1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Clinical disease

Measles, one of the most infectious viral diseases of humans affecting >95% of exposed individuals in the absence of vaccination, is spread by the respiratory route and remains a major cause of mortality in children, particularly in developing countries (Hope-Simpson, 1952; van den Ent *et al*., 2011). Although other primates are also susceptible to measles and develop similar clinical disease, these populations are not large enough to support sustained transmission of the virus and thus humans are the only natural reservoir (Griffin, 2007). Measles has an incubation period of approximately 10-12 days, followed by a 3-4 day prodromal period of fever, anorexia, malaise and one or more of the three C's (cough, coryza, conjunctivitis). During this prodromal period, Koplik's spots (small, bright red spots with a blueish-white centre) appear on the buccal mucosa. These spots are considered to be pathognomonic for measles diagnosis prior to onset of rash (Moss & Griffin, 2009). The prodrome is followed by a characteristic erythematous maculopapular rash that appears first on the face and behind the ears, spreads to the trunk and extremities, and fades after 3-4 days (Figure 1.1).

People with measles are infectious for several days before and after the onset of rash. In uncomplicated measles, clinical recovery begins soon after onset of rash, and results in viral clearance and lifelong immunity. As a consequence of the immunosuppression induced by measles infection, and the direct damage to the respiratory tract (loss of cilia), secondary bacterial, viral and parasitic infections may occur. In developed countries, complications such as otitis media, gastroenteritis, pneumonia, myocarditis and pericarditis occur in approximately 10% of measles cases, with encephalitis occurring in a very small subset of cases. In developing countries, complication rates may reach 80%. Pneumonia and gastroenteritis due to secondary infections are the most common fatal complications, especially in malnourished and immunocompromised children (Duke & Mgone, 2003; Griffin, 2007). Up to 15% of immunocompetent adults with measles may also experience pneumonia as a direct result of the measles virus (MV) infection, as opposed to a secondary infection. Childhood blindness associated

with keratitis and corneal lesions and exacerbated by vitamin A deficiency, is a frequent complication of measles, especially in developing countries.

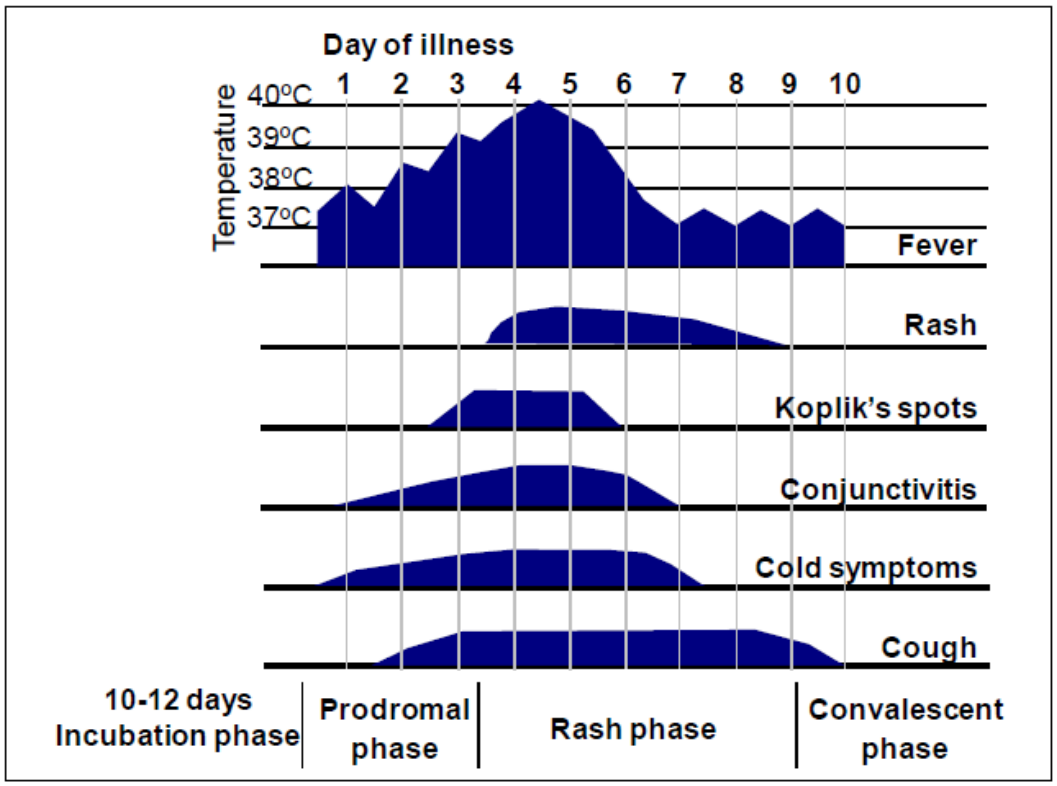

Figure 1.1 Clinical features of typical measles time-course from onset of illness (WHO, 2007).

Acute disseminated encephalomyelitis (ADEM), also known as acute post-infectious measles encephalomyelitis, is an autoimmune-mediated demyelinating disease, usually occurring within two weeks of the primary measles infection in immunocompetent individuals over two years of age and is the most common neurologic complication of measles. Most survivors of ADEM suffer neurological sequelae. In rare instances, MV is able to enter the brain and replicate in neurons and glial cells, resulting in two possible types of neurologic disease: measles inclusion body encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE). MIBE is a progressive, generally fatal MV infection of the brain in immunocompromised patients; symptoms generally occur within a few months of the initial MV infection and results in death within eight weeks of onset. MV ribonucleic acid (RNA) and antigens are readily detectable in brain biopsy and autopsy specimens, and eosinophilic intranuclear and intracytoplasmic inclusions can be seen in glial cells and neurons. SSPE may manifest within ten years of the primary infection in

children who were infected with measles before the age of two. The onset is insidious, with progressive loss of cortical function, development of myoclonus, characteristic electroencephalographic changes, ataxia and loss of vision. Periods of remission are common, but death generally occurs within three years of onset (Moss & Griffin, 2009). It is not known why MV persists in some individuals, but it is likely to be host-related as there have been case reports of SSPE in siblings and twins (Moulin *et al*., 2011).

Some features of the clinical presentation of SSPE resemble Dravet syndrome (severe myoclonic epilepsy of infancy) which is associated with mutations in the SCN1A gene (encoding the alpha subunit of the sodium channel protein), as are several other types of acute or subacute encephalopathic syndromes. It was recently postulated that there may be SCN1A gene abnormalities in children who develop SSPE following measles infection (Garg, 2012). Besides involvement of host-related factors in the development of SSPE, there are also special features about the strains of MV that are able to replicate in brain: whole genome sequence analysis of SSPE viruses has shown that they differ from wild-type (WT) viruses mainly in the accumulation of mutations in three viral proteins (matrix, fusion, haemagglutinin) which results in poor expression of viral envelope proteins and prevents release of infectious viral particles from infected neuronal cells (Ayata *et al*., 2002; Oldstone *et al*., 2005; Jiang *et al*., 2009; Moulin *et al*., 2011). While other Morbilliviruses such as canine distemper virus and phocine distemper virus often invade the central nervous system of their host causing encephalitis, this is not a common feature in MV and rinderpest virus infections.

