut-off controls were considered negative.

Samples with absorbance value below 20% of the mean of the cut-off and above 10% below the mean of the cut-off were treated as equivocal and repeated. If afterwards still having absorbance value below that considered positive were regarded as negative.

3.4.3 Parvovirus B19 IgM ELISA

The DGR Parvovirus B19 IgM ELISA Kit is a solid phase enzyme-linked Immunosorbent assay. The Microtiter plate as the solid phase is coated with parvovirus B19 antigen. The samples are diluted with sample diluent and additionally incubated with IgG-RF-Sorbent, containing hyper-immune anti-human IgG-class antibody to eliminate competitive inhibition from specific IgG and to remove rheumatoid factors. This pre-treatment prevents false negative or false positive results.

Sample Dilution

Prior to the ELISA, each sample is first to be diluted with Sample Diluent. The pre-diluted samples then have to be incubated with IgG-RF-Sorbent.

Procedure

Each sample was diluted with a ratio factor of 1:50 with sample diluent and mixed well. The pre-diluted samples were further diluted 1:1 with IgG-RF-Sorbent and mixed well.

Afterwards the samples were incubated at room temperature for 15 minutes. 100µl of these diluted samples was used for the ELISA assay.

ELISA Procedure

Well A1 was left empty as the blank well. One hundred microliter of negative control was dispensed into well B1and 100µl of cut-off control was dispensed into well C1 and D. Also 100µl of positive control was dispensed into well E1, afterwards 100µl of the pre-treated samples were dispensed into all the remaining wells with new disposable tips for each sample. The ELISA plate was covered with foil and incubated at room temperature for 60 minutes. After the incubation the foil covering was removed and the wells washed in the ELISA washer using the reconstituted wash solution six times. The plate was gently tapped to an absorbent paper to remove residual droplets from the wells. Afterwards, 100µl of conjugate was dispensed into each well except well A1. The plate was then covered with foil and incubated for 30 minutes at room temperature. After the incubation period the foil was removed and the plate was washed and gently tapped on absorbent paper to remove residual droplets. One hundred microlitre of Substrate solution was dispensed into all the wells. The plate was then covered with foil and incubated for 15 minutes at room temperature in the dark and 100µl of stop solution was dispensed into all the wells. The optical density was then read at 450nm with a Microtiter plate reader within 30 minutes after adding the stop solution.

Validation of the Test Run

The test run is considered valid if:

Substrate in well A1 - Absorbance value of substrate blank in well A1 is lower than 0.100

Negative in well B1 - Absorbance value of negative control is lower than 0.200

Cut-Off in well C1 and D1 - Absorbance value of cut of control is between 0.300-0.600

Positive in well E1 - Absorbance value of positive control is greater than 0.600

Interpretation of Result

All samples with absorbance values more than 20% above that of the mean of the cut-off controls were considered positive

All samples with absorbance value more than 10% below the mean of the cut-off controls were considered negative.

Samples with absorbance value below 20% of the mean of the cut-off and above 10% below the mean of the cut-off were treated as equivocal and repeated. If afterwards still having absorbance value below that considered positive were regarded as negative.

3.5 ISOLATION OF VIRAL RNA

Total RNA was extracted from throat swab and urine samples of children that had measles IgM antibody by using QIAmp viral RNA isolation mini kit by QIAGEN, Hilden, Germany according to the manufacturers' instruction (Spin Protocol).

3.5.1 Principle of the Test

The principle of the kit is based on the selective properties of silica gel based membrane and the speed of microspin technology. The samples were first lysed by chaotropic salts to inactivate the RNases in them. Carrier RNA and guanidine thiocyanate were added to ensure isolation of intact viral RNA. Buffering conditions were then adjusted to allow optimum binding of the RNA to the QIAmp membrane, and the samples were loaded into the QIAmp Mini spin column. The RNA binds to the membrane, and contaminants were efficiently washed away in two steps using two different wash buffers. High quality RNA was eluted in

a special RNase-free buffer, ready for direct use or safe storage. The purified RNA was free of protein, nucleases, contaminants and inhibitors.

3.5.2 Reagents

The following reagent were supplied with the RNA Isolation kit: -

I. Buffer AVE:- This is for dissolving and stabilising lyophilised carrier RNA and for eluting the isolated RNA
II. Buffer AVL: - This contains chaotropic agents and guanidine thiocyanate
III. Buffer AW1: - This also contains chaotropic agent and guanidine hydrochloride
IV. Buffer AW2: - This contains sodium azide
V. Carrier RNA
VI. Elution Buffer

The following reagent were used but not supplied with the kit:-

I. 98% Ethanol

3.5.3 Reconstitution of Reagents

Some of the reagents supplied with the RNA isolation kit were reconstituted as follows according to the manufacturer's instruction.

I. To prepare buffer AW1 needed for 100 samples, 50ml of 98% ethanol was added to 38ml of buffer AW1 concentrate to make a final volume of 88ml
II. To prepare buffer AW2 needed for 100 samples, 60ml of 98% ethanol was added to 26ml of buffer AW2 concentrate to make a final volume of 86ml

III. For preparation carrier RNA, 310µl of buffer AVE was added to a tube containing 310µg of lyophilised carrier RNA to obtain a final concentration of 1µg/µl. This was allowed to dissolve completely and stored at -20^{0}C until use.

IV. To prepare Buffer AVL for 100 samples, Buffer AVL-carrier RNA mix needed was calculated as follows

N = number of samples

Y = calculated volume of buffer AVL

N x 0.56ml = yml

For 100 samples, 100 x 0.56m=56ml

Thus 56ml of Buffer AVL was needed

The volume of carrier needed was calculated thus:

Y x 10µl/ml

56 x10µl = 560µl of reconstituted carrier RNA

Thus 560µl of reconstituted carrier RNA was added to 56ml of Buffer AVL.

The solution was then gently mixed by inverting the tubes several times.

3.5.4 Protocol

Using pipette 560µl of prepared Buffer AVL containing carrier RNA was dropped into a 1.5mL micro centrifuge tube. Exactly 140µL of processed specimen was added to the buffer AVL-carrier RNA in the micro centrifuge tube. The solution was mixed thoroughly by pulse – vortexing (for 15 seconds) until a homogenous solution was obtained. The resulting homogenate was incubated at room temperature (15-25C) for 10 minutes. This ensures

complete lysis of viral particles. The tube was briefly centrifuged to remove drops from inside of the lid. Five hundred and sixty microliter of ethanol (98% vol) was added to the sample and mixed by pulse vortexing for 15 seconds. 630µL from the resulting solution was carefully added to the QIAmp spin column (in a 2mL collection tube) without wetting the rim. After closing the cap, the tube was centrifuged at 6000g (8000 r.p.m) for 1 minute. The spin column was placed into a clean 2mL collection tube, and the tube containing the filtrate was discarded. The QIAmp spin column was carefully opened and 630µL from the sample was carefully added to repeat the centrifugation step. After the repeat, 500µL of Buffer AW1 was added to the QIAmp Mini spin column and the cap was closed. The column was centrifuged at 6000×g (8000rpm) for 1minute. The QIAmp Mini spin column was placed in a clean 2mL collection tube and the tube containing the filtrate was discarded. The QIAmp Mini spin column was carefully opened and 500µL of Buffer AW2 was added. After closing the cap, the column was centrifuged at full speed (20,000 × g; 14,000rpm) for 3 minutes. The QIAmp Mini spin column was placed in a new 2mL collection tube. The old collection tube with the filtrate was discarded. The spin column was again centrifuged at full speed for 1minute. The QIAmp Mini spin column was placed in a clean 1.5mL micro centrifuge tube. The old tube containing the filtrate was discarded. The QIAmp spin column was carefully opened and 60µL of Buffer AVE equilibrated to room temperature was added. This is for elution of the viral RNA. The spin column cap was closed and incubated at room temperature (15-25^{0}C) for 1 minute. Thereafter, the column was centrifuged at 6000 × g (8000rpm) for 1min. The resulting eluate is the total, highly purified viral RNA from the processed specimen.

Positive and negative control was included in the RNA extraction process.

3.6 REVERSE TRANSCRIPTION

3.6.0 Complementary DNA (cDNA) Synthesis

3.6.1 Principle and Application of Reverse - Transcription (RT)

Reverse Transcription (RT reaction) is a process in which single stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total RNA or poly(A) RNA, a reverse transcriptase enzyme (a polymerase that synthesizes DNA from RNA), a primer, dNTPs and RNase inhibitor. The resulting cDNA can be used in RT-PCR reaction. Three types of primers can be used in RT reaction these are oligo (dT) primers, random (Hexamer) and gene specific primers. The RNA template used in RT reaction is usually 1-2µg in concentration. Other components used includes RT buffer which contains ions and cations such as $MgCl_2$ required by the enzyme.

3.6.2 Procedure

The cDNA synthesis was done using SCRIPT cDNA synthesis kit by Jena Bioscience, Germany according to the manufacturer's instruction. It makes use of SCRIPT reverse transcriptase which is a genetically engineered version of M-MLV Reverse Transcriptase (M-MLV RT) with eliminated RNase H activities and increased thermal stability. The reaction was performed on each sample in a micro amp tube using the protocol in Table 3.3.

The reaction tube was incubated in a Thermal cycler (Master cycler gradient Eppendorf, Hamburg, Germany) using the following condition;

42^0C for 10min

55^0C for 60min

The cDNA was kept at -20^0C until used

3.7 POLYMERASE CHAIN REACTION (PCR)

3.7.1 Principle and Application of Polymerase Chain Reaction

PCR is a revolutionary method developed by Kary Mullis in the 1980s. It is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. It is an extremely sensitive technique and possible to achieve a sensitivity of down to 1 DNA molecule in a clinical specimen.

Component of PCR reaction includes the followings:-

DNA template: - the sample DNA that contains the target sequence.

DNA polymerase: - a type of enzyme that synthesizes new strands of DNA complementary to the target sequence.

Primers - short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.

Nucleotides (dNTPs or deoxynucleotide triphosphates): - single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands.

Buffer solution: - this provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.

Divalent Cations: -this could be Magnesium or Manganese ions

Monovalent Cations: - Potassium ions.

3.7.1 Primers Used

Primer sequence published by Mosquera *et al.* (2002) and Shimizu *et al.* (1993) and repeated by Kremer *et al.* (2010) was obtained from a commercial company Jena Biosciences, Jena, Germany were used for the PCR assay. The primers used were targeted at the conserved region of measles virus nucleoprotein and matrix gene and the conserved region of rubella virus glycoproteins. The nucleotide sequence of the primers is shown in Table 3.4

3.7.2 Multiplex PCR

3.7.2.1 Principle of Multiplex PCR

Multiplex PCR is a modification of PCR that uses multiple pairs of primers in a single PCR mixture with primer pairs being specific to different DNA sequences. By targeting multiple genes at once, a single PCR can provide the information that otherwise would require several times the amount of reagents and take longer to perform.

3.7.2.2 Procedure

Five microliters of the prepared cDNA of each sample was used in the multiplex reaction using PCR master mix from Jena Biosciences and 1st round primers for measles and rubella virus to make a 25µl reaction mix as described in the table 3.5. The PCR master mix contains a premix of PCR buffer, Magnesium Chloride, dNTPs, and Taq Polymerase enzyme in optimised concentrations. Nucleotide sequence of the primers is as shown in table 3.4. Micro amps tubes containing the PCR reaction mixes were placed in a thermal cycler (Master cycler gradient Eppendoff, Hamburg, Germany) programmed to run as follows:

There was an activation of the Taq polymerase enzyme at 95^{0}C for five minutes followed by 34 cycles of denaturation of the double stranded DNA at 95^{0}C for 30 seconds, primer

annealing at 55^0C for 60 seconds, and an elongation of 60 sec at 72^0C. There was a final extension time at 72^0C for five minutes.

Table 3.3: Protocol for complementary DNA synthesis from RNA isolated from Throat swabs and urine samples

Component	Stock Conc	Final Conc	Volume in 20µl reaction
RNase free water			13µl
RNA Template		Total RNA: 10pg	5µl
Primer	100 µM	50 pmol (300ng)	0.5µl
SCRIPT Complete Buffer	5X	1X	4µl
dNTP Mix	10 mM each	500 nM each	1µl
DTT Stock Solution	100 mM	5mM	1µl
RNase Inhibitor	40 unit/µl	40 units	1µl
SCRIPT Reverse Transcriptase	200 unit/µl	100 units	0.5µl

Conc = concentration

Table 3.4: Nucleotide sequence of the primers used to screen for measles and rubella viruses in RNA isolates from throat swab and urine samples

Oligonucleotide Primer	**Gene**	**Map Position**	**Sequence (5'-3')**
MN5 (Measles 1st round)	Nucleocapsid	nt 1113 – 1134	GCCATGGGAGTAGGAGTGGAAC
MN6 (Measles1st round)	Nucleocapsid	nt 1773 – 1754	CTGGCGGCTGTGTGGTCAG
Nfla (Measles 2nd round)	Nucleocapsid	nt 1199-1224	CGGGCAAGAGATGGTAAGGAGGTCAG
Nr7a (measles 2nd round)	Nucleocapsid	nt 1725-1703	AGGGTAGGCGGATGTTGTTCTGG
RV98f (Rubella)	Glycoprotein	nt × 98	CGTCTGGCAACTCTCCG
RV413v (Rubella)	Glycoprotein	nt × 413	CGTATGTGGAGTCCGCAGT

Table 3.5: Preparation of the master mix for the multiplex PCR for measles and rubella viruses.

Reagent	Concentration In Stocks	Concentration In PCR	µL / Sample
Distilled water			12.5µl
PCR Master Mix	5×	1×	5µl
MN5	10µM	0.25µM	0.625µl
MN6	10µM	0.25µM	0.625µl
RV98f	10 µM	0.25µM	0.625 µl
RV413v	10 µM	0.25 µM	0.625 µl

3.7.3 Measles First Round PCR

The throat swab and tissue culture materials were then further screened for measles virus alone by using 5µl of the corresponding prepared cDNA of each sample in a 25µl PCR reaction using PCR master mix from Jena Biosciences and measles virus primers spanning the matrix and nucleoprotein gene as shown in table 3.6. The PCR master mix contains a premix of PCR buffer, magnesium chloride, dNTPs, and Taq polymerase enzyme in optimised concentrations. Micro amps tubes containing the PCR reaction mixes were placed in a thermal cycler (Master cycler gradient Eppendorf, Hamburg, Germany) programmed to run as follows:

There was an activation of the Taq polymerase enzyme at 95^0C for five minutes followed by 34 cycles of denaturation of the double stranded DNA at 95^0C for 30 seconds, primer annealing at 55^0C for 60 seconds, and an elongation of 60 sec at 72^0C. There was a final extension time at 72^0C for five minutes.

3.7.4 Measles Nested PCR

To further the targeted non-coding region of the carboxyl end of the measles virus nucleocapsid (N) gene, a nested PCR was performed using 2µl of the 1st round PCR product and primers Nfla and Nr7a in a 25µl as described in the table. Micro amps tubes containing the PCR reaction mixes were placed in a thermal cycler (Master cycler gradient Eppendorf, Hamburg, Germany) programmed to run as follows:

There was an activation of the Taq polymerase enzyme at 95^0C for five minutes followed by 34 cycles of denaturation of the double stranded DNA at 95^0C for 30 seconds, primer annealing at 55^0C for 60 seconds, and an elongation of 60 sec at 72^0C. There was a final extension time at 72^0C for five minutes.

Table 3.6: Preparation of the Master Mix for 1st Round PCR for Measles

Reagent	Concentration In Stocks	Concentration In PCR	µL / Sample
Distilled water			12.5µl
PCR Master Mix	5×	1×	5µl
MN5	10Mm	0.25µM	0.625µl
MN6	10µM	0.25µM	0.625µl

MN5: Measles Nucleocapsid Gene first round forward primer

MN6: Measles Nucleocapsid Gene first round reverse primer.

Table 3.7: Description for preparation of master mix for measles nested PCR

Reagent	Concentration In Stocks	Concentration In PcR	µL / Sample
Distilled water			12.5µl
PCR Master Mix	5×	1×	5µl
Nfla	10µM	0.25µM	0.625µl
Nr7a	10µM	0.25µM	0.625µl

3.8 IDENTIFICATION OF THE AMPLIFIED PRODUCTS BY GEL ELECTROPHORESIS

3.8.1 Preparation of Reagents

Tris Acetate EDTA (TAE) Buffer

TAE buffer was used both as running buffer and in preparation of Agarose in Agarose gel electrophoresis. A stock solution of TAE buffer is prepared by dissolving 242g TRI base in deionised water, adding 57.1ml glacial acetic acid and 1000ml of 500mM EDTA (the pH should be 8.0). The final volume was made up to 1 litre by adding the appropriate volume of deionised water. The stock solution was then diluted 50 times with deionised water to make a working solution of 1X. This working solution contains 40nM Tris, 20nM acetic acid and 1M EDTA

Ethidum Bromide

Ethidium bromide is a common dye used to make DNA bands visible for agarose gel electrophoresis. It fluoresces under UV light when intercalated into the major groove of DNA. It was prepared by dissolving 10mg of the ethidium bromide powder supplied by Sigma in 1ml of double distilled water. Five microliter of the prepared ethidium bromide was added to the melted Agarose gel

Loading Buffer

This is usually mixed with DNA samples for use in agarose gel electrophoresis. It contains a dye to assess how fast the gel is running and a reagent to make the DNA samples denser than the running buffer. It was prepared by adding 3ml of 30% glycerol, 25mg of bromophenol blue and 10ml of deionised water. One microliter of the loading buffer was added to each 5µl of PCR amplicons.

3.8.2 Agarose Gel

Two grams of Agarose was diluted in 100ml of 1x working solution of TAE buffer to prepare a 2% Agarose gel. The agar was melted in melted in microwave for 2 minutes and allowed to cool to 45^0C. Five microliter of ethidium bromide was added to the melted agar before being poured into a horizontal gel plate containing template comb mounted vertically. The agar was allowed to solidify for 20 to 30 minutes before the template was carefully removed. The gel was placed in the electrophoresis unit with TAE buffer added to submerge the gel. Five microliters of each PCR amplicons was added to 1µl of loading buffer dye was loaded into each well and 5µl of standard DNA marker was also added to the first and last wells of each gel.

The electrical leads were attached to the electrophoresis unit and 150 volts and 500 mill amperes was applied for 45 minutes to allow the DNA to migrate towards the anode. At the completion of the electrophoresis resulting DNA band was examined and photographed under the ultraviolet light using the Trans-illuminator. Positive samples showed the expected DNA band of 560 base pairs. Samples that showed no band were regarded as negative.

3.9 SEQUENCING OF THE NON-CODING REGION OF THE CARBOXYL END OF THE NUCLEOPROTEIN GENE

All samples that were positive for measles virus at the end of the second round or nested PCR were purified from Agarose gel and quantified before cycle sequencing.

3.9.1 Cutting and Purification of PCR Product from Gel

This process was used to obtain pure PCR amplicons by removing primer dimers, excess primers, nucleotides, proteins, salts, agarose, ethidum bromide and other impurities. Fifty microliters of positive PCR amplicons from second round PCR for measles virus was loaded

with 5µl of loading buffer into wells of Agarose gel prepared as described above. Five microliters of standard DNA marker was also loaded into the first and last well of each gel and electrophoresis was then run on the samples as described above. At the end of the electrophoresis the corresponding bands to the 560 base pairs in the DNA markers were cut and put into new clean and labelled 1.5ml tubes.

Each tube was weighed before and after the addition of the cut gel to determine the weight of the cut gel band. The PCR amplicons were then purified using the gel purification kit by Jena Bioscience according to the manufacturer's description. Three volumes of extraction buffer were added to 1 volume of the sliced gel. (300µl of extraction buffer was added to 100µg of the sliced gel). The tubes were incubated at 60^0C in a heating block for 10 minutes with occasional mixing to ensure gel dissolution.

Spin columns were placed in 2ml collection tubes and 100µl of activation buffer was added into the each spin column. The columns centrifuged at 10,000g for 30 seconds in a micro centrifuge. The sample buffer mixture was added into the each activated spin column and centrifuged at 10,000g for 30 seconds in a micro centrifuge. The flow through were discarded, and 700µl of washing buffer was added into each spin column and further centrifuged at 10,000g for 30 seconds. The flow though was also discarded and the process was repeated a second time. The spin column was then centrifuged again for 2 minutes at 10,000g to remove the residual washing buffer. The purified amplicons were eluted by adding 30µl of the elution buffer into the spin column that has been placed in a new 1.5ml tube and incubating at room temperature for 1 minute before centrifuging for 1 minute at 10,000g.

