MOLECULAR EPIDEMIOLOGY OF MEASLES VIRUS IN GHANA

BY

LORRETA ANTWI

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DECLARATION

The work described in this dissertation is an independent investigation conducted at the Department of Medical Laboratory Sciences (MSc), School of Biomedical and Allied Health Sciences, College of Health Sciences and University of Ghana under supervision. All the work recorded in this dissertation is original, unless otherwise acknowledged in the text or by reference cited. This work also has not in its present form or otherwise been submitted to this or any other university for the award of a higher degree.

LORRETA ANTWI

(ID: 10551115)

ALEXANDER MARTIN-ODDOM, PhD …….

(SUPERVISOR)

RICHARD HARRY ASMAH, DPhil …….

(SUPERVISOR)

DEDICATION

To my children and all other children around the world

ACKNOWLEDGEMENT

To God be the Glory! I thank God for how far He has brought me.

To my Supervisors, Dr. Alexander Martin-Odoom and Dr. Richard Harry Asmah, I thank you so much for all the help you gave me and for your patience.

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TABLE OF CONTENTS

PAGE No.

3.0 MATERIALS AND METHODS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C- Degree Celsius

µl-Microliter

CI- Consistency index

CDC- Centers for Disease Control

COOH- Carboxyl

DNA-Deoxyribonucleic Acid

ELISA- Enzyme-linked immunosorbent assay

F protein- Fusion protein

H protein- Haemagglutinin protein

IgG- Immunoglobin G

IgM- Immunoglobin M

MP- Maximum Parsimony

M protein- Matrix protein

MeV- Measles Virus

mRNA- Messenger Ribonucleic acid

N gene-Nucleoprotein gene

NPHRL- National Public Health and Reference Laboratory

PCR-Polymerase Chain Reaction

POD- Peroxidase

RF- Rheumatoid Factor

RI- Retention index

RNA-Ribonucleic Acid

RNase P- Ribonuclease P

RT-PCR- Reverse transcription polymerase chain reaction

SIA- Supplementary Immunization Activity

SPR- Subtree-Pruning-Regrafting

UV- Ultraviolet

WHO-World Health Organization

Abstract

Background: Measles (Rubeola) remains one of the leading causes of death among children under the age of 5 years globally, and Ghana is no exception. Despite the availability of a safe and effective vaccine approximately, 134, 200 people died from measles in 2015 globally.

Aim: The aim of this study was to investigate the epidemiology and genetic diversity of measles virus in Ghana.

Materials and Methods: This study was a cross sectional study using archived samples stored at the National Public Health and Reference Laboratory. Archived samples of acute measles infection cases as determined by the presence of measles specific IgM in sera confirmed at the National Public Health and Reference Laboratory was analyzed by genotyping and sequencing. Epi Info was used to analyze data generated and Chi-square was performed to compare any difference in variables such as age, date of specimen collection, district, and region of origin.

Results:

From 2014-2016, a total of 183 archived laboratory confirmed measles positive serum samples were analyzed and of these, 95 were males (51.9%) and 88 (48.1%) were females. The age ranged from 2 months to 43 years. The median age was 1year and the mean age was 5.6 years. Of the positive cases that had vaccinated status (50 cases), majority were in the age group 9-17 months (66%) followed by 18 months-5 years (18%). The vaccinated age group was 10-13 years (6%).

The phylogenetic tree obtained from sequences from the Ghanaian strain after genotyping and DNA sequencing (MeV-M072/19), belongs to the B3 genotype cluster.

Conclusion

The study confirms the presence of the endemic sub-Saharan genotype (B3) in Ghana. The phylogenetic tree presented showed that sequences from the Ghanaian strain (MeV-M072/19) belongs to the B3 genotype cluster as confirmed by the presence of Ibadan.NGE/0.97, one of the reference sequences for genotype B3.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The Measles virus is a single, negative-stranded RNA that belongs to the family called Paramyxoviridae and genus Morbillivirus. Measles (Rubeola) is a highly contagious disease caused by a Morbillivirus. The disease remains one of the leading causes of death among young children globally, despite the availability of a safe and cost-effective vaccine in 2018 (WHO. Key Fact, measles. 2019). Approximately 134 200 people died from measles in 2015 globally – mostly children under the age of 5 years (World Health Organization (WHO), Measles Facts 2016). It is one of the most contagious respiratory virus known (Center for Disease Control and Prevention (CDC), 2012) and can circulate in a population with up to 95% immune-protected individuals thus hindering the current measles elimination programmes forcing the World Health Organization to postpone to 2020 (WHO, 2012).

Even though a safe and cost-effective vaccine is available, in 2018, the World Health Organization (WHO) estimated that there were more than 140 000 measles deaths globally, mostly among children under the age of five

Although a high coverage of routine and supplementary measles virus immunization programmes has been achieved (one dose of measles at nine months and Supplementary Immunization

Activities (SIA), large epidemic outbreaks still occur in rural and urban populations worldwide. This is in part due to the lower titre of neutralizing antibodies at delivery in vaccinated mothers, and the low vaccination efficiency in children under 12 months of age and the presence of a vaccine non-responder population (Muñoz-Alía *et al*., 2015).

The major problem caused by the Measles virus for infected persons is that the virus weakens the immune system and opens the door to secondary health problems, such as pneumonia, blindness, diarrhoea, encephalitis etc. (WHO, 2011). When one person has measles, 90 percent of the people the patient comes into close contact with will become infected, if they are not already immune to it (Schoenstadt, 2006).

1.2 Problem statement

Although a high coverage of routine and supplementary measles virus immunization programmes has been achieved (one dose of measles at 9 months and SIA), epidemic outbreaks still occur in both urban and rural populations in Africa. In 2002 a molecular epidemiological study observed different measles virus isolates and grouped into classes and genotypes; the major group, which has been identified as indigenous to Africa, is Class B. In Nigeria and Ghana, the virus subgroups B3.1 and B3.2 co-circulate (Kouomou *et al*., 2002). More recently, there is hardly any baseline information on the characteristics of the circulating Measles viruses in Ghana, it is therefore important to re-analyze the genotype of measles virus circulating in Ghana.

1.3 Significance of Study

Measles virus genotyping can play an important role in tracking transmission pathways during outbreak investigations. Genotyping results can help confirm, disprove, or detect connections among cases. If two cases have matching genotypes, they may be connected even if the connection is not obvious (CDC, 2012). Genotyping is also the only way to determine whether a person has wild-type measles virus infection, or a rash caused by a recent measles vaccination. During outbreaks, measles vaccine is administered to help control the outbreak, and in these situations, vaccine reactions may mistakenly be classified as measles cases. A small number of measles vaccine recipients experience rash and fever 10 to 14 days following vaccination (Plan, 2012).

The vaccine strain of measles virus can be distinguished from wild-type viruses by determination of the genotype from clinical samples or virus isolates. Also, measles virus genotyping can help establish which foreign country may be the source of an imported case, since different genotypes circulate in different countries. In Ghana, there is paucity of data on measles virus characterization. In Ghana, there has been only one report since 2002 (Kouomou *et al.*, 2002) on the genome of a measles virus circulating in Ghana. This current study provided a comprehensive genetic analysis of circulating measles genotypes in Ghana. The information could be useful to policy maker in vaccination strategies.

1.4 Aim

The aim of this study was to investigate the epidemiology and genetic diversity of measles virus in Ghana.

1.5 Specific objectives

1. To retrospectively characterize the genetic diversity of Measles virus genotypes in laboratory-confirmed Measles virus cases identified in Ghana from January 2014 to December 2016
2. To differentiate between the wild type and imported viral strains.
3. To compare the IgM positivity rate in the sub- region.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Measles virus

2.1.1 Structure of the Measles virus

The Measles Virus (MeV), a paramyxovirus which belongs to the genus *Morbillivirus* is 120–250 nm in diameter and has a negative-sense single-stranded RNA genome (CDC, 2015). The F (fusion) protein, which is responsible for fusion of virus and host cell membranes, viral penetration, and hemolysis, and the H (hemagglutinin) protein, which is responsible for adsorption or attachment of virus to cells and an antigen against which neutralizing antibodies are formed, are two essential membrane envelope proteins important in pathogenesis (CDC, 2015). The MeV also has an M (matrix) protein which regulates viral RNA synthesis and assembly by interacting with the nuclleocapsid protein (Iwasaki *et al*., 2010).