1.2 Measles in history

Measles is a disease of human civilization – mathematical models indicate that a population size of 250 000 - 400 000 and an annual birth cohort of 5000 – 10 000 is needed in order to sustain circulation of the virus (Keeling & Grenfell, 1997). Populations of this size did not exist until the development of urban civilizations in the Middle East, India and China, around the third millennium BC. Measles therefore appears to be a relatively new disease of humans, and on the basis of phylogenetic analysis of *Morbillivirus* sequences, it is postulated that a zoonotic *Morbillivirus* (an ancestor of modern rinderpest virus) was transmitted to humans within the last 5 000 to 10 000

years, and this eventually evolved into measles virus (Moss & Griffin, 2009). However a recent study estimated that divergence of MV from rinderpest virus may have occurred within the last 1000 years (Furuse *et al*., 2010). Similarly, carnivores could have contracted a *Morbillivirus* infection from their ruminant prey, this pathogen eventually evolving into canine distemper virus (Barrett & Rossiter, 1999).

Measles was already recognized as a disease distinct from smallpox in the ninth century, by Abu Becr (Rhazes of Baghdad) and he traced descriptions of the disease as far back as the sixth century. There are abundant historical records from Europe and the Far East that describe repeated epidemics of rash-related illnesses, and it was documented as a childhood disease in 1224 (Griffin, 2007). In 1670, Thomas Sydenham described a measles outbreak in London, accurately detailed the clinical presentation of the disease, and highlighted pulmonary complications and increased severity in adults. In European literature, it was named *morbilli* from the Latin “little diseases” to distinguish it from plague *il morbo*, however the term morbilli included several illnesses presenting with rash. In 1763, Sanvages defined morbilli as measles (from which the taxonomic nomenclature *Morbillivirus* was derived), but called it *rubeola* (derived from Spanish) which led to subsequent confusion with rubella (Latin for ‘’little red”, commonly referred to as German measles) (Moss & Griffin, 2009).

Epidemics of rash illnesses, associated with abrupt decreases in population size, were recorded in China, India and the Mediterranean region. The European conquest of the New World decimated the indigenous populations, largely due to the introduction of diseases such as smallpox and measles (McNeill, 1976). These historical examples illustrate the extremely high mortality associated with the introduction of measles into virus-naïve populations. In 1846, a Danish physician, Peter Panum, investigated a measles outbreak in the Faroe Islands from which he confirmed the highly contagious nature of the disease, probably with a respiratory route of transmission, an incubation period of 14 days and lifelong immunity following infection (Moss & Griffin, 2009).

Measles was shown to be caused by an infectious agent in 1757 when Francis Home, a Scottish physician, transmitted the disease to naïve individuals, while attempting immunization, by inoculation with blood from a person with the characteristic rash. Hektoen repeated this experiment using blood “free of bacteria” from acute measles

cases to transmit the disease to volunteers in 1905. The viral aetiology of measles was confirmed by Goldberger & Anderson in 1911 when the disease was transmitted to monkeys using filtered respiratory tract secretions from measles patients, and the illness was then serially passed on to other monkeys (Griffin, 2007).

1.3 The Virus

The taxonomy of MV has been assigned as follows (Lamb & Parks, 2007):

Order: Mononegavirales
Family: Paramyxoviridae
Subfamily: Paramyxovirinae
Genus: *Morbillivirus*
Species: measles virus (Type species) (humans and primates)
canine distemper virus (dogs and other mammalian carnivores)
rinderpest virus (domestic cattle, water buffalo, swine, sheep and goats)
peste-des-petits-ruminants virus (sheep and goats)
phocine distemper virus (seals)
cetacean morbillivirus (porpoises and dolphins)

All members of the Mononegavirales are enveloped viruses with a single-stranded, negative-sense RNA genome. Within the Paramyxovirinae, the genus *Morbillivirus* is characterized by the formation of intranuclear inclusion bodies and lack of neuraminidase activity. Virions are pleiomorphic and range in size from 100-300 nm. The surface projections of the viral envelope are composed of viral transmembrane haemagglutinin (H) and fusion (F) glycoproteins (Figure 1.2). The matrix protein (M) lines the interior of the virion envelope. The helical nucleocapsid (up to 1.2 µm in length; diameter of 12-17 nm) is coiled within the envelope and consists of approximately 2500 copies of the nucleoprotein (N) bound to the genomic RNA, and is associated with a small number of copies of the phosphoprotein (P) and large protein (L).

The genome comprises 15 894 nucleotides, but the exact length, especially for laboratory strains, depends on the strain and passage history (Crowley *et al*., 1988). The gene order and transcriptional map of the genome were elucidated to be 3'–N–P/C/V–M–F–H–L–5' (Rima *et al*., 1986). There are six transcriptional units which code for the six structural proteins of the virus (N, P, M, F, H, L) as well as at least two non-structural proteins (C, V) which are encoded within the P gene (Figure 1.2; Table 1.1).

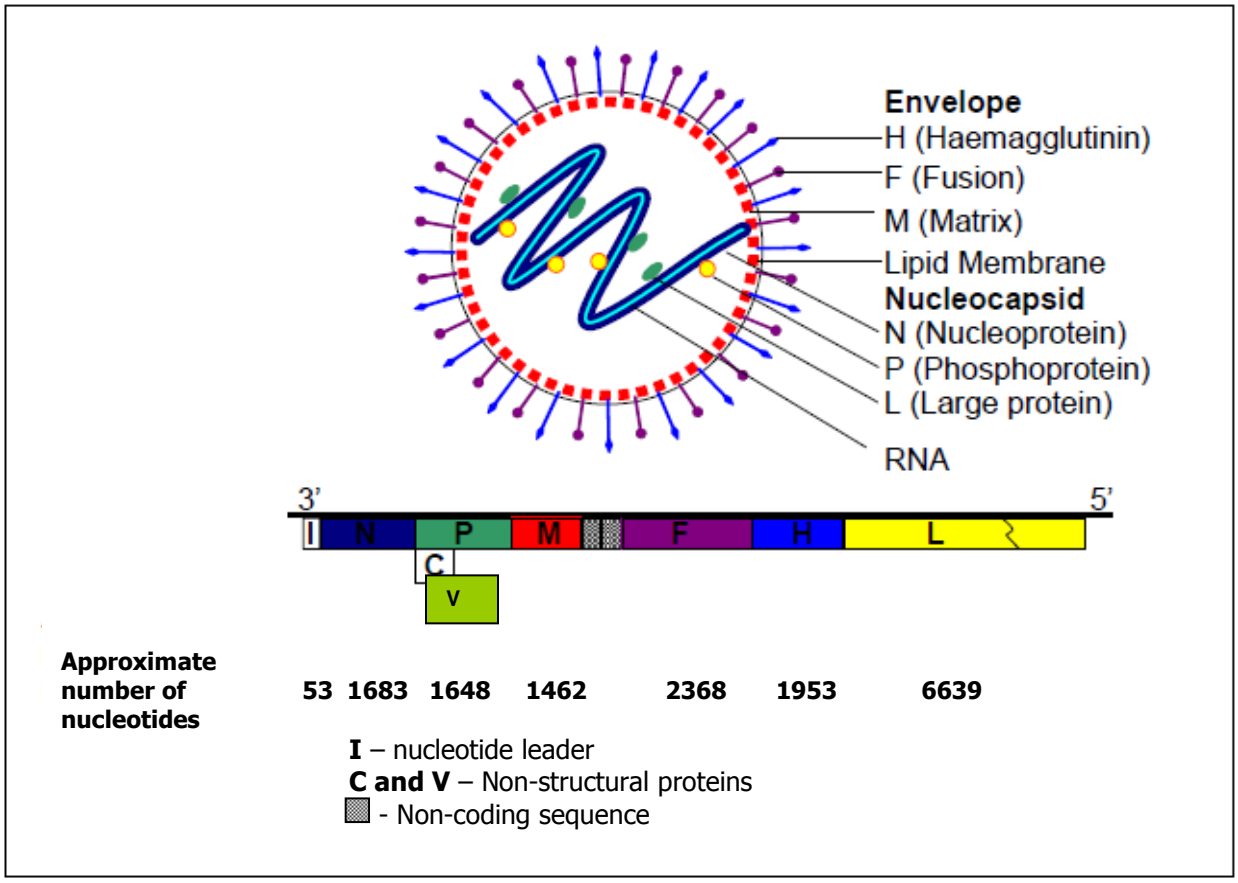

Figure 1.2 Diagram of a measles virus particle and correlation with the genetic map (Murray *et al*, 1998; Griffin, 2007).