3.9.2 Quantification of the Amplicons

The purified amplicons were quantified using the Nano drop curvet free spectrophotometer. The instrument allows for fast measurement of micro-volume of DNA. The instrument was first blanked to calibrate it by using the elution buffer that was used to elute the DNA during purification. One microliter of elution buffer was dispensed into the Nano-drop curvet to calibrate it and this is measured as Nano-gram per microliter. Subsequently 1µl of each purified PCR amplicons was then measured in the Nano-drop. The corresponding value given is the concentration of DNA in the product.

3.9.3 Cycle Sequencing of PCR Products

The sequencing reaction was carried out using the DNA cycle sequencing kit by Jena Bioscience, Jena Germany. The kit is based on the Sanger Method (dideoxy chain termination using fluorescent- labelled primers). The composition of the reaction mix for the sequencing is shown in Table 3.8. The prepared mix was aliquoted into a micro micro amp plate, sealed and transferred into the thermal cycler. DNA sequencing was carried out with an enzyme activation period of 95^0C for two minutes, and a 35 cycles of denaturation for 30 seconds at 95^0C, primer annealing for 60 seconds at 55^0C and elongation for 60 seconds at 75^0C followed by one time extension of five minutes at 72^0C.

The sequencing was carried out in both forward and reverse direction using primers Nfla and Nr7a.

Table 3.8: Preparation of sequencing master mix

Reagent	Concentration In Stocks	Concentration In PCR	µl / Sample
Distilled water			2µl
Sequencing Buffer	10×	1×	4µl
Sequencing Polymerase		4units/µl	1µl
Primer (Nr7a/Nfla)	10µM	0.25µM	1µl
Terminator Nucleotides Mix	80µM each	8Mm	4µl
PCR Product		5ng/µl	8µl

3.9.4 Cleaning of Sequenced Products

The sequenced products were purified using nucleotide dye removal kit by Jena Bioscience, Germany. The kit was designed for fast and easy separation of unincorporated dye-labelled, marker-labelled or unlabelled nucleotide from DNA. The kit contains ready to use spin columns preloaded with a gel filtration resin.

The spin column was gently vortexed to re-suspend the resin and the cap of the column was loosened a quarter turn to avoid vacuum inside the spin column. The bottom closure of the spin column was snapped off and the spin column was placed in the 2ml collection tubes. This was then centrifuged at 1,000g for 1 min and the flow through was discarded. The spin column was transferred to a new 2ml micro-centrifuge tube.

The Micro Amp plate containing sequenced amplicons was removed from the thermal cycler and the foil seal was removed. The DNA samples were carefully loaded to the centre of the gel surface of the spin column without touching the gel-bed with the pipet tip. The spin column was then centrifuged at 1000g for 1 minute. The spin column was removed and the eluate which is the purified DNA was transferred into a new micro-amp plate and placed in the sequence analyser.

3.10 Analysis of the Sequenced Fragments.

The query DNA Sequences generated from 12 measles isolates were extracted in Fasta format and manually edited using MEGA 5.05 (Tamura *et al.,* 2011) software. The sequences similarity to measles virus sequences in the Genbank was checked using BLAST (Basic Local Alignment Search Tool) program in the National Centre for Biotechnology Information (NCBI) database. WHO measles reference sequence was downloaded from NCBI and a multiple alignment with the sequences generated was carried out using MEGA 5.05 software.

Phylogenetic analysis was performed by Neighbour-Joining method using the Clustal W package in MEGA 5.05 and Maximum Likelihood trees were generated after correcting for multiple substitutions, complete removal of positions that contained gaps and estimating reliability by 1000 bootstraps. Multiple alignment and phylogenetic analysis of the sequence sample sequences with previous sequences from Nigeria and Ghana was also done.

3.11 Molecular Analysis of the 150 Amino Acid Sequence of the Carboxyl Terminal of Measles Virus N Gene

The nucleotide sequences generated from the samples were translated into amino acid sequences from the open reading frames within each sequence using MEGA 5.05 software. An alignment of the amino acid residue of the carboxyl end of the nucleotide gene of the 12 measles isolates was compared with the WHO reference amino acid residues of all the measles virus genotypes and maximum likelihood trees were generated after correcting for multiple substitutions, complete removal of positions that contained gaps and estimating reliability by 1000 bootstraps.

3.12 Statistical Analysis

Data from the questionnaires administered and others generated in the study were analysed using SPSS version 20.0.

CHAPTER FOUR

RESULTS

4.1 Characteristics of the Study Population

A total of 1200 blood samples were collected from children (557 males and 641 females) aged five months to five years (mean age = 2.4, and STD = 1.08) presenting with fever and maculopapular rash in health centres in Lagos and Oyo State. Four hundred and fifty eight (38.2%) of the children in this study had received at least one dose of measles vaccination, while 742 (61.8%) of them had not received any form of measles vaccination. Table 4.1 shows the characteristics of the study population.

4.2 Serology

4.2.1 Prevalence of measles virus IgM Antibody in Febrile Children with Maculopapular Rash

Table 4.2 shows the prevalence of measles virus IgM antibody in children presenting with fever and maculopapular rash in Ibadan and Lagos between 2007 and 2011. One thousand two hundred samples were tested for measles virus IgM, 557 (46.4%) males and 641 (53.6%) females. Of the 1200 tested samples, a total of 133 (11.1%) had measles IgM antibodies, 56 (42.1%) males and 77 (57.9%) females. In 2007, 33 of the 301 (11%) children tested were positive for measles IgM and in 2008, 17 (11.5%) of the 148 children tested positive. Also in 2009, of the 251 tested, 26 (10.4%) had measles IgM antibodies while in 2010, 31 (10.4%) of the 298 tested had the antibodies and in 2011, 21 (10.4%) of the 202 children tested were positive for measles IgM.

A total of 34 (25.6%) of the children who tested positive to measles virus specific IgM had received at least one dose of measles vaccination. Table 4.3 shows the vaccination history of

the children who tested positive to measles virus IgM antibody. Of the 458 vaccinated children 34 (7.4%) had measles virus IgM antibody while 99 (13.3%) of the unvaccinated children had measles virus IgM antibody. It was also observed that 26.6% of the children who had measles virus IgM antibody had previously received at least on dose of measles vaccination. There was a significant difference of measles infection among vaccinated and unvaccinated children (p=0.02) with more of the unvaccinated children having measles virus IgM antibody.

Figure 4.1 shows the overall age distribution of measles IgM antibody among the children. The highest rate was found among children age 1-3 years while the lowest was found among children aged 3-5 years. There was a significant difference in the rate of infection across the different age groups with an overall p value of 0.027.

Table 4.1: Characteristics of the study population

Characteristics	Frequency (%)
Age Group	
<9 months	258 (21.5%)
>9 months -2 years	307 (25.6%)
>2-3 years	338 (28.2%)
>3-5 years	232 (19.3%)
Missing	65 (5.4%)
Gender	
Male	557 (46.4%)
Female	641 (53.4%)
Vaccination History	
Vaccinated	458 (38.2%)
Unvaccinated	742 (61.8%)

Table 4.2: Annual and gender distribution of measles IgM antibodies in sera from febrile children with maculopapular rash in Ibadan and Lagos, 2007-2011

Year	Male		Female		Total	
	No Screened	No (%) Positive	N0 Screened	No (%) Positive	No Screened	No (%) Positive
2007	120	11(9.2%)	181	22 (12.2%)	301	33(11%)
2008	63	7(11%)	85	10 (11.8%)	148	17(11.3%)
2009	137	12(8.8%)	114	14 (12.3%)	251	28(10.4%)
2010	139	13(9.4%)	159	18 (11.3%)	298	31(10.3%)
2011	98	13(11.3%)	104	13 (12.5%)	202	24(11.9%)
Total	557	56 (10.1%)	643	77 (11.9%)	1200	133 (11.1%)

Table 4.3: Vaccination history and gender distribution of Measles virus IgM in serum samples collected from febrile children with maculopapular rash in Ibadan and Lagos, 2007-2011

	Vaccinated children		Unvaccinated children	
Gender	No Tested	No (%) +ve	No Tested	No (%) +ve
Male	207	14 (6.7%)	350	42 (12%)
Female	251	20 (7.9%)	392	57 (14.5%)
Total	458	34 (7.4%)	742	99 (13.3%)

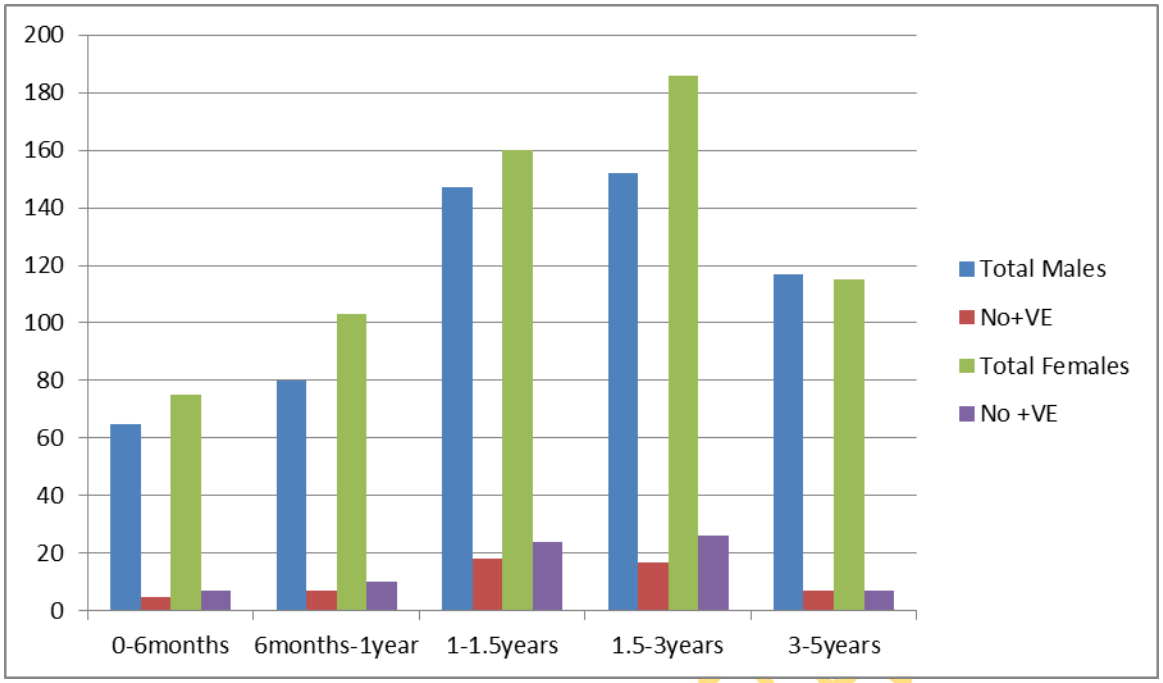


Figure 4.1: Measles Virus IgM Positivity and Age Distribution of Children with Fever and Maculopapular Rash between 2007 and 2011

4.2.2 Prevalence of Rubella Virus IgM Antibody in Febrile Children with Maculopapular Rash

The Table 4.4 shows the prevalence of rubella IgM antibody in with fever and maculopapular rash in Ibadan and Lagos, 2007-2011. A total of 1081 samples from children with fever and maculopapular rash that tested negative for measles specific IgM antibodies were tested for rubella virus IgM. Of these 509 were males and 572 were females.. Two hundred and sixty seven (27.8%) were positive for rubella IgM, 123 males (46.1%) and 144 (53.9%) females. There was no significant difference in the gender distribution of rubella IgM antibody among the study population (p=0.7). Sixty five (23.6%) of the 276 children were positive for rubella virus IgM antibody in 2007 and 35 (26.3%) of the 133 were positive in 2008. In 2009, out of the 224 children, 55 (24.6%) were positive for rubella virus IgM while 65 (24.2%) of the 269 and 47 (26.3%) of the 179 children were positive in 2010 and 2011, respectively. The highest prevalence of rubella infection was in 2008 and 2011. There was also no significant difference in the seroprevalence of rubella IgM antibodies across the years between 2007 and 2011 (p=0.2).

Figure 4.2 shows the overall age distribution of rubella virus IgM positivity among the children. The highest prevalence was found among children aged 1-3 years while the lowest was found among children aged 3-5 years and this difference is significant with a p value of 0.032.

Table 4.4: Annual Distribution of rubella virus IgM in children with fever and maculopapular rash between 2007 and 2011

Year	Male		Female		Total	
	No. Screened	No. (%) Positive	No. Screened	No. (%) Positive	No. Screened	No. (%) Positive
2007	109	24(22%)	167	41(24.6%)	276	65(23.6%)
2008	58	15(25.9%)	75	20(26.7%)	133	35(26.3%)
2009	125	30(24%)	99	25(25.3%)	224	55(24.6%)
2010	127	32(25.2%)	142	40(28.2%)	269	72(26.8%)
2011	90	22(24.4%)	89	25(28.1%)	179	47(26.3%)
Total	509	123(24.2%)	572	151(26.4%)	1081	274(25.3%)

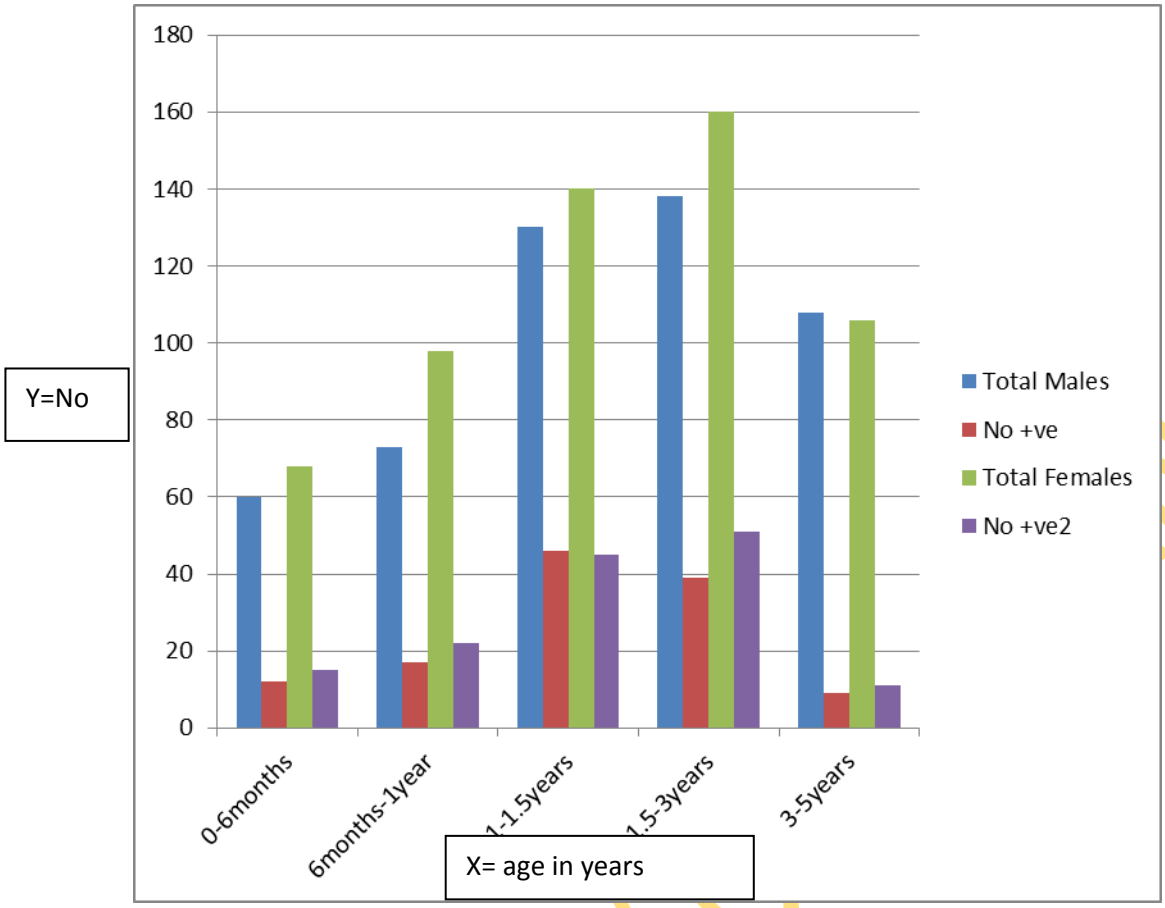


Figure 4.2: Prevalence of Rubella Virus IgM antibody and age distribution of children with fever and maculopapular rash between 2007 and 2011

4.2.3 Prevalence of Parvovirus B19 IgM Antibody in Febrile Children with Maculopapular Rash

Table 4.5 shows the prevalence of parvovirus B19 IgM antibody in children with fever and maculopapular rash in Lagos and Ibadan, 2007-2011. A total of 807 blood samples from children with fever and maculopapular rash were screened for parvovirus B19 IgM from 2007 to 2011 of which 386 were males and 421 were females. Ninety six of these children (11.9%) were positive for parvovirus B19 IgM, 45 (46.8%) male and 51 (53.1%) females. There was no significant difference in the prevalence of parvovirus B19 IgM antibody across the gender of the tested children (p=0.8). Thirty (14.2%) of the 211 and 16 (16.2%) of the 98 were positive for rubella virus IgM in 2007 and 2008 respectively. In 2009, 15 (8.9%) out of the 169 children were positive for parvovirus B19 IgM while 20 (10.2%) of the 197 were positive in 2010 and 15 (11.4%) of the 132 children were positive in 2011.

Figure 4.3 shows the overall age distribution of Parvovirus B19 IgM positivity among the children. The highest rate was found among children aged 1-3 years (13.1%) while the lowest was found among children aged >3-5 years (7.4%). The difference in the prevalence between the age groups was not significant (p=0.43). The mean age of children with parvovirus B19 IgM antibody was 2.9 years (age range 8 months to 5 years). Among the <1 and 1-3 years, old children very little variation in parvovirus B19 IgM antibody between the gender was observed while more males than females were positive among >3-5 year old children (8.6 versus 5.9%, P=0.108).