The nucleocapsid which covers the viral RNA is made up of large protein (L) and nucleoprotein (N). Approximately 16,000 nucleotides which are enclosed in a lipid-containing envelope derived from the host cell makes up the RNA genome (Aryal, 2018*)*. The genome begins with a leader, which is made up of a 52 nucleotide non-coding region and a trailer, which is made up of a 37 nucleotide non-coding region, both of which are essential for the transcription and the replication of the genome (Aryal, 2018).

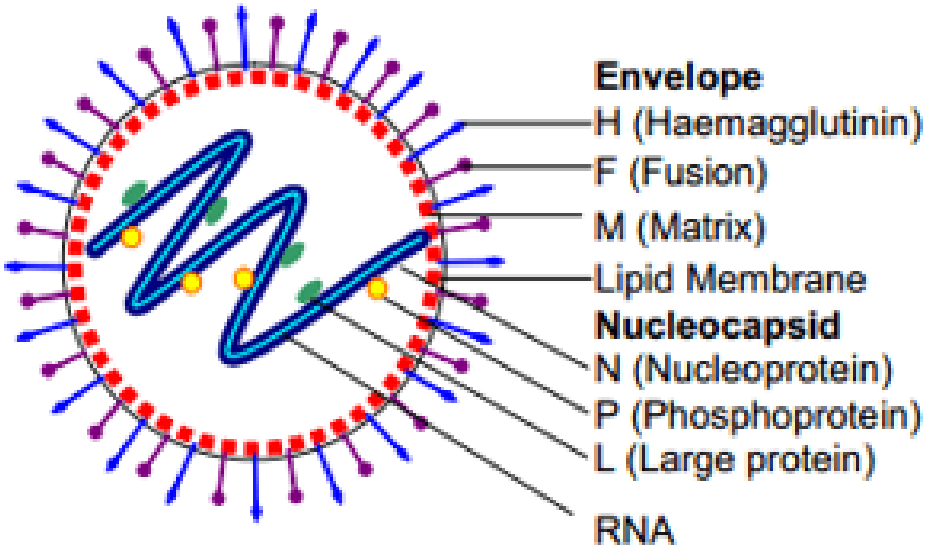

Figure. 1*: Measles virus (adapted from microbeonline.com)*

2.1.3 Classification and Epidemiology of Measles virus

Measles occurs worldwide and is the fifth most common cause of death in children <5 years of age (Stein et al., 2003). For molecular epidemiologic purposes, the genotype designations are considered the operational taxonomic unit, whereas related genotypes are grouped by clades (Rota *et al*., 2011).

The World Health Organization (WHO) currently recognizes 8 clades, designated A, B, C, D, E, F, G, and H. Within these clades, there are 23 recognized genotypes based on the nucleotide sequence of their Haemagglutinin (H) and nucleoprotein (N) genes. The genotypes are 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, and 1 provisional genotype, d11 making 24 (Rota *et al*, 2011). Viruses with related sequences within some of the genotypes (example, B3 and H1) are referred to as clusters (Rota *et al*., 2011*)*. The WHO recommends that the 450 nucleotides coding for the carboxyl (COOH) terminal 150 amino acids of the nucleoprotein (N-450) are the minimum amount of sequence data required for assigning a measles genotype (Rota *et al*., 2011). Identified measles vaccines (example, Moraten, Edmonston-Zagreb) belong to genotype A (https://www.cdc.gov/measles/lab-tools/genetic-analysis.html)This is a genotype that is not associated with recognized endemic transmission in any part of the world. Because serologic methods (example, ELISA) cannot distinguish between a vaccine-induced antibody response and from normal disease antibody derived, molecular characterization of viral isolates provides the only method to differentiate between natural infection and vaccine-induced adverse events. In elimination phase, rapid confirmation of these vaccine reactions is important to ensure that a public health action is not started unnecessarily.

In general, three patterns of measles genotype distribution have been defined (Rota *et al*., 2011). The first one is in countries that still have endemic transmission of measles; secondly, in countries that have eliminated measles, but small numbers of cases are caused by several different genotypes giving an indication of imported virus. The third pattern occurs in countries or regions that have had very good measles control but are experiencing an increase in the numbers of susceptible individuals because of failure to maintain high vaccination coverage rates.

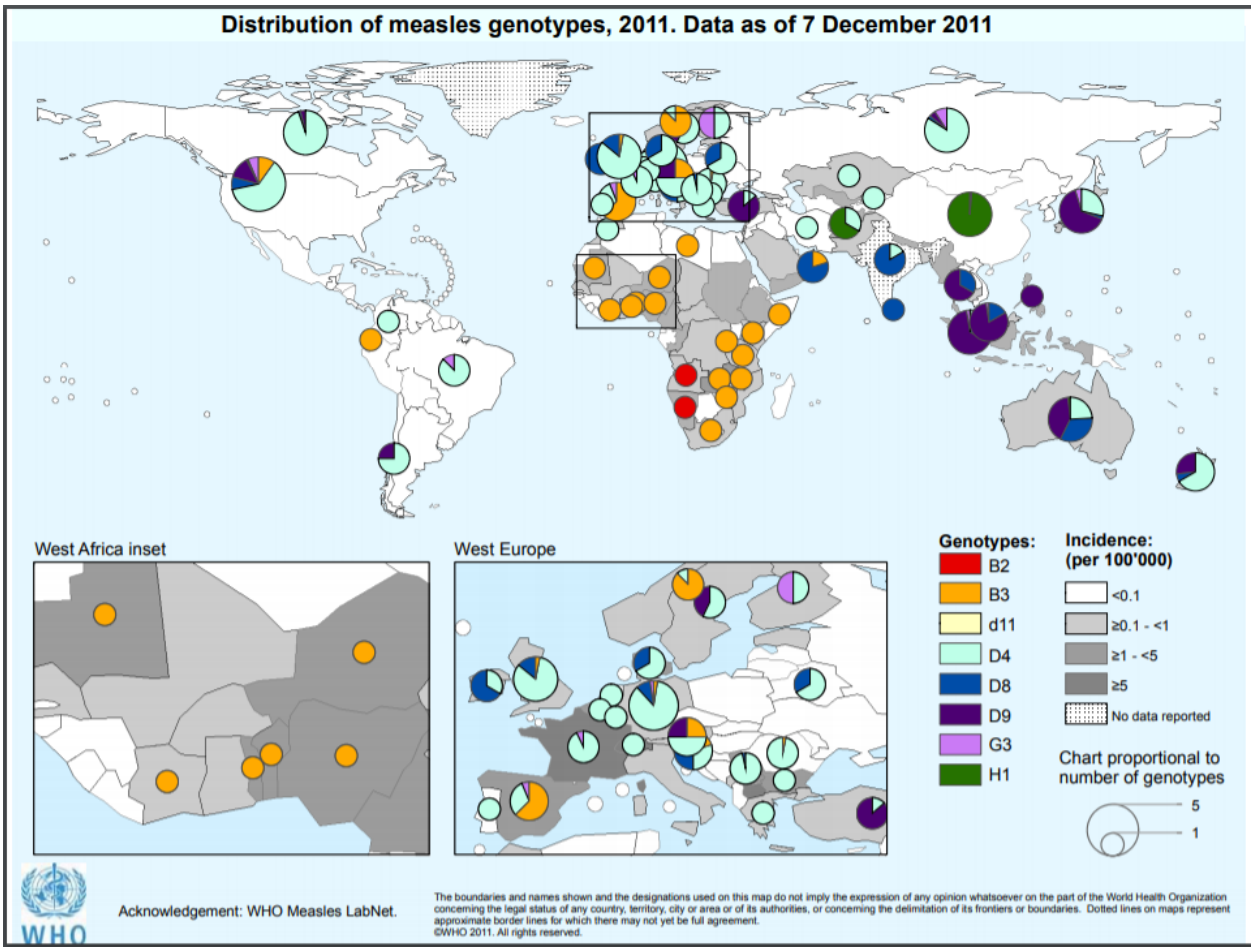

Figure 2: Global Distribution of Measles Genotypes and Incidence (WHO, 2011)

Genotype B3 is the endemic MeV genotypes circulating in most African countries. In 2010, genotype B3 was also found to be circulating in Liberia, Malawi and Mauritania (Rota *et al.*, 2011) except for the Northern African countries in the Eastern Mediterranean Region. Genotypes D2, D4, and D10 had been the most frequently detected genotypes in the Southern and Eastern parts of the African continent (Muwonge *et al.*, 2005) although more recent outbreaks in Kenya, Uganda, Burundi, and Tanzania have been caused by genotype B3 viruses (Rota *et al.*, 2006). Data on the MeV genotype in Gabon in 1984 confirmed that only

the B2 genotype was circulating in that region (Demanou *et al*., 2013). Since 1984, genotype B2 has not been found elsewhere (Demanou *et al*., 2013).