Although there is genetic variation in all six genes of MV (Baczko *et al*., 1991; Baczko *et al*., 1992; Rota *et al*., 1992; Komase *et al*., 1995), the highest degree of variation is in the carboxyl-terminus of the nucleoprotein, followed by the H and P proteins. The 150 carboxyl-terminal amino acid (AA) residues of the nucleoprotein satisfy the requirements

for phylogenetic differentiation of strains (Baczko *et al*., 1992; Rima *et al*., 1995). Transcription is more efficient at the 3' end of the genome, with a transcriptional gradient from N to L, the slope of which varies in different host cells (Horikami & Moyer, 1995). This transcriptional gradient is a major determinant of the relative abundance of the viral proteins, and indeed, the nucleoprotein is the most abundant. N occurs in the cytoplasm as a soluble N-P complex (Huber *et al*., 1991). However, when P is present in reduced amounts (late in infection and in strains isolated from SSPE brain tissue) or entirely absent (e.g. when N is expressed from plasmids), N is an insoluble protein that migrates to the nucleus where it assembles into nucleocapsid-like structures without encapsidated viral RNA that can be seen as intranuclear inclusion bodies (Bohn *et al*., 1990; Baczko *et al*., 1992). The conserved amino-terminal portion of N is necessary for self-assembly into nucleocapsids and for RNA binding (Karlin *et al*., 2002). The ribonucleoprotein complex serves as the template for both transcription and viral replication (Griffin, 2007).The carboxyl-terminal portion of N is more variable, has an intrinsically disordered structure, and shares features with acidic activation domains of cellular transcription factors (Longhi *et al*., 2003).

The phosphoprotein associates with the L protein to form the viral RNA-dependent RNA polymerase; together with N they comprise the replicase complex (Table 1.1). The P-gene also encodes two non-structural proteins, C and V. C is a basic protein, transcribed from an overlapping reading frame of the P-gene (Bellini *et al*., 1985). V is produced by editing of the P mRNA during transcription by insertion of a non-templated guanosine residue that shifts the reading frame (Cattaneo *et al*., 1989), therefore the amino terminal part of V is identical that of the P protein, while its carboxyl-terminal 70 AA residues are unique. This carboxyl-terminal region of V is rich in cysteine residues and shares features with zinc-finger proteins (Baczko *et al*., 1992). Although neither C nor V are necessary for MV replication, they interact with cellular proteins, interfering with innate immune responses by inhibition of interferon signaling pathways (Valsamakis *et al*., 1998; Palosaari *et al*., 2003). A diverse range of host evasion activities, such as prevention of apoptosis, cell cycle alterations and inhibition of double stranded RNA signaling, have also been ascribed to the V-proteins of other members of the *Paramyxoviridae* (Lin & Lamb, 2000; He *et al*., 2002; Wansley & Parks, 2002). These immune suppression activities contribute to the pathogenicity of wild-type MV and to the attenuation in vaccine strains (Palosaari *et al*., 2003).

Table 1.1 Structural and regulatory proteins of measles virus (Patterson *et al*., 2000; Palosaari *et al*., 2003; Bankamp *et al*., 2005; Moss & Griffin, 2009).

MV protein	AAs	Location within infected cell	Function of protein
Nucleoprotein (N)	525	Cytoplasm	Binds to RNA and P protein; required for transcription, replication and self-assembly
Phosphoprotein (P)	507	Cytoplasm	Regulation of transcription and replication; polymerase co-factor, linking L to N to form the replicase complex
C	186	Cytoplasm, nucleus	Regulation of transcription; down-regulates alpha, beta and gamma interferons in the antiviral pathway
V	298	Cytoplasm	Regulation of transcription; down-regulates alpha, beta and gamma interferons in the antiviral pathway
Matrix (M)	335	Inner layer of plasma membrane; cytoplasm in persistent infections	Virion assembly, maturation and budding; transcriptional regulator
Fusion (F)	553	Endoplasmic reticulum, golgi, plasmalemma	Type 1 transmembrane glycoprotein; membrane fusion; virus entry in conjunction with H
Haemagglutinin (H)	617	Endoplasmic reticulum, Golgi, plasmalemma	Type 2 transmembrane glycoprotein; receptor binding; haemagglutination activity
Large (L)	2213	Cytoplasm	Provides the catalytic component of RNA-dependant RNA-polymerase, when complexed with P; viral transcription and replication

The M protein is associated with the nucleocapsid, the inner layer of the cell membrane, and the intracytoplasmic regions of one or both of the transmembrane glycoproteins (F and H). Deletion of M increases cell-to-cell fusion and decreases production of infectious virus (Cathomen *et al*., 1998). Defects in M are associated with persistent infections, which are characterised by a marked decrease in the amount of infectious virus released, accumulation of intranuclear and intracytoplasmic nucleocapsids and decreased virus-induced cytopathic effect (CPE) compared with lytic infections. SSPE strains produce no infectious virus and persistent infection appears to be maintained by cell-to-cell transmission (Patterson *et al*., 2001).

The fusion protein mediates the fusion of viral and target cell membranes, a prerequisite for virus entry. F is synthesized and glycosylated in the endoplasmic reticulum as an inactive precursor (F_0) which is subsequently cleaved by furin-like proteases in the Golgi apparatus into F_1 and F_2 subunits that are covalently linked to produce the fusion-competent, type 1 transmembrane protein. The F_2 subunit contains the signal peptide as well as all the predicted glycosylation sites necessary for stability of the mature fusion protein (Griffin, 2007). The F_1 subunit, derived from carboxyl-terminus of F_0, is anchored in the viral envelope, and has a cytoplasmic tail that contains 14 terminal AAs which are highly conserved among the morbilliviruses. The fusion peptide, a hydrophobic region of 25 AA at the amino-terminus of F_1, interacts with the host cell membrane to induce fusion, is also highly conserved in the paramyxovirus family. However, F alone cannot induce fusion: interaction of H with the cellular receptor is necessary to bring the fusion peptide region of the F_1 subunit into contact with the cell membrane (Richardson & Choppin, 1983).

The H-protein is a type-2 transmembrane glycoprotein of the virion that mediates attachment to cellular receptors and is therefore the major determinant of cellular tropism. Interaction of H with a cellular receptor and F results in fusion of the viral and cellular envelopes, entry of the ribonucleocapsid into the cytoplasm and initiation of the viral replication cycle (Wild & Buckland, 1995). The ability of MV to agglutinate monkey erythrocytes resides in a region of H that is close to, yet distinct from, the receptor-binding sites, as antibodies that block haemagglutination can neutralize viral infectivity. Extensive glycosylation and oligosaccharide modifications are necessary for H to be correctly assembled as disulfide-linked homodimers that accumulate as tetramers on the viral envelope (Griffin, 2007). By comparison with the known 3-dimensional structure of the H-protein of newcastle disease virus, structural models of MV H suggest a stem and six-β-sheet propeller structure. Receptor-binding sites in the predicted model appear to map to β-sheets 4 (the CD46 binding site) and 5 (SLAM binding site) while neutralizing epitopes map to the top loops protruding from the blades of the propeller (Vongpunsawad *et al*., 2004).