Table 4.5: Annual and Gender Distribution of Parvoviru B19 IgM Anitibody in Serum Samples Collected from Febrile Children with maculopapular rash in Lagos and Ibadan, 2007-2011

Year	Male		Female		Total	
	No. Screened	No. (%) +ve	No. Screened	No. (%) +ve	No. Screened	No. (%) +ve
2007	85	12(14.1%)	126	18(14.2%)	211	30(14.2%)
2008	43	7(16.3%)	55	9(16.4%)	98	16(16.3%)
2009	95	9(9.5%)	74	6(8.1%)	169	15(8.9%)
2010	95	9(9.5%)	102	11(10.8%)	197	20(10.2%)
2011	68	8(11.3%)	64	7(10.9%)	132	15(11.4%)
Total	386	45(11.7%)	421	5112.1%)	807	96(11.9%)

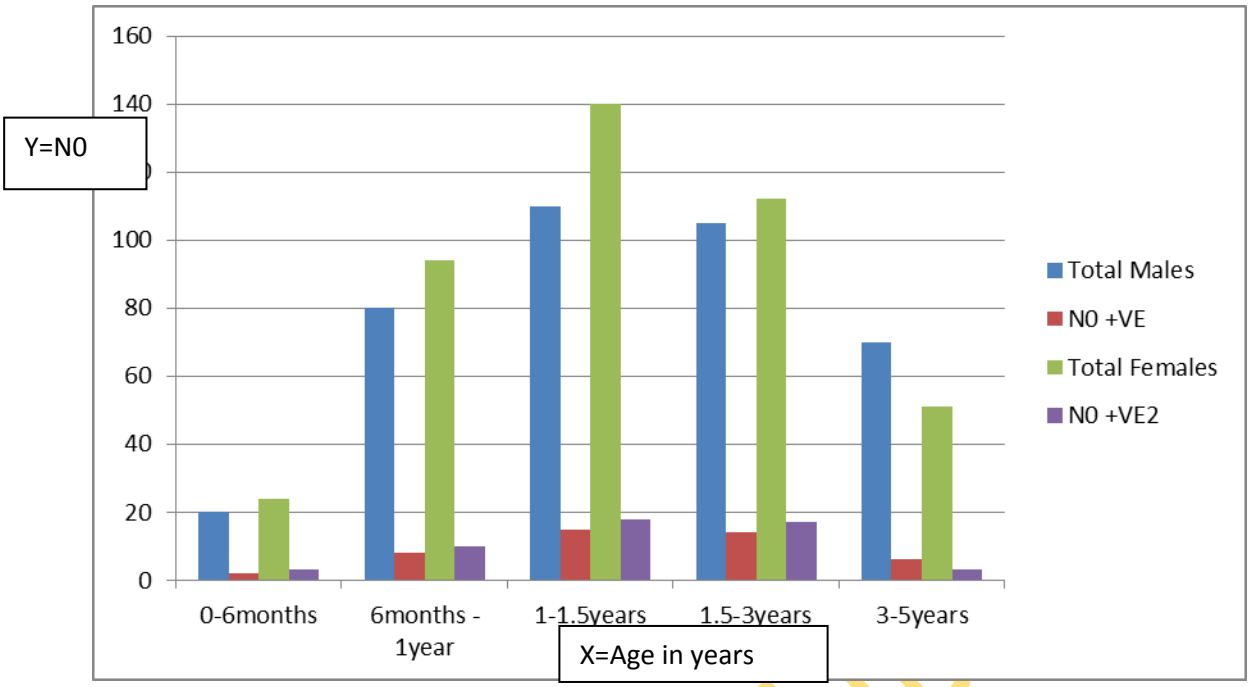


Figure 4.3: Prevalence of Parvovirus B19 IgM antibody and age distribution of children with fever and maculopapular rash between 2007 and 2011

4.3.0 Polymerase Chain Reaction for Measles Virus RNA In Throat Swab and Urine Samples Collected from Febrile Children with Maculopapular Rash in Southwestern Nigeria, 2007-2011

4.3.1 Samples Positive for Measles Virus RNA in Nested PCR

Table 4.6 shows the annual distribution of samples from febrile children that were positive for measles virus nucleic acid in nested PCR. A total of 286 Throat swab and 264 urine samples were tested for measles virus by RT-PCR using primers targeted at the carboxyl end of the N gene of measles virus genome. Twenty three samples had the expected 560 base pair band size after the nested reaction both in the throat swab and urine samples. The throat swab and urine samples that tested positive for measles virus RNA were from the same children. All the children who tested positive for measles virus RNA had not received any form of measles vaccination and they all tested positive for measles virus IgM antibody. Table 4.7 shows the age and gender distribution of measles virus RNA detected. Figure 4.4 and 4.5 shows the picture of agarose gel electrophoresis of the positive amplicons before and after purification respectively.

Figure 4.5 shows the picture of agarose gel electrophoresis of purified measles virus amplicons from throat swab and urine samples. After purification of the PCR amplicons, 21 samples were positive for measles RNA.

4.4 QUANTIFICATION OF PCR AMPLICONS

After purification the 21 samples showing the expected band size were quantified. Eleven samples had DNA concentration that is more than 20ng/µl

Table 4.6: Annual Distribution of Measles Virus PCR Positive Samples Collected from Febrile Children with Maculopapular rash in Lagos and Ibadan, 2007-2011

Year	Throat swab		Urine	
	No Screened	No. +ve	No Screened	No +ve
2007	70	0	62	0
2008	33	0	24	0
2009	63	1	61	1
2010	68	2	67	2
2011	52	20	50	20
Total	286	23	264	23

Table 4.7: Age and gender distribution of measles virus RNA detected.

Age Group (Years)	Male (%)	Female (%)	Total (%)
0-1	5 (21.7%)	6 (11.3%)	11 (47.8%)
>1-3	4 (17.4%)	4 (17.4%)	8 (34.8%)
>3-5	3 (13%)	1 (4.3%)	4 (17.4)
Total	12 (52.2%)	11 (47.8%)	23 (100%)

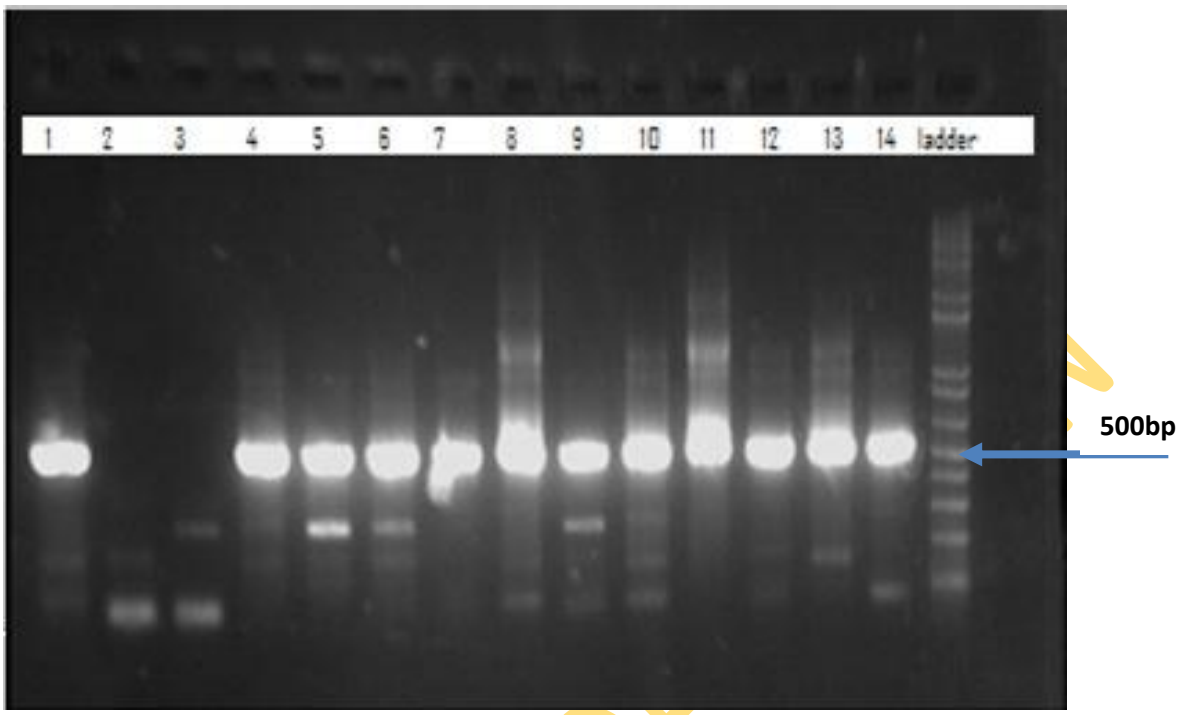


Figure 4.4: Agarose gel electrophoresis of the positive amplicons before purifications

Legend: Lane 1 is Positive control

Lane 2 is Negative control

Lane 3-14 is measles positive samples

Lane 15 is 100 bases pair ladder

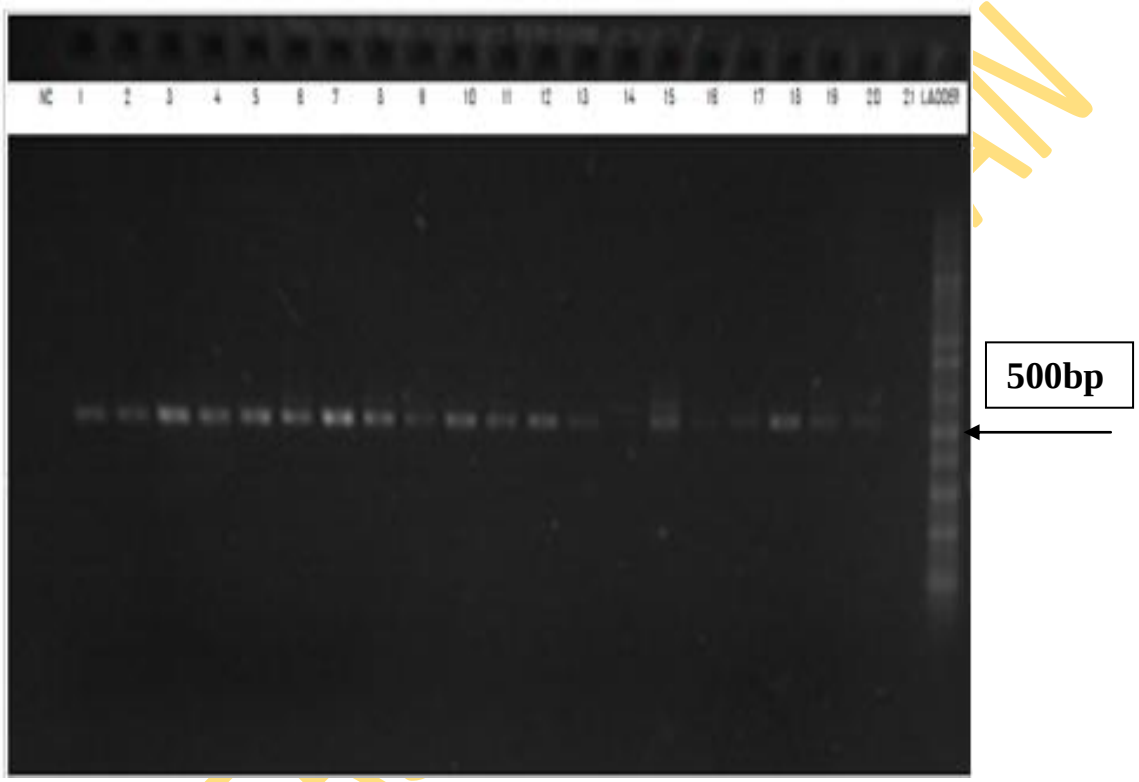


Figure 4.5: Agarose gel electrophoresis after purification of the measles virus positive amplicons Throat Swabs collected from febrile children with maculopapular rash in Lagos and Ibadan, 2007-2011.

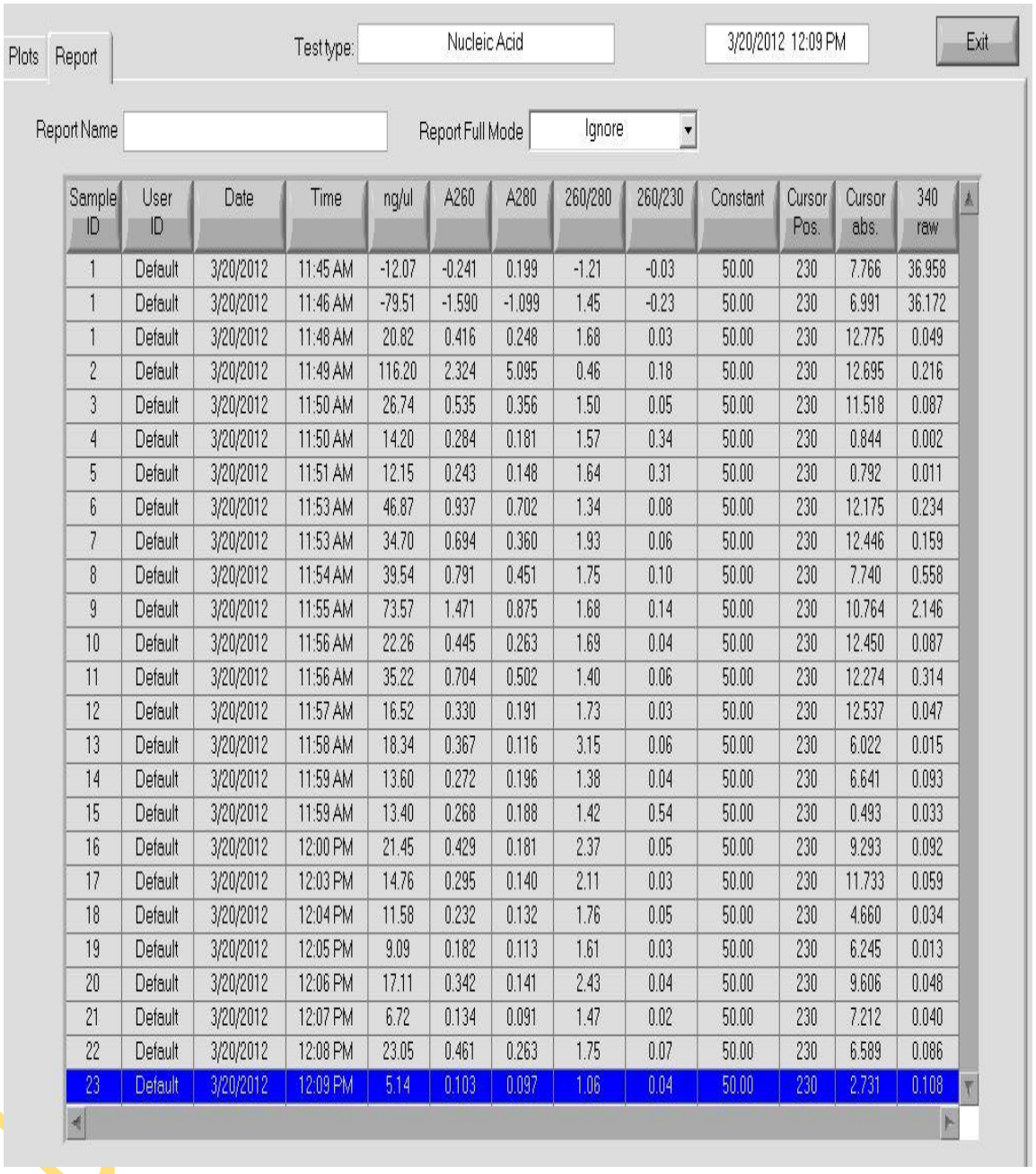
Legend

Lane 1 is Negative control

Lane 2-21 is purified amplicons

Lane 22 is 100 base pair ladder

Table 4.8: Quantification of the Purified Measles Virus Amplicons from Throat Swab and urine Samples Collected from Febrile Children with Maculopapular Rash in Lagos and Ibadan, 2007-2011



Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1	Default	3/20/2012	11:45 AM	-12.07	-0.241	0.199	-1.21	-0.03	50.00	230	7.766	36.958
1	Default	3/20/2012	11:46 AM	-79.51	-1.590	-1.099	1.45	-0.23	50.00	230	6.991	36.172
1	Default	3/20/2012	11:48 AM	20.82	0.416	0.248	1.68	0.03	50.00	230	12.775	0.049
2	Default	3/20/2012	11:49 AM	116.20	2.324	5.095	0.46	0.18	50.00	230	12.695	0.216
3	Default	3/20/2012	11:50 AM	26.74	0.535	0.356	1.50	0.05	50.00	230	11.518	0.087
4	Default	3/20/2012	11:50 AM	14.20	0.284	0.181	1.57	0.34	50.00	230	0.844	0.002
5	Default	3/20/2012	11:51 AM	12.15	0.243	0.148	1.64	0.31	50.00	230	0.792	0.011
6	Default	3/20/2012	11:53 AM	46.87	0.937	0.702	1.34	0.08	50.00	230	12.175	0.234
7	Default	3/20/2012	11:53 AM	34.70	0.694	0.360	1.93	0.06	50.00	230	12.446	0.159
8	Default	3/20/2012	11:54 AM	39.54	0.791	0.451	1.75	0.10	50.00	230	7.740	0.558
9	Default	3/20/2012	11:55 AM	73.57	1.471	0.875	1.68	0.14	50.00	230	10.764	2.146
10	Default	3/20/2012	11:56 AM	22.26	0.445	0.263	1.69	0.04	50.00	230	12.450	0.087
11	Default	3/20/2012	11:56 AM	35.22	0.704	0.502	1.40	0.06	50.00	230	12.274	0.314
12	Default	3/20/2012	11:57 AM	16.52	0.330	0.191	1.73	0.03	50.00	230	12.537	0.047
13	Default	3/20/2012	11:58 AM	18.34	0.367	0.116	3.15	0.06	50.00	230	6.022	0.015
14	Default	3/20/2012	11:59 AM	13.60	0.272	0.196	1.38	0.04	50.00	230	6.641	0.093
15	Default	3/20/2012	11:59 AM	13.40	0.268	0.188	1.42	0.54	50.00	230	0.493	0.033
16	Default	3/20/2012	12:00 PM	21.45	0.429	0.181	2.37	0.05	50.00	230	9.293	0.092
17	Default	3/20/2012	12:03 PM	14.76	0.295	0.140	2.11	0.03	50.00	230	11.733	0.059
18	Default	3/20/2012	12:04 PM	11.58	0.232	0.132	1.76	0.05	50.00	230	4.660	0.034
19	Default	3/20/2012	12:05 PM	9.09	0.182	0.113	1.61	0.03	50.00	230	6.245	0.013
20	Default	3/20/2012	12:06 PM	17.11	0.342	0.141	2.43	0.04	50.00	230	9.606	0.048
21	Default	3/20/2012	12:07 PM	6.72	0.134	0.091	1.47	0.02	50.00	230	7.212	0.040
22	Default	3/20/2012	12:08 PM	23.05	0.461	0.263	1.75	0.07	50.00	230	6.589	0.086
23	Default	3/20/2012	12:09 PM	5.14	0.103	0.097	1.06	0.04	50.00	230	2.731	0.108

4.5. Sequencing Results

Twenty one isolates were sequenced but only 11 isolates (52.4%) had readable sequences. Table 4.9 shows the list of measles virus isolates with readable sequences, the location and year of their isolation. Figure 4.6 shows the picture of the sequence electropherographs of measles virus isolates from throat swabs and urine samples of febrile children with maculopapular rash in Lagos and Ibadan, 2007-2011.

4.5.1 Nucleotide Sequences Alignment of Study isolates and WHO Reference Strains Sequence

Figure 4.8 shows the multiple sequence alignment of the study isolates and figure 13 shows the multiple sequence alignment of the study isolates and the WHO reference strains.

4.5.2 Informative Sites

Table 4.10 shows the difference in the informative sites of the aligned sequences of measles virus isolates from this study and WHO reference sequence when compared to the generated consensus sequence. The sequences of the measles virus isolates were aligned with WHO reference sequences and the informative sites were determined and are shown in table 4.10. There was an average of 35 nucleotide difference in the sequences of measles virus isolates from this study compared to the generated consensus.

4.5.3 Phylogenetic Analysis of Study Isolates and WHO Reference Strains Sequences

Figure 4.9 shows phylogenetic analysis of the sequences of the COOH terminal of the N-gene of the of measles virus isolates from this study and WHO measles reference strains sequences by maximum likelihood. The generated phylogram showed that 10 of the isolated sequences clustered with genotype B3 reference strain and one isolates clustered with

genotype A reference strain. Estimates of evolutionary of the isolates shows an overall mean distance of 0.08 within the isolates and 0.075 when compared with WHO reference stains. Table 4.11 and table 4.12 shows the evolutionary divergence of the measles virus isolates from this study and the WHO reference strains.