The analysis of isolates from Cameroon in 2001 show that only genotype B3 was found (Kouomou *et al*., 2002). Genotype B1 was identified in Cameroon in 1983 and has not been found since (Kouomou *et al*., 2002). It appears to have been replaced by genotype B3 (Demanou *et al*., 2013), or it may be present in areas that have not been investigated. In Morocco, only genotype C2 was found during the 2003 epidemic. An imported case of MV of genotype C2 from Morocco was found during the same year in Spain (Kouomou *et al*., 2002). Since the beginning of molecular characterization of MV in Morocco in 1998 (Kouomou *et al*., 2002) only genotype C2 has been identified. It probably represents the endemic strain in that country.

In Ghana there is paucity of data on the current circulating genotype of the measles virus. Geographical distribution for the period 1993-2001 of the MV shows that B3.1(Genotype B3, cluster 1) was found from the Sudan to Nigeria and Ghana extending south to the Cameroon, whereas the B3.2 genotype (Genotype B3, cluster 2) was found to be limited in West Africa (Kouomou *et al*., 2002). A B3 genotype found to be circulating in Accra in 1998 as well as in a 1994 case was linked to Kenya (Hanses *et al.,* 1999)

2.2 Measles Infection

2.2.1 Pathogenesis

Aerosal inhalation or contacts (into conjunctiva) with respiratory secretions are the two main ways by which the measles virus is transmitted between humans. Immune cells such as T and B cells, macrophages and dendritic cells that express CD150 which serves as an entry receptor are the main target cells (Erlenhὸfer *et al*., 2002). CD46 which is expressed on most cells can also be used by some wild-type strains, but mainly vaccine strains (Clifford *et al*., 2012). The measles virus amplifies massively in the regional lymph nodes which causes immunosuppression. Two to three days after invasion and replication in the respiratory epithelium and regional lymph nodes, a primary viremia occurs with subsequent infection of the reticuloendothelial system (*virology-online.com/ / viruses* ***MEASLES****4.htm*). Following further viral replication in regional and distal reticuloendothelial sites, a second viremia occurs 5–7 days after initial infection. During this viremia, there may be infection of the respiratory tract and other organs (the skin, the viscera, kidney and bladder). Measles virus is shed from the nasopharynx beginning with the prodromal phase until 3–4 days after rash onset. (*https://www.cdc.gov/vaccines/pubs/pinkbook/downloads/meas.pdf*).

2.2.2 Manifestation of Measles Infection

Measles infection takes an average of 10-12 days of incubation from the time of infection to the prodromal stage and from exposure to rash onset averages 14 days (range, 7–21 days). The prodromal phase lasts 2–4 days (range 1–7 days) and this is characterized by fever, which increases in stepwise fashion, often peaking as high as 39.4°C –40.6°C. This is then followed by the onset of cough, coryza (runny nose), or conjunctivitis (Xavier *et al*, 2015; Etenna *et al*., 2019*).* A Koplik spot, a rash present on mucous membranes, is pathognomonic for measles (Xavier *et al*., 2015). It occurs 1–2 days before the rash to 1–2 days after the rash and appears as punctate blue-white spots on the bright red background of the buccal mucosa (Xavier *et al*, 2015*)*.

The measles rash is a maculopapular eruption that usually lasts 5–6 days after it appears. It begins at the hairline, and then involves the face and upper neck. During the next 3 days, the rash gradually proceeds downward and outward, reaching the hands and feet. The maculopapular lesions are generally discrete, but may become confluent, particularly on the upper body (Robert *et al*., 2004; https://www.cdc.gov/measles/symptoms/signs-symptoms.html 2019;). Initially, lesions blanch with fingertip pressure. By 3–4 days, most do not blanch with pressure. Fine desquamation occurs over more severely involved areas. The rash fades in the same order that it appears, from head to extremities. Other symptoms of measles include anorexia; diarrhea, especially in infants; and generalized lymphadenopathy (Robert *et al*., 2004; https://www.cdc.gov/measles/symptoms/signs-symptoms.html).

2.2.3 Transmission of Measles

Measles is a viral infection of the respiratory system. Measles is a very contagious disease that can spread through contact with infected mucus and saliva. An infected person releases the infection into the air as droplets when they cough or sneeze and settle on surfaces, therefore anyone within close proximity can become infected when they breathe in the contaminated air or touch the infected surface, then touch their eyes, noses, or mouths. The measles virus can live on surfaces for several hours (WHO Facts, 2012) therefore drinking from an infected person's glass or cup, or sharing eating utensils with an infected person, increases one's risk of infection. The measles virus can be spread from 4 days through 4 days after the onset of rash. (https://www.cdc.gov/measles/symptoms/signs-symptoms.html*)*.

2.3 Diagnosis of Measles Infection

2.3.1 Clinical Diagnosis

Clinicians can confirm measles by examining the skin rash and checking for symptoms that are characteristic of the disease, such as Koplik's spots (punctate blue-white spots on the bright red background) which are found in the cheek or buccal mucosa after two or three days of illness, maculopapular rash (a reddish brown rash which develops after about twenty-four hours of the onset of the Koplik's spots (Xavier *et al.*, 2015*)*. The rash starts from the hair line and spreads down the body to the feet which normally lasts for about five days.), with watery eyes, sneezing, photophobia (sensitivity to light), generalized body aches, fever,

conjunctivitis, cough, and sore throat. If they are unable to confirm a diagnosis based on observation, they may order a blood test to check for the measles virus (Etenna *et al.*, 2019).

