GENETIC CHARACTERIZATION OF WILD-TYPE MEASLES VIRUSES CIRCULATING IN KENYA

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Infectious Disease Diagnosis) in the school of Pure and Applied Sciences, Kenyatta University.
DECLARATION

I Francis Muturi Mbugua do hereby declare that, this research is my original work and has not been presented to any University for any other award.

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Signature... Date... 26/11/2008

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DEDICATION

I dedicate this work to my mother Milka Wanjiku Mbugua for inculcating the virtues of hard work in me since the early days of my life. Her incessant prayers for my success were a source of inspiration throughout this study.
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### Glossary of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>DHMT</td>
<td>District health management team</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme Immunoassay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>H Gene</td>
<td>Haemagglutinin gene</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>KEPI</td>
<td>Kenya Expanded Program on Immunization</td>
</tr>
<tr>
<td>MV</td>
<td>Measles Virus</td>
</tr>
<tr>
<td>N Gene</td>
<td>Nucleoprotein gene</td>
</tr>
<tr>
<td>PSG</td>
<td>Penicillin, streptomycin and geneticin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase Polymerase chain reaction</td>
</tr>
<tr>
<td>SLAM</td>
<td>Signaling lymphocyte activation molecule</td>
</tr>
<tr>
<td>UNICEF</td>
<td>United Nations Children’s Emergency Fund</td>
</tr>
<tr>
<td>VTM</td>
<td>Virus transport medium</td>
</tr>
<tr>
<td>VTSM</td>
<td>Virus transport swab medium</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>Vero/SLAM</td>
<td>Vero/Signaling lymphocyte activating molecule</td>
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ABSTRACT

Measles is an acute illness caused by measles virus from the genus *Morbillivirus*, a member of the family *Paramyxoviridae*. The virus has a single-stranded negative-sense RNA genome. Measles is one of the most infectious human diseases. The virus can be transmitted in the air, in respiratory droplets, or by direct contact with nasal and throat secretions of infected persons. Complications of measles include otitis media, pneumonia, diarrhoea, blindness and encephalitis. Although a vaccine-preventable disease, the World Health Organization (WHO) estimates that worldwide in 2004, there were 20-30 million cases of measles and 453,000 deaths, one-third of all vaccine-preventable childhood deaths. Molecular genotyping of measles virus strains is based on analyzing the genetic variability of nucleoprotein (N) and haemagglutinin (H) genes. Nomenclature for wild-type measles viruses has been standardized by WHO. WHO has designated two measles strain banks; the measles virus section of the Centres for Disease Control and Prevention (CDC), USA and the Health Protection Agency (HPA), to acquire, analyse, store and dispense representative strains. In 1998, WHO published guidelines for uniform nomenclature for designating wild type measles viruses and describing genotypes. The sequence of the 450 nucleotides that code for the COOH-terminal 150 amino acids of the nucleoprotein (N) is the minimum amount of data required for determining the genotype of a measles virus. If a new genotype is suspected, a viral isolate and the entire 1855 nucleotide haemagglutinin (H) gene sequence should be obtained in addition to the 456 nucleotide-carboxyl terminal coding region of the gene. The terms Clade and genotype are used to describe the genetic characteristics of wild-type measles viruses. For molecular epidemiological purposes, the genotype designations are the operational taxonomic unit, while the clades are used to indicate the genetic relationship between the various genotypes. The WHO has recognized 8 clades (A to H) and 23 measles genotypes (A, B1-B3, C1-C2, D1-D10, E, F, G1-G3 and H1-H2). The study was based on 300 samples comprising 92 (30.7%) nasopharyngeal swabs (NPS) and 208 (69.3%) urine samples. Serum samples numbering 294 (98%) were taken for IgM test by ELISA technique. Measles viruses were recovered from 36 out of 92 NPS (39.1%) NPS and from 97 out of 208 (46.6%) urine samples in Vero/SLAM cells. Serum samples numbering 117 out of 294 (39.8%) were ELISA positive. All the 133 measles virus isolates were RT-PCR positive. The alignment and phylogenetic analysis of the sequences of nucleoprotein (N) and haemagglutinin (H) genes of the isolates were performed using the p-distance neighbour-joining algorithm in the MEGA version 3.1 software packages. Measles genotypes D4, B2 and B3 were detected. Genotype D4 was detected in 2005 and 2005. Genotypes B3 and B2 were detected for the first time in 2005. More measles viruses were recovered from children who had received one dose of measles vaccine. This study has observed the interruption of transmission of D4 measles strain by mass immunization campaign against measles that took place in Kenya in 2002 and subsequent high routine immunization coverage that followed. Measles viruses isolated from Somali refugees who had camped in Eastleigh in Nairobi and Dadaab Camp in North Eastern Province indicated the likelihood of the B3 strain having been imported into the country from Somalia. The data was analyzed by using EpiInfo Programme.
CHAPTER ONE
INTRODUCTION

1.1 Background information

Measles is a highly infectious viral disease caused by the measles virus from the genus *morbillivirus* for which humans are the only reservoirs. Wild-type measles viruses have been divided into distinct genetic groups according to the nucleotide sequences of their haemagglutinin and nucleoprotein genes. It is expected that molecular epidemiological studies will become a powerful tool for evaluating strategies to control, eliminate, and eventually eradicate measles. Molecular characterization of measles viruses is an important component of measles surveillance because these studies enhance the ability to identify the source and transmission pathways of the virus. Molecular surveillance is most beneficial when it is possible to observe the change in measles virus genotypes over time in a particular region. Such information can help to document the interruption of transmission of measles virus genotypes and thus provide an important tool for assessing the effectiveness of vaccination programs. It is recommended that molecular surveillance of measles virus be conducted during all phases of measles control and be expanded to give an accurate description of the global distribution of measles genotypes.

The role of the laboratory in measles surveillance is to confirm clinically suspected measles outbreaks by IgM serology and to determine measles genotypes for molecular epidemiological purposes (WHO, 2000). The genotyping of measles viruses is important for mapping transmission pathways, differentiating vaccine from wild-type strains, and documenting the elimination of a particular endemic measles
strain(s) from a geographical area (WHO, 2001; 2003). In addition, it is important to collect measles genotyping data over time in order to monitor for possible changing of endemic measles strains in a particular geographical area (Chibo et al., 2000; Santibanez et al., 2002; Kubo et al., 2003).

In most cases, genetic characterization of wild-type measles viruses has been conducted by sequencing the genes coding for the haemagglutinin (H) protein or the nucleoprotein (N). Of the six genes on the viral genome, the H and N genes are the most variable. Over their protein coding regions, the H and N genes vary by up to 7% at the nucleotide level. The single most variable part of the measles genome is the 450 nucleotides that code for the COOH-terminus of the N protein, where nucleotide variability between various wild-type measles viruses can approach 12%. The World Health Organization (WHO) has established a standardized genotyping system and nomenclature for the characterization of wild-type measles viruses (WHO, 2001a; 2001b; 2003). Currently there are 8 measles clades (A through H), which have been divided into 23 genotypes [WHO, 2003]. Clades C, D, G and H each contain multiple genotypes (B1-B3, C1-C2, D1-D10, E, F, G1-G3, H1—H2). Clades A, E and F each contain a single genotype (genotype A, genotype E and genotype F). Over the past several years, there has been significant progress in understanding the global distribution of measles virus strains including the discovery and designation of new genotypes (Chibo et al., 2000; Truong et al., 2001; Nigatu et al., 2001; Chibo et al., 2002; Chibo et al., 2003; Rota and Bellini, 2003), as well as the documentation of the elimination of the endemic strains from several countries (Rota et al., 2002; Tipples et al., 2003; Mbugua et al., 2003).
The present study was undertaken in order to culture, isolate and characterize circulating wild-type measles strains in Kenya and monitor the transmission pathways of both endemic and imported strains in the country.

1.2 Problem statement and justification

Measles is still endemic in Kenya despite the availability of measles vaccine for over two decades. This failure to completely control measles is mainly the result of inadequate coverage of measles immunization, which is given as a routine single dose of vaccine at 9 months of age in Kenya. The Ministry of Health (MOH) has adopted the World Health Organization (WHO) and the United Nations Children’s Fund (UNICEF) Measles Mortality and Morbidity reduction strategies (2001-2005). The strategies outlined in this report include reducing measles mortality by increasing routine immunization coverage and ensure that all children have a second opportunity for measles vaccination. The Report also recommended that catch-up campaigns targeting children 9 months through 14 years of age be carried out after every three years in order to catch up with the children who never seroconverted during the initial stages of measles vaccination and those children who missed the opportunity to be vaccinated. Also recommended in the WHO/UNCEF report is the enhancement of measles surveillance with integration of molecular epidemiology and the improvement of the management of every measles case by adopting case-based surveillance system.

The challenges for the future include the achievement and maintenance of high routine immunization coverage in all districts, a reduction of measles morbidity and mortality by 90% and 95% respectively and implementation of measles case-based
surveillance. In July 2002 Kenya conducted a catch-up campaign for children aged 9 months to 14 years. The campaign reached over 14 million children with measles vaccine, with a national coverage of 99.8%. This campaign resulted in a decrease in reported laboratory confirmed measles cases from 35% to 1% in 2004. Case-based measles surveillance was established in 2003 and by December 2004, more than 80% of districts were investigating measles cases according to the measles case-based surveillance guidelines that were rolled out to the districts. The molecular surveillance of measles virus that was carried out before and after the catch up campaign of 2002 detected measles virus genotype D4 as the endemic strain circulating in Kenya. A new measles genotype D10 was detected in Uganda in 2005 (Muwonge et al., 2005). This strain was not detected in Kenya in this study.

Genetic characterization is an important laboratory surveillance procedure that can differentiate between vaccine and wild-type associated measles cases. This molecular information, in conjunction with standard case reporting and investigation, is useful in assessing the effectiveness of vaccination programmes. Genetic characterization of wild-type measles virus is a key component of laboratory surveillance activities in all phases of measles control. To facilitate genetic characterization of measles viruses, a uniform nomenclature and analysis protocol was recommended by the WHO. WHO currently recognizes 23 genotypes of measles virus and has established guidelines for the designation of new genotypes. Continuous molecular epidemiological surveillance in Kenya will eventually document the elimination of circulating endemic and imported measles strains in the country. Measles vaccine is composed of genotype A.
1.3 Research question
What are the current measles genotypes circulating in Kenya?

1.4 Hypothesis
There is no difference in the genotypes of measles virus strains circulating among the Kenyan populations.

1.5 OBJECTIVES
1.5.1 Main objective
To determine the measles virus genotypes circulating in Kenya.

1.5.2 Specific objectives
i. To isolate and genetically characterize the wild-type measles viruses circulating in Kenya.
ii. To detect and monitor transmission pathways of imported measles virus strains.
iii. To define regional distribution of measles vaccination programmes.
iv. To create a database of endemic and imported measles strains.
v. To monitor transmission pathways of measles genotype(s) following the importation of new virus strain(s) into the country.
vi. To define regional distribution of measles virus genotypes in Kenya.
vii. To monitor transmission of measles virus during and after outbreaks.
viii. To detect interruption of indigenous virus circulation.
ix. To monitor efficiency of vaccination strategies.
x. To determine the sources of virus genotypes circulating in Kenya.
1.6 Significance and anticipated output

The proposed study will determine the genotypes of the wild measles viruses currently circulating in Kenya. This will help to monitor any future importation of new measles virus genotypes into the country if the information on the measles strains currently in circulation is known. The analysis and results of this study may be useful to the Ministry of Health for appropriate policy formulation and implementation of case-based surveillance strategies. The knowledge on the endemic measles strains will facilitate the monitoring of elimination of measles strains through successful vaccination programmes. Sequencing of measles virus haemagglutinin (H) and nucleoprotein (N) genes remains the “gold standard,” and this methodology will facilitate measles virus molecular surveillance in Kenya and other African countries where molecular epidemiological work has not been carried out comprehensively and should therefore contribute to enhanced integrated measles virus control and elimination strategies as recommended by the World Health Organization.
2.1 The measles virus

Measles virus is a negative-sense, single-stranded RNA virus within the family *paramyxoviridae*. The virion lacks neuraminidase enzyme and thus it is grouped into a separate genus, the *Morbillivirus*. The envelope consists of haemagglutinin protein embedded in the lipid bilayer. The haemagglutinin protein acts as a means of attachment to susceptible cells. The negative-sense, single-stranded RNA genome is contained within a helical nucleocapsid in the virion. The genome consists of 15,894 nucleotides, which code for the six structural proteins (nucleoprotein [N], phosphoprotein, matrix, fusion, hemagglutinin [H], large protein (L) and two nonstructural proteins.