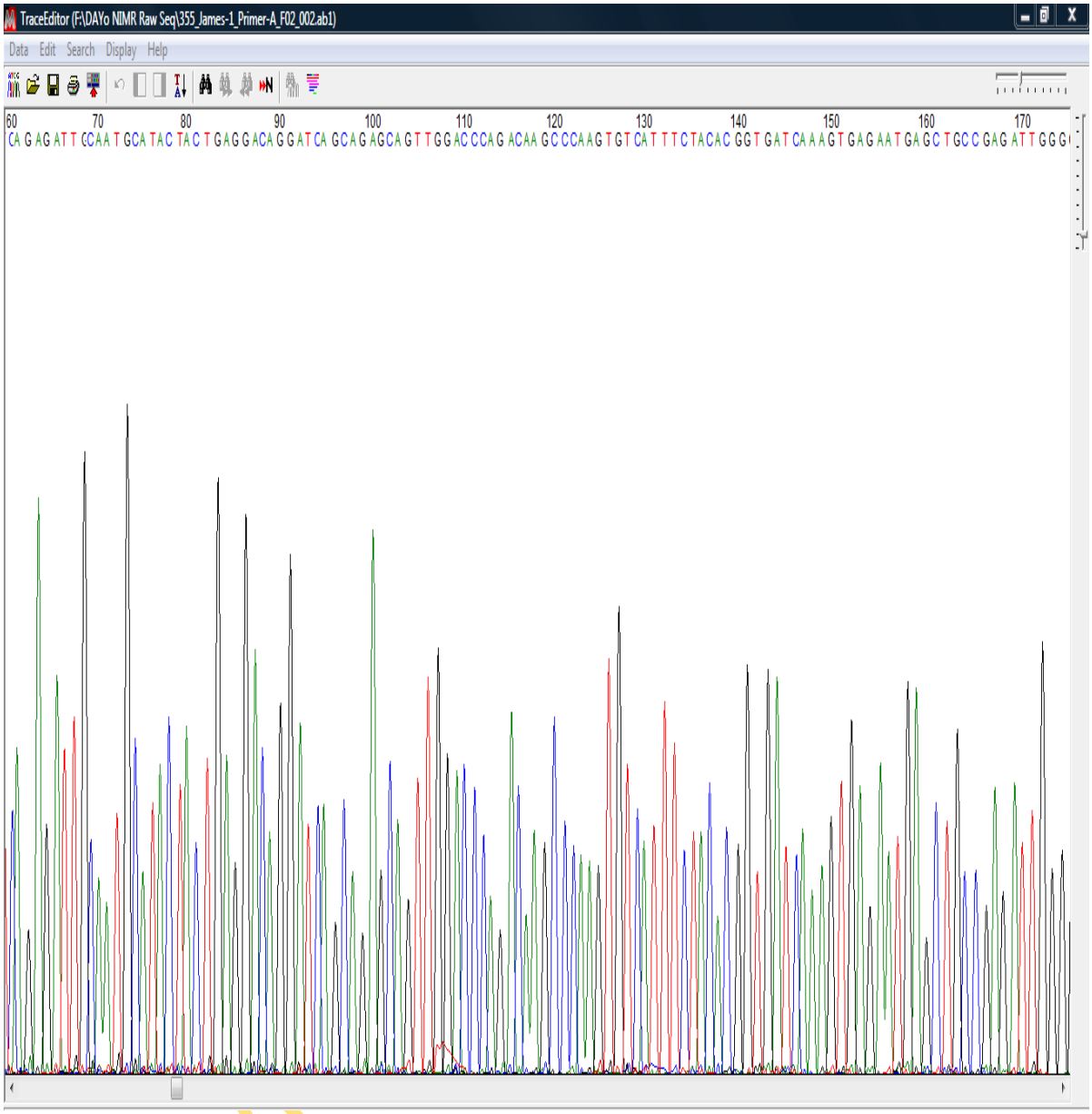


Figure 4.6: Sequence electropherographs of measles virus isolates from throat swabs and urine samples of febrile children with maculopapular rash showing peak and length of read

Table 4.9: Measles virus isolates with readable sequence, year and the location of isolation

S/N	Name of Isolate	Location of Isolation
1	MVs/Ibadan.NIE/07.11/2	Ibadan
2	MVs/Ibadan.NIE/02.11/2	Ibadan
3	MVs/Ibadan.NIE/05.11/2	Ibadan
4	MVs /Lagos.NIE/05.11/1	Lagos
5	MVs/Ibadan.NIE/07.11/1	Ibadan
6	MVs/Ibadan.NIE/03.11/1	Ibadan
7	MVs/Lagos.NIE/07.11/12	Lagos
8	MVs/Ibadan.NIE/03.11/2	Ibadan
9	MVs/Ibadan.NIE/05.11/1	Ibadan
10	MVs/Lagos.NIE/07.11/1	Lagos
11	MVs /Ibadan.NIE.11.10	Ibadan

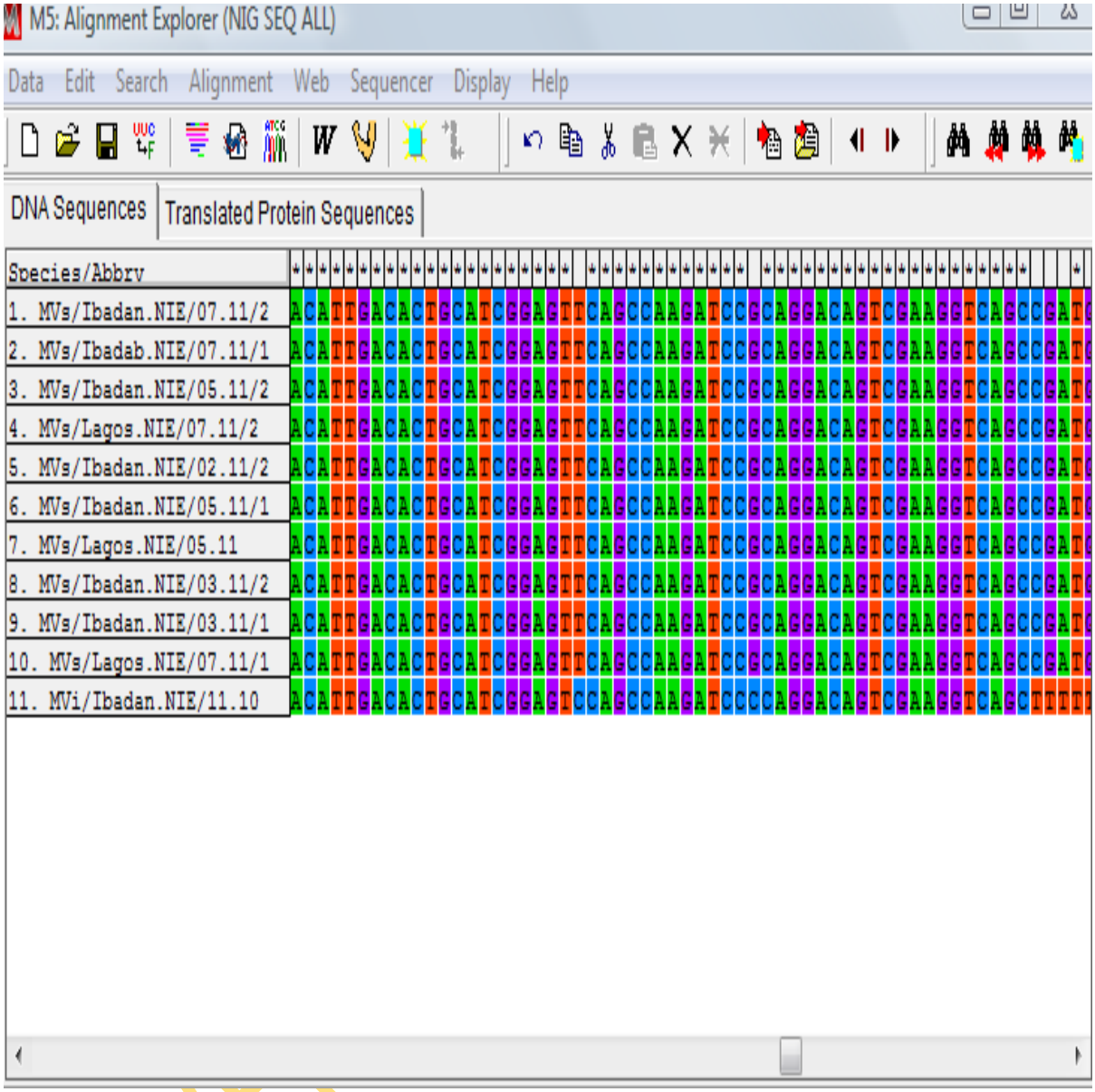


Figure 4.7: Multiple Nucleotide Sequence Alignment of measles virus isolates from Lagos and Ibadan, Southwest Nigeria.

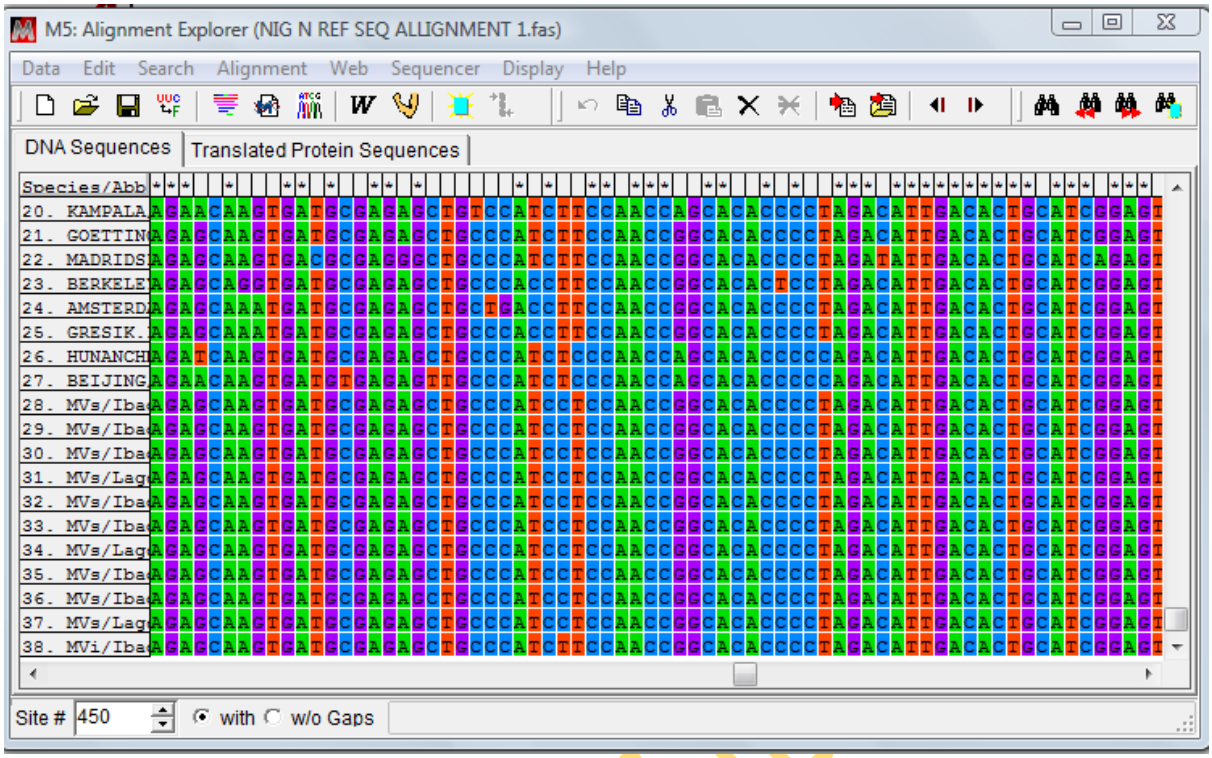
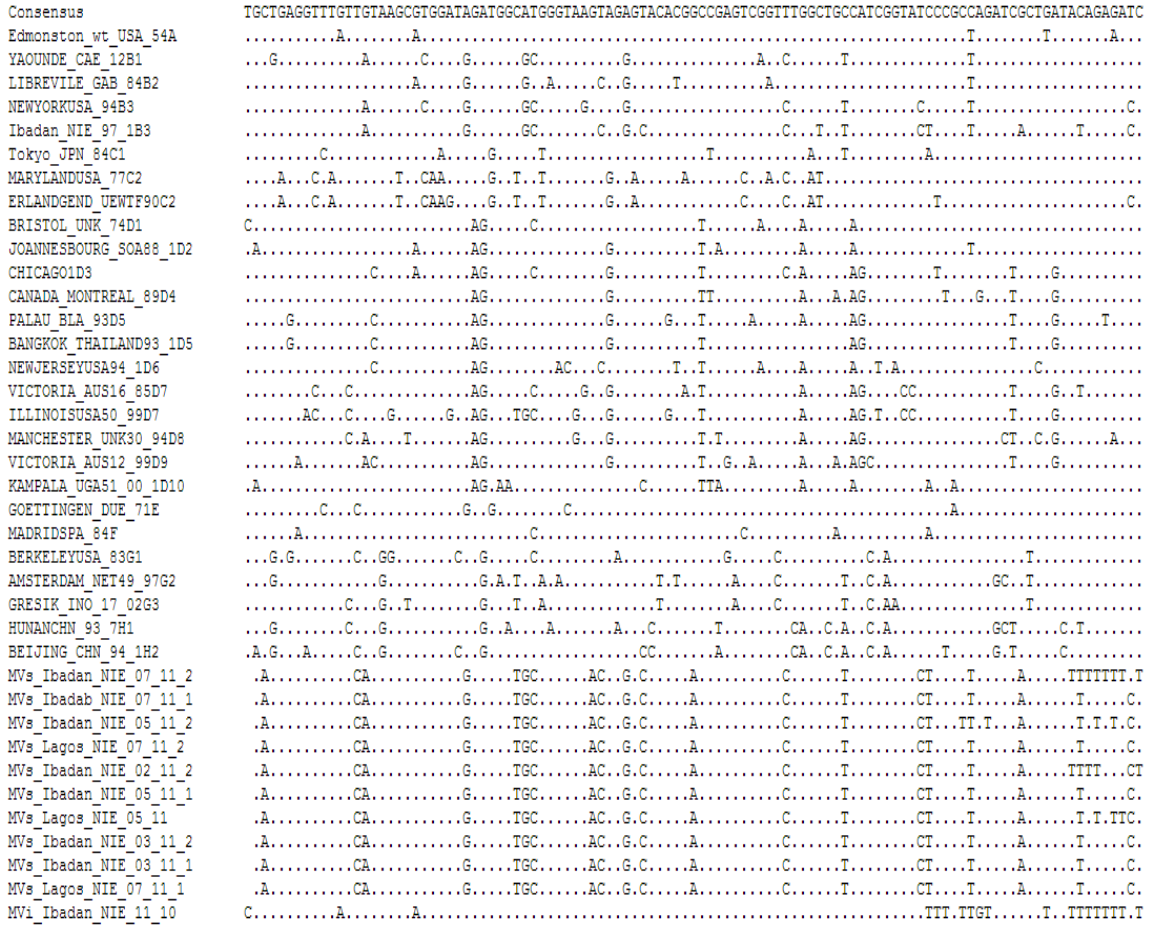


Figure 4.8: Multiple nucleotide sequence alignment of measles virus isolates from Lagos and Ibadan and WHO reference measles virus stains

Table 4.9: Informative sites of Alignment of Nigerian measles virus Isolates and WHO Reference sequence



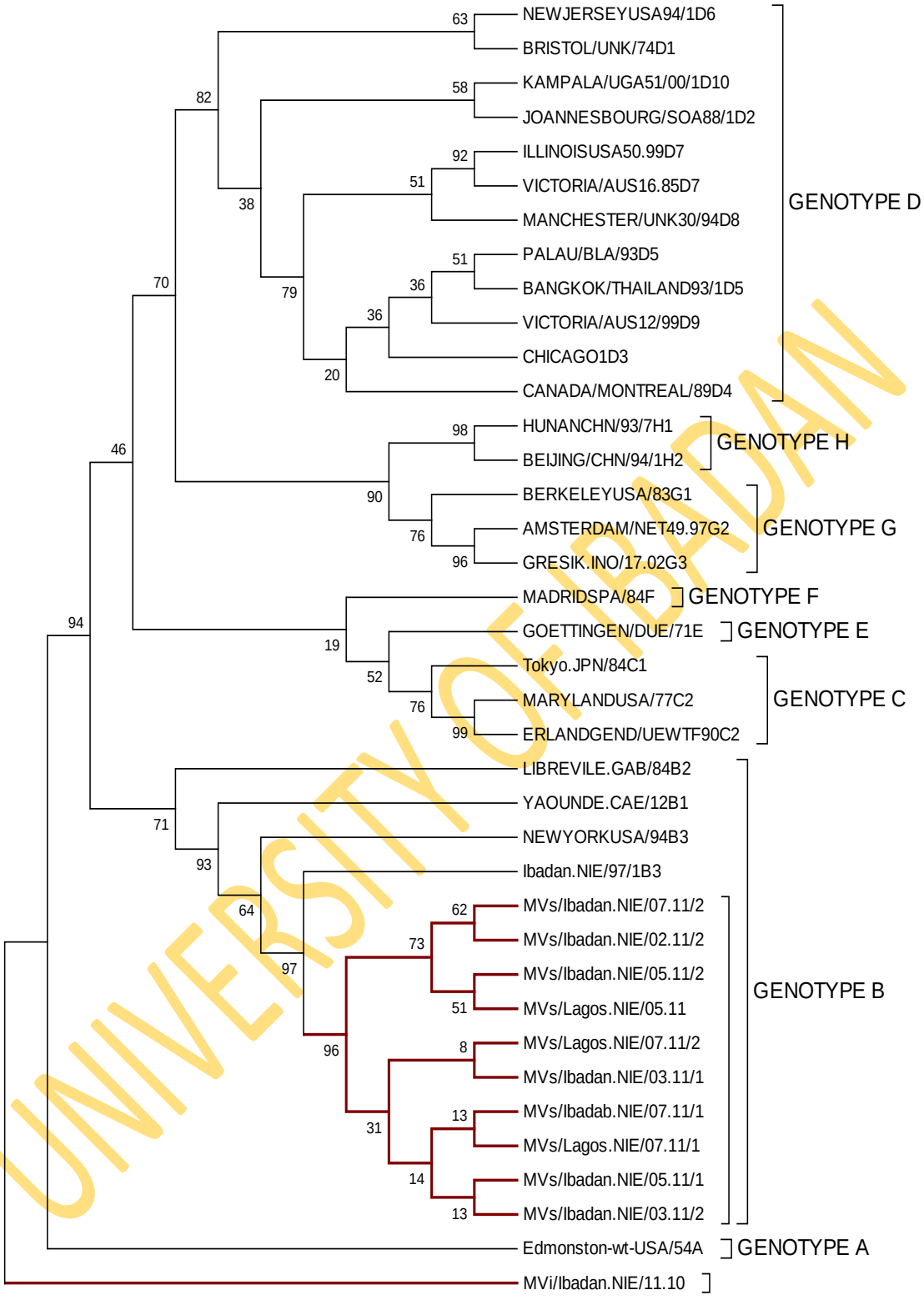


Fig 4.9: Phylogram of the sequences of the isolates from this study and of WHO reference strains sequence

Table 4.11: Estimates of Evolutionary Divergence between Sequences of the 11 measles virus isolates from throat swab of febrile children with maculopapular rash in Lagos and Ibadan, 2007-2011.