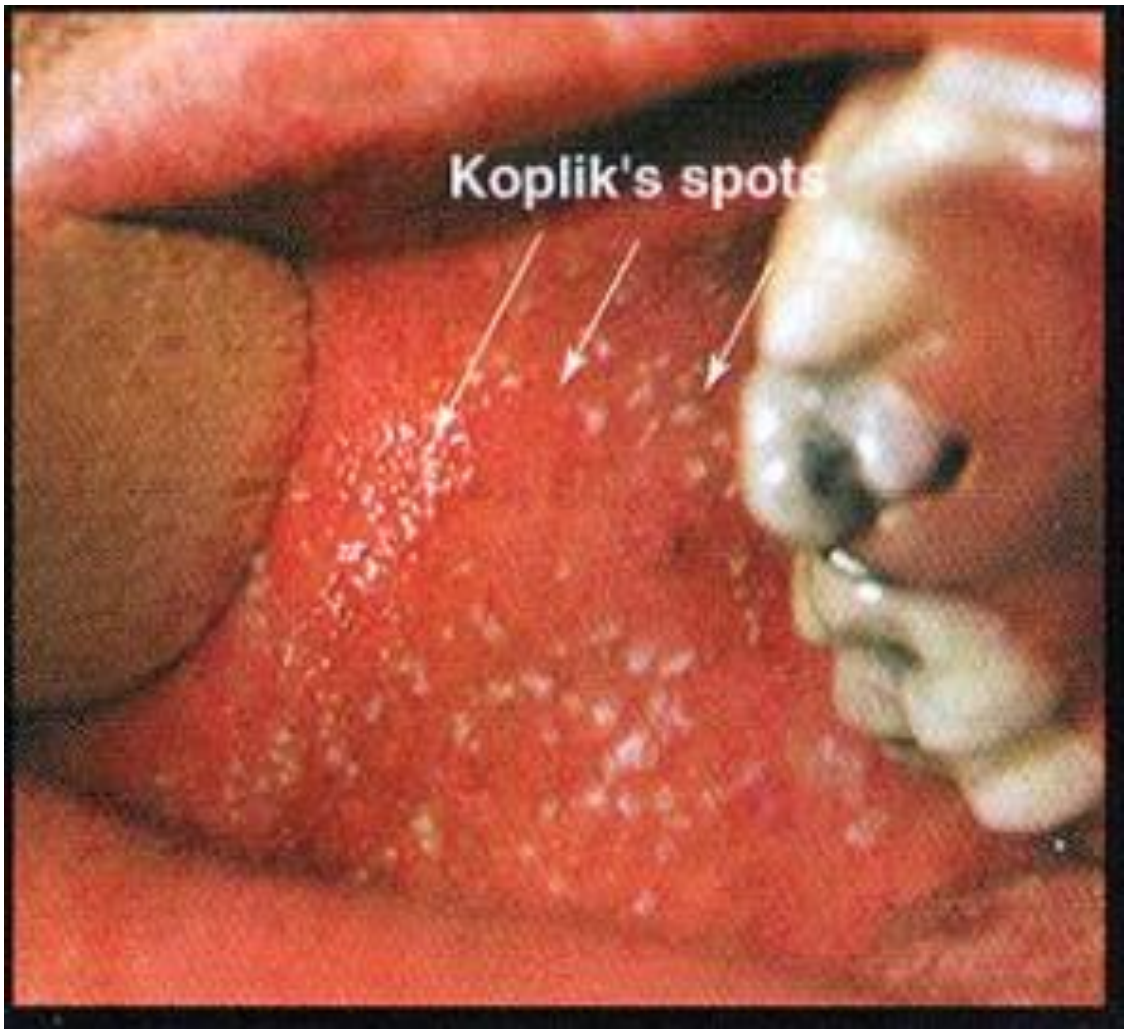

Figure 3: *Koplik's spots (Adapted from www.2.med.wayne.edu)*

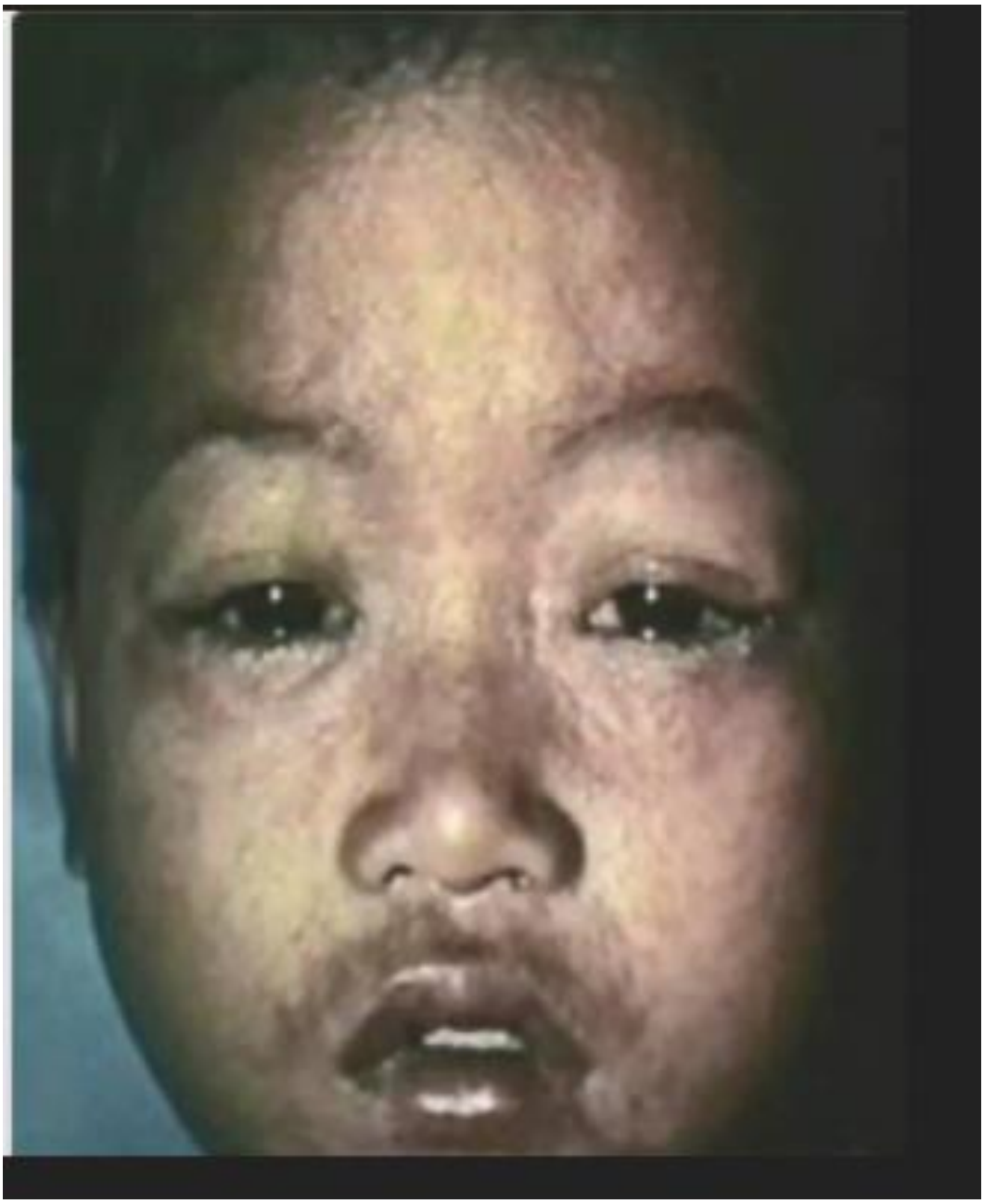

Figure 4: *Measles Rash* https://www.cdc.gov/measles/symptoms/photos.html

July 10, 2020 19:41 GMT

2.3.2 Laboratory Diagnosis

Laboratory confirmation of suspected measles cases is important, and this includes both serology Enzyme-linked immunosorbent assay (ELISA) and direct detection Polymerase Chain Reaction (PCR).

Enzyme-linked Immunosorbent Assay (ELISA)

Serum or plasma samples of suspected measles patients are tested to determine the presence of measles specific IgM in sera using an ELISA. Approximately 90% of patients are expected to have an IgM response 3 days after rash onset, peaking after 7-10 days (https://www.who.int/immunization/monitoring_surveillance/burden/laboratory/manual_section1.3/en/)

Enzygnost anti-measles virus/IgM test kit can be used. The assay is performed and interpreted according to the manufacturer's instructions (Enzygnost® Anti-Measles-Virus/IgM kit; Siemens Diagnostics Products, Marburg, Germany). It is performed by the following principle: the rheumatoid factors (RF) Absorbent binds to the IgG present in the test sample. Any RF in the sample binds to the resulting immune complexes and is eliminated. This enhances the sensitivity of the IgM test. The IgM antibodies in the sample which are specific for measles virus bind to the antigen in the wells of the test plate. The anti-human IgM/POD conjugate binds to these specific antibodies. The enzyme component of the conjugate catalyzes the working chromogen solution, producing a blue colour. This reaction

is terminated by the addition of stopping solution POD during which the colour changes to yellow. IgM directed against cellular antigens is measured in the same way in the well coated with control antigen. The difference between the colour intensities in the well coated with antigen and in the well coated with control antigen is a measure of the concentration and immunochemical reactivity of the antibodies detected in the sample. False positive IgM results can occur and in those who have previously received Measles Mumps Rubella (MMR) vaccine, the IgM response may be attenuated *(www.immunopaedia.org.za/wp-content/uploads/2014).*