Measles is an antigenically stable virus. There is one serotype only and there are very little differences between different isolates but genetic variation in the hemagglutinin (H) and nucleoprotein (N) genes can be analyzed by molecular techniques to study transmission patterns (Rota *et al.*, 1996; Griffin *et al.*, 2001). Of the six measles virus structural proteins, three are complexed to the RNA and the other three are associated with the viral membrane envelope. Two of the membrane envelope proteins are most important in pathogenesis. They are: 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 of virus to cells. There is only one antigenic type of measles virus.
The virus is rapidly inactivated by heat, light, acidic pH, ether, and trypsin. The virus has a short survival time (less than 2 hours) in the air, or on objects and surfaces.

2.2 Transmission of measles virus

The virus can be transmitted in the air, in respiratory droplets, or by direct contact with nasal and throat secretions of infected persons. The primary site of infection is the respiratory epithelium of the nasopharynx. Two to three days after invasion and replication in the respiratory epithelium and regional lymph nodes, a primary viraemia occurs with subsequent infection of the reticuloendothelial system. Following further viral replication in regional and distal reticuloendothelial sites, there is a second viraemia, which occurs 5 to 7 days after initial infection. During this viraemia, there may be infection of the respiratory tract and other organs. Measles virus is shed from the nasopharynx beginning with the prodromal phase of illness until 3-4 days after rash onset.

2.3 Clinical manifestations

After an incubation period of 7-14 days, the patient enters the prodromal stage with fever, malaise, sneezing, rhinitis, congestion, conjunctivitis and cough. Koplik’s spots, which are pathognomonic of measles appear on the buccal and lower labial mucosa opposite the lower molars. The distinctive maculopapular rash appears about 4 days after the prodromal phase of illness and starts behind the ears and the forehead and then the rash spreads to involve the whole body. Although a vaccine-preventable disease, the World Health Organization (WHO) estimates that worldwide, there were more than 30 million measles cases, resulting in 777,000 deaths in the year 2000, half of which occurred in Africa.
2.4 Complications of measles disease

Complications due to secondary bacterial infection include otitis media, bronchopneumonia, diarrhoea, blindness and death. Malnutrition, Vitamin A deficiency and infection with human immunodeficiency virus are risk factors for complications and mortality.

Measles pneumonia is a giant cell pneumonia, which occurs mainly in immunocompromised patients. This is a severe infection with an often protracted and fatal course. Measles infection is thus a serious threat in immunocompromised and debilitated patients. Acute measles encephalitis is a severe complication with a frequency of around 1 in 1000-5000. The mortality rate is around 15% and 20-40% of patients are left with residual neurological sequelae.

Sub-acute measles encephalitis mainly occurs in immunosuppressed patients. It is most common in children with leukemia undergoing radiation therapy. The condition commences with focal convulsions, hemiplegia and coma. This condition is frequently confused with subacute sclerosing panencephalitis (SSPE). SSPE is a rare slowly progressing fatal degeneration of the brain. It is seen in children and young adults and occurs 6-8 years after the initial attack of measles. The incidence is of the order of 1 in 100,000 cases of acute measles. Myocardial deaths have been reported during the prodrome and the acute phase of measles. Measles in pregnancy usually results in a high rate of spontaneous abortion and premature delivery.
2.5 Laboratory diagnosis

Diagnosis of measles infection is made by detection of measles specific IgM antibody in single serum samples drawn during the acute phase of infection and within 28 days of rash onset (Helfand et al., 2001). IgM antibody due to wild-type measles virus infection should be differentiated from vaccine related IgM antibody. Vaccine-related IgM is detected within 30 days of vaccination against measles. Wild type measles IgM antibody is confirmed if vaccination took place earlier than 30 days. However, it is important to perform genetic characterization on representative measles virus isolates to facilitate molecular epidemiological studies.

The most convenient specimens for virus isolation include specimens such as urine, nasopharyngeal aspirates, throat swabs, nasal swabs and peripheral blood monocytes (PBMC). It is acceptable to collect both urine and respiratory samples at the same time (Kobune and Sagiura 1990). Nasopharyngeal aspirates and heparinized blood samples are also excellent sources of virus. Specimens for virus isolation should be obtained as soon as possible after the onset of rash but not more than 5 days after rash onset. Microscopy can be used to demonstrate the production of multinucleate giant cells with inclusion bodies, which are pathognomonic for measles. Direct and indirect immunofluorescence is used to demonstrate measles virus antigens in the infected cells from nasopharyngeal secretion (NPS) specimens. This technique can also be applied to urine sediments as such cells may be present in the urine 2 to 5 days after the appearance of rash. Measles virus can be isolated from a variety of sources, such as conjunctival washings, sputum as well as peripheral lymphocytes. Primary human kidney cells and continuous cell lines such as Vero/SLAM and B95a cells lines can be used successfully for measles virus isolation (Kobune et al., 1990;
A CPE develops between 2 to 15 days and consists of either a broad syncytium with inclusion bodies visible or the presence of measles infection in cell cultures can be confirmed by haemadsorption. Isolation is most likely to be successful from specimens taken in the prodromal phase but not in the later stages after the rash has developed. Serological diagnosis of measles infection can be made if the IgG antibody titre rises by 4 fold between the acute and the convalescent phase of illness or if measles-specific IgM is detected in unvaccinated individuals. The serological methods that can be used for diagnosis include haemagglutination inhibition (HAI), complement fixation (CF), neutralization and enzyme-linked immunosorbent assay (ELISA) tests. The detection of the presence of measles specific antibodies in the cerebral spinal fluid (CSF) is the most reliable means of laboratory diagnosis of SSPE.

Laboratory confirmation of measles virus can also be carried out by reverse transcriptase polymerase chain reaction (RT-PCR). Genetic characterization of measles virus is carried out by sequencing the 456 nucleotides of carboxyl terminal coding region of nucleoprotein (N) gene and the entire 1855 nucleotide haemagglutinin (H) gene. The World Health Organization (WHO) has established a standardized genotyping system and nomenclature for the characterization of wild-type measles viruses (WHO: 2001a, 2001b; 2003). Currently there are 8 WHO recognized measles clades (A through H) and 23 genotypes (WHO, 2003). Over the past several years, there has been significant progress in understanding the global distribution of measles virus strains, including the discovery and designation of new genotypes (Truong et al., 2001; Nigatu et al., 2001; Chibo et al., 2002). There are 23 measles genotypes: A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9,
D10, E, F, G1, G2, G3, H1, and H2. Designation of new genotypes is based on both molecular and epidemiological criteria. The WHO criteria for defining a new genotype includes N-gene sequences to be greater than 2.5% divergent and H-gene sequences to be greater than 2% divergent. WHO has designated two measles strain banks; the measles virus section of the Centres for Disease Control and Prevention (CDC), USA, and Health Protection Agency (HPA), UK, to acquire, analyse, store and dispense representative strains to support measles molecular surveillance.

2.6 Control of measles disease

In the majority of patients, measles is an acute self-limiting disease that will run its course without the need for specific treatment. However, it is far more serious in the immunocompromised, the undernourished, and children with chronic debilitating diseases. Such patients can be protected by the administration of human anti-measles gammaglobulin if given within the first 3 days after exposure. Alternatively, the exposed individual can simply be vaccinated within 72 hours of exposure. Antibiotics may be indicated in cases of secondary bacterial pneumonia or otitis media. The treatment of acute measles encephalitis is only symptomatic and supportive. A wide variety of treatment has been tried for SSPE but no convincing effects have been demonstrated. With no animal reservoir, it must be possible to eradicate the virus through a controlled vaccination campaign. In the USA, where vaccination of all children is required before commencing school, case reports have fallen by over 99% but eradication has not been achieved.
The following vaccines are available:

i  **Inactivated Vaccine**

This vaccine was intended for use in young children less than 1 year of age who are most prone to severe complications. The inactivated vaccine is by killing the whole virus normally by using formolsaline. However, this type of measles vaccine was found to impart short-term immunity against the measles virus. It was observed that at least 3 doses were needed to elicit a protective antibody response but the antibody levels soon waned. This left the vaccinated person open to attack by the natural virus. In some cases, the nature of the partial immunity led to serious hypersensitivity reactions to infection (atypical measles). The exact mechanism is still uncertain but it was thought that the vaccine lacked an important antigen of the virus and thus immunity was not complete. In view of the above and the fact that antibody levels decline rapidly after administration of the killed vaccine, live vaccination is now generally recommended and individuals previously immunized with the killed vaccine should be reimmunized with the live vaccine. The killed vaccine has now been withdrawn.

ii  **Live attenuated vaccine**

Live vaccines are now currently used. Live vaccines are made by retaining the antigenicity of the virus but reducing its pathogenicity. The seroconversion rate is 95% and the immunity lasts for at least 10 years or more, possibly lifelong. The virulence of the attenuated strain now in use is so low that encephalitis has only been noted in 1 in 1 million recipients. SSPE has been reported in children given the live vaccine. However, the rate is lower than that following natural infection. Therefore the vaccine is safe for use in very young children. The live attenuated vaccine is
now incorporated as part of the Measles Mumps and Rubella (MMR) vaccine and is currently used in USA and the rest of the world. This triple vaccine is yet to be incorporated into the Kenya Expanded Programme on Immunization (KEPI). Single measles vaccine is routinely used for children at 9 months of age. As vaccine-induced measles antibody develops more rapidly than following natural infection, MMR, vaccine can be used to protect susceptible contacts during a measles outbreak. To be effective, the vaccine must be administered within three 3 days of exposure. If there is doubt about a child’s immunity, vaccine should be given since there are no ill effects from immunizing individuals who are already immune. Immunoglobulin should be given to those for whom the vaccine is contraindicated.

2.7 Molecular epidemiology in countries that have eliminated measles

Endemic transmission of measles has been eliminated in many areas of the world, including most of the countries in the Western Hemisphere. Both virological and epidemiological data collected in the United States between 1989 and 2000 indicated that interruption of transmission of the genotype D3 viruses that were associated with the measles resurgence of the early 1990s was achieved in 1993 and subsequently maintained (Rota et al., 1996). Analysis of viruses isolated from measles cases and outbreaks in the United States between 1994 and 2001 failed to detect ongoing transmission of an endemic genotype. Rather, the diversity of genotypes detected in the last 7 years was indicative of multiple, imported sources of virus (Rota et al., 1998). Likewise, the diversity of genotypes detected in Australia, Canada, and the United Kingdom was similar to that observed in the United States, suggesting frequent importation of measles and lack of endemic circulation of virus (Tipples et al., 2003).
Though virological surveillance has improved recently in South America, there is no record of the endemic genotypes that circulated before Pan American Health Organization (PAHO) launched its very successful measles elimination efforts in the early 1990s. However, recent molecular epidemiological studies have demonstrated interruption of circulation of genotype D6 viruses that were responsible for the large measles outbreak in Sao Paulo in 1997 and subsequent outbreaks in Rio de Janeiro, Argentina, Chile, Bolivia, Haiti, and the Dominican Republic (Oliveira et al., 2002). The record low number of cases and the identification of genotypes other than D6 in association with measles cases imported into South and Central America were consistent with regional elimination strategies by the Pan American Health Organization (Hersh et al., 2000).

During 2002, indigenous transmission in the Americas was limited to a large outbreak (12,000 cases) that started in Venezuela and spread to Colombia. Viruses isolated in Venezuela were found to be members of genotype D9. Genotype D9 viruses are circulating in Java, Indonesia, and have been associated with measles cases imported into Australia (Chibo et al., 2000).

2.8 The Kenyan situation

The Kenya Expanded Programme on Immunization (KEPI) was established as a unit within the Ministry of Health in 1980 with the goal of immunizing all children in the country against the 6 traditional vaccine preventable diseases; namely tuberculosis, polio, diphtheria, pertussis, tetanus and measles. Before the establishment of KEPI, immunization in Kenya was given in some municipalities (Lema et al., 1975). Even with the availability of immunization, measles epidemics still occurred in urban areas and mainly affected children below 5 years of age. Kenya started routine single dose
of measles vaccination in children at 9 months of age throughout the country in 1980 at the inception of KEPI. Measles vaccine coverage was 43% countrywide before 1980, 70% in 1987 and approximately 79% in 1998; (Central Bureau of Statistics, 1998).