	1	2	3	4	5	6	7	8	9	10	11
1. MVs/Ibadan.NIE/07.11/2		0.007	0.008	0.007	0.005	0.007	0.006	0.007	0.008	0.007	0.018
2. MVs/Ibadab.NIE/07.11/1	0.019		0.006	0.000	0.005	0.000	0.005	0.000	0.003	0.000	0.019
3. MVs/Ibadan.NIE/05.11/2	0.026	0.016		0.006	0.007	0.006	0.006	0.006	0.007	0.006	0.019
4. MVs/Lagos.NIE/07.11/2	0.019	0.000	0.016		0.005	0.000	0.005	0.000	0.003	0.000	0.019
5. MVs/Ibadan.NIE/02.11/2	0.009	0.009	0.021	0.009		0.005	0.006	0.005	0.006	0.005	0.019
6. MVs/Ibadan.NIE/05.11/1	0.019	0.000	0.016	0.000	0.009		0.005	0.000	0.003	0.000	0.019
7. MVs/Lagos.NIE/05.11	0.014	0.009	0.016	0.009	0.014	0.009		0.005	0.006	0.005	0.019
8. MVs/Ibadan.NIE/03.11/2	0.019	0.000	0.016	0.000	0.009	0.000	0.009		0.003	0.000	0.019
9. MVs/Ibadan.NIE/03.11/1	0.024	0.005	0.021	0.005	0.014	0.005	0.014	0.005		0.003	0.020
10. MVs/Lagos.NIE/07.11/1	0.019	0.000	0.016	0.000	0.009	0.000	0.009	0.000	0.005		0.019
11. MVi/Ibadan.NIE/11.10	0.116	0.138	0.135	0.138	0.127	0.138	0.133	0.138	0.144	0.138	

The number of base substitutions per site from between sequences are shown diagonally on the right side of the table. Standard error estimate(s) are shown above the diagonal and were obtained by using analytical formulas. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 482 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Table 4.12: Estimates of Evolutionary Divergence between the sequences of the measles virus isolates from throat swab of febrile children with maculopapular rash in Lagos and Ibadan, 2007-2011and WHO reference measles virus strains Sequences

MS: Pairwise Distances (C:\Users\Deewan\AppData\Local\Temp\PhyloAnalysis-25.meg)

File Display Average Caption Help

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1. MVs/Ibadan.NIE/8.98/10	0.000	0.010	0.006	0.008	0.006	0.007	0.008	0.007	0.007	0.007	0.012	0.010	0.012	0.010	0.011	0.010	0.009	0.009	0.008	0.008	0.009	0.009	0.008	0.011	0.010	0.011	0.010	0.020
2. MVs/Ibadan.NIE/8.98/4		0.010	0.006	0.008	0.006	0.007	0.008	0.007	0.007	0.007	0.012	0.010	0.012	0.010	0.011	0.010	0.009	0.009	0.008	0.008	0.009	0.009	0.008	0.011	0.010	0.011	0.010	0.020
3. MVs/Ibadan.NIE/9.98/11	0.036		0.008	0.004	0.008	0.009	0.009	0.009	0.009	0.008	0.010	0.007	0.009	0.007	0.008	0.007	0.005	0.005	0.004	0.004	0.005	0.005	0.004	0.008	0.007	0.008	0.007	0.019
4. MVs/Ibadan.NIE/10.98/2	0.017	0.029		0.007	0.002	0.006	0.006	0.005	0.005	0.005	0.012	0.009	0.011	0.009	0.010	0.009	0.008	0.008	0.007	0.007	0.008	0.008	0.007	0.010	0.009	0.010	0.009	0.019
5. MVs/Ibadan.NIE/8.98/2	0.029	0.007	0.021		0.007	0.008	0.008	0.008	0.008	0.007	0.009	0.005	0.008	0.005	0.007	0.005	0.005	0.002	0.000	0.000	0.002	0.003	0.000	0.007	0.005	0.006	0.005	0.018
6. Ibadan.NIE/8.98/12	0.014	0.026	0.002	0.019		0.005	0.006	0.005	0.005	0.005	0.011	0.009	0.011	0.009	0.010	0.009	0.007	0.007	0.007	0.007	0.007	0.008	0.007	0.010	0.009	0.009	0.009	0.019
7. MVs/Lagos.NIE/97	0.021	0.034	0.014	0.026	0.012		0.004	0.002	0.002	0.006	0.012	0.010	0.012	0.010	0.011	0.010	0.008	0.008	0.008	0.008	0.008	0.009	0.008	0.011	0.010	0.010	0.010	0.019
8. MVs/Accra.GHA/9.98/1	0.024	0.036	0.017	0.029	0.014	0.007		0.003	0.003	0.007	0.012	0.009	0.012	0.009	0.011	0.009	0.008	0.008	0.008	0.008	0.009	0.009	0.008	0.011	0.009	0.010	0.009	0.019
9. MVs/Accra.GHA/9.98/2	0.019	0.031	0.012	0.024	0.009	0.002	0.005		0.000	0.006	0.012	0.009	0.012	0.009	0.011	0.009	0.007	0.007	0.008	0.008	0.008	0.008	0.008	0.011	0.009	0.010	0.009	0.019
10. MVs/Accra.GHA/98	0.019	0.031	0.012	0.024	0.009	0.002	0.005	0.000		0.006	0.012	0.009	0.012	0.009	0.011	0.009	0.007	0.007	0.008	0.008	0.008	0.008	0.008	0.011	0.009	0.010	0.009	0.019
11. MVs/Lagos.NIE/10.98	0.019	0.026	0.012	0.019	0.009	0.017	0.019	0.014	0.014		0.011	0.009	0.011	0.009	0.010	0.009	0.007	0.007	0.007	0.007	0.007	0.008	0.007	0.010	0.009	0.009	0.009	0.018
12. MVs/Ibadan.NIE/07.11/2	0.061	0.038	0.053	0.031	0.051	0.058	0.056	0.056	0.056	0.051		0.007	0.008	0.007	0.005	0.007	0.010	0.009	0.009	0.009	0.009	0.009	0.009	0.006	0.007	0.008	0.007	0.018
13. MVs/Ibadab.NIE/07.11/1	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019		0.006	0.000	0.005	0.000	0.008	0.006	0.005	0.005	0.006	0.006	0.005	0.005	0.000	0.003	0.000	0.019
14. MVs/Ibadan.NIE/05.11/2	0.058	0.033	0.051	0.028	0.048	0.056	0.053	0.053	0.053	0.048	0.026	0.016		0.006	0.007	0.006	0.010	0.009	0.008	0.008	0.009	0.009	0.008	0.006	0.006	0.007	0.006	0.019
15. MVs/Lagos.NIE/07.11/2	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019	0.000	0.016		0.005	0.000	0.008	0.006	0.005	0.005	0.006	0.006	0.005	0.005	0.000	0.003	0.000	0.019
16. MVs/Ibadan.NIE/02.11/2	0.051	0.029	0.043	0.021	0.041	0.048	0.046	0.046	0.046	0.041	0.009	0.009	0.021	0.009		0.005	0.009	0.008	0.007	0.007	0.008	0.008	0.007	0.006	0.005	0.006	0.005	0.019
17. MVs/Ibadan.NIE/05.11/1	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019	0.000	0.016	0.000	0.009		0.008	0.006	0.005	0.005	0.006	0.006	0.005	0.005	0.000	0.003	0.000	0.019
18. MVs/Ibadan.NIE/10.98/1	0.031	0.009	0.024	0.012	0.021	0.024	0.026	0.021	0.021	0.021	0.043	0.024	0.041	0.024	0.033	0.024		0.006	0.005	0.005	0.006	0.006	0.005	0.009	0.008	0.008	0.008	0.019
19. MVs/Lagos.NIE/11.98/2	0.031	0.009	0.024	0.002	0.021	0.024	0.026	0.021	0.021	0.021	0.033	0.014	0.031	0.014	0.024	0.014	0.014		0.002	0.002	0.003	0.004	0.002	0.008	0.006	0.007	0.006	0.018
20. MVs/Ibadan.NIE/7.98/3	0.029	0.007	0.021	0.000	0.019	0.026	0.029	0.024	0.024	0.019	0.031	0.012	0.028	0.012	0.021	0.012	0.012	0.002		0.000	0.002	0.003	0.000	0.007	0.005	0.006	0.005	0.018
21. MVs/Ibadan.NIE/97/2	0.029	0.007	0.021	0.000	0.019	0.026	0.029	0.024	0.024	0.019	0.031	0.012	0.028	0.012	0.021	0.012	0.012	0.002	0.000		0.002	0.003	0.000	0.007	0.005	0.006	0.005	0.018
22. MVs/Ibadan.NIE/8.98/6	0.031	0.009	0.024	0.002	0.021	0.029	0.031	0.026	0.026	0.021	0.033	0.014	0.031	0.014	0.024	0.014	0.014	0.005	0.002	0.002		0.004	0.002	0.008	0.006	0.007	0.006	0.018
23. MVs/Lagos.NIE/8.98	0.034	0.012	0.026	0.005	0.024	0.031	0.034	0.029	0.029	0.024	0.036	0.017	0.033	0.017	0.026	0.017	0.017	0.007	0.005	0.005	0.007		0.003	0.008	0.006	0.007	0.006	0.019
24. MVs/Ibadan.NIE/8.98/1	0.029	0.007	0.021	0.000	0.019	0.026	0.029	0.024	0.024	0.019	0.031	0.012	0.028	0.012	0.021	0.012	0.012	0.002	0.000	0.000	0.002	0.005		0.007	0.005	0.006	0.005	0.018
25. MVs/Lagos.NIE/05.11	0.051	0.028	0.043	0.021	0.041	0.048	0.046	0.046	0.046	0.041	0.014	0.009	0.016	0.009	0.014	0.009	0.033	0.024	0.021	0.021	0.024	0.026	0.021		0.005	0.006	0.005	0.019
26. MVs/Ibadan.NIE/03.11/2	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019	0.000	0.016	0.000	0.009	0.000	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.009		0.003	0.000	0.019
27. MVs/Ibadan.NIE/03.11/1	0.046	0.024	0.038	0.016	0.036	0.044	0.041	0.041	0.041	0.036	0.024	0.005	0.021	0.005	0.014	0.005	0.029	0.019	0.016	0.016	0.019	0.021	0.016	0.014	0.005		0.003	0.020
28. MVs/Lagos.NIE/07.11/1	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019	0.000	0.016	0.000	0.009	0.000	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.009	0.000	0.005		0.019
29. MVs/Ibadan.NIE/11.10	0.141	0.130	0.133	0.124	0.130	0.133	0.132	0.130	0.130	0.124	0.116	0.138	0.135	0.138	0.127	0.138	0.133	0.121	0.124	0.124	0.127	0.130	0.124	0.133	0.138	0.144	0.138	

The number of base substitutions per site from between sequences are on the right side of the table. Standard error estimate(s) are shown above the diagonal and were obtained by using analytical formulas. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 38 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 430 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

4.5.4. Phylogenetic Analysis of Sequences of Measles Virus Isolates from Throat Swab of Febrile Children with Maculopapular Rash in Lagos and Ibadan, 2007-2011 and Previous Isolates from Nigeria and Ghana of Genotype B3 Cluster 1 And 2

Figure 4.10 shows Phylogenetic analysis of measles virus isolates from this study and previous measles virus isolates from Nigeria and Ghana. Ten of the sequences of the isolates clustered with those of the reference strains and other isolated from Nigeria that belongs to Genotype B3 cluster 1. Estimation of the evolutionary divergence of the measles virus isolates from this study and previous isolates from Nigeria of genotype B3 of cluster 1 and 2 showed an overall mean distance of 0.024.Table 4.13 showed the pairwise comparison of the sequences of measles virus isolates from this study and previous measles isolate from Nigeria and Ghana of genotype B3 cluster 1 and 2. Estimates of the pairwise distance between the isolates ranges from 0.001 to 0.144.

4.5.5 Phylogenetic Analysis of Sequences Measles Virus Isolates from this study and Sequences of Other Strains of Genotype B3 Cluster 1

Figure 4.11 shows the phylogram of the sequences of measles virus isolates from this studies and previous isolates from Nigeria of genotype B3 cluster 1. All the isolates clustered together except one that formed an outlier. Estimation of the evolutionary divergence of the measles virus isolates from this study and previous isolates from Nigeria of genotype B3 cluster 1 showed an overall distance 0.018 while the pairwise distance ranges from 0.001 t0 0.144. Table 4.14 shows the estimation of the evolutionary divergence between measles virus isolates from this study and previous isolates from Nigeria of genotype B3 cluster 1.

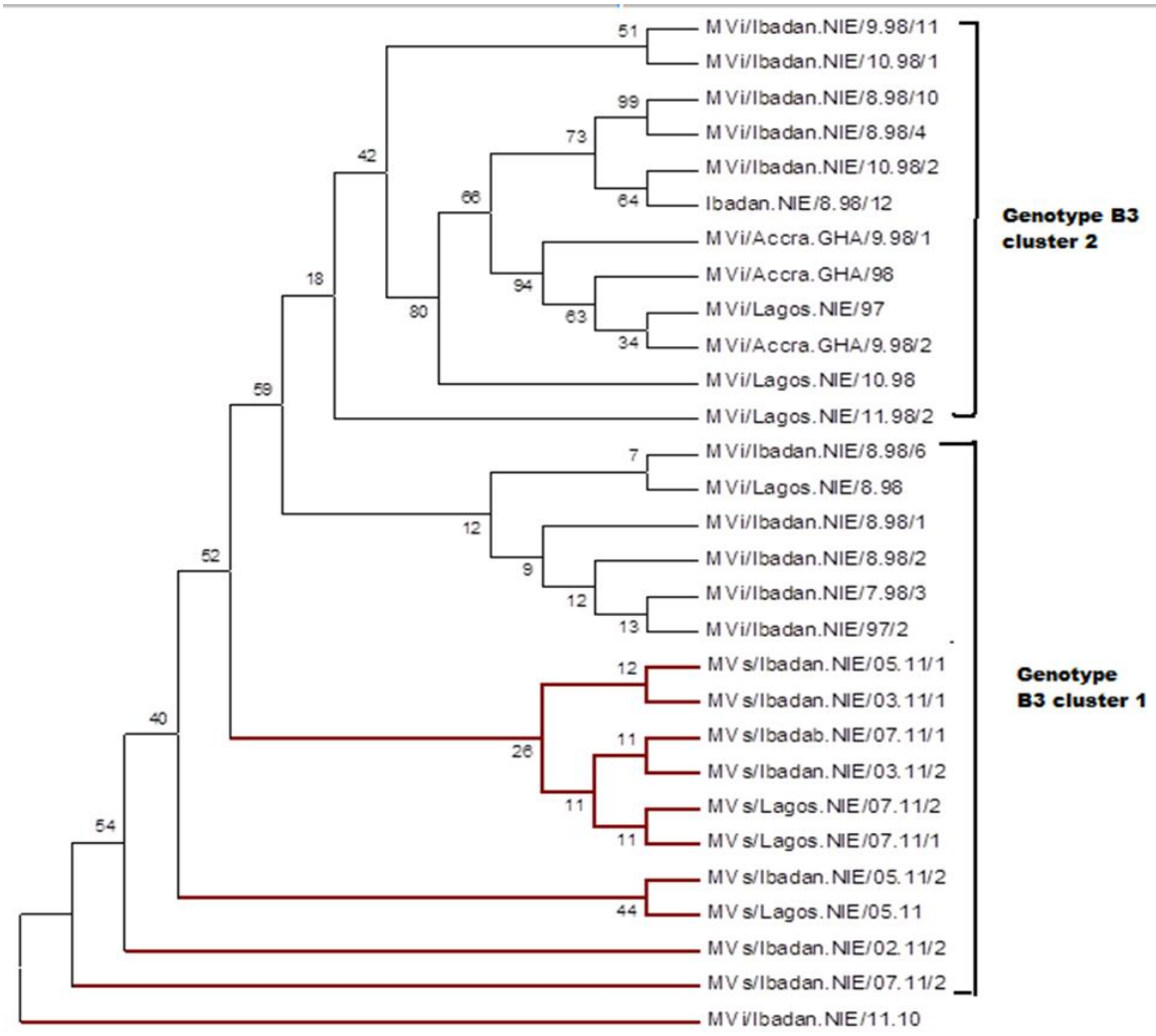


Fig 4.10: Phylogram of the sequences of measles virus isolates from this study and previous isolates from Nigerian and Ghana isolates of genotype B3 cluster 1 and 2

Table 4.13: Estimates of evolutionary divergence between study isolates and previous isolates from Nigeria and Ghana genotype B3 Sequences

M5: Pairwise Distances (C:\Users\Deewan\AppData\Local\Temp\PhyloAnalysis-37.meg)

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1. MVi/Ibadan.NIE/10.98/1		0.006	0.005	0.005	0.006	0.006	0.005	0.009	0.009	0.005	0.008	0.005	0.007	0.008	0.008	0.007	0.007	0.007	0.010	0.008	0.010	0.008	0.009	0.008	0.009	0.008	0.008	0.008
2. MVi/Lagos.NIE/11.98/2	0.014		0.002	0.002	0.003	0.004	0.002	0.009	0.009	0.005	0.008	0.002	0.007	0.008	0.008	0.007	0.007	0.007	0.009	0.006	0.009	0.006	0.008	0.006	0.008	0.006	0.007	0.006
3. MVi/Ibadan.NIE/7.98/3	0.012	0.002		0.000	0.002	0.003	0.000	0.008	0.008	0.004	0.007	0.000	0.007	0.008	0.008	0.008	0.008	0.007	0.009	0.005	0.008	0.005	0.007	0.005	0.007	0.005	0.006	0.005
4. MVi/Ibadan.NIE/97/2	0.012	0.002	0.000		0.002	0.003	0.000	0.008	0.008	0.004	0.007	0.000	0.007	0.008	0.008	0.008	0.008	0.007	0.009	0.005	0.008	0.005	0.007	0.005	0.007	0.005	0.006	0.005
5. MVi/Ibadan.NIE/8.98/6	0.014	0.005	0.002	0.002		0.004	0.002	0.009	0.009	0.005	0.008	0.002	0.007	0.008	0.009	0.008	0.008	0.007	0.009	0.006	0.009	0.006	0.008	0.006	0.008	0.006	0.007	0.006
6. MVi/Lagos.NIE/8.98	0.017	0.007	0.005	0.005	0.007		0.003	0.009	0.009	0.005	0.008	0.003	0.008	0.009	0.009	0.008	0.008	0.008	0.009	0.006	0.009	0.006	0.008	0.006	0.008	0.006	0.007	0.006
7. MVi/Ibadan.NIE/8.98/1	0.012	0.002	0.000	0.000	0.002	0.005		0.008	0.008	0.004	0.007	0.000	0.007	0.008	0.008	0.008	0.008	0.007	0.009	0.005	0.008	0.005	0.007	0.005	0.007	0.005	0.006	0.005
8. MVi/Ibadan.NIE/8.98/10	0.031	0.031	0.029	0.029	0.031	0.034	0.029		0.000	0.010	0.006	0.008	0.006	0.007	0.008	0.007	0.007	0.007	0.012	0.010	0.012	0.010	0.011	0.010	0.011	0.010	0.011	0.010
9. MVi/Ibadan.NIE/8.98/4	0.031	0.031	0.029	0.029	0.031	0.034	0.029	0.000		0.010	0.006	0.008	0.006	0.007	0.008	0.007	0.007	0.007	0.012	0.010	0.012	0.010	0.011	0.010	0.011	0.010	0.011	0.010
10. MVi/Ibadan.NIE/9.98/11	0.009	0.009	0.007	0.007	0.009	0.012	0.007	0.036	0.036		0.008	0.004	0.008	0.009	0.009	0.009	0.009	0.008	0.010	0.007	0.009	0.007	0.008	0.007	0.008	0.007	0.008	0.007
11. MVi/Ibadan.NIE/10.98/2	0.024	0.024	0.021	0.021	0.024	0.026	0.021	0.017	0.017	0.029		0.007	0.002	0.006	0.006	0.005	0.005	0.005	0.012	0.009	0.011	0.009	0.010	0.009	0.010	0.009	0.010	0.009
12. MVi/Ibadan.NIE/8.98/2	0.012	0.002	0.000	0.000	0.002	0.005	0.000	0.029	0.029	0.007	0.021		0.007	0.008	0.008	0.008	0.008	0.007	0.009	0.005	0.008	0.005	0.007	0.005	0.007	0.005	0.006	0.005
13. Ibadan.NIE/8.98/12	0.021	0.021	0.019	0.019	0.021	0.024	0.019	0.014	0.014	0.026	0.002	0.019		0.005	0.006	0.005	0.005	0.005	0.011	0.009	0.011	0.009	0.010	0.009	0.010	0.009	0.009	0.009
14. MVi/Lagos.NIE/97	0.024	0.024	0.026	0.026	0.029	0.031	0.026	0.021	0.021	0.034	0.014	0.026	0.012		0.004	0.002	0.002	0.006	0.012	0.010	0.012	0.010	0.011	0.010	0.011	0.010	0.010	0.010
15. MVi/Accra.GHA/9.98/1	0.026	0.026	0.029	0.029	0.031	0.034	0.029	0.024	0.024	0.036	0.017	0.029	0.014	0.007		0.003	0.003	0.007	0.012	0.009	0.012	0.009	0.011	0.009	0.011	0.009	0.010	0.009
16. MVi/Accra.GHA/9.98/2	0.021	0.021	0.024	0.024	0.026	0.029	0.024	0.019	0.019	0.031	0.012	0.024	0.009	0.002	0.005		0.000	0.006	0.012	0.009	0.012	0.009	0.011	0.009	0.011	0.009	0.010	0.009
17. MVi/Accra.GHA/98	0.021	0.021	0.024	0.024	0.026	0.029	0.024	0.019	0.019	0.031	0.012	0.024	0.009	0.002	0.005	0.000		0.006	0.012	0.009	0.012	0.009	0.011	0.009	0.011	0.009	0.010	0.009
18. MVi/Lagos.NIE/10.98	0.021	0.021	0.019	0.019	0.021	0.024	0.019	0.019	0.019	0.026	0.012	0.019	0.009	0.017	0.019	0.014	0.014		0.011	0.009	0.011	0.009	0.010	0.009	0.010	0.009	0.009	0.009
19. MVs/Ibadan.NIE/07.11/2	0.043	0.033	0.031	0.031	0.033	0.036	0.031	0.061	0.061	0.038	0.053	0.031	0.051	0.058	0.056	0.056	0.056	0.051		0.007	0.008	0.007	0.005	0.007	0.006	0.007	0.008	0.007
20. MVs/Ibadab.NIE/07.11/1	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.041	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019		0.006	0.000	0.005	0.000	0.005	0.000	0.003	0.000
21. MVs/Ibadan.NIE/05.11/2	0.041	0.031	0.028	0.028	0.031	0.033	0.028	0.058	0.058	0.033	0.051	0.028	0.048	0.056	0.053	0.053	0.053	0.048	0.026	0.016		0.006	0.007	0.006	0.006	0.006	0.007	0.006
22. MVs/Lagos.NIE/07.11/2	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.041	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019	0.000	0.016		0.005	0.000	0.005	0.000	0.003	0.000
23. MVs/Ibadan.NIE/02.11/2	0.033	0.024	0.021	0.021	0.024	0.026	0.021	0.051	0.051	0.029	0.043	0.021	0.041	0.048	0.046	0.046	0.046	0.041	0.009	0.009	0.021	0.009		0.005	0.006	0.005	0.006	0.005
24. MVs/Ibadan.NIE/05.11/1	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.041	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019	0.000	0.016	0.000	0.009		0.005	0.000	0.003	0.000
25. MVs/Lagos.NIE/05.11	0.033	0.024	0.021	0.021	0.024	0.026	0.021	0.051	0.051	0.028	0.043	0.021	0.041	0.048	0.046	0.046	0.046	0.041	0.014	0.009	0.016	0.009	0.014	0.009		0.005	0.006	0.005
26. MVs/Ibadan.NIE/03.11/2	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.041	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019	0.000	0.016	0.000	0.009	0.000	0.009		0.003	0.000
27. MVs/Ibadan.NIE/03.11/1	0.029	0.019	0.016	0.016	0.019	0.021	0.016	0.046	0.046	0.024	0.038	0.016	0.036	0.044	0.041	0.041	0.041	0.036	0.024	0.005	0.021	0.005	0.014	0.005	0.014	0.005		0.003
28. MVs/Lagos.NIE/07.11/1	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.041	0.041	0.019	0.034	0.012	0.031	0.039	0.036	0.036	0.036	0.031	0.019	0.000	0.016	0.000	0.009	0.000	0.009	0.000	0.005	
29. MVi/Ibadan.NIE/11.10	0.133	0.121	0.124	0.124	0.127	0.130	0.124	0.141	0.141	0.130	0.133	0.124	0.130	0.133	0.132	0.130	0.130	0.124	0.116	0.138	0.135	0.138	0.127	0.138	0.133	0.138	0.144	0.138