The L protein is the catalytic subunit that interacts with P to form the viral RNA-dependent RNA polymerase. It contains six domains that are highly conserved in the RNA polymerases of the minus-strand RNA viruses (Moss & Griffin, 2009)

Nearly 11% of the MV genome is composed of non-coding RNA located between each of the viral genes; these regions appear to function as *cis*-acting elements which specify, organize and control gene expression and genome replication. This usually occurs through interaction with viral and host cell proteins (Parks *et al*., 2001). The genome has a 53 nucleotide leader sequence at the 3' end, which is highly complementary to the 40 nucleotide trailer sequence at the 5' end of the genome, theoretically permitting the formation of a stable, panhandle structure. It is likely that these leader and trailer sequences contain motifs that promote encapsidation of the genomic RNA by the nucleoprotein, as well as binding sites for L, the enzymatic subunit of the viral RNA polymerase (Moss & Griffin, 2009). An intergenic spacer of three nucleotides (GAA) is located between each gene except between the H and L genes where a GCA spacer has been identified. In addition, there is a non-coding GC-rich region of about 1000 nucleotides between the M and F genes. The mRNA for *Morbillivirus* matrix proteins contain approximately 400 nucleotides of unknown function at the 3' end, while the mRNA for the precursor of the fusion protein contain up to 585 nucleotides of this non-coding sequence at the 5' terminus (Richardson *et al*., 1986).

1.4 Virus isolation and development of vaccines

MV was first isolated in 1954 (Enders & Peebles, 1954) using peripheral blood leukocytes and respiratory secretions collected from a child with measles (David Edmonston) by serial passage in primary human kidney cells and subsequently also in primary monkey kidney cells (Griffin, 2007). However, the increasing difficulty in obtaining primary cells and the potential contamination of such cells with monkey viruses led to the use of less sensitive continuous cell lines (e.g. Vero and CV-1 cells originally from the kidneys of African green monkeys, chick embryo fibroblasts CEF, dog and sheep kidney). The Edmonston isolate was further attenuated by multiple passages through a variety of cell lines and plaque purifications, and developed into vaccines, the first of which was licensed in 1963 (Bellini *et al*., 1994; Yanagi, 2001). These vaccine strains (Zagreb, AIK-C, Schwarz, Moraten, Edmonston-B, Rubeovax) differ genetically because of their passage history but are nevertheless derived from the original isolate. Other live-attenuated vaccines (e.g. CAM-70, Leningrad-4, Changchun-47, Shanghai-191) were developed independently in Japan, Russia and China from different WT

progenitors by similar methods. Nevertheless all vaccine strains cluster in genotype A, possibly because this was the most widely distributed genotype in the 1950s (Riddell *et al*., 2005; Bankamp *et al*., 2011).

In 1990, a Japanese group developed an immortalized (transformed with Epstein-Barr virus) Marmoset lymphocyte cell line B95-8 and a derivative adherent cell line, B95-a, that was superior for the primary isolation of MV from clinical samples (Kobune *et al*., 1990). Marmosets had been shown to be extremely sensitive to MV infection and closely mimicked human clinical disease presentation. MV isolated from B95-a cells retained pathogenicity for monkeys, whereas strains isolated in Vero cells did not. The B95-a cell line was susceptible to both WT and vaccine strains of measles virus, whereas WT virus grew very poorly or not at all in Vero cells.

MV replication in cell culture results in three types of CPE:

- altered cell shape – uninfected cells are typically polygonal in monolayer cultures, whereas infected cells generally have stellate-, dendritic- or spindle-shapes, and increased refractility (Griffin, 2007);
- multinucleate giant cells (syncytia) – these are formed as a result of fusion of infected cells with uninfected cells, resulting in syncytia with 50 or more nuclei within a single cell membrane. This is facilitated by the viral glycoproteins H and F. Nuclei in the centre of the syncytia have marginated chromatin, which is one of the ultrastructural features of apoptosis (Esolen *et al*., 1995a);
- inclusion bodies – both spindle-shaped cells and syncytia can contain intracytoplasmic and intranuclear (Cowdry type A) inclusion bodies. Intranuclear inclusion bodies are smaller, lack viral RNA and differ ultrastructurally from the intracytoplasmic bodies (Bohn *et al*., 1990).

1.5 Cellular receptors and pathogenesis

In 1993, human CD46, also known as membrane cofactor protein, was identified as a cellular receptor for the vaccine strains of MV (Dorig *et al*., 1993; Naniche *et al*., 1993). CD46 is a complement regulatory protein expressed on all nucleated cells and preferentially on the apical surface of polarized epithelial cells. Its main function is to

protect cells from complement-mediated attack (i.e. self versus non-self protection). However, the ubiquitous distribution of CD46 does not explain the lymphoid tropism of MV, nor the fact that WT viruses grow poorly in Vero cells which do express CD46.

Signaling Lymphocyte Activation Marker (SLAM) was identified in 1995 as a novel receptor of the immunoglobulin superfamily that is involved in the activation of T-cells and natural killer cells (Cocks *et al*., 1995). Functional expression cloning experiments using extracts of B95-a cells, showed that SLAM (CD150) was a major cellular receptor for MV (Tatsuo *et al*., 2000). SLAM is only expressed on a subset of immune cells: immature thymocytes; mature dendritic cells; activated monocytes; activated T-cells and B-cells (Yanagi *et al*., 2006). This distribution of SLAM coincides with the tropism of MV for cells of the immune system, and the utilization of SLAM as a receptor was thought to explain some of the pathology of MV infection, such as lymphopenia and immunosuppression. Two other Morbilliviruses (canine distemper virus and rinderpest virus) have been shown to use the canine and bovine analogs of SLAM respectively, as receptors (Tatsuo *et al*., 2001; Baron, 2005).

The lymphocytic B95-a cell line, although very sensitive for MV isolation because of SLAM expression, unfortunately persistently produces Epstein-Barr virus and therefore poses a risk of infection to laboratory staff. It is also difficult to distribute the cell line to laboratories undertaking MV isolation as there are shipping restrictions for infectious materials. For this reason, B95-a is no longer the preferred cell line for the isolation of MV, and has been replaced by the Vero-hSLAM cell line (WHO, 2007). This is a genetically engineered Vero cell line transfected with a plasmid encoding the gene for human SLAM (hSLAM) (Ono *et al*., 2001). The sensitivity of Vero-hSLAM cells for the isolation of WT MV is equivalent to that of B95-a cells (Kouomou & Wild, 2002). Vero-hSLAM cells are also sensitive to infection by laboratory-adapted strains including vaccine viruses. Dr. Yusuke Yanagi (Kyushu University, Japan) who developed the Vero-hSLAM cell line has magnanimously allowed the World Health Organisation (WHO) Measles and Rubella Laboratory Network (LabNet) to make use of this cell line with the proviso that it may not be distributed outside of the LabNet without permission.

Until recently, it was thought that the initial MV infection was established in the respiratory tract and oropharynx, with viral replication in the epithelial cells and

pulmonary macrophages, and subsequent spread to local lymphatic tissues where further replication occurs (Kamahora & Nii, 1965; Sakaguchi *et al*., 1986; Hummel *et al*., 1998; Hilleman, 2001; Shingai *et al*., 2005). Replication within the lymph nodes results in the appearance of giant, multinucleate cells (containing up to 100 nuclei) of lymphoid or reticuloendothelial origin, referred to as Warthin-Finkeldey cells. These cells are a consistent pathological feature of MV infection. Further dissemination to other sites (skin, conjunctivae, kidney, lung, gastrointestinal tract, genital mucosa, liver) is mediated by infected monocytes and results in a viraemic phase (White & Boyd, 1973). In these secondary sites, replication occurs primarily in endothelial cells, epithelial cells, monocytes and macrophages (Moench *et al*., 1988; Esolen *et al*., 1993; Griffin *et al*., 1994; Esolen *et al*., 1995b; Takahashi *et al*., 1996). This replication, together with the immune response, is responsible for the prodromal signs and symptoms. Giant, multinucleate epithelial cells are evident in nasal secretions and conjunctivae. MV-infected epithelial cells are also shed into the urine (Lightwood & Nolan, 1970; Scheifele & Forbes, 1972).