From a global perspective of accelerated measles control and elimination, Kenya is still at the measles outbreak control phase. Morbidity data from the Ministry of Health shows that in 1994 alone, 65,000 cases of measles were reported countrywide and measles was the 12th most common admission by cause. Measles surveillance by KEPI between July 1998 and March 1999 recorded 1,500 cases in 16 out of the total 63 administrative districts of Kenya.

In June 2002, Kenya conducted national immunization days for children 9 months through 14 years of age. Measles vaccination coverage of at least 98% was attained (Wanyama et al., 2002). In May and July 2006, the Ministry of Health (MOH) conducted mass measles immunization campaign in the country after the widespread measles outbreaks that had occurred countrywide.

2.9 The distribution of measles genotypes in Africa

Measles genotyping information from Africa indicates the circulation of several genotypes in different areas of the continent. Early measles genotyping information is from Rota et al. (1994) who reported on genotype B1 and B2 measles viruses from Cameroon and Gabon respectively. Measles genotype B1 and B2 viruses had not been detected since 1983 – 1984 and were therefore considered inactive (WHO, 2003). Genotype B3 measles viruses have been shown to be circulating in Nigeria
and Ghana in 1997-1998 (Hances et al., 1999), Gambia in 1993 and Cameroon in 2001 (Kouomou et al., 2002), Sudan in 1997-2000 (El Mubarak et al., 2002) and Burkina Faso in 2001 (Mulders et al. 2003). Kreis et al. (1997) reported on the detection of measles genotype D2 over the years 1986-1995 and D4 in 1994-1995 in South Africa. In Ethiopia both D4 and D8 measles viruses were detected in 1999, although the D8 virus was from a sporadic case (Nigatu et al., 2001). Measles genotype C2 viruses were found in outbreaks in Morocco in 1998-1999 (Alla et al., 2002). Rota et al. (2002) reported the importation into the United States of a genotype C2 virus from Zimbabwe in 1998 and D4 viruses from Ethiopia in 2000. Mbugua et al., 2003 sequenced 4 virus isolates and extracted measles RNA genome from 6 specimens obtained from clinical measles cases in Kenya and confirmed the circulation of D4 genotype in Kenya.
2.10 Global distribution of measles virus genotypes

Where relatively extensive virological surveillance has been done, two general patterns of measles genotype distribution have been observed. In countries that still have endemic transmission of measles, most cases are caused by relatively few endemic genotypes. In countries that have eliminated measles, the small numbers of cases are caused by a number of different genotypes that reflect various imported sources of virus and suggest the lack of sustained transmission of an endemic genotype. These paradigms will be discussed in greater detail below.
2.11 Global distribution of measles genotypes in measles-endemic areas

Virus surveillance is still incomplete, and isolates have not been obtained from many parts of the world, including many areas with endemic measles. It will be important to characterize viruses from all parts of the world in the next few years in an effort to develop a complete genetic baseline before accelerated measles control programs are initiated. Most developing countries have endemic or widespread measles outbreaks. These counties have been identified as the source of importation of a particular genotype into other countries. In these latter cases, the circulation of a genotype has not been verified by virological surveillance in the source country but was inferred on the basis of a consistent pattern of importations. For example, although genotype D3 viruses have never been isolated in the Philippines, there have been several instances of genotype D3 being detected in measles cases imported into the United States from the Philippines. In each of these cases, standard case investigation confirmed that the individuals were traveling in the Philippines during the incubation period. Because of the relatively low vaccination coverage rates in many countries, measles continues to circulate in Western Europe. The most frequently isolated measles genotypes in Europe have been C2 and D6 (Hanses et al., 2000). Genotype D7 (Santibanez et al., 2002) has been shown to circulate widely in the western part of Germany. Because of the frequency of travel to and from Europe, genotypes C2, D6, and D7 are often associated with measles cases imported from Europe to other parts of the world (Rota et al., 2002)

Several measles genotypes have been identified in Africa. Clade B viruses are endemic in the central and western parts of sub-Saharan Africa, and recent analysis of a large number of recent measles isolates from Nigeria, Ghana, The Gambia,
Cameroon, and Sudan supports the division of Clade B into 3 genotypes, B1, B2, and B3 (El Mubarak et al., 2000). Genotype B3 has been divided into two clusters. Genotype B3 cluster 1 viruses have been isolated from Cameroon, Ghana, and Nigeria and from as far east as Sudan, suggesting that Clade B viruses are widely distributed in sub-Saharan Africa.

The circulation of genotype B3 cluster 2 viruses appears to be more limited to western Africa. In contrast, genotypes D2 and D4 have been the most frequently detected genotypes in the southern and eastern parts of the African continent (Nigatu et al., 2001). Virus isolates from only one northern African country, Morocco, have been characterized. The Moroccan viruses were all in genotype C2, suggesting that the pattern of measles genotypes in northern Africa may be more related to the European pattern than to the pattern seen in other parts of Africa.

Measles is endemic on the Indian subcontinent. Genotype D4 and D8 viruses have been isolated in India and Nepal, and genotype D4 was detected in Pakistan (Wairagkar et al., 2002). D4 viruses have also been detected in measles cases imported into the United States from both Pakistan and India (Rota et al., 2001).
## Table 1: Global Distribution of Genotypes of Wild-type Measles Viruses: 2003-07

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Areas with endemic transmission and importation</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3</td>
<td>Congo, DR Congo, Gambia, Cameroun, Sudan, Nigeria, Ghana, Niger, Libya, Tunisia, Somalia, Kenya, Morocco, Western Europe, USA, Mexico, Algeria, Canada</td>
</tr>
<tr>
<td>C2</td>
<td>Morocco</td>
</tr>
<tr>
<td>D2</td>
<td>South Africa</td>
</tr>
<tr>
<td>D3</td>
<td>Philippines, Papua New Guinea,</td>
</tr>
<tr>
<td>D4</td>
<td>India, Pakistan, Ethiopia, South Africa, Russia, Nepal, Kenya, Syria, Iran, Sudan, Egypt, Romania Germany, Switzerland, Portugal, Italy, Poland, Greece, Albania Denmark.</td>
</tr>
<tr>
<td>D5</td>
<td>Thailand, Cambodia, Viet Nam</td>
</tr>
<tr>
<td>D6</td>
<td>Turkey, Ukraine, Belarus, Bulgaria, Estonia, Germany, Latvia, Russian Fed, Spain, UK, Azerbaijan, Uzbekistan, Kazakhstan, Denmark, Lux, Greece</td>
</tr>
<tr>
<td>D7</td>
<td>Germany, Italy, France, Morocco</td>
</tr>
<tr>
<td>D8</td>
<td>India, Nepal, Bangladesh, Morocco</td>
</tr>
<tr>
<td>D9</td>
<td>Indonesia, Japan</td>
</tr>
<tr>
<td>D10</td>
<td>Uganda, UK</td>
</tr>
<tr>
<td>G2</td>
<td>Indonesia, Malaysia, Thailand</td>
</tr>
<tr>
<td>G3</td>
<td>East Timor, Indonesia</td>
</tr>
<tr>
<td>H1</td>
<td>China, R of Korea, Mongolia, Japan, Viet Nam, Russia, DPR Korea</td>
</tr>
<tr>
<td>H2</td>
<td>Vietnam</td>
</tr>
</tbody>
</table>

**Source:** Centres for Disease Control and Prevention

Extensive virological surveillance in Japan has demonstrated that genotypes D3 and D5 have been co-circulating in Japan for at least 10 years (Katayama et al., 1997). Mostly genotype D5 viruses have been associated with many measles cases imported...
from Japan. These data provide evidence that multiple genotypes of measles can co-circulate if there are sufficient numbers of susceptible individuals in the population to sustain transmission. The data also suggest that measles genotypes do not displace one another, as do new variants of influenza virus.

Elsewhere in Asia, sequence analysis of wild type measles viruses isolated in several provinces in the People’s Republic of China show widespread distribution of viruses in genotype H1 (Xu et al., 1998). Viruses that were indistinguishable from the Chinese genotype H1 viruses were isolated during the outbreak of measles in Korea in 2000–2001. Virological surveillance was not conducted in South Korea before the outbreak, so it was not possible to determine if H1 viruses were endemic to South Korea or were recently introduced by importation from China. Wild type measles viruses in Vietnam are also classified as Clade H, but they are sufficiently different from the Chinese viruses to be designated as a separate genotype, H2 (Liffick et al., 2001). It is interesting to note that wild type measles viruses isolated in Thailand are in genotype D5 (WHO, 2001) and more closely related to viruses circulating in Japan than to viruses circulating in other parts of Asia. Until recently, the only measles viruses representing Clade G had been isolated in 1983, and this Clade was considered to be inactive. In 1997, a virus belonging to Clade G was isolated from an Indonesian child who was being treated at a Dutch hospital. The H and N sequences of this virus were sufficiently different from the reference strain for it to be considered a new genotype (G2) within Clade G (de Swart et al., 2000). Viruses belonging to genotype G2 have recently been isolated in Indonesia and Malaysia (Rota et al., 2000). In addition, viruses from two proposed new genotypes have been isolated in Indonesia and East Timor. RT-PCR and sequence analyses of clinical
specimens obtained from measles cases imported into Australia indicated that viruses circulating in East Timor represent a third genotype within Clade G, G3 (Chibo et al., 2002).

Figure 2: Worldwide Distribution of Measles Genotypes: 1995-2006

Source: Centres for Disease Control and Prevention (CDC)
Atlanta, Georgia, USA. (Weekly Epidemiological Review No. 51/52, 2006, 81, 15 December 2006 474-480)

2.12 Summary of global distribution of measles virus genotypes

The recent identification of new measles genotypes and the rate at which these new genotypes have been found suggest that our understanding of the extent of genetic heterogeneity present among wild type measles viruses is still far from complete. Virus isolates have not been obtained from many parts of the world that still have endemic measles and, clearly, the quality of virological surveillance needs to be
improved in all areas. The recent standardization of the molecular techniques and the continuing expansion of the WHO Global Measles Laboratory Network will facilitate rapid advances in virological surveillance activities. An important activity of the laboratory network will be to develop methods to improve measles surveillance in remote areas. In particular, establishing protocols for collection of specimens that can be stored and transported at ambient temperature, such as the dried blood spots on filter paper (Helfand et al., 2001; Katz et al., 2002) of measles will facilitate expansion of laboratory-based surveillance to areas that lack the infrastructure to use standard methods.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Design

Measles specimens for virus isolation and molecular genotyping were obtained at the health facilities from clinically diagnosed measles cases that constituted measles outbreaks in various districts of Kenya as shown in Table 4. Samples were obtained from cases that met measles clinical definition of any person with generalized maculopapular rash and fever plus one of the following: cough, coryza and conjunctivitis or any person in whom a clinician suspected measles infection.

3.1.1 Study population

The study was conducted on persons meeting clinical definition of measles virus infection or any case suspected by a clinician to have been caused by measles virus. Measles being mainly a childhood disease, the main focus on the study was infants aged 2 years and below and children aged from 2 to 18 years. Adults who contracted were also considered for inclusion in the study. Specimens were taken from clinically diagnosed measles cases at the health facilities.

3.1.2 Inclusion criteria

Persons meeting clinical case definition of measles were counseled and prepared for participation in this study. Consent was sought from parents/guardians if the study participants were infants or children.
3.1.3 Exclusion criteria

Suspected measles cases that did not meet clinical case definition were disqualified from the study. Participants who were not willing to take part in the research were also excluded from this study.

3.1.4 Sample size determination

The sample size was calculated by the use of the formula that was described by Fisher et al. (1997) where

\[ N = \frac{Z^2 P (1-P) D}{d^2} \]

Where \( N \) = minimum number of specimens for measles isolation and molecular genotyping

\( Z \) = standard error for confidence interval (CI) at 95% (1.96)

\( P \) = Prevalence of measles estimated at 5.2% for persons below 5 years and 2% for persons above 5 years

\( d \) = Allowable error, which is 0.05

\( D \) = Design effect

Using the measles prevalence in Kenya of 5.2% for persons below 5 years, and 2% for persons 5 years of age and above.