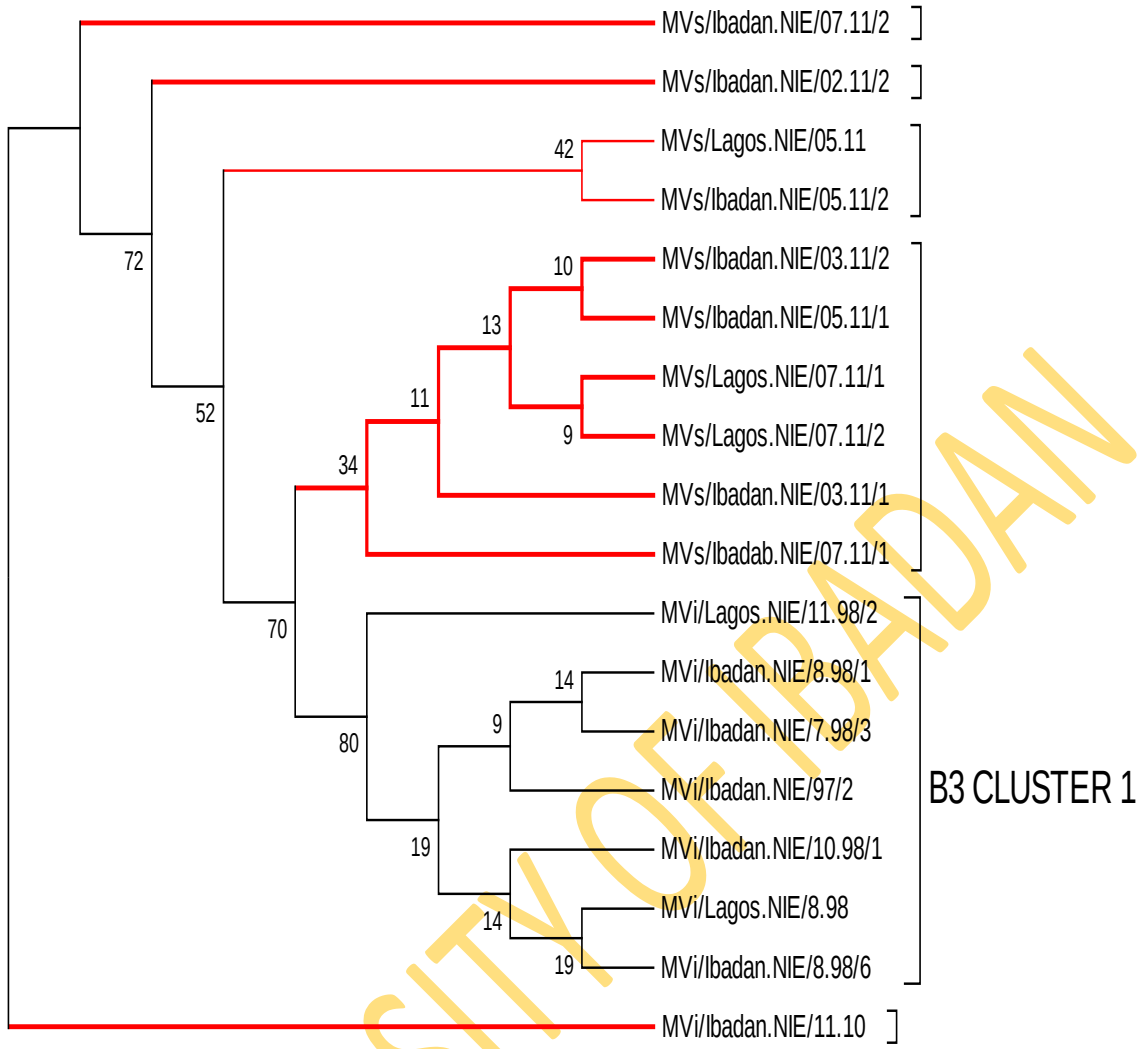

Fig 4.11: Phylogram of sequences measles isolates from this study and previous isolates from Nigeria of genotype B3 cluster 1

Table 4.14: Estimates of Evolutionary Divergence between study isolates and previous Nigerian isolated of genotype B3 cluster 1

M5: Pairwise Distances (C:\Users\Deewan\AppData\Local\Temp\PhyloAnalysis-42.meg)

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. MVi/Ibadan.NIE/10.98/1		0.006	0.005	0.005	0.006	0.006	0.005	0.010	0.008	0.010	0.008	0.009	0.008	0.009	0.008	0.008	0.008	0.019
2. MVi/Lagos.NIE/11.98/2	0.014		0.002	0.002	0.003	0.004	0.002	0.009	0.006	0.009	0.006	0.008	0.006	0.008	0.006	0.007	0.006	0.018
3. MVi/Ibadan.NIE/7.98/3	0.012	0.002		0.000	0.002	0.003	0.000	0.009	0.005	0.008	0.005	0.007	0.005	0.007	0.005	0.006	0.005	0.018
4. MVi/Ibadan.NIE/97/2	0.012	0.002	0.000		0.002	0.003	0.000	0.009	0.005	0.008	0.005	0.007	0.005	0.007	0.005	0.006	0.005	0.018
5. MVi/Ibadan.NIE/8.98/6	0.014	0.005	0.002	0.002		0.004	0.002	0.009	0.006	0.009	0.006	0.008	0.006	0.008	0.006	0.007	0.006	0.018
6. MVi/Lagos.NIE/8.98	0.017	0.007	0.005	0.005	0.007		0.003	0.009	0.006	0.009	0.006	0.008	0.006	0.008	0.006	0.007	0.006	0.019
7. MVi/Ibadan.NIE/8.98/1	0.012	0.002	0.000	0.000	0.002	0.005		0.009	0.005	0.008	0.005	0.007	0.005	0.007	0.005	0.006	0.005	0.018
8. MVs/Ibadan.NIE/07.11/2	0.043	0.033	0.031	0.031	0.033	0.036	0.031		0.007	0.008	0.007	0.005	0.007	0.006	0.007	0.008	0.007	0.018
9. MVs/Ibadab.NIE/07.11/1	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.019		0.006	0.000	0.005	0.000	0.005	0.000	0.003	0.000	0.019
10. MVs/Ibadan.NIE/05.11/2	0.041	0.031	0.028	0.028	0.031	0.033	0.028	0.026	0.016		0.006	0.007	0.006	0.006	0.006	0.007	0.006	0.019
11. MVs/Lagos.NIE/07.11/2	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.019	0.000	0.016		0.005	0.000	0.005	0.000	0.003	0.000	0.019
12. MVs/Ibadan.NIE/02.11/2	0.033	0.024	0.021	0.021	0.024	0.026	0.021	0.009	0.009	0.021	0.009		0.005	0.006	0.005	0.006	0.005	0.019
13. MVs/Ibadan.NIE/05.11/1	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.019	0.000	0.016	0.000	0.009		0.005	0.000	0.003	0.000	0.019
14. MVs/Lagos.NIE/05.11	0.033	0.024	0.021	0.021	0.024	0.026	0.021	0.014	0.009	0.016	0.009	0.014	0.009		0.005	0.006	0.005	0.019
15. MVs/Ibadan.NIE/03.11/2	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.019	0.000	0.016	0.000	0.009	0.000	0.009		0.003	0.000	0.019
16. MVs/Ibadan.NIE/03.11/1	0.029	0.019	0.016	0.016	0.019	0.021	0.016	0.024	0.005	0.021	0.005	0.014	0.005	0.014	0.005		0.003	0.020
17. MVs/Lagos.NIE/07.11/1	0.024	0.014	0.012	0.012	0.014	0.017	0.012	0.019	0.000	0.016	0.000	0.009	0.000	0.009	0.000	0.005		0.019
18. MVi/Ibadan.NIE/11.10	0.133	0.121	0.124	0.124	0.127	0.130	0.124	0.116	0.138	0.135	0.138	0.127	0.138	0.133	0.138	0.144	0.138	

4.4.6 Phylogenetic Analysis of Amino Acid Sequences of Measles Virus Isolates from Throat Swab and Urine of Febrile Children with Maculopapular Rash in Lagos and Ibadan, 2007-2011 and Who Reference Strains

Figure 4.12 shows the multiple sequence alignment of the amino acid sequences from the 450 nucleotide sequences of the carboxyl terminal of the nucleoprotein gene of measles virus isolates from throat swab and urine of febrile children with maculopapular rash in Lagos and Ibadan, 2007-2011 and WHO measles reference strains. A general 16 amino acid differences was observed when the amino acid sequence of the isolates from this study was compared to the generated consensus sequence. Figure 4.13 shows the generated phylogenetic tree of the above mentioned isolates. As observed with nucleotide sequence analysis, ten of the isolates clustered with WHO reference isolates for genotype B3 and one of the isolate clustered with the reference isolate for measles virus of clade A. An overall mean distance of 0.015 was observed in the estimates of evolutionary divergence of the amino acid sequence of the isolates.

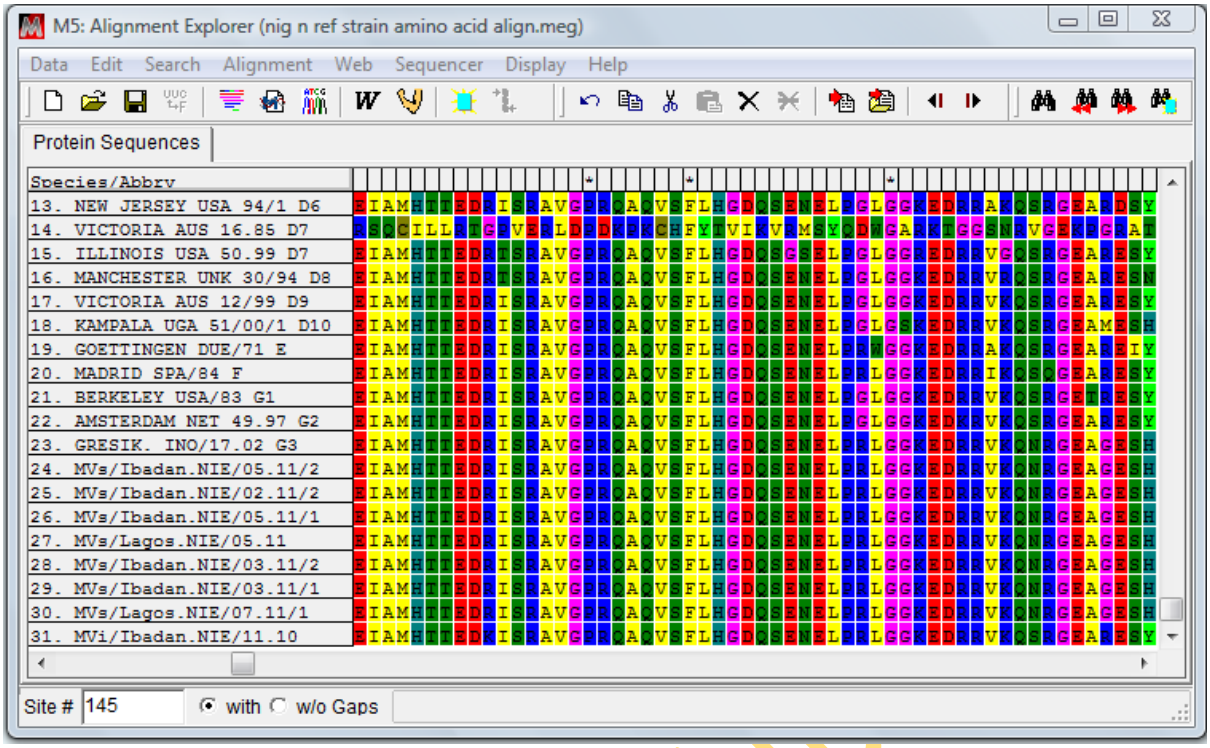


Fig 4.12: Multiple sequence alignment of amino acid sequence of the carboxyl terminal of measles virus nucleoprotein gene of measles virus isolates from throat swab and urine samples from febrile children with maculopapular rash in Lagos and Ibadan, 2007-2011 and WHO reference measles virus strains

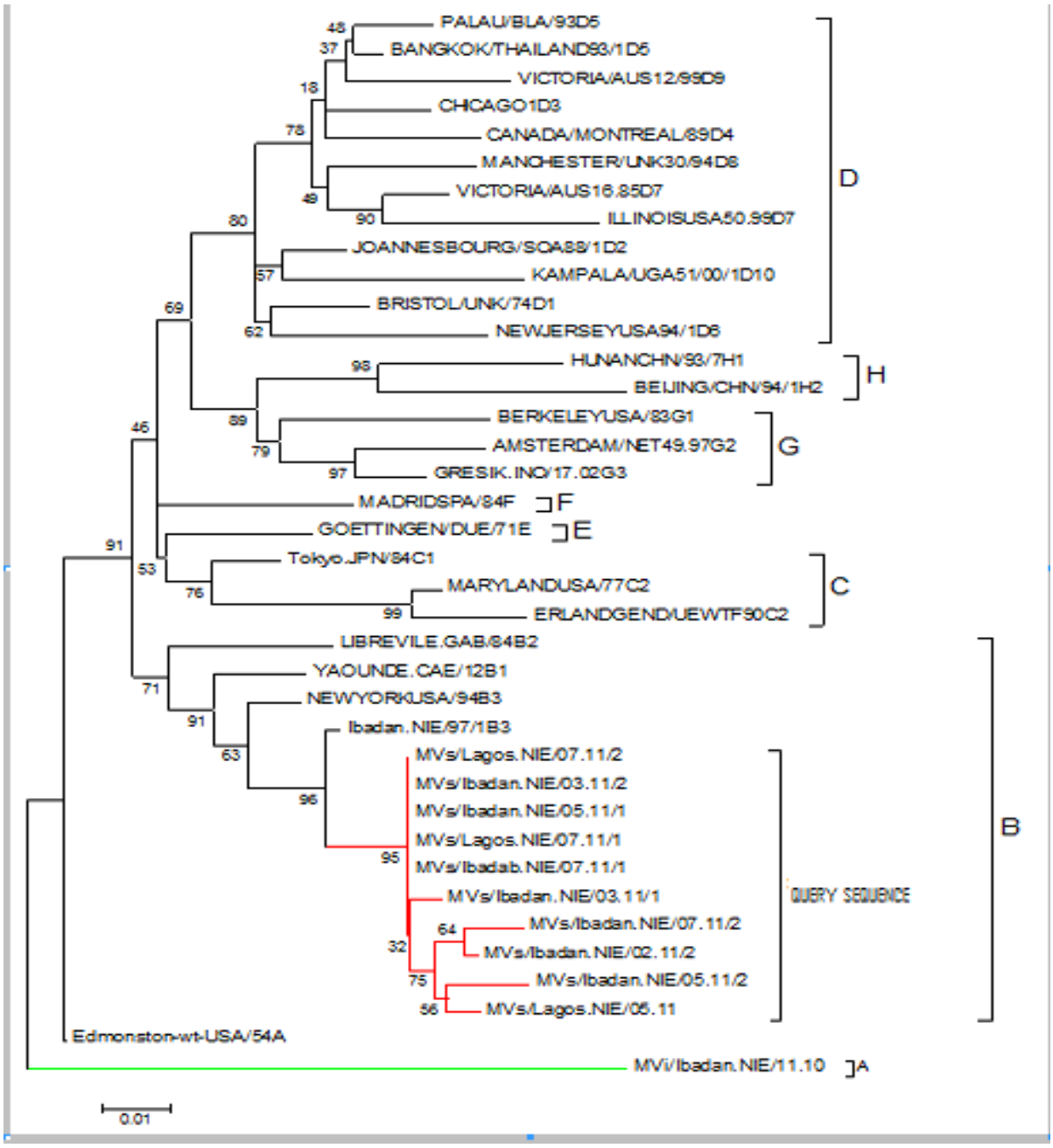


Fig 4.13: Phylogram of amino acid sequence of the carboxyl terminal of measles virus nucleoprotein gene of measles virus isolates from throat swab and urine samples from febrile children with maculopapular rash in Lagos and Ibadan, 2007-2011 and WHO reference measles virus strains

4.5.7 Blast Search of Sequence of the Divergent Isolates

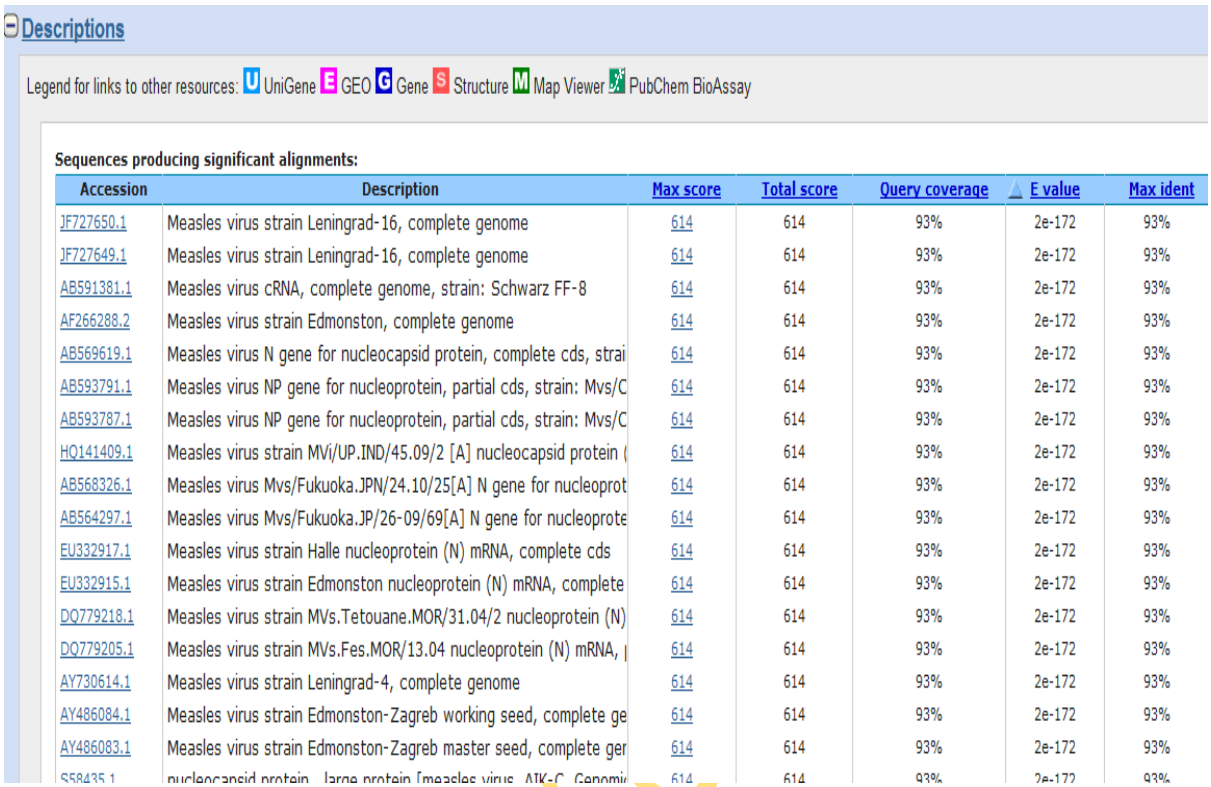
A blast search of the sequence of the divergent isolate showed it to be a measles virus isolate of genotype A with a maximum indent of 93%. Figure 4.14 shows the result of the nucleotide blast search of the divergent isolate. It shows closest similarity to measles virus of clade A with a maximum score of 614 and an E value of 2e-172.