PCR

Oral fluid is sampled at an early stage (day 0-4) after the onset of the rash, and 80% of patients are positive for the measles virus IgM antibodies as well as molecular testing. Viral RNA is extracted directly from Elisa positive serum samples or from OF samples using the commercial QIAmp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, USA), per the manufacturer's instructions. Detection of measles virus (MeV) RNA is performed by real-time reverse transcription polymerase chain reaction (RT-PCR) using the SuperScript III Platinum OneStep qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA) to detect a 75bp nucleotide fragment of the N gene (Hummel et al, 2006). RNA samples that are positive by real-time PCR are used as template in an endpoint RT-PCR assay to amplify 634bp of the MeV N gene containing N-450 required for MeV genotyping. The RT-PCR assay is carried out using the Qiagen one-step RT-PCR kit (Qiagen Inc., Valencia, CA, USA) with the forward primer MeV216 (5' TGG AGC TAT GCC ATG GGA GT 3') and reverse primers MeV214 (5' TAA CAA

TGA TGG AGG GTA GG 3') (Bankamp *et al*., 2013). Also, nasopharyngeal aspirates samples are preferred for detection by PCR

(*www.health.wa.gov.au/diseasewatch/vol16_issue5/docs/**measles**_breakout.pdf*).

Microscopy

Nasopharyngeal secretions (NPS) can also be examined microscopically. Production of multinucleate giant cells with inclusion bodies is pathognomonic for measles. During the prodromal phase, such cells are detectable in the NPS. This is more rapid and practical than virus isolation (*virology-online.com/viruses/**MEASLES**4.htm 22/12/16*). Also, direct and indirect immunofluorescence has been used extensively to demonstrate MV antigens in cells from NPS specimens. This technique can also be applied to the urine as such cells may be present in the urine 2 to 5 days after the appearance of the rash (*virology-online.com/viruses/**MEASLES**4.htm 22/12/16*).

2.4 Treatment and control

There is no specific treatment for an established measles infection since it is an acute self-limiting disease that will run its course without the need for specific treatment. There are however some measures that can be taken by individuals who have been exposed to the virus. It is important to contact a clinician immediately if one suspects he/she has measles or has been exposed to the virus *(www.health.wa.gov.au/diseasewatch/vol16_issue5/docs/measles_breakout.pdf)*.

One has to visit the clinician to receive a measles vaccine within 72 hours of contact to prevent infection. A person who has not received a measles vaccine should not come into contact with an infected person. A dose of immunoglobulin can be taken within six days of contact with an infected person *(www.emro.who.int/health-topics/**measles**/disease-and-**epidemiology**.html 29/12/16).* Fever reducers such as acetaminophen (Paracetamol, others), or ibuprofen (Advil, others) can be given to help relieve the fever that accompanies measles. If bacterial infection such as pneumonia or ear infection develops while an individual has measles, the clinician may prescribe an antibiotic. Generally, a large dose (200,000 international units-IU) of vitamin A may be given to children (older than a year) with low levels of the vitamin to reduce the severity of the measles infection *(www.mayoclinic.org/diseases.conditions).*

All children in developing countries diagnosed with measles should receive two doses of vitamin A supplements, given 24 hours apart. This treatment restores low vitamin A levels during measles that occur even in well-nourished children and help prevent eye damage and blindness. Vitamin a supplement has been shown to reduce the number of deaths from measles by 50% (https://www.afro.who.int/health-topics/measles August 10, 2020)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

The study was conducted at the National Public Health and Reference Laboratory (NPHRL). The laboratory is a WHO certified National Measles laboratory for Ghana. Serum samples were collected from all measles suspected cases in the country as part of measles surveillance program using the guidelines from the WHO Regional Office for Africa (AFRO) 2004 and were sent to the NPHRL. A suspected measles case was defined as any person in whom a clinician suspected measles, or any person with fever, maculopapular rash, and coryza, conjunctivitis, or cough.

3.2 Study population

Archived laboratory confirmed positive samples for the presence of Measles IgM from all regions in Ghana between 2014-2016 was used. The samples collected (183) from suspected measles cases were tested for anti-MeV-IgM using the ELISA Anti-Measles-Virus/IgM test kit (Enzygnost anti-measles virus/IgM test by Siemens, Marburg, Germany). The assays were performed and interpreted according to the manufacturer's instructions.

3.3 Inclusion criteria

All serum samples that were laboratory confirmed positive for measles IgM stored at -80 ℃ at the National Public Health and Reference laboratory between 2014-2016.

3.4 Exclusion criteria

All serum samples that were laboratory confirmed negative (2483) or equivocal (248) for measles IgM stored at the National Public Health and Reference laboratory between 2014-2016.

3.5 Study design

The study was cross sectional using archived samples. These samples were stored at the National Public Health and reference Laboratory.

3.6 Measles Virus (MeV) RNA Detection

Viral RNA was extracted directly from Elisa positive serum samples using QiaAmp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA was eluted in 60 µl Azide Viral Elution (AVE) buffer (0.04% sodium azide in RNase-free water) and stored at -70°C for further analysis.

Real-time reverse transcription polymerase chain reaction RT-PCR assay was used to detect the measles virus N Gene RNA and Human RNase P mRNA (control for the integrity of the RNA) using SuperScript III Platinum OneStep qRT-PCR kit (Invitrogen, Carlsbad, CA, USA) according to the protocol (CDC protocols for the molecular epidemiology of measles virus and rubella virus; version of 01/26/2015) to detect a 75bp nucleotide fragment of the N gene as previously described.

For the detection of the MeV RNA, the following primer sequences were used

i. Forward Primer (MVN1139-F): 5' TGG CAT CTG AAC TCG GTA TCA C 3'
ii. Reverse Primer (MVN1213-R): 5' TGT CCT CAG TAG TAT GCA TTG CAA 3'
iii. Probe (MVNP1163-P): 5' FAM CCG AGG ATG CAA GGC TTG TTT CAG A BHQ 3' (Touria *et al.*, 2016)

For the detection of human RNA, the following primer sequences were used

i. Forward Primer (HURNASE-P-F): 5' AGA TTT GGA CCT GCG AGC G 3'
ii. Reverse Primer (HURNASE-P-R): 5' GAG CGG CTG TCT CCA CAA GT 3'
iii. Probe (BHQ1 HURNASE-P): 5' FAM TTC TGA CCT GAA GGC TCT GCG CG BHQ1 3' (Touria *et al*., 2016).

The CDC protocol included two internal positive controls; MeV control RNA (high concentration control) which contains synthetic MeV RNA (MeV-N3in) and total human RNA and MeV control RNA (low concentration control) which also contains synthetic MeV RNA (MeV-N3in) and total human RNA. This control contains less synthetic MeV RNA than the high control, but the same amount of total human RNA. These controls can be used for both MeV and RNase P reactions. Thermal cycling was performed by using the ABI 7500 Real-time system Software v.2.0.4. The Thermocycler was programmed for cycling under the following conditions: Reverse transcription step: 48 °C for 30 minutes, activation step: 95 °C for 5 minutes, Amplification step (40 cycles): 95 °C for 15 seconds then 60 °C /1 minute. Results were interpreted according to the CDC protocol.