Minimum Sample Size (<5 years) \( = \frac{1.96^2 \times (1-0.052) \times (1)}{0.05^2} \) = 75.75 \( \approx \) 75.8

Minimum sample size (> 5 years) \( = \frac{1.96^2 \times 0.02 \times (1-0.02) \times (1)}{0.05^2} \) = 30.11 \( \approx \) 30.1

The total minimum sample size = 75.8 + 30.1 = 105.9 \( \sim \) 106

300 samples were used in this study
3.2 Sample collection

Respiratory and urine samples for virus isolation were collected at the same time with blood samples. Specimens for virus culture were taken during the acute phase of illness and within 5 days of rash onset. Serum samples were obtained within 28 days of rash onset. The blood samples were tested for measles specific IgM antibody by enzyme immunoassay (EIA). Respiratory and urine samples were cultured in Vero/SLAM cells for virus isolation.

3.3 Collection of blood specimens for measles IgM serology

Three to five milliliters of blood were collected by venipuncture into a sterile tube labeled with patient identification and collection date. Blood was then stored at 4-8°C for up to 24 hours to allow clotting and subsequent separation of serum. The blood was centrifuged at 1500 rpm for 10 minutes to separate the serum. If there was no centrifuge at the health facility, blood was kept overnight in the +4°C refrigerator until there was complete retraction of the clot from the serum. The serum was removed carefully, avoiding extracting red blood cells, and transferred aseptically to a sterile container. The container was labeled with the patient’s identifier, date of collection and specimen type. The serum was kept at 2-8°C until it was ready for shipment. The investigation forms were filled with the patient’s biodata including:
(1) Date of last measles vaccination, (2) Date of rash onset,
(3) Date of sample collection, (4) Date of shipment of sample to KEMRI Measles Laboratory.
3.3.1 Shipment of blood specimens

Specimens were shipped to laboratory immediately while placed in zip lock or plastic bags. Styrofoam boxes or a thermos flask with frozen ice packs were used during transportation. Specimens and investigation forms were placed on separate plastic bags and then taped to inner top of Styrofoam box before shipment. The shipping boxes were appropriately labelled with addresses of sender and recipient and decontaminated on the outside before shipping.

3.3.2 Measles specific IgM assay

The Enzygnost ® Anti-Measles Virus/IgM enzyme immunoassay diagnostic kit (Dade Behring: Germany) for qualitative detection and quantitative determination of IgM antibodies to measles virus in human serum and plasma was used for serological assay of measles IgM antibodies, as recommended by World Health Organization (WHO). Briefly, 200 μl of rheumatoid factor (RF absorbent) was added to 10 μl of serum sample already pre-diluted in 200 μl of sample buffer and incubated at 18-25 °C for 15 minutes. The mixture was then vigorously shaken (on a shaker) and 150μl of the mixture dispensed in duplicates into measles antigen and antigen-control wells on the microtitre plastic plate and incubated at 37 °C for one hour while covered with a foil (provided in the kit). The wells in the plate were then washed four times with diluted washing buffer to remove unbound immunoglobulin before 100 μl of working conjugate was added and incubated at 37 °C for one hour. After washing four times with washing buffer to remove unbound conjugate, 100 μl of working chromogen substrate (tetramethylbenzidine) was added and incubated in the dark at 18-25 °C for 30 minutes while covered with a foil. 100 μl of Stop Solution was added to stop colour development and the optical density read at 450 nm with a
reference filter of 650 nm. All the samples were ran simultaneously with reference positive and negative controls.

3.3.3 Interpretation of IgM assay results
For test samples to be positive for measles specific IgM, they had to have an optical density reading greater than 0.200 using 450 nm to 500 nm wavelengths in the spectrophotometer. Negative samples gave an optical density reading of less than 0.100 that is required for the test to attain quality assurance standards. Equivocal samples gave optical density readings of between 0.100 and 0.200. Absorbance of the reference negative control samples gave an optical density reading of less than 0.100 for the test to be valid. The test cannot detect IgM antibody from samples taken 30 days after rash onset or during the incubation period. IgM antibody levels peak after about 7-10 days and then decline rapidly, being rarely detectable after 30 days.

3.4 Measles virus isolation
3.4.1 Culture media preparation
Dulbecco’s Modified Eagle Medium (DMEM) was prepared by adding appropriate amount of penicillin G/Streptomycin solution (100-μg/ml penicillin G/ streptomycin) and 4 ml of Geneticin per 500ml of DMEM. The resultant medium (DMEM-PSG) was used for all procedures with Vero/SLAM cells. The cells were grown in flasks with appropriate volumes preferably 75cm² flasks or tissue culture test tubes. The cells were trypsinized and passaged in the same way as with any adherent cell line.
3.4.2 Trypsinization procedure

The cell monolayer was washed once with 5 ml pre-warmed (37 °C) trypsin solution or warm phosphate buffered saline (PBS) for about 30 sec to 1 minute. The wash medium was then discarded. 5 ml of pre-warmed trypsin solution was added and the flask placed in 37°C incubator. The flask was observed after every few minutes to see if the cells were detached. When the cells were detached, trypsin was decanted and the cap screwed down tightly on the flask. The flask was then quickly hit with palm of hand to dislodge cells. The dislodged cells were resuspended in 5 ml of DMEM-PSG plus 10% FBS. The solution was then pipetted up and down to break up cell clumps. The suspension of the cells was seeded into flasks containing DMEM-PSG and 10% FBS using split ratios of up to 1:5.

3.4.3 Specimen collection and processing for virus isolation

Nasopharyngeal, urine and throat swabs for measles virus isolation were taken from measles clinical cases during the acute phase of illness that normally occurs 4 days before rash onset. These specimens were also taken within five to seven days of rash onset during which time the virus is normally excreted. With very young patients, nasopharyngeal or throat swabs were easier to obtain than urine. Nasopharyngeal swabs proved ideal for virus isolation. For this study, the specimens of choice were obtained from the nasopharynx. Nasopharyngeal swab if well taken is normally rich in virus infected epithelial cells.

The use of the Virocult Virus Transport Swab/Medium (VTSM) (Medical Wire and Equipment Co. Ltd., Corsham, Wilts, England) was recommended by WHO for nasopharyngeal swab specimens as it incorporated virus transport medium (VTM).
The patient opened the mouth wide and said “ah”. Nasopharyngeal swab sample was then obtained by firmly rubbing the nasopharyngeal passage and throat with sterile cotton swab incorporated in VTSM to dislodge epithelial cells. Throat swab specimens were collected ensuring that the back of the throat was vigorously swabbed. Tongue depressors were used when necessary. The swab was placed in a viral transport tube ensuring that it was immersed in the sponge containing the VTM. The tube was labeled with the patient’s identifier. Specimens were sealed in appropriate zip-lock bags or plastic containers. Transportation of specimens to laboratory was carried out in vaccine carriers containing frozen ice packs arranged inside to maintain the reverse cold chain of 4°C to 8°C. In the laboratory, the swabs were broken into a screw-capped Bijou bottle containing 2-3 ml of VTM. A pipette was used to transfer the remaining liquid from the sponge to the Bijou bottle and mixed by vortexing. Correct labeling was done on the Bijou bottles containing specimens. Inoculation of the specimens for virus isolation was carried out in Vero/SLAM cell lines for this study. Vero/SLAM cells are Vero cells that are transfected with a plasmid encoding the gene for the human SLAM molecule (signaling lymphocyte activation molecule, (Ono et al., 2001). SLAM has been shown to be a receptor for both wild type and laboratory-adapted strains of measles.

The sensitivity of Vero/SLAM cells for isolation of measles virus is equivalent to that of B95a cells (Kobune and Suguira 1990). The advantage of the Vero/SLAM cells is that they are not persistently infected with virus and therefore are less of biological hazard than B95a cells which are usually infected with Epstein Barr virus. The disadvantage of the Vero/SLAM cells is that they must be cultured in medium containing antibiotic Geneticin to retain SLAM expression.
3.4.4 Inoculation of specimens for virus isolation

T-25 flasks were used for inoculation of the specimens. Briefly, growth medium was decanted and 5 ml of DMEM-PSG plus 2% FBS added into the T-25 flasks. 0.5 - 1 ml of specimen suspension in virus transport medium (VTM) was inoculated into the culture flask and incubated at 37°C for 1 hour to allow for virus adsorption. The cells were observed under the inverted microscope to ascertain if the specimen was toxic to the cells (e.g. rounding of cells or cells floating). When the cells were observed to be okay maintenance medium (Dulbecco’s modified essential medium) was added to the flasks and incubated at 37°C. The inoculated cell monolayer was observed for cytopathogenic effects (CPE) on a daily basis. The cells were fed continually by replacing the medium with fresh DMEM-PSG containing 2% FBS until visible CPE became extensive. Infected cells were passaged 1-2 times to allow the infection to spread before cells became overgrown. When CPE was visible over at least 50-75% of the cell monolayer the virus was assumed to have reached a suitable titre for harvesting of the infected monolayer for viral stock preparation.

3.4.5 Preparation of viral stock from infected cell monolayer

The infected cell monolayer was scraped to remove the cells attached to the walls of the flask into the medium in order to make viral suspension. The viral suspension was placed into 2-ml plastic cryovials. The viral suspension was then stored at -70°C to -80°C. The viral stock would be used for a second isolation attempt if isolation procedures failed to yield the virus in the original specimen.
3.4.6 Extraction of measles RNA

The measles RNA genome was extracted from specimens obtained from IgM confirmed cases and specimens yielding viral isolates viral isolates. Cellular fractions of urine and viral culture harvests isolates were obtained by centrifugation of 1.5ml from each sample at 2300xg for 2 min in a variable-speed centrifuge. The deposited pellets were resuspended in 140μl sterile deionized water and spin-column procedure of the QIAmp Viral RNA Mini Kit (Qiagen, Germany) was used on the pellet suspensions. Great care was taken to avoid inadvertently introducing ribonucleases (RNAses) into the RNA sample during or after the isolation procedure. This was meant to eliminate any chance of RNA degradation. The RNA was eluted in 50μl of the kit elution buffer. 50μl aliquots were immediately stored at -70°C until use.

3.4.7 Gene sequencing

PCR amplicons were purified using 3M sodium acetate and Ethanol precipitation. DNA sequencing was done using the ABI 310 Genetic Analyzer (Applied Biosystems: USA) with the BigDye Terminator Cycle Sequencing System. The generated sequences were analyzed using BLAST and Treeview software for determination of their genotypes. The WHO criteria for defining a new measles genotype includes N-gene sequences to be >2.5% divergent and H-gene sequences to be > 2% divergent (World Health Organization, 2001).

3.4.8 Primers, RT-PCR and post-amplification manipulation

Following extraction of the RNA, a one-step reverse Transcriptase Polymerase chain reaction (RT-PCR) was performed using the Titan One Tube RT-PCR System (Roche, Germany). Primers N16 (nt 1066-1095) and MV 63 (nt 1681-1701, were
used to amplify a 636 base pair (bp) fragment from the 3′ region of the measles nucleoprotein (N) gene from all specimens (numbering according to the Edmonston strain of MV). When the amplification did not yield visible products, these reactions were further amplified in a hemi-nested PCR using primers MV60 (nt1108-1131) and MV63 to generate a 594 bp fragment. PCR products were resolved by electrophoresis through 2% agarose (containing 0.2-μg/ml ethidium bromide) in Tris-Borate-EDTA (TBE) buffer and visualized by UV-transillumination. Positive bands were excised from the agarose gels and the amplicons were recovered using the Wizard SV Gel and PCR clean-up system (Promega, USA).

3.4.9 Sequencing reactions

The amplicons were sequenced using the Big Dye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, USA). Unincorporated Big Dye terminators were removed from the reactions by ethanol precipitation in 96 well plates. The fragments were resolved by capillary electrophoresis on an ABI PRISM 310 genetic analyser (Applied Biosystems, USA).

3.4.10 Analysis of sequences

The N and H gene sequences were assembled and edited using Sequencer version 4.1.4 software (Gene Codes Corporation, USA).

3.4.11 Alignment and phylogenetic analysis

Alignments and phylogenetic analyses were performed by using the p-distance neighbour-joining algorithm in the MEGA version 3.1 software package by comparison with WHO reference sequences.
3.4.12 Genotyping

Extraction of measles RNA genome from respiratory and urine specimens was carried out when these specimens failed to yield measles virus on culture. Extraction was carried out when their corresponding serum samples were positive for measles IgM by enzyme immunoassay technique. Measles RNA extraction was not done on specimens whose corresponding serum specimens were negative for measles IgM. The serological detection of measles IgM is recommended by WHO for confirmation of measles outbreaks. Vaccine related IgM antibody is detectable within 30 days of measles vaccination. Reverse transcription reaction was done in order to generate complementary DNA (cDNA). This was done by using an RT-PCR kit with platinum Taq according to manufacturer’s instructions (Invitrogen, Carlsbad, Ca. USA).