4.5.8 Phylogenetic Analysis of The Sequences of The Divergent Isolates (MVi/Ibadan.NIE/11.10) and other Genotype A Strains from The Genebank.

Phylogenetic analysis of the sequences of the divergent isolate (MVi/Ibadan.NIE/11.10) and other genotype **A** strains from GenBank is shown in Figure 4.15 and Table 4.15. Only isolate MV1/Ibadan.NIE/11.10 clustered with other isolates of genotype A in the phylogenetic tree constructed. Other isolates from this study clustered together separately from isolates of clade A. the pairwise distance of the sequences of measles virus isolate MV1/Ibadan.NIE/11.10 and other isolates of clade A showed an average distance of 0.1 as against 14% observed with isolates of genotype B3.

4.5.9 Phylogenetic Analysis of Amino Acid Sequence of Genotype A Strains

All the downloaded genotype A sequences was translated to amino acids and phylogenetic analysis was done on the amino acid sequences by constructing a phylogenetic tree and estimating the divergence. Figure 4.16 showed the phylogram of the amino acid sequence of isolate MVi/Ibadan.NIE/11.10 and other isolates of clade A. There is an overall mean distance of 1.98 within the amino acids and a pairwise distance ranging from 0.0 to 0.4



Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JF727650.1	Measles virus strain Leningrad-16, complete genome	614	614	93%	2e-172	93%
JF727649.1	Measles virus strain Leningrad-16, complete genome	614	614	93%	2e-172	93%
AB591381.1	Measles virus cRNA, complete genome, strain: Schwarz FF-8	614	614	93%	2e-172	93%
AF266288.2	Measles virus strain Edmonston, complete genome	614	614	93%	2e-172	93%
AB569619.1	Measles virus N gene for nucleocapsid protein, complete cds, strai	614	614	93%	2e-172	93%
AB593791.1	Measles virus NP gene for nucleoprotein, partial cds, strain: Mvs/C	614	614	93%	2e-172	93%
AB593787.1	Measles virus NP gene for nucleoprotein, partial cds, strain: Mvs/C	614	614	93%	2e-172	93%
HQ141409.1	Measles virus strain MVi/UP.IND/45.09/2 [A] nucleocapsid protein (	614	614	93%	2e-172	93%
AB568326.1	Measles virus Mvs/Fukuoka.JPN/24.10/25[A] N gene for nucleoprot	614	614	93%	2e-172	93%
AB564297.1	Measles virus Mvs/Fukuoka.JP/26-09/69[A] N gene for nucleoprote	614	614	93%	2e-172	93%
EU332917.1	Measles virus strain Halle nucleoprotein (N) mRNA, complete cds	614	614	93%	2e-172	93%
EU332915.1	Measles virus strain Edmonston nucleoprotein (N) mRNA, complete	614	614	93%	2e-172	93%
DQ779218.1	Measles virus strain MVs.Tetouane.MOR/31.04/2 nucleoprotein (N)	614	614	93%	2e-172	93%
DQ779205.1	Measles virus strain MVs.Fes.MOR/13.04 nucleoprotein (N) mRNA,	614	614	93%	2e-172	93%
AY730614.1	Measles virus strain Leningrad-4, complete genome	614	614	93%	2e-172	93%
AY486084.1	Measles virus strain Edmonston-Zagreb working seed, complete ge	614	614	93%	2e-172	93%
AY486083.1	Measles virus strain Edmonston-Zagreb master seed, complete ger	614	614	93%	2e-172	93%
S58435.1	nucleocapsid protein, large protein [measles virus, AIK-C, Genomi	614	614	93%	2e-172	93%

Figure 4.14: Nucleotide Blast result of the divergent isolate (MVi/Ibadan.NIE/11.10)

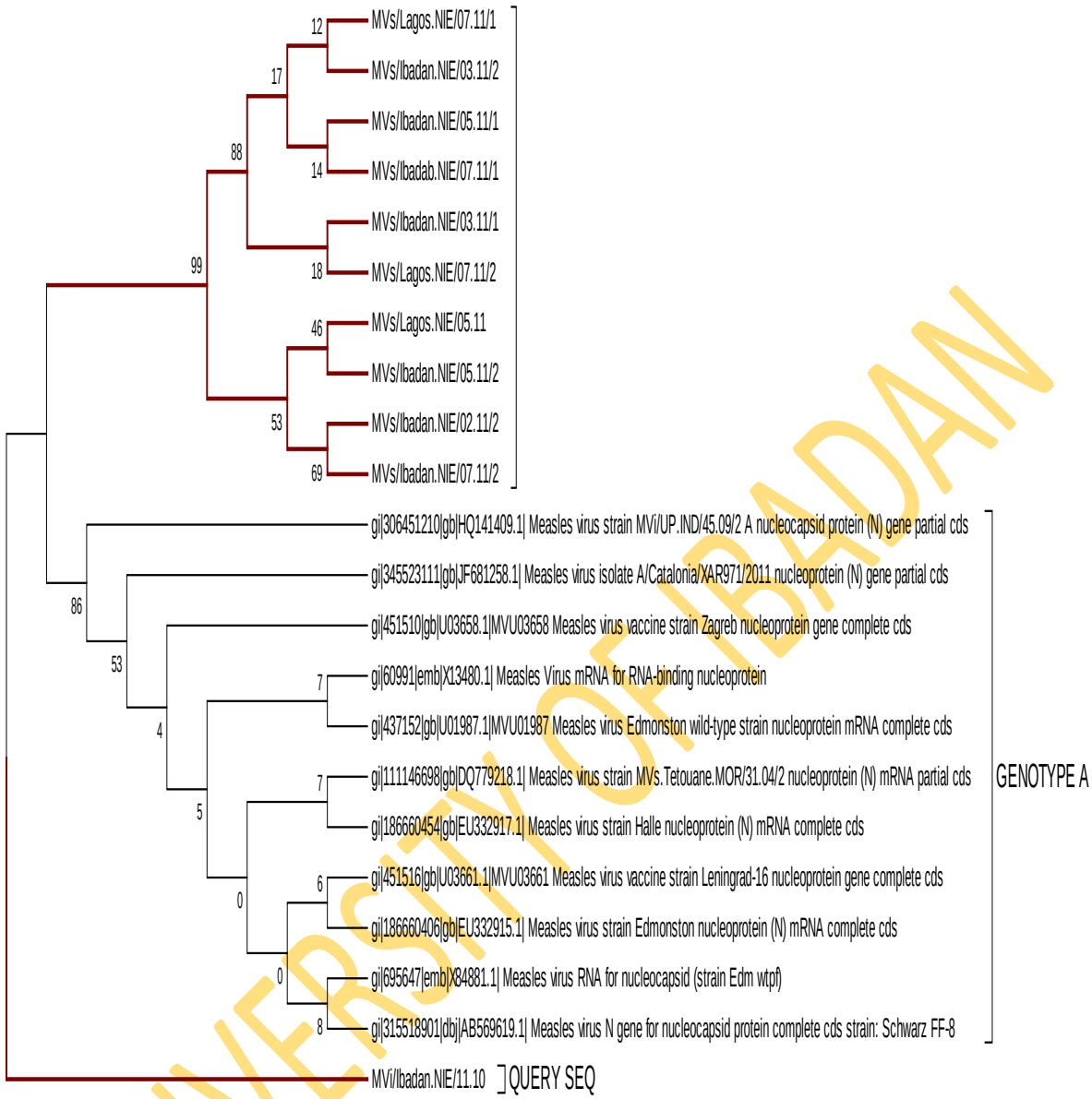
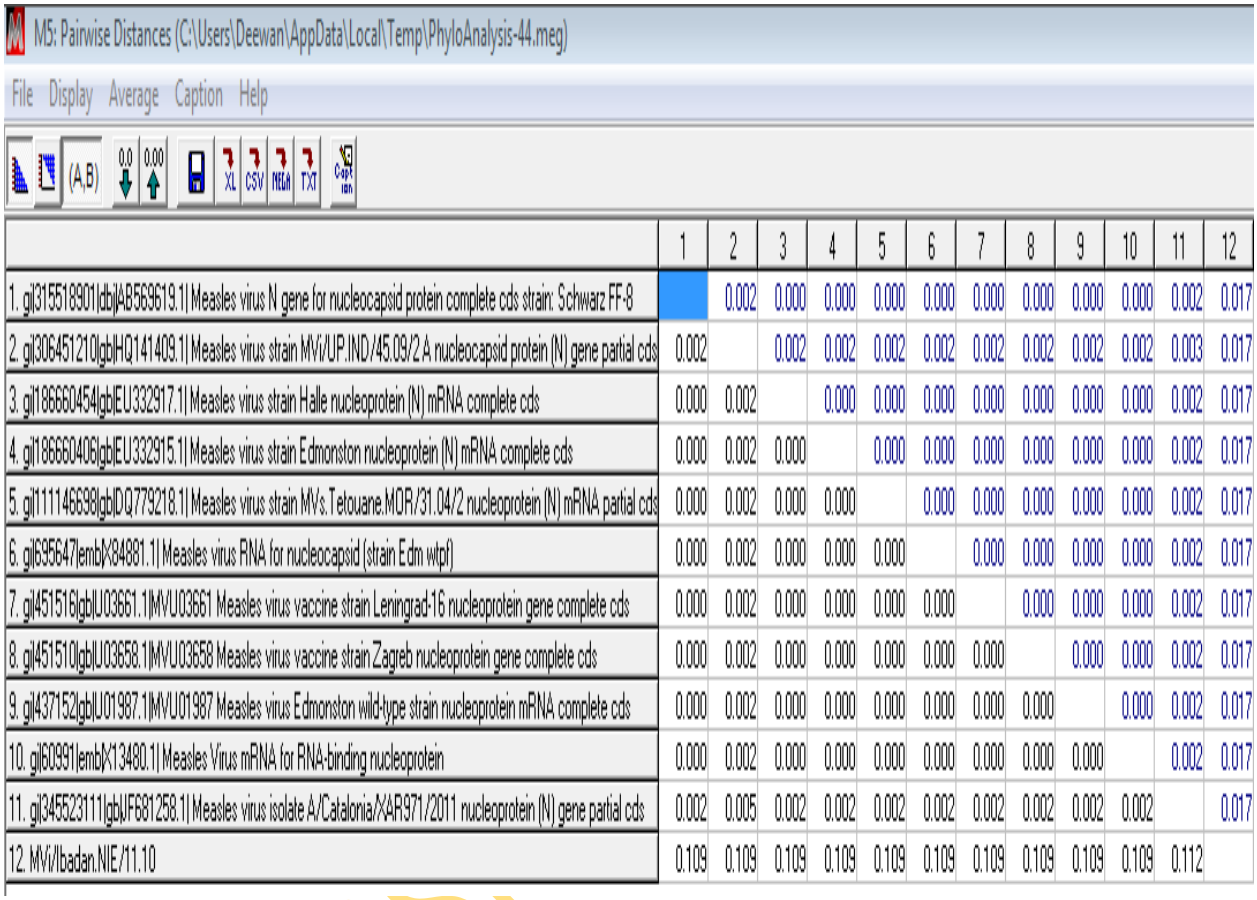


Fig 4.15: Phylogram of sequences of study isolates and measles strains of Clade A

Table 4.15: Estimate of Evolutionary Divergence between sequences of Nigerian measles virus of genotype A (MV1/Ibadan.NIE/11.10) and other isolates of genotype A


M5: Pairwise Distances (C:\Users\Deewan\AppData\Local\Temp\PhyloAnalysis-44.meg)

File Display Average Caption Help

	1	2	3	4	5	6	7	8	9	10	11	12
1. gi|315518901|dbj|AB569619.1| Measles virus N gene for nucleocapsid protein complete cds strain: Schwarz FF-8		0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.017
2. gi|306451210|gb|HQ141409.1| Measles virus strain MVi/UP.IND/45.09/2.A nucleocapsid protein (N) gene partial cds	0.002		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.017
3. gi|186660454|gb|EU332917.1| Measles virus strain Halle nucleoprotein (N) mRNA complete cds	0.000	0.002		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.017
4. gi|186660406|gb|EU332915.1| Measles virus strain Edmonston nucleoprotein (N) mRNA complete cds	0.000	0.002	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.017
5. gi|111146698|gb|DQ779218.1| Measles virus strain MVs.Tetouane.MOR/31.04/2 nucleoprotein (N) mRNA partial cds	0.000	0.002	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.002	0.017
6. gi|69564|emb|X84881.1| Measles virus RNA for nucleocapsid (strain Edm wtpf)	0.000	0.002	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.002	0.017
7. gi|451516|gb|U03661.1|MVU03661 Measles virus vaccine strain Leningrad-16 nucleoprotein gene complete cds	0.000	0.002	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.002	0.017
8. gi|451510|gb|U03658.1|MVU03658 Measles virus vaccine strain Zagreb nucleoprotein gene complete cds	0.000	0.002	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.002	0.017
9. gi|437152|gb|U01987.1|MVU01987 Measles virus Edmonston wild-type strain nucleoprotein mRNA complete cds	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.002	0.017
10. gi|60991|emb|X13480.1| Measles Virus mRNA for RNA-binding nucleoprotein	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.002	0.017
11. gi|345523111|gb|JF681258.1| Measles virus isolate A/Catalonia/X4AR971/2011 nucleoprotein (N) gene partial cds	0.002	0.005	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002		0.017
12. MVi/Ibadan.NIE/11.10	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.112	

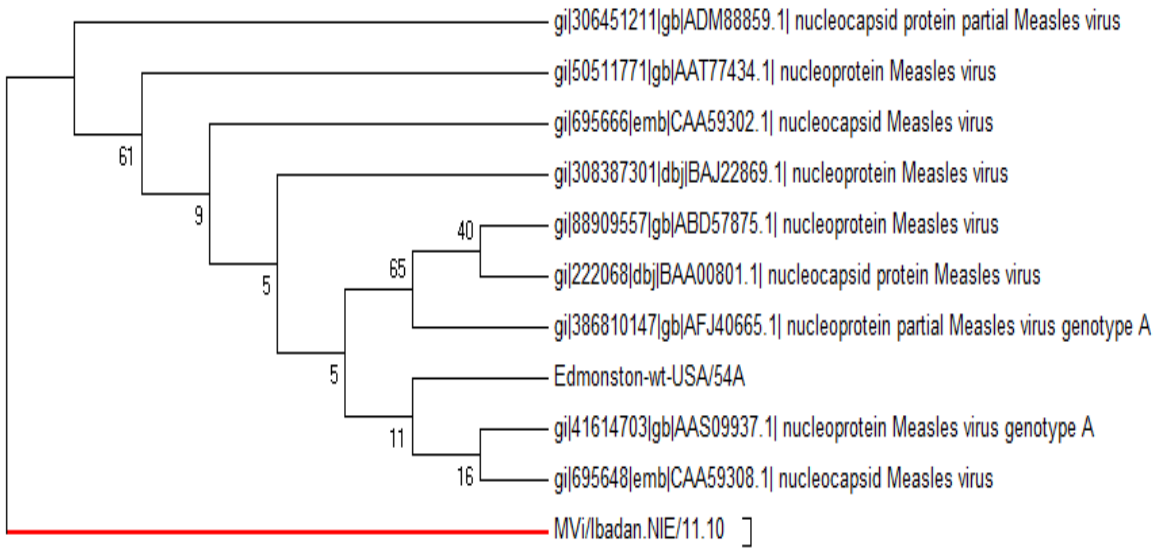


Figure 4.16: Phylogram of amino acid sequence of isolate MVi/Ibadan.NIE/11.10 and isolates of Clade A

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CHAPTER FIVE

DISCUSSION

Eleven of the 23 measles virus isolated in this study had readable nucleotide sequences. The phylogram generated from the aligned nucleotide sequences of the eleven measles virus isolates and sequences of the WHO reference strains showed 10 of the isolates clustering with the reference strain of genotype B3 cluster 1 thus indicating that the isolates belong to genotype B3.cluster1.

This is similar to the findings of Haness *et al.* (1999) and Kremer *et al.* (2010). They found a repeated circulation of genotype B3 in Nigeria between 1997 to 1998 and 2004 to 2005 respectively. Rota *et al.*, (2011) in a review of global distribution of measles genotypes observed that measles genotype B3 is the most frequently reported genotype in the Africa region and was detected in 197 (89%) of 220 reported sequences from 21 countries where viral genotype studies has been done. Genotype B3 is the endemic genotype in most of the African continent except in the Northern African countries (Rota *et al.*, 2011) and in Ethiopia where measles genotype D4 is main circulating strain (Nigatu *et al.*, 2001). Genotype B3 cluster 1 viruses have also been previously reported from Cameroon, Ghana, Kenya and Tanzania (Global Distribution of Measles Genotypes, 2011). The continuous circulation and isolation of measles virus of genotype B3 in Nigeria suggests an uninterrupted endemic transmission of the virus genotype in the country from 1997 to 2011

The nucleotide and amino acid differences observed within the in the measles virus strains isolated in this study showed a high level of diversity. There were 35 nucleotide differences found within the sequences of the isolates when compared with the generated consensus sequence and the overall mean distance of 0.08 within the isolates as well as 0.075 when aligned with WHO reference sequences. This shows a high variability of the isolates.

Analysis of the amino acid sequences showed a general 16 amino acid differences from the consensus sequence and an overall mean distance of 0.015 from the aligned amino acid sequences from the virus strains isolated in this study as well as a mean distance of 0.09 when the amino acid sequences of these isolates and WHO reference strains. The amino acid differences found demonstrated that the nucleotide differences found are not just silent differences but translated to amino acid differences.

The genetic heterogeneity of the virus isolates in this study is not a result of increased mutation rates but is due to the presence of multiple co-circulating lineages which is sustained by presence of high population of susceptible children in Ibadan and Lagos where the isolates were obtained. This shows that multiple lineages of genotype B3 cluster I are co-circulating in the region and thus consistent with an endemic genotype. This is similar to the report by Akhalesh *et al.*, (2012) where 0.2 to 0.3% differences were found in the sequences of viruses isolated in India between 2009 and 2011.

The consistently less than optimal vaccination coverage among Nigerian children during the duration of the study as reported by the WHO (2011) could also be responsible for the high variability found in this study as the mutations found among the isolates appears to be random and not driven by vaccine pressure or immune responses. WHO reported that 9% of the 20 million children who had not received first dose of measles vaccine are from Nigeria (WHO Fact sheet 2011).

Importation of measles virus from neighbouring countries could also be responsible for the high diversity found in the nucleotide and amino acid sequences of the virus isolates in this study. Phylogenetic comparison of genotype B3 strains from Nigeria with those from other countries in Africa such as Ghana and Congo suggests that transmissions of measles virus between these countries and Nigeria are frequent. Consequently the diversity of genotype B3

in Nigeria reflects the overall genetic diversity of measles genotype B3 in Africa (Kremer *et al.,* 2010).

If the level of measles vaccination could be increased in Nigeria, there will be a reduction in available susceptible individuals and thereby limiting the lines of transmission of different virus strains. This was observed in Burkina Faso where low variability (1.5%) of the measles viruses isolated in 2001 was attributed to higher vaccination coverage (Mulders *et al.,* 2003, Kremer *et al.,* 2010).

The high diversity of the measles viruses in Nigeria as seen in this study was similar to what is found in the People's Republic of China when wild measles viruses isolated between 1995 and 2003 were analysed. Multiple lineages of measles virus genotype H1 with a diversity of 5.3% in the high variable region of N gene was observed and the genotype was found to be circulating without geographic restriction (Zhang *et al.*, 2007).

It was observed that one of the measles virus isolates (MVi/Ibadan.NIE/11.10) had the most variability compared to the others with a 44 nucleotide difference and 26 amino acid differences from the generated consensus nucleotide and amino acid sequences respectively. This indicates an isolate of different genotype from the other isolates within the group. The Isolate clustered with the WHO reference measles virus isolate for clade A in the generated phylogram of both nucleotide and amino acid sequences. Similarly the isolate also showed 93% homology to other isolates of clade A in the GenBank.