Unfortunately, the hypothesis of initial infection of epithelial cells and subsequent viraemia mediated by infected monocytes is an unlikely sequence of events given that neither of these cell types express SLAM which is required for entry of WT MV (de Swart *et al*., 2007). It was recently shown that the initial target cells infected by MV are macrophages and dendritic cells of the lower respiratory tract which express SLAM (Ferreira *et al*., 2010; Leonard *et al*., 2010; Lemon *et al*., 2011). These cells then cross the respiratory epithelium and transport the infection to bronchus-associated lymphoid tissue, where there is abundant viral replication (de Swart *et al*., 2007) and subsequent systemic dissemination by viraemia (Lemon *et al*., 2011). This was demonstrated using a recombinant MV clone (Takeda *et al*., 2000) containing an enhanced green fluorescent protein (EGFP) inserted upstream of the N-gene. Expression of EGFP permitted the process of pathogenesis to be followed by fluorescence both in live monkeys (rhesus and cynomolgus macaques) as well as in tissue samples. Peripheral monocytes did not sustain productive MV infection (de Swart *et al*., 2007).

Nevertheless, none of these studies was able to address how virions are able to infect epithelia from the basolateral (abluminal) surface, in order to replicate and exit from the apical (luminal) epithelial surface into the respiratory tract (Ludlow *et al*., 2009). Several

studies have alluded to the existence of a receptor on polarized epithelial cells that can be used by MV for attachment and subsequent fusion of membranes, for entry and for viral shedding (Leonard *et al*., 2008; Tahara *et al*., 2008; Yanagi *et al*., 2009; Ludlow *et al*., 2010). This receptor was recently identified as nectin-4 (polio virus receptor-like-4, PVRL4), a member of the immunoglobulin superfamily of proteins, which is expressed on the adherens junctions of epithelial cells (Muhlebach *et al*., 2011; Noyce *et al*., 2011). Infection of macaques with a mutant MV that was unable to bind to nectin-4 resulted in clinical symptoms of measles and detectable viraemia, but the inability to infect respiratory epithelial cells via the nectin-4 receptor prevented this virus from being released and spread via respiratory secretions as aerosolized droplets (Leonard *et al*., 2008; Noyce *et al*., 2011). Wild-type MV is able to infect adenocarcinoma cell lines derived from the glandular epithelia of a variety of lung, breast and colon tumours, because these cell lines have high levels of nectin-4 expression. Blocking of this receptor prevents MV infection, thus confirming the role of nectin-4 for receptor-mediated entry. As nectin-4 is a cellular marker of several types of cancer, MV is also being considered as an oncolytic agent to target cancerous cells (Takano *et al*., 2009; Galanis *et al*., 2010; Noyce *et al*., 2011).

1.6 Immune reponses

The generation of immune responses to a viral infection is primarily dependent on the dynamic interactions between viral factors and host determinants. Viruses are subject to control by the innate immune system before an adaptive immune response is generated, and like many other human viruses, MV encodes proteins that function to evade or delay the innate immune response. In particular, the V and C proteins encoded by the P-gene of WT MV strains function as interferon antagonists (Naniche *et al*., 2000; Ohno *et al*., 2004; Haralambieva *et al*., 2007) whereas these proteins in attenuated, vaccine strains are unable to significantly suppress interferon pathways and signaling. Gene-expression profiling experiments have revealed significant cytokine suppression at the transcriptional level for viruses encoding the functional WT P-gene compared to attenuated vaccine strains (Haralambieva *et al*., 2010). Cytokines are important regulators of both innate and adaptive immune responses to viral infections. Measles-specific cytokine and lympho-proliferative responses can be used as surrogate

measures of cell-mediated immunity (CMI) following measles vaccination and disease (Griffin & Ward, 1993; Naniche, 2009; Howe *et al*., 2005a; Howe *et al*., 2005b).

MV-specific immune responses are essential for recovery from measles, and may be directly responsible for a number of the clinical manifestations of measles and its complications. Immune responses can be detected during the prodrome period and are well developed by the time the rash appears (Figure 1.3). Marked immune activation is evidenced by T- and B-cell activation and proliferation, as well as increased levels of cytokines. MV-induced immune suppression occurs concurrently with immune activation, resulting in impaired delayed-type hypersensitivity skin test responses to recall antigens, in reduced humoral and CMI responses to new antigens and in lowered natural killer cell function thus increasing susceptibility to secondary infections (Moss *et al*., 2004). Measles patients exhibit a marked neutropenia, i.e. decreased levels of polymorphonuclear neutrophils (Kim *et al*., 2002). Neutrophils display defects in chemotaxis, adherence, metabolic burst and killing capacity, but have normal serum opsonic activity (Anderson *et al*., 1976; Yetgin & Altay, 1980; Thatte *et al*., 1991; Toraldo *et al*., 1992). Individuals with compromised CMI (congenital or acquired) can present with an atypical rash or even without the characteristic rash (Moss *et al*., 1999; De Carvalho *et al*., 2003) and are susceptible to slowed viral clearance (Permar *et al*., 2001), progressive disease and a significantly increased risk of death due to giant-cell pneumonia and encephalitis (Kaplan *et al*., 1992; Moss & Griffin, 2009; WHO, 2007). Contrastingly, persons with deficits in antibody production (e.g. agammaglobulinaemia) generally recover normally. This emphasizes the importance of a competent CMI for recovery from infection.

Both IgM and IgG antibodies are produced during the primary immune response and can be detected in serum within a few days of onset of rash (Figure 1.3). The IgM antibody levels peak seven to ten days after onset of rash and disappear after six to eight weeks. IgG antibodies are initially of low avidity, but this increases with isotype switching from IgG2 and IgG3 to high avidity IgG1 and IgG4 within a few months after rash onset as the adaptive immune response matures, and this results in lifelong immunity (Paunio *et al*., 2003). Antibodies are produced against most of the viral proteins, proportionally to the protein abundance, thus most antibodies recognise various epitopes of the viral nucleoprotein (Graves *et al*., 1984). Neutralizing antibodies are primarily directed against

the H-protein, and to a lesser extent, against the fusion protein (de Swart *et al*., 2005).