The aliquots of the finished reverse-transcription reactions (cDNA) were added to the PCR master mix for amplification of cDNA using specific primers for measles N and H genes. The PCR was carried out with Taq DNA polymerase. One-step RT-PCR was performed using Titan One RT-PCR System (Roche, Germany). The carboxyl-terminal coding region of the N gene was amplified with primers MVN1109 (5’-GCTATGCCATGGGAGTAGGAGTGG-3’ and MVN1698R (5’-GGCCTCTCGCACCTAGTCTAGTCTAG-3’). The H gene was amplified with primers MVH7271 (5’-ATGTCACCACAAACGAGACC-3’) and MVH9172R (5’-GTATGCCCTGATGTCTGGGTGAC-3’). The PCR products were analyzed by 2% agarose gel electrophoresis to identify the positive samples and then visualized by staining with ethidium bromide.
Primers N16 (nucleotide 1066-1095) and MV63 (nucleotide 1681-1701) were used to amplify a 636 base pair (bp) from the 3' region of the measles nucleoprotein (N) gene from the specimens numbering according to the Edmonston strain of MV. Amplifications not yielding visible products were further amplified in a hemi-nested PCR using primers MV60 (nt. 1108-1131) and MV63 to generate a 594bp fragment. PCR products were resolved by electrophoresis through 2% agarose containing 0.2μg/ml ethidium bromide in Tris- Borate-EDTA (TBE) buffer. Visualization of PCR products was done by UV-transillumination and positive bands were excised from the agarose gels. The amplicons were recovered using the Wizard SV Gel and PCR clean-up system Promega, USA. The amplicons were sequenced using the BigDye Terminator Cycle Sequencing Kit 3.1 (Applied Biosystems, USA). Unincorporated BigDye terminators were removed from the reactions by sodium acetate and ethanol precipitation. The fragments were resolved by capillary electrophoresis on an ABI PRISM 310 genetic analyzer (Applied Biosystems, USA). The N and H genes were assembled and edited using Sequencer version 4.1.4 software (Gene codes Corporation, USA). Using the P-distance neighbour-joining algorithm MEGA version 3.1 software packages by comparison with the World Health Organization reference strains, alignment and phylogenetic tree analyses were performed.
3.4.13 Data management and analysis

EpilInfo program was used to analyze data according to WHO/AFRO Region procedures for measles surveillance and outbreak control systems. The data was saved and stored in computer Flash disk, Floppy disk and Hard copy. The sequences generated were analyzed by the Basic Local Alignment Search Tool (BLAST) and then aligned using CLUSTAL W. Phylogenetic comparisons were done using the Treeview Phylogenetic programme.

3.4.14 Ethical considerations

The subjects in the study were not placed in any danger due to their participation in the study. All the participants were subjected to a consenting process. The Kenya Medical Research Institute Ethical Review Committee granted permission for this study.
CHAPTER FOUR

RESULTS

4.1 Serology
Out of the 300 nasopharyngeal and urine specimens, 294 (98%) of these specimens had corresponding serum specimens taken for serological assay of measles IgM antibody to determine acute infection by the virus. A total of 117 (39.8%) were laboratory confirmed for wild- measles specific IgM antibody by enzyme immunoassay (EIA).

4.2 Virus isolation
A total of 300 samples of urine and nasopharyngeal swabs were taken from clinically diagnosed measles cases. A total of 92 (30.7%) nasopharyngeal swabs (NPS) and 208 (69.3%) urine samples were collected for virus isolation. Measles viruses were isolated in Vero/SLAM cells from 36 (39.1%) and 97(46.6%) of NPS and urine samples respectively. Out of the 300 samples, 139 (46.3%) were from female patients and 161(53.7%) from male patients. Out of the 300 samples collected from measles clinical cases 163 (54.3%) had been vaccinated against measles, 77 (25.7%) had not been vaccinated and 60 (20%) had their vaccination status unknown. Measles viruses were isolated from a total of 133 cases, 55.(41.4 %) of whom had already received the first dose of measles vaccine. 6 (4.5%) cases had received a second dose of the measles vaccine and 5 (3.8%) measles infected cases had already received three doses of the vaccine. A total of .33 (24.8%) of the laboratory confirmed measles cases were not vaccinated, whereas 34 (25.6%) had their vaccination status unknown. The minimum age from whom the specimens were obtained was 2 months with the maximum age being 34 years. The mean age was 10.14 years.
4.3 RT-PCR and post-amplification manipulation

All 133 virus isolates were RT-PCR positive for measles. The alignment and phylogenetic analysis of the sequences of Nucleoprotein (N) and Haemagglutinin (H) genes of the isolates were performed using the p-distance neighbour-joining algorithm in the MEGA version 3.1 software packages. The nucleotide sequences for the N and H genes of the Kenyan measles virus isolates were similar with other WHO established reference strains. 128 measles viruses were isolated from Nairobi and Central Provinces. They all belonged to Clade B that was identical to WHO reference sequences of B3 genotype (Figure 4.2e). The same B3 strains were also isolated from specimens obtained from Rift Valley, Eastern and Coast Provinces. Four measles virus isolates had similar nucleotide sequences with WHO D4 reference strains; Pennsylvania USA/14.03 (dq398068), Omdurman.SUD/30.04/1 (dq023697) and Queensland.AUS/12.04 (dq398068) and were therefore classified as genotype D4. These D4 strains were found circulating in Eastern, Nairobi and Rift Valley Provinces. One measles virus isolate was found to have similar nucleotide sequences with WHO reference strains; Gauteng. ZAF/25.06, Cabinda.AGO/24.06/1, Soyo.AGO/45.05 and Kinshasa.COD/19.05/2. This strain was classified as B2 genotype. It was detected circulating in Kajiado district of Rift Valley Province.
Table 2: Virus isolation by gender from urine and nasopharyngeal specimens (NPS)

<table>
<thead>
<tr>
<th>Gender</th>
<th>Urine Specimens</th>
<th>Virus isolates</th>
<th>NPS</th>
<th>Virus isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>80</td>
<td>41</td>
<td>59</td>
<td>24</td>
</tr>
<tr>
<td>Male</td>
<td>100</td>
<td>42</td>
<td>61</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>83</td>
<td>120</td>
<td>50</td>
</tr>
</tbody>
</table>

The above table shows the total number of urine and NPS specimens that were obtained from measles clinical cases and the number of viruses that were successfully isolated.
Figure 4: Virus isolation laboratory confirmed measles cases by sex. The bar graph above indicates that the highest number of laboratory confirmed measles cases was from males (51%) compared to 49% in females.
**Figure 5: Virus recovery by age.** The graph above indicates that the highest numbers of laboratory confirmed measles cases were in persons aged above 14 years.

**Table 3: Measles virus isolation from vaccinated and unvaccinated persons**

<table>
<thead>
<tr>
<th>Measles Vaccine Doses</th>
<th>Virus isolates</th>
<th>Percentage Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Dose</td>
<td>56</td>
<td>42.1%</td>
</tr>
<tr>
<td>Second Dose</td>
<td>6</td>
<td>4.5%</td>
</tr>
<tr>
<td>Third Dose</td>
<td>4</td>
<td>3.0%</td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>33</td>
<td>24.8%</td>
</tr>
<tr>
<td>Vaccination Status Unknown</td>
<td>34</td>
<td>25.6%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>133</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>
Figure 6: The relationship of measles virus isolation to vaccine dosage administration

The highest measles isolation percentage was from persons who had received only one dose of the measles vaccine while the lowest was in persons who had received a third dose. More viruses were isolated from unvaccinated persons and those whose vaccination status was unknown compared to those who had been vaccinated.
Figure 7: Virus isolation by districts. The highest number of measles cases was reported in Kilifi district while the lowest cases were reported in Thika and West Pokot districts.
Figure 8: Visualization of RT-PCR products of measles isolates. Results of RT-PCR for the measles virus isolates were visualized by gel electrophoresis.

Key:

- Lanes 1 and 12 = Molecular Weight Marker (MWM)
- Lane 2 = KEN-095
- Lane 3 = KEN-096
- Lane 4 = KEN-117
- Lane 5 = KEN-121
- Lane 6 = KEN-122
- Lane 7 = KEN-124
- Lane 8 = KEN-128
- Lane 9 = KEN-130
- Lane 10 = Negative control
- Lane 11 = positive control
Table 4: A summary of measles viruses isolated and genotyped in various districts of Kenya

<table>
<thead>
<tr>
<th>Province</th>
<th>District</th>
<th>Frequency of Virus Isolates</th>
<th>Measles Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B2</td>
</tr>
<tr>
<td>Nairobi</td>
<td>Central</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dagoretti</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Embakasi</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kasarani</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kibera</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Makadara</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pumwani</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Westlands</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Coast</td>
<td>Kilifi</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kwale</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mombasa</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Eastern</td>
<td>Kitui</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Machakos</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Makueni</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Moyale</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Central</td>
<td>Kiambu</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Thika</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Rift Valley</td>
<td>Kajiado</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Turkana</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Uasin Gishu</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>West Pokot</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>133</td>
<td>1</td>
</tr>
</tbody>
</table>

In the above table it was observed that B3 genotype was more prevalent in Kilifi and Dagoretti districts. The B3 strain was also responsible for most of the measles outbreaks that occurred in the country in 2005 and 2006.
Table 5: Summary of the measles viruses isolated in the five provinces of Kenya.

<table>
<thead>
<tr>
<th>PROVINCE</th>
<th>DISTRICT</th>
<th>FREQUENCY OF VIRUS ISOLATES</th>
<th>MEASLES GENOTYPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAIROBI</td>
<td></td>
<td>46</td>
<td>-</td>
</tr>
<tr>
<td>COAST</td>
<td></td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>EASTERN</td>
<td></td>
<td>17</td>
<td>15 2</td>
</tr>
<tr>
<td>CENTRAL</td>
<td></td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>RIFT VALLEY</td>
<td></td>
<td>13</td>
<td>1 11 1</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>135</td>
<td>1 128 4</td>
</tr>
</tbody>
</table>

In the measles epidemic season during the study period followed by the scale measles virus genotypes B2 were isolated in a measles outbreak in Nandi district, Riff Valley Province.
Figure 9: The current distribution of measles genotypes in Kenya. B3 genotype is the most dominant measles virus strain circulating in the country followed by D4 strain. Measles virus genotype B2 was detected in a measles outbreak in Kajiado district, Rift Valley Province.
Figure 10: Nucleotide sequences of measles genotype B2 #K47_(Rift_Valley.KEN/9.06). Identities = 448/450 (99%), Gaps = 0/450 (0%)

A BLAST result indicating that the virus isolate was closely related to a virus isolate from Kinshasa i.e. 99 % homology (Source: NCBI BLAST Search).
Figure 11: An un-rooted phylogenetic tree analysis of Kenyan genotype B2 against other genotypes isolated from other countries. The analysis showed that the B2 isolate from Kenya was closely associated with isolates from Southern Africa i.e. Cabinda, Soyo (Angola) and Gauteng South Africa.
Figure 12: An un-rooted phylogenetic tree analysis of Kenyan genotype B3. Comparison with other WHO established reference strains. One isolate clustered with Group A isolates from Gauteng. Some isolates clustered with group B (an isolate from Lusaka) in E above while other isolates clustered with Group C isolate from Niger. Two isolates did not directly cluster with any isolates closely i.e. group C and D as shown in the phylogenetic tree above.
Figure 13: Wild-type measles strains (B3) isolated from refugees in Eastleigh Nairobi from Somali. They were compared with other WHO reference strains and clustered closely with a reference sequence from Benin as shown above.
Figure 14: Wild-type measles strains isolated in Kenya. They clustered in 3 main groups. Group A was made up of D4 sequences that clustered with a reference sequence from Michigan USA, while group B sequences clustered with a reference sequence from Virginia USA and group C sequences clustered with a D4 sequence from Omdurman, Sudan.
Figure 15: Unrooted phylogenetic tree of D4 measles viruses isolated in 2002. (Mbugua et al., 2003)

The figure above shows an un-rooted tree generated using isolates that were collected from the Kenyan population in 2002 between May and August. The isolates shown here were D4 isolates which clustered with reference isolates circulating in USA.
Figure 16: Unrooted tree showing two groups of D4 isolates generated in Kenya

The figure above shows an un-rooted tree generated using another group of isolates that were collected from the Kenyan population in 2002 (Mbugua et al., 2002). The isolates shown here were D4 isolates which clustered with reference isolates circulating in USA (Group A) and South Africa (Group B).
The table above shows a summary of the wild type measles viruses isolated in Kenya in the 2002 outbreak. All of the isolates were D4 subtypes. The viruses were genetically analyzed in this study so as to determine what virus strains were circulating before and after mass measles immunization campaign of June 2002. It was observed that all the strains circulating then belonged to D4 genotype.