The isolation of a virus that has high sequence homology to viruses of clade A indicated that viruses of different genotypes from the known genotype B3clusters 1 and 2 that has been reported could be circulating in Nigeria. However the virus could also have been imported into the country, although the patient from whom the virus was isolated had no history of travelling outside the country.

Measles virus of genotype A has previously been detected in acute cases of measles in South and North America, China, Japan, Eastern Europe, Finland and the UK and South Africa over the last 40 years (Michaela *et al.*, 2005), but there had not been any report of the clade in West Africa. It is important to note that all the measles virus live attenuated vaccine viruses belong to the clade A whether they are derived from the Edmonston strain (Attenuvax, Edmonston-Zagreb, AIKC, Scwarz) or from temporally and geographically independent wild type isolates (Shanghai-191, China, Changchun-47, China, CAM-70 among others). Thus reports of isolation of wild type virus of clade A is always treated with caution since it is difficult to distinguish wild-type viruses in genotype A from vaccine strains and some of the sequences may have been derived from vaccine associated cases or the result of laboratory contamination (Michaela *et al.*, 2005). Genotype A measles viruses have been isolated from a few sporadic measles cases in the past, but there have not been any reports of the genotype been associated with any large outbreaks. The viruses isolated in this study are from children who had never received any form of measles vaccination and there was no activities involving measles virus vaccine in the laboratory as at the time of the virus isolation. Thus this virus strain could have been possibly imported to Nigeria from other regions or could be an unreported circulating strain.

Isolation of measles virus genotypes different from the known genotypes circulating in a region has previously been reported. Demanou *et al.*, (2013) isolated measles virus of genotype B3 cluster 3 in Cameroon which previously was isolated only in Libya before then. genotype B3 cluster 1 and 2 that was previously reported in Cameroon. Although the different virus strain that was isolated in Cameroon could be traced to Libya through Chad, the different strain isolated in this study could not be traced to any other African country.

In this study, measles virus was isolated from children presenting at health centres in south western Nigeria with fever and rash between 2007 and 2011. Molecular characterization of

the isolates was done using the WHO recommendation for analysis of the 450 nucleotides region which codes for the carboxyl terminal 150 amino acids of measles virus N protein the most variable region of the virus with a diversity that could be as high as 12% between wild type viruses (Xu *et al.*, 1998).

Twenty one (9.2%) measles isolates were obtained from throat swab and urine samples from 286 children using primers targeted at the carboxyl end of the measles virus N gene. All the isolates were from children whose serum samples were measles virus IgM positive. The low positivity rate by RT-PCR testing for measles RNA compared to the specific IgM antibodies could be due to the nature of measles virus RNA which is very thermolabile and can easily be destroyed. Both throat swab and urine samples had equal positivity rate as all the samples that tested positive to measles RNA in throat swab also tested positive in urine. The fact that measles RNA could be detected in both throat and urine samples indicated that both samples could be used alternatively in testing for measles virus RNA and is in line with WHO recommendation in WHO manual for the laboratory diagnosis of measles virus infection (Dec 1999), but throat swab was the easiest to collect of all the samples as it could be collected from the affected children anytime with minimal discomfort.

Of the 1200 children presenting with fever and maculopapular rash included in this study, 10.7% had measles specific IgM antibodies. Detection of measles IgM remains the gold standard for diagnosis measles infection as recommended by the WHO in surveillance guidelines for measles, in the WHO African Region (WHO 2009). The seropositivity rate of 10.7 % of measles IgM found is similar to the 9.9% reported by Umeh and Ahaneku in 2013. However, the seropositivity rate of 10.7% found in this study is lower than the 17% reported by Mohammed *et al.*, (2010) among children presenting with measles related 2006 and 2009 in Nigeria. The lower rate observed in this study could be because only children presenting at health centres and general hospitals were recruited and this does not take into account

infections that were not reported in hospitals as against the study by Mohammed *et al.*,. that included not only children presenting at hospitals but also other contacts of these children that did not report to an hospital and health centre during their study period (Mohammed *et al.*, 2010). Another reason for lower rate than reported by Mohamed *et al.*'s study could be because the study of Mohamed *et al.*, (2010) involved all states and region in Nigeria including the Northern region where a high number of measles cases are reported each year as against the south-western region with fewer reported cases.

The measles virus IgM prevalence of 10.7% found in this study showed that measles infection is still a major challenge to Nigerian health system. Nigeria still ranked high among countries reporting highest measles infections yearly as seen in the WHO (2012). Nigeria reported 18, 843 cases of measles in 2011 along with DRC (134, 042 cases), Ethiopia (3255 cases) France (14, 949 cases) and India (29, 339 cases) (WHO measles report 2012).

Although Nigeria is one of the countries committed to measles elimination by 2020 (WHO 2012), recurrent outbreaks still experienced in the country and this poses a serious challenge to the elimination efforts and it shows that the national health systems need strengthening so that they can provide effective immunization services and laboratory-supported surveillance for vaccine-preventable diseases to all children especially measles cases and deaths. The outbreaks also indicate the need to ensure that parents are fully aware of the benefits of immunization and the risks associated with not vaccinating children as 39% of the children recruited in this study has never received at least one dose of measles vaccination despite the ongoing measles vaccination program in the EPI and the supplementary immunization campaign that took place within the study period.

It was observed that 26.5% of the children who tested positive to measles specific IgM antibodies had received at least one dose of measles vaccination. This finding is similar to the

report by Bassey *et al.,* (2011) where they found 26.2% of the children with measles IgM antibodies to have received at least one dose of measles vaccination. Measles infection among previously vaccinated children has been reported to be milder and usually subclinical (Ababy *et al.,* 1986) and is probably due to vaccine failure. It is not known from this study whether primary vaccine failure or secondary was the major cause as there was no data available on the seroconversion status of the children after vaccination. Causes of primary vaccine failure could be failure of the cold chain, inadequate viral dose and host immune factors such as persistence of maternal immunity (WHO, 2014). While nutritional status of the children as well as presence of other underlying diseases such as malaria and HIV among others could be responsible for secondary vaccine failure. Malaria is still endemic in Nigeria and the infection is known to interact with EPI vaccines. Treatment with drugs known to interact with the measles vaccines may also cause vaccine failure (Bill, 2012). Also, further studies on the interactions between HIV treatment and measles vaccination in the country are needed. Increase in vaccine failures will reduce the herd immunity thereby aiding the endemicity of measles in the country.

Although, not significant (p=0.547) when tested by ANOVA, a higher percentage of female children (11.3%) were infected with measles compared to males (10.3%) in this study. This is similar with previous reports by Bassey *et al.,* (2010). They found more female (37.4%) children being infected than male (27.7%) and a significant association was found among female children having higher infection rate compared to their male counterparts. The higher positive rate in female observed in this study is in line with reports that female children are slightly more susceptible to measles infection (Martins *et al.,* 2009).

The highest rate of measles IgM seropositivity was found among children aged less than nine months (11.5%) when measles vaccination is administered in Nigeria. This agrees with the findings by Ntshoe *et al.,* (2013) that children under five years are more susceptible to

measles infection in Africa. They found a high proportion of children <1 year to be mostly affected by measles in a 3 years survey in South Africa.

The high risk of infection among this age group could be because this is the age range at which most children start attending day care and crèche and thus mix with other children and thereby become exposed to measles infection. A considerable number of less than a year old children were also found to be infected with measles (10.4%) in this study. This may be due to waning maternal antibodies for those not vaccinated and primary vaccine failure among those vaccinated. The early clearing of maternal antibodies could be because much of the nursing mothers in present day Nigeria would be protected from measles infection by vaccination rather than by natural infection. Since measles is endemic in Nigeria, vaccinating at an earlier month than nine months may not make much difference as the children are likely to become exposed to the virus very soon after vaccination. In addition, children age >3-5 years was found to have the least rate of infection. This is because most children would have either received measles vaccine or infected with the wild virus before attaining this age. Measles infection confers lifelong immunity on infected children thus children under five year of age are the target population for measles infection in Africa (Moss, 2008).

Although not significant there was a slight decline in the prevalence of measles from 2008 (11.3%) to 2011 (10.5%) was obtained in this study and this could be due to the effect of improvement in measles vaccination and the supplementary immunization campaign that occurred during the period of the study.

Children who tested negative for measles specific IgM antibodies were investigated for other viral causes of febrile rash. Rubella virus IgM antibodies were detected in 24.7% of the 1081 measles IgM negative children. The prevalence of 24.7% obtained in this study is higher than the 12.0% obtained in a survey of rubella IgM in individuals with febrile rash illness in Akwa

Ibom state Nigeria (Enya *et al.*, 2011), but lower than the findings from in Jos Nigeria where rubella IgM seropositivity of 45.2% (Junaid *et al.*, 2011) was reported. James *et al.*, (2011) also reported a prevalence of 24% seropositivity to rubella IgM in suspected measles cases between 2002 and 2009 in the WHO African region. Children less than three years were seen to be more affected than those older and this is similar to what was observed in the study conducted in Jos Nigeria (Junaid *et al.*, 2011) and this may be due to risk factors like frequent exposures to already infected people in congregate environments like schools and play ground.

The first step in determining an appropriate immunization policy for rubella virus for a given population is to carry out a serologic survey in order to determine the sero-prevalence of rubella antibodies (WHO, 2009). Nigeria is among the countries that are yet to set rubella virus elimination goals and does not have an established integrated measles, rubella and congenital rubella syndrome surveillance programme thus the country have not included rubella vaccine in their routine immunization programme.

Parvovirus B19, another known cause of febrile rash was also investigated among the children in this study that tested negative to measles specific antibodies. A seropositivity rate of 11.6% was found for B19 specific IgM among the 807 children that tested negative for both measles and rubella virus. The varied rate of positivity to B19V IgM antibodies across the years found in this study could be as a result of an outbreak as parvovirus B19 outbreaks has been reported to occur in every 3-6 years in some regions. (Wildrig *et al.*, 2007) Although the highest percentage of infection was found in 2008, there was a consistent high level of infection ranging from 8.9 to 16.3 % over the years. This shows that the virus is endemic in Nigeria. There was no significant difference in the rate of infection across the gender in this study as also found by Berns *et al.*, (2000).

The 11.6% prevalence of parvovirus B19 IgM found in the study is lower than 39.5% reported among healthy children of 0-15 years (Akinsulie *et al.,* 2007) This variance in positivity rate could be because the children recruited in this study are those presenting at hospitals and health centres and B19V infection in most children are often very mild and does not require medical care The age group one to three years found to have the highest rate of infection is consistent what was obtained by Akinsulie *et al., (*2007) where the children less than 3 years of age had the highest rate of current infection even among healthy children. This indicated that most Nigerian children get exposed to the infection at early age.

The prevalence of 11.6% of parvovirus B 19 specific IgM antibodies observed in this study is lower than 35% reported by Candotti *et al.,* (2004) among Ghanaian children and 39.5% by Akinsulie *et al.,* (2007) but similar to 12.7% that was reported in a study conducted in Brazil by Mendonca *et al.,* (2005), among patients with febrile exanthematic diseases.

A large proportion of the children with fever and maculopapular rash tested negative to measles, rubella and parvovirus B19 specific IgM in this study. This suggests that other viral pathogens such as dengue, human herpes virus 6 or 7, West-Nile virus and non-polio Enteroviruses (Coxsackie and Echo virus) may be responsible for such clinical symptoms presented by the children (Siennicka *et al.,* 2011 and Ivna de Melo *et al.,* 2011,).

In summary, 11 measles virus isolates were sequenced and genotype in this study. Ten of the isolates were found to belong to clade B (genotype B3 cluste 1) while one isolate has a high sequence similarity to measles virus of clade A. A prevalence of 10.7% for measles virus specific IgM was also found among children presenting with fever and maculopapular rash as well as 24.7% for rubella virus and 11.9% for parvovirus B19.

CHAPTER SIX

SUMMARY AND CONCLUSION

In this study it was observed that there is a high prevalence of measles virus infection in febrile children with maculopapular rash in Lagos and Ibadan, Nigeria, 2007-2011 as against the impression that the disease is predominant in the Northern part of the country. It was also noted that prevalence of measles infection in southwestern Nigeria remains the same between 2007 and 2011despite supplemental measles immunization activities carried out in 2008 and 2011. Female children were more affected by the disease than male although the difference is not significant.

Twenty three measles isolates was obtained from throat swab and urine by polymerase chain reaction in this study. Eleven of the 23 isolates were successfully sequenced. Ten of the 11 sequenced isolates clustered with WHO reference measles virus strain of genotype B3 cluster 1 and the predominant circulating strains were of genotype B3. This suggest that there is likelihood of uninterrupted transmission of endemic viruses in this part of the country. One of the isolates showed high sequence similarity with strains of clade A. This is the first report of measles virus detection with high sequence similarity with clade A viruses in West Africa. The isolates could have been imported into the region or may have been circulating without being detected.

Two other viruses: rubella virus and parvovirus that present with fever and maculopapular rash were also found to be endemic in southwestern part of Nigeria.

In the context of the WHO measles control programme, this study has shown the need for enhanced molecular surveillance of measles viruses circulating in Nigeria. Therefore it is necessary to include laboratory investigation for rubella and parvovirus B19 in the routine

diagnosis and surveillance for measles in Nigeria, as all the children enrolled for the study were presumed to have measles virus infection.

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Appendix 1

- MVs/Ibadab.NIE/07.11/2

CGGTATTACCCCATTGGCCTCTGAACTCGGTATAACTGCCGAGGATGCAAAGCTT
GTTTCAGAGATTGCAATGCATACTACTGAGGACAGGATCAGCAGAGCAGTTGGA
CCCAGACAAGCCCAAGTGTCATTTCTACACGGTGATCAAAGTGAGAATGAGCTG
CCGAGATTGGGGGGTAAGGAGGACAGGAGGGTCAAACAGAACCGAGGAGAAGC
CGGGGAGAGCCACAGAGAAACCGAGCCCAGCAGAGCAAGTGATGCGAGAGCTG
CCCATCCTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGTTCAGCCA
AGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGGCTGCAAGCCAT
GGCAGGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCTAGAGTGTACAA
TGATAGAGACCTTCTAGACTAGGTGCGAGGGGCCGAGGACCAGAACAACATCCG
CCTACCCTAGGGGAGGTGGTCC----------

- MVs/Ibadan.NIE/05.11/2

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AAGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACAGGATCAGCAGAGC
AGTTGGACCCAGACAAGCCCAAGTGTCATTTCTACACGGTGATCAAAGTGAGAA
TGAGCTGCCGAGATTGGGGGGTAAGGAGGACAGGAGGGTCAAACAGAACCGAG
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AGAGCTGCCCATCCTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGT
TCAGCCAAGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGGCTGC
AAGCCATGGCAGGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCTAGA
GTGTACAATGATAGAGACCTTCTAGACTAGGTGCGAGGGGCCGAGGACCAGAAC
AACATCCGCCTACCCTAAAGGGGAGGGGGCC—

- MVs/Ibadabn.NIE/05.11/2

CTGGAAGGTCATCTCCATTGGCATCTGAACTCGGTATAACTGCCGAGGATGCAAG
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TGGACCCAGACAAGCCCAAGTGTCATTTCTACACGGTGATCAAAGTGAGAATGA
GCTGCCGAGATTGGGGGGTAAGGAGGACAGGAGGGTCAAACAGAACCGAGGAG
AAGCCGGGGAGAGCCACAGAGAAACCGAGCCCAGCAGAGCAAGTGATGCGAGA
GCTGCCCATCCTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGTTCA
GCCAAGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGGCTGCAAG
CCATGGCAGGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCTAGAGTGT
ACAATTATATATTCCTTCTAGACTAGGTGCGAGGGGCCGAGGACCAGAACAACA
TCCGCCTACCCTACATAGAGTGG---------

- MVs/Lagos.NIE/05.11/1

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TTGGACCCAGACAAGCCCAAGTGTCATTTCTACACGGTGATCAAAGTGAGAATG
AGCTGCCGAGATTGGGGGGTAAGGAGGACAGGAGGGTCAAACAGAACCGAGGA
GAAGCCGGGGAGAGCCACAGAGAAACCGAGCCCAGCAGAGCAAGTGATGCGAG
AGCTGCCCATCCTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGTTC
AGCCAAGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGGCTGCAA
GCCATGGCAGGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCTAGAGTG
TACAATGATAGAGACCTTCTAGACTAGGTGCGAGGGGCCGAGGACCAGAACAAC
ATCCGCCTACCCTAAAGGGAATA----------

- Mvs/Ibadan.NIE/07.11/1

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ATGCAAGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACAGGATCAGCA
GAGCAGTTGGACCCAGACAAGCCCAAGTGTCATTTCTACACGGTGATCAAAGTG
AGAATGAGCTGCCGAGATTGGGGGGTAAGGAGGACAGGAGGGTCAAACAGAAC
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TGCGAGAGCTGCCCATCCTCCAACCGGCACACCCCTAGACATTGACACTGCATCG
GAGTTCAGCCAAGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGG
CTGCAAGCCATGGCAGGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCT
AGAGTGTACAATGTTTTAGTACTTTCTAGACTAGGTGCGAGGGGCCGAGGACCA
GAACAACATCCGCCTACCCTACGGGGAGTAGA

- MVs/Ibadan.NIE/03.11/1

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AGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACAGGATCAGCAGAGCA
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GAGCTGCCCATCCTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGTT
CAGCCAAGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGGCTGCA
AGCCATGGCAGGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCTAGAGT
GTACAATGATAGAGACCTTCTAGACTAGGTGCGAGGGGCCGAGGACCAGAACAA
CATCCGCCTACCCTAAGAGAGAAGGGG------

- MVs/Lagos.NIE/07.11/112

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AGCTGCCCATCCTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGTTC
AGCCAAGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGGCTGCAA
GCCATGGCAGGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCTAGAGTG
TACAATGATAGAGACCTTCTAGACTAGGTGCGAGGGGCCGAGGACCAGAACAAC
ATCCGCCTACCCTAACTGGAGT----------

- MVs/Ibadan.NIE/03.11/2

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GCCATGTTTTTGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCTAGAGT
GTACAATGATATATACCTTCTAGACTAGGTGCGAGGGGCCGAGGACCAGAACAA
CATCCGCCTACCCTAAGAGGGAGGGC-------

- MVs/Ibadan.NIE/05.11/1

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AGCTGCCGAGATTGGGGGGTAAGGAGGACAGGAGGGTCAAACAGAACCGAGGA
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AGCCAAGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGGCTGCAA
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ATCCGCCTACCCTAGGGGAGCG----------

- MVs/Lagos.NIE/07.11.1

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AGCCAAGATCCGCAGGACAGTCGAAGGTCAGCCGATGCCCTGCTTAGGCTGCAA
GCCATGGCAGGAATCTCGGAAGAACAAGACTCAGACACGGACACCCCTAGAGTG
TACTATGTTTTTTTTTTTCTAGACTAGGTGCGAGGGGCCGAGGACCAGAACAACA
TCCGCCTACCCTAGAGGAGGGTA---------

- MVs/Ibadan.NIE.11.10

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TTTCACGAGATTGCAATGCATACTACTGAGGACAAGATCAGTAGAGCGGTTGGA
CCCAGACAAGCCCAAGTATCATTTCTACACGGTGATCAAAGTGAGAATGAGCTA
CCGAGATTGGGGGGCAAGGAAGATAGGAGGGTCAAACAGAGTCGAGGAGAAGC
CAGGGAGAGCTACAGAGAAACCGGGCCCAGCAGAGCAAGTGATGCGAGAGCTG
CCCATCTTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGTCCAGCCA
AGATCCCCAGGACAGTCGAAGGTCAGCTTTTTTTTTTTTTTTAGGGGGGGGGGAT
GTCAGGAATCTCGGAAGAACAAGGCTCAGACACGGAACCCCCTATAGTTGTTTTT
TTTTTTTTAATC----------

APPENDIX 2

Components of the ELISA Kits

1. Microtiter wells (96 wells; wells coated with rubella virus antigen)
2. Sample Diluent (ready to use)
3. IgG-RF-Sorbent (ready use)
4. Positive Control (ready to use)
5. Negative Control (ready to use)
6. Cut-Off Control (ready to use)
7. Enzyme Conjugate (ready to use)
8. Substrate (ready to use)
9. Stop Solution (ready to use)
10. Wash solution (20X concentrated for 600ml)