MeV Genotyping

RNA samples that were positive by real-time PCR were used as template in an endpoint RT-PCR assay to amplify 634bp of the MeV N gene containing N-450 required for MeV genotyping using the Qiagen One-Step Kit (Touria *et al.*, 2016) according to manufacturer's instructions. *(CDC protocols for the molecular epidemiology of measles virus and rubella virus; version of 01/01/2018).* Extracted MeV RNA was used for this method. MeV primer sequences used are as follows: Forward Primer (MeV216): 5' TGG AGC TAT GCC ATG GGA GT 3', Reverse Primer (MeV214): 5' TAA CAA TGA TGG AGG GTA GG 3'

The CDC protocol includes Negative controls (water control: 5 µl nuclease-free water was added instead of RNA and Extraction control: mock-extracted RNA obtained by extraction of water), and Positive control (MeV control RNA). Cycling was carried out under the following conditions for Qiagen One-Step Kit

- Reverse transcription 50 °C 30 min (1 cycle)
- Denaturation step 95 °C for 15 min (1 cycle)
- Denaturation 95 °C 30 sec (40 cycles)
- Annealing 55 °C 30 sec (40 cycles)
- Extension 72 °C 30 sec (40 cycles)
- Final extension 72 °C 10 min (1cycle)
- Storage 4 °C hold (1 cycles)

3.8 Gel electrophoresis

The PCR products were analyzed on 2% agarose gel with ethidium bromide for visualizing the RNA following the CDC protocols for the molecular epidemiology of measles and rubella virus: version of 001/20/2015 instructions. The principle of the Gel electrophoresis is the negatively charged RNA molecules migrate through the 2% agarose gel pores towards the positive charge anode under the influence of constant current. The resolution band was 600 base pairs (CDC protocols for the molecular epidemiology of measles and rubella virus; version of 001/20/2015)

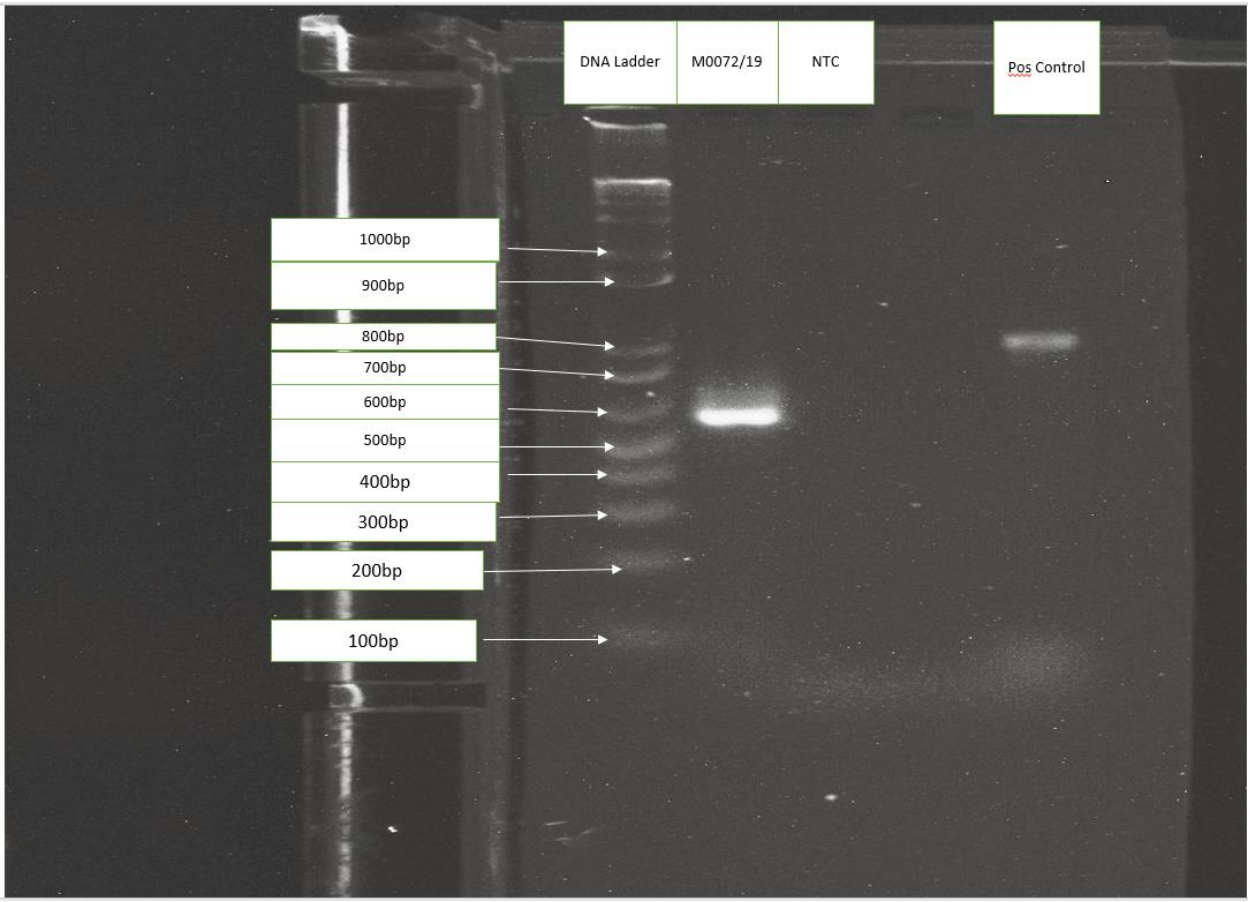

Figure 5: Picture of the PCR product

3.9 Sequencing

3.9.1 Molecular Studies

Primers and control RNA were supplied by the Centers for Disease Control and Prevention as a kit. RNA was extracted from serum samples in 60µl of AVE buffer, this is RNase-free water containing 0.04% sodium azide. If samples give a positive result in the real-time RT-PCR assays, the RNA was tested in a measles genotyping RT-PCR assay to amplify the target for sequence analysis and genotyping. The sample MeV-M072/19 was shipped to the Regional Measles Reference Laboratory in the Ivory Coast where the genotyping reactions were completed. Sequences of the 450 nucleotides in the 3' hypervariable region of the N gene were obtained from 1 (MeV-M072/19) out of 3 (MeV-M072/19,MeV-M143/16, MeV-M225/17) measles IgM positive sera samples using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and a DNA sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The typeable sample was within 1–3 days of rash onset. It was not possible to amplify the N gene fragment from 2 (MeV-M143/16, MeV-M225/17) samples due to poor quality of the clinical specimens.

3.9.2 Sequence Analysis

The evolutionary history was inferred using the Maximum Parsimony (MP) method. This is an optimality criterion in phylogenetic analysis under which the phylogenetic tree that minimizes the total number of character-state changes is to be preferred. In other words, the optimal tree is the shortest possible tree that explains the dataset. The metrics used in this tree selection process

included the consistency index (CI) which measures the consistency of a tree to a set of data (a measure of the minimum amount of homoplasy implied by the tree). The retention index (RI) also measures the extent of homoplasy and it is calculated as an improvement on the CI metric.

The analysis involved 29 nucleotide sequences obtained from the sequencing. Codon positions included were 1st, 2nd, 3rd and Noncoding regions. All positions containing gaps and missing data were eliminated. There was a total of 450 positions in the final dataset. The analyses of sequencing data were conducted in MEGA version 7.

The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei *et al*., 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The tree was drawn to scale, with branch lengths calculated using the average pathway method (Nei *et al*., 2000) and are in the units of the number of changes over the whole sequence.

3.10 Data Analysis

Soft copy data was stored in *EPI info version 3.5.1*and all hard copy stored in a locker. The data was analyzed using *EPI info version 3.5.1*. exploratory analysis was carried out to obtain descriptive statistics. Categorical variables were compared using Chi square test. The analyses of sequencing data were conducted in MEGA version 7.

3.11 Ethical Consideration

Permission was obtained from the Head of the National Public Health and Reference Laboratory. The proposal was submitted to the Ethics and Protocol Review Committee of the School of Biomedical and Allied Health Sciences for consideration and was approved before the study commenced.

CHAPTER FOUR

4.1 Results

From 2014-2016, a total of 183 archived laboratory confirmed measles positive serum samples were analyzed and of these, 95 were males (51.9%) and 88 (48.1%) were females. The age ranged from 2 months to 43 years. The median age was 1year and the mean age was 5.6 years (Table 1). Eighty-eight (48.1%) of the cases which were vaccinated. However, vaccination status of 95 (51.9%) of the cases were not known. About 28% of the cases were not due for vaccination.