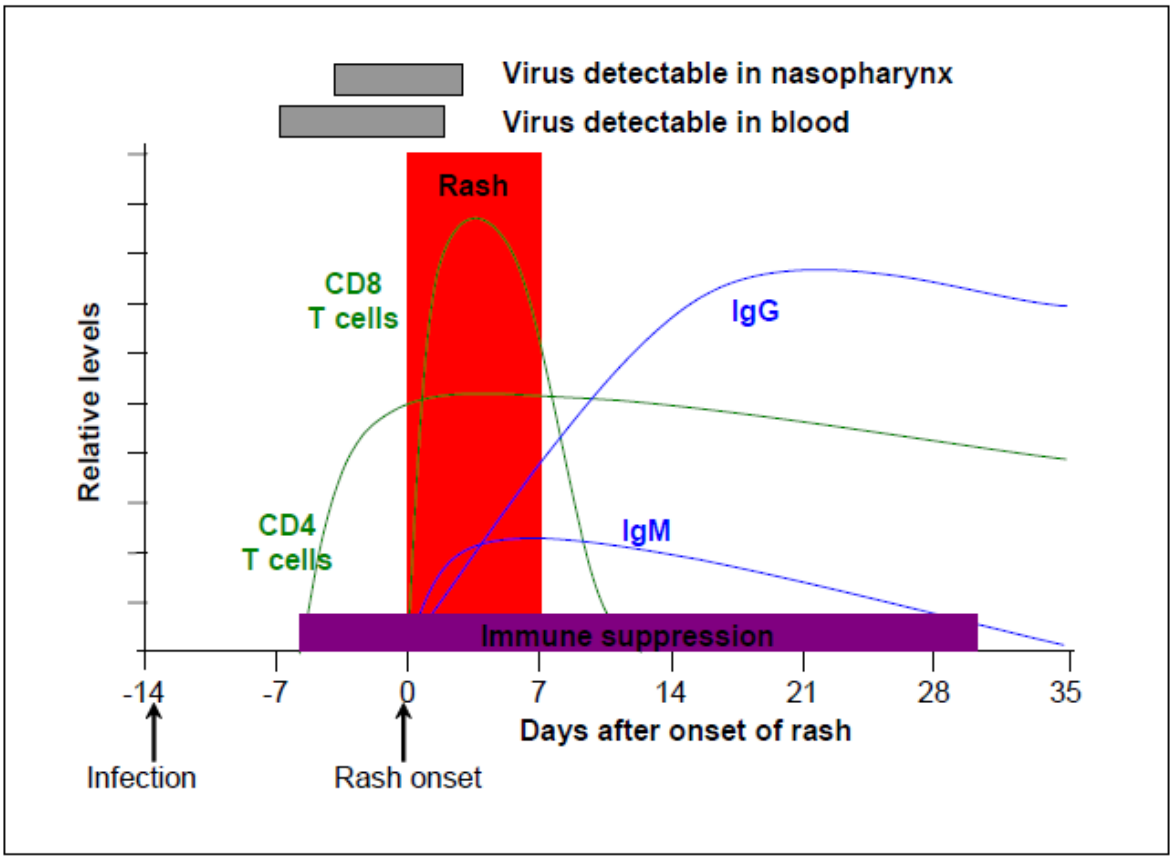

Figure 1.3 Immune responses in acute measles infection (Redd *et al*., 1999).

1.7 Measles control programmes

It is estimated that before the global use of measles vaccines, there were approximately 2.6 million measles-related deaths annually, and that more than 90% of children would have been exposed to measles by 15 years of age (Levin *et al*., 2011). A two-dose vaccination schedule for measles-containing vaccine (MCV: vaccines containing attenuated viruses are available in several formulations: measles only; measles and rubella MR; measles, mumps and rubella MMR) is the most efficient way to achieve the level of population immunity (~95%) required to interrupt measles transmission (Gay, 2004). Approximately 16% of children vaccinated at nine to eleven months of age, and about eight percent vaccinated at ≥12 months, fail to develop immunity to measles when

given one dose of MCV (Uzicanin & Zimmerman, 2011). This primary vaccine failure is generally overcome by the administration of a second dose of MCV (MCV2) either as part of the routine Expanded Programme for Immunization (EPI) childhood vaccination schedule or during mass campaigns known as supplementary immunization activities (SIAs). SIAs greatly enhance vaccination equity by reaching more unvaccinated children than routine vaccination alone but follow-up campaigns must be conducted every two to four years to prevent outbreaks until routine coverage reaches at least 90% (Vijayaraghavan *et al*., 2007; WHO, 2009). Implementation of this strategy allowed the Americas to eliminate measles in 2000 (Paunio *et al*., 1999; de Quadros *et al*., 2008). By 2008 all WHO member nations (193) offered one dose of measles vaccine (MCV1) although the timing of the schedules varied, 132 countries offered MCV2 as part of the routine schedule and 152 countries conducted SIAs with the result that 192 countries offer a second dose of MCV either as a routine second dose, or SIAs or both (Hall & Jolley, 2011). By 2010, all member countries offered MCV2, with 139 countries offering it as part of the routine schedule (CDC, 2012). The level of vaccine coverage varies greatly between countries/regions and in 2009 the average global measles vaccination coverage was only 82% (Simons *et al*., 2011), and increased from 58% in 2000 to 78% in 2010 in the 47 priority countries (CDC, 2012). In the post-vaccine era, measles outbreaks occur in regions where there is suboptimal vaccination coverage. For example, in Africa, susceptible individuals are generally those who have not been offered vaccination, or are too young to be vaccinated (under 9 months of age). In Europe, many susceptible individuals live in communities comprising immigrants originating from countries in which there is low vaccine coverage, or in communities that choose not to be vaccinated, and/or have restricted access to healthcare due to religious convictions; they are generally surrounded by highly vaccinated populations and therefore benefit from the herd immunity of these populations (Muscat, 2011).

In South Africa, a single dose of MCV at 9 months of age has been provided since 1975, with a second dose at 18 months of age being implemented since 1995. Towards achieving the goal of eliminating measles in South Africa by 2002, the strategy of the Pan American Health Organisation was used: strengthening of routine immunization; a catch-up campaign conducted in 1996 and 1997 targeting all children aged nine months to fourteen years; periodic follow-up SIAs every three to four years (2000, 2004), targeting children aged nine months to five years. Despite the reported 92%

administrative coverage, an investigation of the measles outbreak in 2003-2005, indicated that this figure was probably an over-estimate as a result of inaccurate census estimates of the target populations (McMorrow *et al*., 2009).

The aim of the Millennium Development Goal 4 (MDG4) is to reduce the overall number of deaths among children by two thirds by 2015 compared with the level in 1990 (Strebel *et al*., 2011). Global mortality attributed to measles has decreased by 78% from 2000 to 2008 (92% reduction in the African Region in 2001-2009) which represents approximately a 23% of the overall decrease in childhood mortality since 1990 and 24% since 2000, averting an estimated 4.3 million deaths (CDC, 2009; Masresha *et al*., 2011; van den Ent *et al*., 2011; CDC, 2012). This progress, especially in the 47 high-measles-burden countries, was made possible by the financial and technical support of the Measles Initiative, launched in 2001 (CDC, 2012). The Measles Initiative is a partnership founded and led by the American Red Cross, the Centers for Disease Control and Prevention (CDC), UNICEF (United Nations Children's Fund) and the WHO. However, due to global financial constraints, financial support to the Measles Initiative decreased from US$ 150m in 2009 to US$ 35m in 2010, and many priority countries have been unable to raise the required 50% of the operational costs, resulting in the postponement of scheduled SIAs and cutbacks in the range of targeted age groups. It is estimated that the gains in measles mortality reduction will be lost unless the financial situation can be improved, and indeed, there has been a resurgence of measles affecting 28 counties in sub-Saharan Africa from the latter part of 2009 resulting in >200 000 reported cases and >1400 reported deaths (but as there is under-reporting of both cases and deaths, it is estimated that the figures are likely to be 10-20 fold higher) (Strebel *et al*., 2011). Despite decreases in measles case reporting in 2010 in three of the WHO regions (Western Pacific, Eastern Mediterranean, South East Asia), global incidence increased as a result of outbreaks in Africa and the European region (CDC, 2012).