Table 6: Summary of measles viruses isolated in 2002 and genotyped in 2005

<table>
<thead>
<tr>
<th>Lab#</th>
<th>Strain Name</th>
<th>Sub-strain</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Vaccination status</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>MVi/Central.KEN/23.02 [D4]</td>
<td>a</td>
<td>15</td>
<td>F</td>
<td>yes</td>
</tr>
<tr>
<td>14</td>
<td>MVi/Nairobi.KEN/23.02 [D4]</td>
<td>a</td>
<td>16</td>
<td>M</td>
<td>no</td>
</tr>
<tr>
<td>15</td>
<td>MVi/Coast-Kalifi.KEN/24.02 [D4]</td>
<td>b</td>
<td>3</td>
<td>F</td>
<td>no</td>
</tr>
<tr>
<td>40</td>
<td>MVi/Coast-Malindi.KEN/24.02/1 [D4]</td>
<td>a</td>
<td>3</td>
<td>F</td>
<td>no</td>
</tr>
<tr>
<td>63</td>
<td>MVi/Coast-Kwale.KEN/31.02/1 [D4]</td>
<td>a</td>
<td>15</td>
<td>F</td>
<td>no</td>
</tr>
<tr>
<td>67</td>
<td>MVi/Coast-Kwale.KEN/31.02/2 [D4]</td>
<td>a</td>
<td>23</td>
<td>F</td>
<td>not known</td>
</tr>
<tr>
<td>76</td>
<td>MVi/Coast-Malindi.KEN/26.02</td>
<td>b</td>
<td>17</td>
<td>M</td>
<td>yes</td>
</tr>
<tr>
<td>50</td>
<td>MVi/Coast-Malindi.KEN/26.02</td>
<td>a</td>
<td>15</td>
<td>M</td>
<td>not known</td>
</tr>
<tr>
<td>19</td>
<td>MVi/Coast-Malindi.KEN/24.02/2 [D4]</td>
<td>b</td>
<td>20</td>
<td>M</td>
<td>yes</td>
</tr>
<tr>
<td>41</td>
<td>MVi/Coast-Malindi.KEN/24.02/2 [D4]</td>
<td>a</td>
<td>3</td>
<td>F</td>
<td>no</td>
</tr>
</tbody>
</table>
Figure 17: A map of Kenya showing the distribution of measles genotypes isolated in 2002 (Mbugua et al., 2002).
The genotypes circulating then were exclusively D4.
Figure 18: A map of Kenya showing the current distribution of wild-type measles genotypes currently circulating in Kenya. The arrows indicate that measles genotype B3 was imported from neighbouring Southern Sudan and Somalia into Kenya.
Figure 19: Phylogenetic analysis of Kenyan B3 and D4 wild-type measles strains isolated in this study. The sequences of Kenyan isolates were compared with the WHO reference strains as indicated in the Phylogenetic tree above. The unrooted tree shows 2 groups of clusters. The cluster A was made up of isolates that clustered with B3 reference strains while the cluster B was made up of isolates that clustered with D4 who reference strains.
Figure 20: Unrooted phylogenetic tree of the Kenyan measles viruses isolated from 2005 to 2007 compared with WHO reference strains as shown in the phylogenetic tree. This unrooted tree has 3 clusters. Cluster A isolates were B3 genotypes, cluster B isolate was a B2 genotype while cluster C isolates were D4 genotypes.
CHAPTER FIVE
DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

This measles molecular surveillance study resulted in the detection of three measles virus genotypes that are currently circulating in Kenya. Measles virus genotypes B2, B3 and D4 were found to have been responsible for measles outbreaks that had caused widespread outbreaks of measles disease in the country in late 2005 and early 2006. These outbreaks resulted in many deaths and hospitalization of infected persons in healthcare facilities across the country. Measles genotype B2 and B3 had not been detected before in Kenya. Measles genotype D4 had already been detected earlier in a molecular surveillance study that was carried out in 2002 (Mbugua et al., 2003) and D4 reported to have been imported from Kenya into USA in 1999 and 2001 (Rota et al., 2002). The genotypes B2 and B3 were different from the genotype D4 that was detected in the previous molecular surveillance studies carried out in Kenya.

The information on measles molecular surveillance indicates the circulation of several genotypes in different areas of the continent. Rota et al. (1994) reported the occurrences of genotypes B1 and B2 viruses in Cameroon and Gabon respectively. Genotype B3 viruses had been shown to be circulating in Nigeria and Ghana in 1997-1998 (Hanses et al., 1999), Sudan in 1997-2000 (El Mubarak et al., 2002) and Burkina Faso in 2001. Kreis et al. 1997; Mulders et al., 2001 reported the detection of measles genotype D2 between 1986-1995 and D4 between 1994-1995 in South Africa. Both D4 and D8 measles viruses were detected in Ethiopia in 1999; the D8 virus was from a sporadic case (Nigatu et al., 2001). Measles genotype C2 viruses
were found in outbreaks in Morocco in 1998-1999 (Alla et al., 2002). Rota et al. (2002) reported the importation of genotype C2 virus to the United States from Zimbabwe in 1998 and D4 from Ethiopia in 2000.

Extensive virological surveillance in Japan has demonstrated that genotypes D3 and D5 have been circulating in Japan for at least 10 years (Katayama et al., 1997). Sequence analysis of wild type measles viruses isolated in several provinces in the People’s Republic of China show widespread distribution of viruses in genotype H1(Xu et al.,1998). Wild type measles viruses isolated in Thailand are in Genotype D5. Viruses belonging to genotype G2 have recently been isolated in Indonesia and Malaysia (Rota et al., 2002). Genotype G3 viruses have been detected circulating in East Timor and Australia (Chibo et al., 2002). Measles is endemic in the Indian subcontinent where genotypes D4 and D8 viruses have been isolated in India and Nepal, and genotype D4 detected in Pakistan (Wairagkar et al., 2001). The Moroccan viruses are all in genotype C2, and are said to be more related to the European pattern than to the pattern seen in other parts of Africa (Alla et al., 2000). Genotype B3 has been detected circulating in most European countries (Chibo et al., 2000). The finding of new measles virus genotype B2 and B3 in Kenya implies that the two genotypes were imported into the country. This is supported by the molecular surveillance study carried out in 2002 which revealed the presence of endemic D4 genotype and the absence of B2 and B3 genotypes (Mbugua et al., 2003).

The current study found B2 genotype in Kajiado district. However the genotype was previously thought to have been eliminated. Molecular surveillance studies carried out at the National Institute of Communicable Diseases (NICD), South Africa, have
shown evidence of continued circulation of B2 in Africa. This genotype is endemic in the Democratic Republic of Congo (DRC) and Angola (Kouomou et al., 2002). This genotype was first described in an outbreak in Libreville, Gabon in the 1980s and was labelled as inactive by the World Health Organization in 2003 because it had not been detected for over 15 years (WHO, 2003). However, the findings of the current study in Kenya show that the genotype B2 is still actively circulating in Africa. Furthermore molecular analysis of specimens collected during a recent measles outbreak in Luanda, Angola provided another evidence for the circulation of genotype B2. Since the first three cases in Cape Town outbreak were from Angolan citizens who had recently arrived from Angola, it appeared likely that the source of virus was Angola. Elsewhere in Africa genotypes B2 and B3 viruses were detected in the Central African Republic from sixty-seven urine samples collected during measles outbreaks in Bangui in 2000 and 2004 and used for genotyping studies. From these reports and the findings of our study, genotype B2 is still active in Africa and its apparent inactivity is merely the result of insufficient molecular/virological surveillance in the region. The B2 virus strain that is endemic in the Democratic Republic of Congo (DRC) and Angola and detected in Kajiado district, Rift Valley province, from a sporadic case may have been imported by influx of refugees from these areas.

The measles genotype B3 strain that is present in West Africa and Sudan (Mulders et al., 2003) was detected in this study. This genotype may have spread from Sudan, which borders Kenya. The D8 strain, previously detected in a sporadic case in Ethiopia was not detected in Kenya (Nigatu et al., 2001). This study detected an epidemiological link between a refugee from Kenya and a Dutch tourist in New Jersey; USA. Identical genotype B3 sequences from patients with contemporaneous
cases in the United States, Canada, and Mexico in November and December 2005 indicated that Kenya was likely to have been the common source of the virus. On November 9, 2005, a 17-year-old man who arrived at the airport in Newark, New Jersey, United States, had symptoms consistent with measles. The man was part of a group of 148 refugees from Eastleigh community in Nairobi, Kenya, who arrived in the United States from November 3 through 15. Genotype B3 was identified from virus samples from this patient. The sequences were identical to sequences from measles viruses isolated in Nairobi and Machakos, Kenya, in October 2005 in our study. So the transmission pathway of B3 genotype was tracked to the U.S.A from Kenya. Although genotype B3 has been the most frequently detected measles genotype in western and central Africa (Kouomou et al., 2002.), this is the first time this genotype has been detected in Kenya.

Molecular surveillance that was carried out in Kenya in the months of May, June July and August 2002 found measles virus genotype D4 as the only one circulating in Kenya. This molecular epidemiological study observed that genotype D4 was endemic in the country. Aside from the report of the importation of D4 viruses from Kenya into the USA in 1999 and 2001 (Rota et al., 2002) and the two molecular surveillance studies of 2002 and 2005 to 2006, a molecular epidemiological study of measles genotypes circulating in Kenya had not been done before in this magnitude. The discovery of genotype D4 in 1999 by Paul Rota of the Centres for Disease Control and Prevention, Atlanta, USA corroborated with the findings of molecular surveillance studies of 2002. Widespread measles outbreaks occurred in the country in 2001 and 2002. These outbreaks had to be countered with mass measles immunization campaigns that were conducted in June 2002 targeting children aged 9
months to 14 years in order to stem the spread of the measles disease. This study has observed that mass measles vaccination campaign of June 2002 that led to the immunization of more than 13 million children and subsequent improved routine immunization coverage, led to the reduction of the incidence of measles virus genotype D4. Mass immunization has been shown to drastically reduce the circulation of measles genotypes. For example in Kenya where 14 million children were vaccinated against measles genotype D4 resulted in a drastic reduction of the incidence of D4 (Mbugua et al., 2003)

This study observed that children who had been vaccinated against measles failed to be protected against measles infection. This meant that they were vaccinated but never seroconverted since virus isolations were made from vaccinated children. There were more virus isolations from children who had received one dose of measles vaccine compared to those who had received two and three doses respectively. According to National Immunization Schedule in Kenya, children are vaccinated against measles at the age of 9 months. The efficacy of measles vaccine given at 9 months of age is 85%. Measles specific IgG maternal antibodies that are still found in babies and children less than two years of age adversely affect the live attenuated measles vaccine. The implication of the importation of measles genotypes is seen when the susceptible population is large in a particular region due to low routine immunization coverage. Poor cold chain management has been found to be responsible for increased susceptibility to measles infection as well as prolonged period before the next mass measles catch-up immunization campaigns. The generally low immunization coverage experienced in most districts in Kenya result in a build up of large susceptible population. The importation of the new measles
genotype B3 occurred when a build up of large population susceptible to measles virus had taken root in the country. A child given a second opportunity of being vaccinated against measles has a higher chance of seroconversion with a corresponding reduction of susceptible population to measles infection. Poor cold chain management may affect the potency of vaccines leading to low quality. This study found more positive males compared to their female counterparts. There were no apparent reasons for this finding.