Table 1: Demographic Characteristics of Serological Measles Confirmed Cases at NPHRL, 2014-2016

Characteristics	Frequency	Percentage
Sex		
Male	**95**	**51.9**
Female	**88**	**48.1**
Age Group		
<9 months	**38**	**20.8**
9-17 months	**59**	**32.2**
18months-5 years	**31**	**16.9**
6-9 years	**18**	**9.8**
10-13 years	**10**	**5.5**
14-17 years	**9**	**4.9**
18-21 years	**8**	**4.4**
>21 years	**10**	**5.5**
Vaccination Status		
Yes	**50**	**27.3**

Unknown	**95**	**51.9**
NA	**38**	**27.8**

Of the positive cases that had vaccinated status (50 cases), majority were in the age group 9-17 months (66%) followed by 18 months-5 years (18%). The vaccinated age group was 10-13 years (6%).

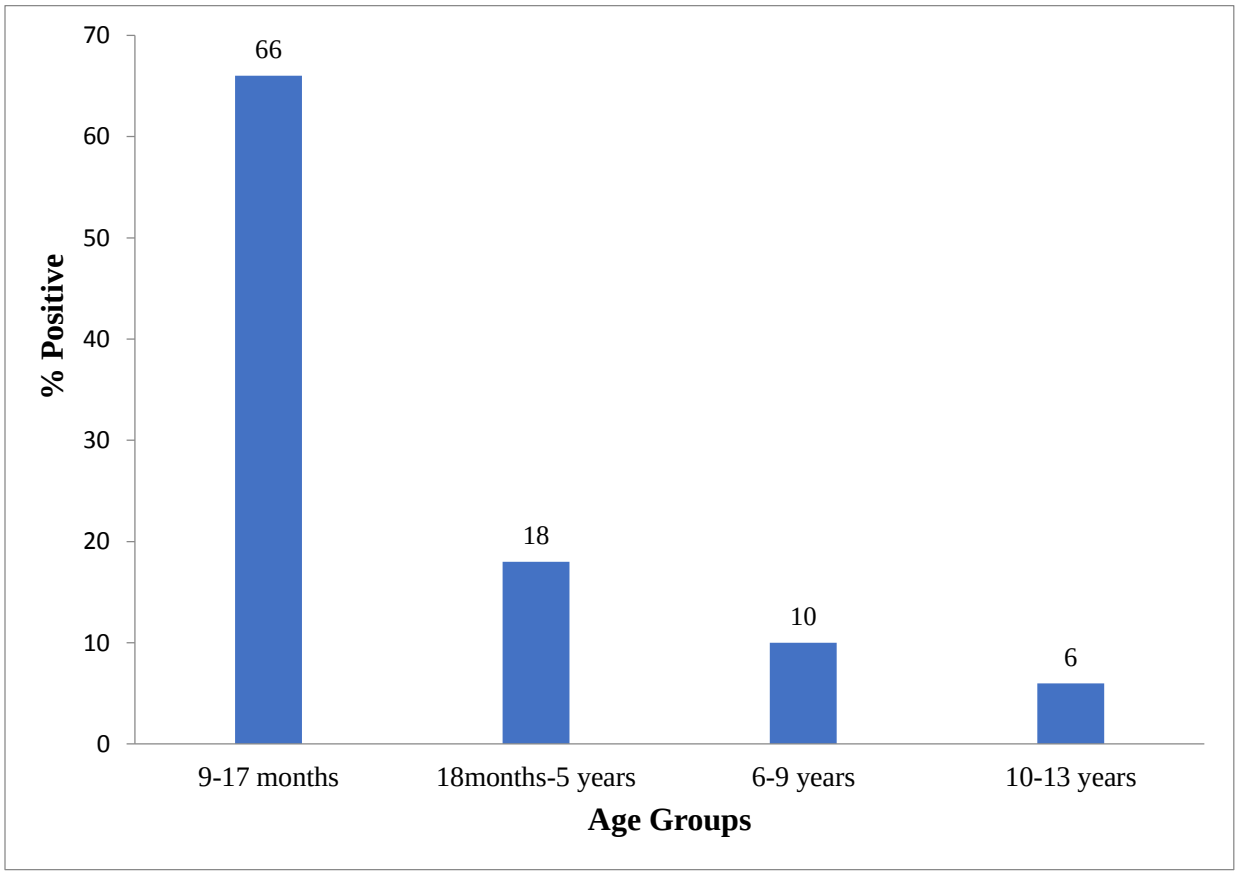

Figure 6: Measles Positivity rate among Vaccinated cases by Age, 2014-2016

Majority of the positive cases came from Brong Ahafo region (40.4%) followed by Northern (15.9%) and Upper East. Central region had the least positivity rate of 1.1%.

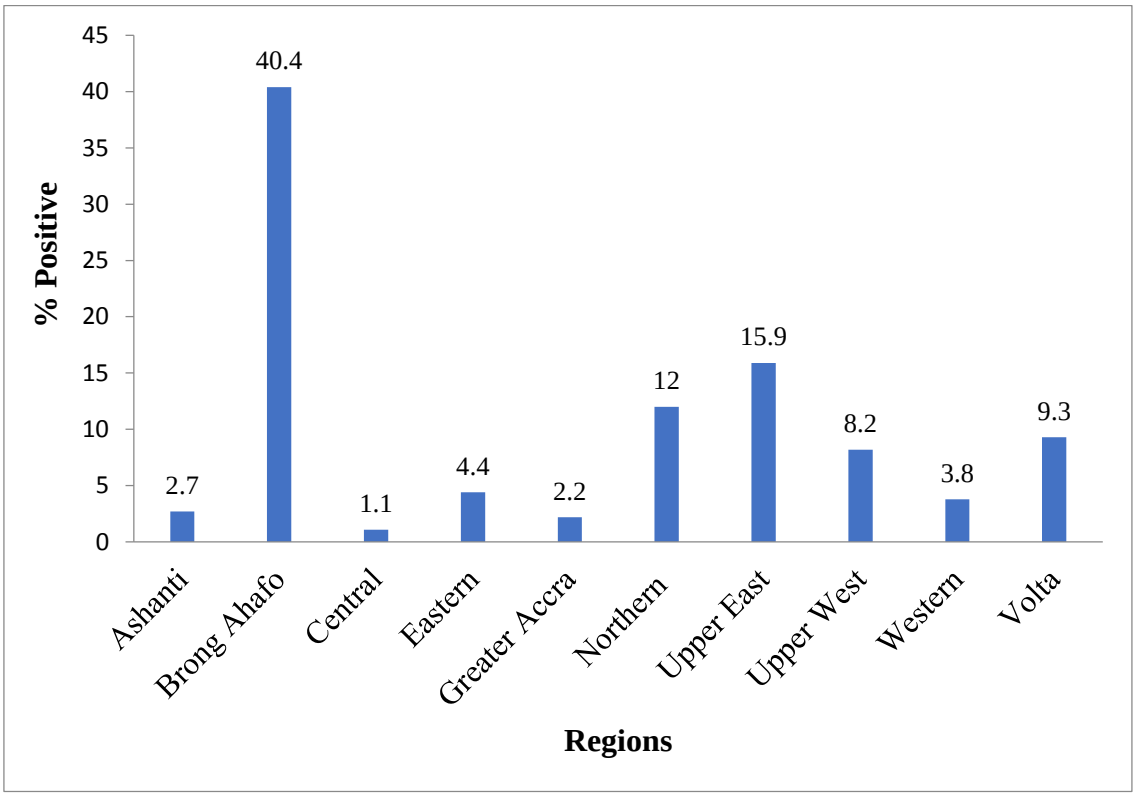

Figure 7: Distribution of Positive Measles Cases by Region, 2014-2016

4.1.1 Molecular Characterization of Measles Strains

Sequence data were analyzed using the Mega version 7 (Kumar *et al.*, 2016).

Genotypes were assigned to Ghana MeV strains by comparing them to the WHO MeV reference strains and drawing a phylogenetic tree using the neighbor-joining method. The first tree out of 3 most parsimonious trees (length = 293) was selected. The consistency index is (0.539216), the retention index is (0.673611), and the composite index is 0.457504 (0.363222) for all sites and parsimony-informative sites (in parentheses).