Since vaccine-induced immunity is not as robust as that resulting from natural disease, there have been many studies to investigate whether the waning immunity of highly-vaccinated populations in the absence of natural boosting (resulting in antibody levels that drop below accepted protective levels of 120 mIU) makes these populations susceptible to reinfection (Chen *et al*., 1990; Edmonson *et al*., 1990; Ozanne & d'Halewyn, 1992; LeBaron *et al*., 2007). Such cases of "secondary vaccine failure" often

present with modified measles – less severe disease, fever lower and of shorter duration, atypical rash, IgM low or absent but IgG present on day of rash. Because these symptoms are atypical, measles is often not suspected in the initial diagnosis (Smith *et al*., 1982; Helfand *et al*., 1998; Lievano *et al*., 2004; Pannuti *et al*., 2004; Sonoda & Nakayama, 2001). Nevertheless, low or decreasing antibody concentrations do not necessarily imply loss of protective immunity, as investigation of such cases revealed a memory response to re-infection: production of high avidity IgG and generation of very high levels of neutralizing antibodies (Hickman *et al*., 2011; Rota *et al*., 2011a). Although vaccination generates neutralizing antibodies against attenuated genotype A strains (all vaccines are genotype A), all known WT MV are neutralized using serum from vaccinees, albeit to different extents. No escape mutants or vaccine-derived variants have been detected to date despite careful monitoring and investigation (Bellini & Rota, 2011; Sanders *et al*., 2011)

Live-attenuated vaccines are contra-indicated in pregnancy and in infants with severe impairment of cellular immunity, specifically in HIV-1 infected infants with CD4 counts of less than 15% (Moss & Griffin, 2009). When the burden of measles is high, measles vaccination at six months of age is likely to benefit HIV-infected and HIV-exposed-but-uninfected children. This is because transplacental transfer of maternal antibodies is impaired in HIV-infected women, so that their infants become susceptible to MV at a younger age than children of HIV-uninfected mothers (Embree *et al*., 1992; Moss *et al*., 2002; Farquhar *et al*., 2005; Scott *et al*., 2007; Moss *et al*., 2008; Chandwani *et al*., 2011; Scott *et al*., 2011). Since the risk of death following measles is also higher in HIV-infected infants, it is a WHO recommendation that infants living in areas with a high incidence of HIV and measles should receive a dose of MCV at six months of age (as a supplementary dose) followed by the two doses (MCV1 and MCV2) according to the national EPI schedules (WHO, 2009). The findings of a recent study in Malawi supported this WHO recommendation in that all the HIV-infected infants in the study were protected till after 12 months of age but unfortunately only 41% were still protected at age 20 months, so the seroprotection was not sustainable (Fowlkes *et al*., 2011). Other studies also found rapid waning of vaccine-induced antibodies in HIV-infected children, with a median half-life of 18 months (Al-Attar *et al*., 1995; Arpadi *et al*., 1996; Nair *et al*., 2009).

1.8 Laboratory diagnosis and confirmation

Measles is generally correctly diagnosed by clinicians who have seen the disease e.g. in endemic areas or during outbreaks, but clinical diagnosis is difficult when the incidence is low and other pathogens are largely responsible for illnesses causing rash and fever (Moss & Strebel, 2011). As disease prevalence falls, the laboratory plays an increasingly important role in differentiating measles from other diseases with similar clinical presentations (Featherstone *et al*., 2011).

Serology is the most commonly used method for measles diagnosis. Enzyme immunoassay (EIA) detection of MV-specific IgM in a single specimen of serum, oral fluid (OF) which is an exudate of serum around the tooth-gum interface or eluates of dried blood spots (DBS) is considered diagnostic of acute infection, as is a fourfold-or-greater increase in measles-specific IgG antibodies between acute and convalescent serum samples. Commercially available IgM EIA assays are of two types: IgM capture (non-specific capture of IgM antibodies followed by addition of viral antigen, anti-viral antigen antibody conjugated with an enzyme and detection using a chromogen substrate; e.g. the Microimmune kit, Microimmune Ltd., Brentford, UK) or indirect EIA for virus-specific IgM (specimens are pretreated to remove IgG, then virus-specific IgM in remaining serum is bound by measles-antigen coated plate followed by detection with enzyme-conjugated anti-IgM antibody and chromogen detection; e.g. the Dade Behring Enzygnost/Siemens kit, Marburg, Germany) (WHO, 2007). The sensitivity of detection of measles-specific IgM antibodies by indirect EIA has been reported to be 77-90% within three days after rash onset and 100% at four days (Helfand *et al*., 1997; Tipples *et al*., 2003).

Only 12 of the 193 WHO member states in 2009 still need to implement case-based measles surveillance with laboratory confirmation, however serological testing is performed for outbreak confirmation (Featherstone *et al*., 2011). Measles case-based surveillance has been established in 40 of the 46 countries in the African region but surveillance remains suboptimal in several of these countries. Testing of all suspected cases during large outbreaks is not required if countries follow the WHO guidelines: for example, the WHO African regional measles surveillance guidelines define a suspected measles outbreak as the occurrence of ≥ five reported suspected measles cases in a

health facility or district in one month, and a confirmed measles outbreak is defined as ≥ three laboratory-confirmed cases in a health facility or district in one month. Once an outbreak has been identified in a health facility or district, there is no need for further collection of specimens or testing of specimens (Masresha *et al*., 2011).

For molecular testing, preferred samples in the WHO LabNet are throat swabs or OF for direct virus detection of viral RNA by reverse-transcription polymerase chain reaction (RT-PCR). Throat swabs can also used for isolation of MV in cell culture. Urine samples and peripheral blood mononuclear cells can be used for virus culture, but in practice these have proven to be more difficult to collect, transport and process successfully (Featherstone *et al*., 2011). Molecular tests can be used to help confirm the serology result in low-incidence settings. Identification and characterisation of MV provides information about the origin of outbreaks (endemic *versus* imported) which is a requirement for monitoring progress towards elimination. MV RNA can be detected in OF samples for at least two weeks after onset of rash, but for other specimen types it is imperative to obtain the sample for virologic surveillance as soon as possible, usually within three to five days, after onset of rash. However, serum samples are frequently the only samples collected from suspected measles cases (SMC) and therefore provide the only means to identify the genotypes associated with the cases, but this requires the use of very sensitive methods such as nested or real-time PCR (Rota *et al*., 2011c). The ability to detect measles RNA in IgM-positive serum samples varies, and studies have reported detection rates ranging from 18-81% (Djebbi *et al*., 2005; Thomas *et al*., 2007; Woo *et al*., 2010).

A recent study compared the sensitivity of IgM detection, virus isolation and RT-PCR for the diagnosis of measles infection – virus isolation had the lowest sensitivity of the three tests. The sensitivities of virus isolation from nasopharyngeal aspirate, throat swab or combined throat and nasal swab, and urine were 82%, 63% and 67% respectively (less than three days after rash onset) and fell to 74%, 40% and 50% respectively (four to seven days after rash onset); no virus could be isolated from samples collected beyond seven days after rash onset. Nevertheless virus isolation remains important for virus characterization especially if longer sequences, entire genes or full genome information is required. Anti-measles-IgM antibody detection was more sensitive than RT-PCR using serum samples, while RT-PCR using RNA extracted from nasopharyngeal aspirate,

throat swab or combined throat and nasal swab, and urine samples was very much more sensitive than virus isolation using cell culture (Woo *et al*., 2010).

Measles outbreaks eventually burn out when most susceptible persons are exhausted, when ORI (Outbreak Response Immunisation) is conducted late in isolated communities or in those with high background vaccination coverage. ORI may appear successful when in fact the outbreak would have ended even without this intervention (Cairns *et al*., 2011).

1.9 Global surveillance

Despite the widespread use of measles vaccine for many decades, there were still around 279 000 reported cases of measles and an estimated 164 000 measles-related deaths in 2008 (CDC, 2009). There is marked variation in vaccine coverage between the different WHO global regions. Measles has been eliminated in the Americas, but is still endemic in the African and South-East Asian regions where vaccine coverage is less than 80%. These two regions account for approximately 94% of all global measles deaths. Outbreaks continue to occur in other global regions, mainly as a result of MV importation into areas where there is suboptimal vaccine coverage (Warrener *et al*., 2011). Surveillance is a key component of measles elimination or control plans and laboratory confirmation of measles is crucial as clinical diagnosis becomes unreliable when there is a low incidence of measles (Featherstone *et al*., 2003). The detection of measles-specific IgM in serum samples by EIA is the cornerstone of laboratory confirmation (WHO, 2007). More recently, alternative samples such as DBS and OF have also been used for diagnosis by antibody detection. As the collection of an OF specimen is a non-invasive procedure, parents of infants and patients are more likely to give permission for a specimen to be collected (Jin *et al*., 2002).