There were more laboratory confirmed measles cases from 15 years of age and above. This is because most catch-up campaigns target children aged 9 months to 14 years. This results in the shift of measles virus to the age segment above 15 years. More measles isolations were made in persons who were not vaccinated and less isolations were made from persons who were vaccinated. This showed the importance of vaccination against vaccine preventable diseases. The study showed Kilifi district to have highest measles cases followed by Dagoretti. Routine immunization coverage in Kilifi is among the lowest (KEPI Annual Report 2005). A large number of susceptible persons had migrated to Dagoretti district. Measles cases were lowest in Thika and West Pokot districts. In recent years there has been an improvement of measles immunization coverage in these two districts (KEPI Reports 2004, 2005, 2006). This study identified three measles genotypes that were responsible for widespread measles outbreaks that occurred in the country in late 2005 and early 2006. These genotypes were detected by the use of phylogenetic tree analysis. In the phylogenetic tree, genotypes B2, B3, and D4 clustered with WHO B2 (Libreville.GAB/84) reference strain. Genotype B3 strain clustered with WHO
B3 (Ibadan.NIE/97/1 and New York City.USA/16.06 (dq888755) reference strains. The D4 genotype clustered with WHO D4 (Montreal.CAN/89) reference strain.

Measles virus is a monotypic virus, but genetic variability exists among wild type strains (Bellini and Rota 1998a, 1998b). Molecular epidemiological studies have provided an important tool for mapping transmission routes, documenting the elimination of endemic virus strains, and differentiating vaccine from wild-type strains (Bellini and Rota, 2002). The different measles virus genotypes are confined to more or less distinct geographic regions. Molecular characterization of virus isolates has been successfully used to determine epidemiological links between cases and the geographical origin of imported viruses. The findings by this study have recorded the presence of genotypes other than the endemic D4 genotype. This is an indication that three and half years since the first national mass measles immunization catch-up campaign of June 2002 the country has experienced an introduction of new measles virus genotypes (B2 and B3). The nucleotide sequences of these genotypes were similar to other WHO reference strains that are found in different regions of the world.

In July 2006, measles vaccination campaign was conducted in the rest of the country resulting in 100% immunization coverage. Mass measles supplemental immunization activities (MSIAs) took place in Kenya from 17th to 23rd June 2002. Over 14 million children from 9 months through 14 years were targeted for measles vaccination. This vaccination campaign achieved impressive immunization coverage of 98%. Measles molecular genotyping was carried out on four measles isolates and six clinical specimens. The measles RNA genome was extracted from the four isolates. The
RNA was also directly extracted from the six clinical specimens. Both the isolates and the clinical specimens were positive by RT-PCR for measles viruses.

All the specimens from which the RNA genome was extracted were obtained from 5 of 8 provinces in Kenya: Coast (Malindi, Kilifi, and Kwale districts). The specimens were also obtained from Eastern, Central, and Nairobi Provinces. The mean age of measles cases was 13.0 years old with a range 3 to 23 years. All urine samples where measles virus was successfully isolated or RT-PCR amplified were collected within 7 days of rash onset. All ten strains were determined to be closely related to the D4 reference strain, MVi/Montreal. CAN/89[D4], showing ≥ 98.4% identity to the reference across both the 456 bp C-terminal region of the N-gene and the entire 1854 bp H-gene. In addition to comparing the Kenyan D4 measles strains with the WHO reference strains, the Kenyan sequences were also compared with other published D4 strains for the N and H genes. Both the phylogenetic trees based on the measles N-gene and H-gene showed the clustering of the subgroups. Seven of the 10 Kenyan measles sequences clustered with Mvi/Montreal.CAN/89 [D4] and were thus designated as Montreal-like, while the other 3 Kenyan measles sequences clustered with a 1994 South African strain, Mvi/Johannesburg.SOA/94, and were designated as Johannesburg-like. Other subgroups of D4 strains clustered with Indian and Ethiopian D4 subgroups and were designated as India-like, Ethiopia-like. Thus the molecular surveillance resulted in the detection of 4 subgroups within D4 genotype. Further molecular characterization was carried out on the same virus isolates in our study and resulted in the detection of D4 genotype.
5.2: Conclusions

- Genotype D4 was detected in measles viruses isolated in 2002 and the virus isolates of 2005 and 2006.

- B3 measles genotype was responsible for most of the measles outbreaks that occurred in the country in 2005 and 2006.

- The mass measles vaccination campaign and the subsequent scaled up routine immunization helped to interrupt the prevalence and transmission pathways of the D4 strain although it was not eliminated.

- Measles genotype B2 that is endemic in the Democratic Republic of Congo, Angola, Gabon and the Central African Republic was for the first time detected in this study. It may have been imported from DRC Congo into Kenya.

- Measles B3 strain appears to have been introduced into Kenya from Somalia and Southern Sudan. Specimens obtained from Somali and Sudanese refugees yielded B3 genotypes.

- With the current knowledge on measles genotypes circulating in the country, it will be possible to know when new genotypes of measles virus genotypes will have been imported from other geographical regions.

- More virus isolates were obtained from persons who had received first dose of measles vaccine compared to those who had received second and third doses of the vaccine.

- Regional distribution of measles genotypes in the country was done successfully.

- Movement of persons has a big impact on distribution of measles virus genotypes from country to country.
Molecular surveillance was found to be crucial for accelerated measles control strategies already adopted in the country.

5.3 Recommendations

- Molecular surveillance is crucial for measles molecular epidemiology and tracking of transmission pathways.
- Documentation of the elimination of endemic and imported measles strains is imperative. This surveillance strategy can only be achieved through the use of molecular surveillance.
- Molecular surveillance is crucial for monitoring the efficiency of vaccination programmes.
- Monitoring of virus genotypes over time in a particular region requires that molecular surveillance should be put on board.
- It is recommended that measles molecular surveillance should be conducted during all phases of measles control and be expanded to give an accurate description of the global distribution of measles genotypes.
- It is important to conduct virological surveillance before accelerated control measures are initiated so that it will be possible to study the pattern of genotypes present before and after vaccination campaigns.
- Genetic characterization is recommended for the differentiation of wild type measles virus from vaccine strains in cases where vaccine may be implicated in serious illness.
- Molecular epidemiology should be used to complement and extend traditional epidemiological methods of measles contact tracing.
REFERENCES


Appendix i

NUCLEOPROTEIN (N) GENE NUCLEOTIDE SEQUENCES OF WILD-TYPE MEASLES VIRUSES ISOLATED FROM MEASLES OUTBREAKS IN KENYA

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Rift_Valley.KEN/33.06

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Coast.KEN/34.06

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Eastern.KEN/3.06

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Eastern.KEN/13.06

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Central.KEN/13.06

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Rift_Valley.KEN/13.06

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Nairobi.KEN/14.06

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Rift_Valley.KEN/19.06

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Coast.KEN/21.06

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APPENDIX II

MAP OF KENYA SHOWING POSSIBLE MIGRATORY ROUTES FROM NEIGHBOURING COUNTRIES

MAP OF KENYA

TUHAN
UGANDA

ETHIOPIA

TANZANIA

Scale (Km)

0 100 200

Country Border
Town or City
Park or Reserve
Lake or Ocean
Mountain
Road
River
Railway

INDIAN OCEAN
Appendix iii

Standard Operating Procedure for Polymerase Chain Reaction (PCR) Test

1. Safety Precautions

1.1. PCR is a very sensitive method of detecting specific DNA fragments and therefore prone to contamination. In theory, the number of DNA fragments produced is $2^n$ where $n$ is the number of cycles. This means that 35 cycles of PCR can produce $3 \times 10^{10}$ fragments. In reality the number of fragments is much less but a single PCR reaction may still contain millions of copies of the fragment in a 50ul reaction volume. Each of these fragments is a good template for future PCR reactions. If a reaction contains $1 \times 10^6$ copies then only 0.00005ul is required to contaminate a subsequent PCR reaction. Because of this, one must be very careful to follow procedures to avoid contaminating the reactions. These precautions are:

1.1.1. Wash your hands when leaving a post PCR area.

1.1.2. **ALWAYS** wash your hands when entering a clean area.

1.1.3. **NEVER** wear a lab coat or other lab clothing worn in a post PCR area into a pre PCR area. Leave lab coats in the post PCR labs.

1.1.4. **NEVER** take **anything** that has been in a post PCR area into a pre PCR area including reagents and equipment.

1.1.5. When setting up PCR reactions move from the PCR master mix set up area, to the reaction set up and specimen
extraction area, then to the PCR thermocycler area, and finally to the post PCR area. Avoid moving from one area to the next then back again.

1.1.6. After setting PCR reactions turn on the UV light if the hood is so equipped or clean the hood with bleach and ethanol.

1.1.7. If possible, avoid going in and out of a pre PCR area from a post PCR area. I.e. when doing an extraction, stay in the extraction area the entire time.

1.1.8. Change gloves several times during extractions.

1.1.9. Clean the hood with bleach and ethanol both before and after carrying out extractions.

1.2 Wear gloves when setting up PCR reactions and doing specimen extractions. This is to protect the PCR reactions from contaminating Dnases from hands as well as post PCR products.

2. Materials and Equipment

2.1. Microcentrifuge tubes – 1.5ml

2.2. PCR tubes – 0.2ml in strips or single

2.3. Aerosol barrier tips – 10ul, 200ul, 1000ul

2.4. Pipettors

2.5. PCR hoods

2.6. PCR thermocycler

2.7. Agarose gel apparatus

2.8. Pipette tips (unplugged)
2.9. Photo documentation – Polaroid or computer system

3. Reagents

3.1. Platinum Taq and buffer (Gibco cat # 10966-034)

3.1.1. Taq DNA Polymerase @ 5U/ul

3.1.2. Buffer - 200mM Tris-HCL (pH 8.4), 500mM KCl

3.1.3. MgCl₂ – 50mM

3.2. dNTP mix – 10mM each NTP

3.3. Primers – 10uM each

3.4. H₂O – ultra pure from a clean source

3.5. Template DNA

3.6. Agarose

3.7. Ethidium bromide

3.8. DNA marker – 100bp ladder

3.9. Gel loading buffer – 33% glycerol in water with bromophenol blue

4.0 Discussion

4.1 PCR is an amplification procedure where 2 primers are annealed to the “ends” of a single stranded target DNA sequence and extended using a DNA Polymerase. The resulting double stranded product is then denatured, the primers allowed to anneal again and then extended. The result is a doubling of the amount of DNA with every cycle. This means that PCR is a very sensitive method of detecting very small amounts of specific DNA fragments. PCR is therefore prone to contamination. Because of this one must be very
careful to follow procedures to avoid contaminating the reactions. Read the safety precautions.

5.0 Procedure

5.1 Follow The Safety Precautions

5.2 Thaw the reaction components for the PCR reactions.

5.3 Determine the required number of reactions. I.e. the number of samples plus the following controls:

5.3.1 PCR positive.

5.3.1 Extraction control; a sample not containing the target gene but which is carried through the entire extraction procedure.

5.3.2 No template; a tube with only the PCR reaction reagents but no sample.

5.4 Set up the reactions with the following reaction components in a master mix with enough for the number of PCR reactions: (see accompanying table for volumes).

5.4.1 Clean distilled H$_2$O

5.4.2 Buffer

5.4.3 MgCl$_2$

5.4.4 dNTP mix

5.4.5 Primers

5.4.6 Taq DNA Polymerase

Aliquoting the master mix into the required number of PCR reaction tubes.

5.5 Mix sample tubes and centrifuge to collect contents in bottom of tube.
5.6 Add the specimen and control DNA to the reaction tubes. Discard the tips into a container with 2% bleach.