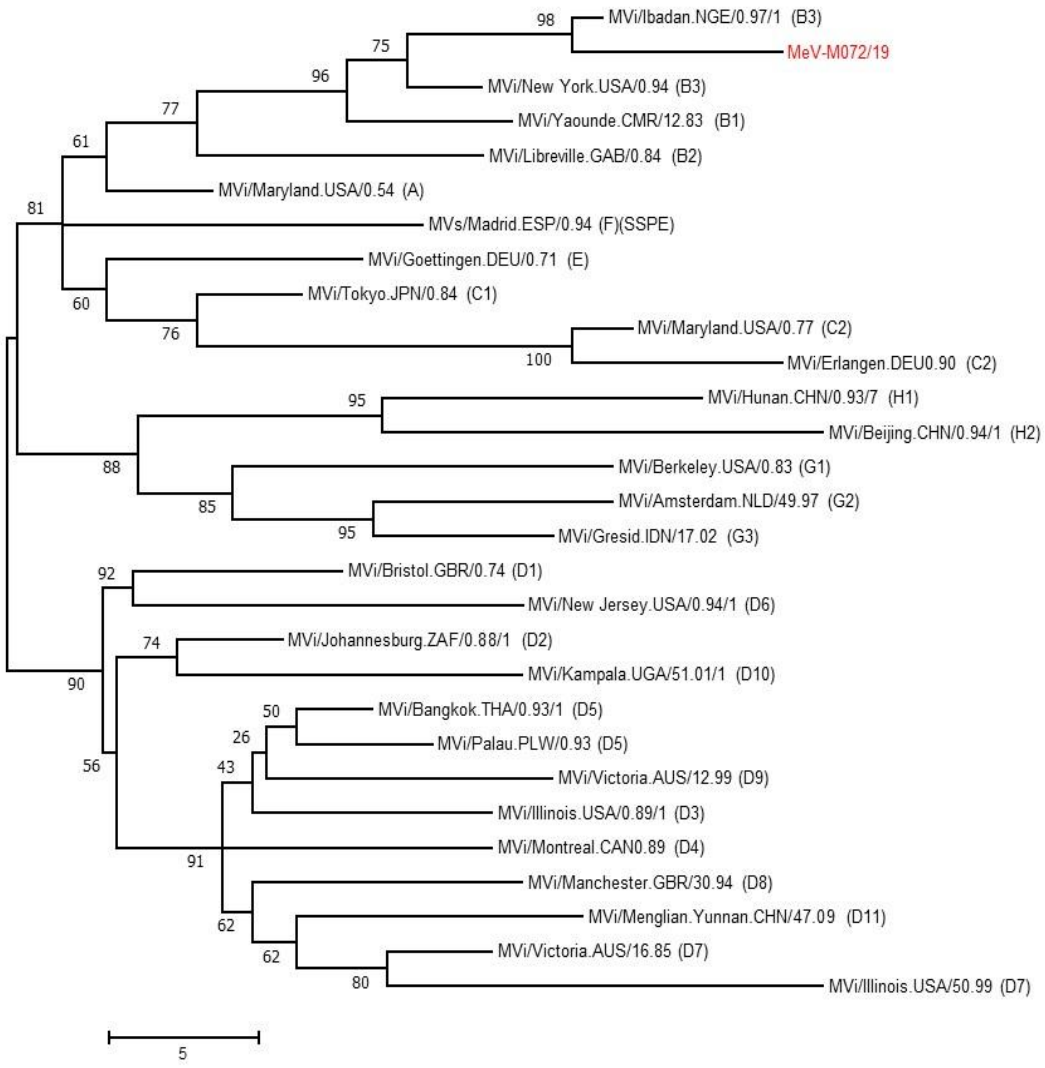

Figure 8: Phylogenetic analysis of strains from Ghana

The horizontal lines depict nucleotide differences between strains as indicated by the scale at the bottom of the tree. The differences between two strains can be calculated by adding the length of the horizontal bars that connect them. In this case, the analysis shows approximately 10 nucleotide differences between the sequenced sample (MeV-M072/19) and the B3 reference strain. Vertical lines are added by the program to facilitate visualization, but they do not indicate nucleotide differences. The nodes show, where

different genotypes branch off. If an unknown sequence branches off the same node as a reference strain, it belongs to the same genotype. The phylogenetic tree presented here showed that sequences from the Ghanaian strain (MeV-M072/19), belongs to the B3 genotype cluster as confirmed by the presence of Ibadan.NGE/0.97, one of the reference sequences for genotype B3. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) was 98%. However, B3 sub genotypes were not determined in this study.

CHAPTER FIVE

5.1 Discussion

This study described the epidemiology of measles virus in Ghana from 2014-2016**.** The phylogenetic tree presented showed that sequences from the strain (MeV-M072/19) from this work, belongs to the B3 genotype cluster as confirmed by the presence of Ibadan.NGE/0.97, one of the reference sequences for genotype B3 as previously described. Previous studies have shown that measles viruses from clade B are endemic in sub-Saharan Africa (Haddad-Boubaker *et al*., 2010) and B3 genotype was particularly characterized in several countries (such as Ghana, Gambia, Nigeria, Libya and Tunisia), elsewhere in Europe (such as France and Germany) and in the USA (Riddell *et al*., 2005). More recently, measles genotype B3 was found in Senegal in 2011-2012 (Dia *et al.*, 2015), in Cameroon during 2003-2016 (Ndambo *et al.*, 2018, Obam *et al.*, 2019) and in Gabon in 2017 (Lekana-Douki *et al.*, 2019)

During 2013–2016, 249 measles virus genotype results were reported from 14 (30%) countries; all were genotype B3 (Masresha *et al*., 2017). However, unlike in Senegal and in many other African countries, studies in Europe show a great diversity of genotypes in the same country, such as the United Kingdom, Germany and Spain (Mosquera *et al*., 2010) despite the fact that data on circulating genotypes is still scarce in Africa. Thus, the findings of this study are consistent with what is known in the extant literature.

Between, 2014-2016 a total of 2914 suspected cases were tested with an overall measles IgM-positivity rate of 6.3%. This IgM positivity rate was similar (6.7%) to a recent study conducted by Baffa et al. (2019) in Nigeria. In contrast to this, the IgM positivity rate of a study conducted in Central African Republic was 24.6% (Farra *et al*., 2019) and one found in Senegal between 2004 and 2013 was 21.4% (Dia *et al*., 2015).

The median age was one year, which is similar that of African continent in pre-vaccine and post-vaccine eras (Goodson *et al*., 2011). The age distribution of measles positive confirmed globally shows that more than 72% of cases were less or 6 years of age. This result is consistent with the 79.7% of the cases found in 9 months to 9 years in this study. This result is also consistent with the 78% of confirmed cases found in 9 months to 9 years in Africa by Goodson *et al.* (2011), 2011 and Wairagkar *et al*. (2011) who found similar proportions in India. In developed countries, positivity rates are mostly lower. In 2013, Europe confirmed cases positive rate from 0-4 years old was 32%, with the large proportion of cases in Romania (Muscat *et al*., 2014). These findings support the fact that measles infection remains high among children less than 5years of age in African countries despite the efforts for an increase in vaccination coverage during the few last years. In this study, low positivity rates were seen in older children and adults. The gender ratio was one (1) and this supports other studies conducted in Africa (Dia *et al*., 2015; Fatiregun *et al*., 2014).

5.2 Limitation

Since archived samples were used, the freeze-thaw may have affected the yield of the MeV RNA hence the low number of positive samples for genotyping and sequencing. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) was 98%. However, B3 sub genotypes were not determined in this study.

5.3 CONCLUSION

The study confirms the presence of the endemic sub-Saharan genotype B3 in Ghana. The phylogenetic tree presented showed that sequences from the Ghanaian strain (MeV-M072/19) belongs to the B3 genotype cluster as confirmed by the presence of Ibadan.NGE/0.97, one of the reference sequences for genotype B3. The IgM positivity rate of measles infection in Ghana is similar to that of other countries in the sub-region.

5.4 Recommendation

Molecular testing should be carried out on all laboratory confirmed Measles Positive IgM samples going forward to know if other genotypes other than the B3 are circulating. Also, B3 sub genotypes should be determined in other studies.

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