5.7 Cap the tubes and place in the thermal cycler and start the program corresponding to the primers used.

5.8 When the cycling is finished, remove the tubes and analyze by agarose gel electrophoresis.

---

### PCR Reagent Vol.

<table>
<thead>
<tr>
<th>PCR Reagent Vol.</th>
<th>1X</th>
<th>2X</th>
<th>3X</th>
<th>4X</th>
<th>5X</th>
<th>6X</th>
<th>7X</th>
<th>8X</th>
<th>9X</th>
<th>10X</th>
<th>20X</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>39 μl</td>
<td>78 μl</td>
<td>117 μl</td>
<td>156 μl</td>
<td>195 μl</td>
<td>234 μl</td>
<td>273 μl</td>
<td>312 μl</td>
<td>351 μl</td>
<td>390 μl</td>
<td>780 μl</td>
</tr>
<tr>
<td>10 X PCR buffer</td>
<td>5 μl</td>
<td>10 μl</td>
<td>15 μl</td>
<td>20 μl</td>
<td>25 μl</td>
<td>30 μl</td>
<td>35 μl</td>
<td>40 μl</td>
<td>45 μl</td>
<td>50 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>50 mM MgCl2</td>
<td>1.5 μl</td>
<td>3 μl</td>
<td>4.5 μl</td>
<td>6 μl</td>
<td>7.5 μl</td>
<td>9 μl</td>
<td>10.5 μl</td>
<td>12 μl</td>
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<tr>
<td>10 mM dNTPs</td>
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<td>3 μl</td>
<td>4 μl</td>
<td>5 μl</td>
<td>6 μl</td>
<td>7 μl</td>
<td>8 μl</td>
<td>9 μl</td>
<td>10 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>Primer #1</td>
<td>1.0 μl</td>
<td>2 μl</td>
<td>3 μl</td>
<td>4 μl</td>
<td>5 μl</td>
<td>6 μl</td>
<td>7 μl</td>
<td>8 μl</td>
<td>9 μl</td>
<td>10 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>Primer #2</td>
<td>1.0 μl</td>
<td>2 μl</td>
<td>3 μl</td>
<td>4 μl</td>
<td>5 μl</td>
<td>6 μl</td>
<td>7 μl</td>
<td>8 μl</td>
<td>9 μl</td>
<td>10 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>cDNA¹</td>
<td>1.5 μl</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Taq*</td>
<td>0.5 μl</td>
<td>1 μl</td>
<td>1.5 μl</td>
<td>2 μl</td>
<td>2.5 μl</td>
<td>3 μl</td>
<td>3.5 μl</td>
<td>4 μl</td>
<td>4.5 μl</td>
<td>5 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>Taq antibody*</td>
<td>0.5 μl</td>
<td>1 μl</td>
<td>1.5 μl</td>
<td>2 μl</td>
<td>2.5 μl</td>
<td>3 μl</td>
<td>3.5 μl</td>
<td>4 μl</td>
<td>4.5 μl</td>
<td>5 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>TOTAL VOLUM</td>
<td>50 μl</td>
<td>100 μl</td>
<td>150 μl</td>
<td>200 μl</td>
<td>250 μl</td>
<td>300 μl</td>
<td>350 μl</td>
<td>400 μl</td>
<td>450 μl</td>
<td>500 μl</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>

*If using Taq/antibody premix, add only Taq amount

Add cDNA in separate room immediately before loading reactions into PCR machine 1.0 μl to 1.5 μl per tube
Measles Virus Genotyping

clinical specimen

→ infect tissue culture cells

→ RNA extraction

→ RT-PCR

→ DNA sequencing

genomic RNA

N  P/CV  M  F  H  L  3'  5'  15.9kb

sequence the C-terminal coding 450 bases of N-gene

sequence the 1.8 kb H-gene
APPENDIX V

SCHEMATIC DIAGRAM OF MEASLES VIRUS

Envelope
- Haemagglutinin (H)
- Fusion (F)
- Matrix (M)
- Lipid Membrane (LM)

Nucleocapsid
- Nucleoprotein (N)
- Phosphoprotein (P)
- RNA
- Large Protein (L)

N = NUCLEOPROTEIN
P = PHOSPHOPROTEIN
M = MATRIX PROTEIN
F = FUSION PROTEIN
H = HAEMAGGLUTININ
L = LARGE PROTEIN
APPENDIX VI

Major Global Measles Virus Transmission Pathways 2006-07

Acknowledgement:
Data provided by WHO Measles/Rubella Laboratory Network

Key
B2 ★
B3 ★
D4 ★★
D5 ★
D6 ★
D8 ★
H1 ★

Transmission pathways with Epi links
Suspected transmission pathways

Use tongue depressor if necessary.
NASOPHARYNGEAL AND THROAT SWAB COLLECTION TECHNIQUE USING VIRUS TRANSPORT SWAB/MEDIUM (VTSM) FROM A MEASLES CASE

Use of the Virocult® Virus Transport Swab/Medium

Collect throat swab, ensuring the back of the throat is vigorously swabbed. Use tongue depressor if necessary.
Measles:
Up to 10 children admitted to KNH daily

Elizabeth Mwai and Allan Kisia

RISING measles cases at Kenya's largest referral facility remained worrying with between seven and 10 children being admitted daily.

Kenyatta National Hospital authorities, however, said the situation was under control, even as a consultant confirmed that most of the children who have died suffered from a complicated strain of the disease.

The strain consisted of severe pneumonia, respiratory infection and malnutrition.

Loise Kimutai, a consultant paediatrician in charge of the measles isolation ward at the hospital, said the facility was receiving between seven and 10 new cases daily.

"We are receiving several new cases everyday and majority are coming from the outpatient wing," Kimutai said.

She said the death toll in the last two weeks stood at seven out of 170 patients who had been admitted in the emergency ward.

Children who are admitted at the facility range between two months old and seven years old, with one 26-year-old.

Kimutai said majority of the patients had not been immunised, owing to their tender age or lack of awareness by their parents.

KNH public relations officer Herman Wawooba said 380 measles patients were treated while 307 have been admitted this year alone.

Francis Mbugua, the Kenya Medical Research Institute Chief Medical Technologist, yesterday expressed concern over the transfer of measles from mother to child.

Mbugua said they had noted that while some mothers had not immunised their children, some were themselves not immunised, hence posing a threat to their children.

"If we wait until the age of two years so that the vaccine can achieve 100 per cent efficacy, the chances of losing the child are very high," Mbugua said.

He said Kemri had conducted a test and identified the measles strain as Genotype B3, which is normally found in Somalia.

He said the strain was more infectious and stronger.

He said the strain had been found in infected children in Nairobi, which has had the highest number of deaths countrywide.

"We have had measles outbreak before, but it has never been this scary," he said.

Mbugua said he conducted a research on measles viruses circulating in Kenya and found the B3 strain in Nairobi's Eastleigh estate, where Somali refugees are dominant.

He said Kemri conducted a research in 2002 and found that the strain circulating then was D4, which is less harmful.

"We want to know the strains so that we can track transmission pathways from country to country," he added.
Appendix ix

DEFINITIONS OF TERMS USED

Measles – an acute viral infection that is transmitted in the air, by respiratory droplets, or by direct contact with the nasal and throat secretions of infected persons.

Measles clinical case definition - any person with fever and maculopapular rash and cough, coryza (runny-nose) or conjunctivitis (red eyes) or any person in whom a clinician suspects measles.

Measles vaccine – live, attenuated virus preparations derived from various measles virus strains. All children aged ≥9 months should receive one dose of measles vaccine. Measles antibodies develop in approximately 85% of children vaccinated at 9 months of age, 95% of children vaccinated at 12 months of age, and 98% of children vaccinated at 15 months of age. Measles vaccine provides life long immunity in most people. A response is given to a second dose by a high proportion of vaccinated persons who lack detectable antibody.

Measles control – reduction of measles morbidity and mortality in accordance with targets. Continued intervention measures are required to maintain the reduction.

Measles elimination – the situation in a large geographical area in which endemic transmission of measles has stopped and sustained transmission does not occur following the occurrence of an imported case; continued intervention measures are required.
Measles eradication – interruption of measles transmission worldwide as a result of deliberate efforts. Intervention methods may no longer be needed. Eradication represents the sum of successful elimination efforts in all countries.

Surveillance – the ongoing systematic collection, analysis and interpretation of outcome-specific data for use in planning, implementation and evaluation of public health practice. Disease surveillance is a critical component of measles control and elimination efforts and is used in the assessment of progress and in making adjustments to programmes as required.

Measles outbreak – the occurrence of clinical overt measles disease is found in more persons than it is normally expected in a given geographical area during the same period of time.

Case-based reporting – reporting of suspect measles cases involving the notification of all measles surveillance stakeholders and shipment of serum to Measles Laboratory, with copies of the Integrated Case-Based Surveillance Form sent to District and Provincial levels, respectively.

Epidemiology – the study of the transmission, control and prevention of disease in a community or a group of persons.

Laboratory confirmed measles case – a case that meets the clinical case definition and is laboratory confirmed by a positive IgM antibody test.
APPENDIX X

A CHILD SUFFERING FROM MEASLES INFECTION

Immunization coverage with measles-containing vaccines in infants, 2009.
APPENDIX XI

Immunization coverage with measles containing vaccines in infants, 2005

192 WHO Member States.
Date of slide: 25 August 2005

- <50% (7 countries or 4%)
- 50-79% (31 countries or 21%)
- 80-89% (39 countries or 20%)
- ≥90% (105 countries or 55%)
Appendix xii

COMPOSITION OF MEDIA AND REAGENTS

1. **Phosphate buffered saline, pH 7.2 (PBS)**

   NaCl ...................... 8.00 gm  
   KCl ...................... 0.20 gm  
   NaHPO₄ .............. 1.15 gm  
   KH₂PO₄ .............. 0.20 gm  

   Dissolve in distilled water. Make up to 800ml. Adjust to pH 7.2 with HCl. Autoclave at 10 PSI for 15 minutes. This gives a working solution of PBS without calcium or magnesium ions. (PBS is also commercially available in powder or liquid form).

2. **PBS-Tween wash solution**

   PBS (# 1 above)  
   Tween 20 (Commercially available)  

   Add 0.05ml Tween 20 per 100 ml PBS. Prepare sufficient volume for one test.

3. **PBS-Gelatin-Tween**

   PBS (above) ............. 1 litre  
   Tween 20 .............. 1.5 ml  
   Gelatine ............... 5.0 gm  

   Mix 5.0 gm gelatin in 1 litre PBS. Heat to dissolve gelatin and add 1.5ml Tween 20. Store at 4°C.

4. **Citrate-Acetate Buffer, 0.1M pH 5.5**

   Sodium acetate, anhydrous .... 8.2 gm  
   1 M Citric acid ............... 4 ml  

   Dissolve sodium acetate in 800 ml DH2O. Add citric acid and adjust to pH 5.5 with additional citric acid. Add distilled H₂O to 1 liter.

5. **Anti-human IgG peroxidase**

   Peroxidase-labeled goat antibody to human IgG (y)  
   Dilute vial in 50% glycerol/PBS to stock conc. 1.3  
   Titer new lot for optimum dilution. Store at -20°C.

6. **Tetramethylenediamine (TMB) Substrate**
(3,3’5, 5’-Tetramethylbenzidine (TMB)-H₂O₂ Chromogen Substrate Reagent)

**Stock TMB Solution, 50X:**

TMB .................... 5.0 mg  
Dimethyl sulfoxide (DMSO) ...... 1.0 ml

Dissolve fresh TMB in fresh dimethyl sulfoxide avoiding contact with skin. Dispense 1 ml volumes and store at -20°C. This reagent is stable for over one year.

**Working TMB solution**

50X TMB ..................... 200 ul  
0.1 M Citrate-acetate buffer ...... 10.0 ml  
30% H₂O₂ .......................... 2. ul

Make working substrate just prior to use in CLEAN container.

6. **Viral Transport Medium**

Hanks' Basal Salt Solution pH 7.4 with HEPES buffer (commercially available 10X)  
Bovine albumin .................. 2.0 gm  
Penicillin/Streptomycin solution (# 9 below) ... 1.0 ml  
Phenol Red, 0.4% ..................... 0.2 ml

Dissolve 2.0 gm bovine albumin in 100ml distilled water. Add 10 ml Hanks’BSS to 80 ml distilled water then add 10ml 2% bovine solution (above) and 0.2 ml phenol red solution. Sterilize by filtration. Add 1 ml penicillin/streptomycin solution. Dispense into sterile vials and store at 4°C.

7. **Penicillin/Streptomycin solution**

Crystalline penicillin G  
Streptomycin sulphate

Dissolve 1 x 10⁶ units of penicillin and 1 gm streptomycin sulphate in 100 ml sterile PBS. Store 5ml aliquots at -20°C. One ml of this solution in 100 ml medium gives a final concentration of 100 units of penicillin and 100 ug of streptomycin per ml.
FINAL CLASSIFICATION OF SUSPECTED MEASLES CASES

Suspected measles case

Adequate serum specimen?

Yes

Measles IgM+ve

Vaccinated in the past 6 weeks

No

Laboratory confirmed measles case

Yes

Measles IgM-ve

Rubella IgM+ve

Discarded measles case

Rubella IgM+ve
APPENDIX XV

VACCINATION STRATEGY: ACHIEVING POPULATION IMMUNITY

500,000 birth cohort
Vaccine coverage = 90%

450,000 vaccinated
Vaccine efficacy = 90%

405,000 immunized

50,000 unvaccinated

45,000 vaccinated but unprotected

405,000 immunized Pop.immunity = 81% 95,000 susceptible