Although MV is serologically monotypic, genetic and antigenic variation of WT viruses have been identified (Taylor *et al*., 1991; Rota *et al*., 1992; Tamin *et al*., 1994). There is far less variation in the M, F and L genes (Rota *et al*., 1994a; Rota *et al*., 1994b; Bankamp *et al*., 1999) than in the N, P and H genes (Rota *et al*., 1992; Rima *et al*., 1997; Bankamp *et al*., 2008). The maximum variation in the genome occurs in the 450

nucleotides encoding the 150 AAs of the carboxyl-terminus of the N protein, referred to as N-450 (Riddell *et al*., 2005). As the most variable part of the genome, N-450 has been shown to vary up to 12% between WT strains of MV (Xu *et al*., 1998). WHO recommends that the N-450 region is the minimum amount of sequence data required for genotyping of MV from cultured isolates or clinical specimens (Rota *et al*., 2009). The entire sequence of the coding region of the H-gene should be obtained from representative isolates and when a new genotype is suspected on the grounds of N-450 phylogenetic analysis (WHO, 1998). New genotypes are proposed if the nucleotide sequence differs from the closest reference sequence by more than 2.5% in N-450 and 2.0% in H, and phylogenetic analysis using at least two different algorithms must produce similar tree topographies (WHO, 1998; WHO, 2001b). Several isolates or specimens should be sequenced and at least one isolate must be available as the reference strain. However, the proposal of a new genotype should not be based solely on the percentage of nucleotide difference to the closest reference strain, but should rather be based on adding epidemiological significance (WHO, 1998).

Analysis of the variation in the nucleotide sequences of WT MV has permitted the use of molecular epidemiology for measles surveillance. The molecular data, when used in conjunction with standard epidemiological investigations, can help to identify links between geographically distinct cases and outbreaks, as well as to track importations of virus (Mulders *et al*., 2001; Rota *et al*., 2002; Chibo *et al*., 2003). Genetic characterisation of specimens or isolates from measles cases is the only means of distinguishing between WT infections and vaccine-associated cases: a small percentage (5%) of vaccine recipients who experience mild symptoms of rash and fever after vaccination (Rota *et al*., 1995; Jenkin *et al*., 1999; Rota *et al*., 2002).

To date, eight clades (A-H), comprising 24 genotypes, of WT MV have been identified by genetic characterization (WHO, 2012). Clades A, E and F contain only a single genotype, whereas clades B, C, D, G and H contain multiple genotypes (B1-B3; C1-C2; D1-D11; G1-G3; H1-H2). All vaccine strains are derived from WT genotype A progenitors. Although there are no known biological differences (severity of disease or likelihood of developing severe complications) between viruses of different genotypes, all measles genotypes can be neutralized *in vitro*, although with varying efficiency, by serum from vaccine recipients (Tamin *et al*., 1994; Klingele *et al*., 2000).

Historically, African measles viruses have been shown to be genetically diverse. Clade B viruses were endemic in the central and western parts of sub-Saharan Africa, and were subdivided into three genotypes: B1, B2 and B3 (Hanses *et al*., 1999; Truong *et al*., 1999; El Mubarak *et al*., 2002; Rota & Bellini, 2003). Genotype B1 is considered inactive as these viruses were last detected in 1983. Genotype B2 was believed to be inactive for the same reason, but was shown to be circulating in Angola (resulting in importation into South Africa in 2002) and in the Central African Republic (Smit *et al*., 2005; Gouandjika-Vasilache *et al*., 2006). More recently, genotype B2 has been detected in samples from the Democratic Republic of the Congo (DRC), Angola and Zambia (Rota *et al*., 2011b). Genotype B3 was divided into two subgenotypes: cluster 1 viruses (B3-1) were widely distributed in sub-Saharan Africa, while cluster 2 viruses (B3-2) had a more limited distribution and were only detected in western Africa (Kouomou *et al*., 2002; Mulders *et al*., 2003). By 2010, genotype B3-1 appeared to be endemic to most of Africa (Rota *et al*., 2006; Rota *et al*., 2011b). In contrast, some clade D viruses (genotypes D2, D4 and D10) circulated endemically in the southern and eastern parts of the African continent (Kreis *et al*., 1997; Mbugua *et al*., 2003; Muwonge *et al*., 2005) with genotype D4 reported from as far north as Ethiopia (Nigatu *et al*., 2001). However, genotypes D4 and D10 have not been detected since 2006 (Baliraine *et al*., 2011; Rota *et al*., 2011b). Genotype C2 viruses were detected in an outbreak in Morocco in 1998-1999 which suggests that northern Africa may be linked to European transmission patterns rather than to African patterns (Alla *et al*., 2002).

1.10 Nomenclature

In 1998, a standard protocol for the designation of measles genotypes was recommended by WHO, with periodic updates to include new genotypes (WHO, 1998; WHO, 2001a; WHO, 2001b; WHO, 2003; WHO, 2005). The general format for naming a MV sequence is:

'Prefix/Location.Country/Epidemiological week.Year/Replicate[genotype]special detail'

where:

- Prefix (MVi or MVs) specifies that the sequence was derived from isolate or clinical specimen, respectively
- Location should be the town or city where the sample was collected, or even the name of the health district or province if specific details are unavailable
- Country is indicated by the three-letter code as specified by the International Organisation for Standardisation (ISO3) (Appendix A). Initially, the epidemiological (EPID) country codes were used, but after 2005, the recommendation was changed to use ISO3 country codes
- Epidemiological week is determined by standard epidemiological definition (the week begins on a Sunday, and week one must have at least four days) and reflects date of onset of rash, but if this information is not available, the date of specimen collection may be used or even the date of receipt in the laboratory. However, in the 2012 measles nomenclature update (WHO, 2012), the epidemiological week is calculated from the first Monday of each week, with week one being the first Monday of the year
- Year – the last two digits of the relevant year
- Replicate – in case of several specimens/isolates were collected from the same location in the same week
- Genotype may be indicated in square brackets, but this is optional; to assign a genotype, sequencing of the minimum N-450 window is required
- Special designation if sequences are derived from MIBE, SSPE or cases with a history of recent vaccination (VAC)

For example, MVi/London.GBR/3.12/2[D4] and MVs/NewYork.USA/17.11[G3]SSPE illustrate the nomenclature.

1.11 Objectives of the study

The genetic characterisation of circulating WT viruses is a critical component of laboratory surveillance for measles, providing support for standard epidemiological studies in order to describe the transmission pathways of MV. Molecular epidemiology provides the only means of monitoring circulation of MV strains, determining interruption of transmission of endemic circulation, and assessing the progress towards measles elimination. Until this study, MV genotype data for Africa was severely limited, with only one publication on South African genotypes (Kreis *et al*., 1997), descriptions of the reference strains for B2 (Gabon in 1984) and B3 (Nigeria in 1997), and several papers prior to 2006 on western, central and eastern Africa (Hanses *et al*., 1999; Nigatu *et al*., 2001; El Mubarak *et al*., 2002; Kouomou *et al*., 2002; Mbugua *et al*., 2003; Mulders *et al*., 2003; Muwonge *et al*., 2005). The objective of this study was to provide molecular characterisation of MV strains in South Africa, necessary for determination of endemic elimination status. With this study's development of successful molecular techniques, as well as access to samples from countries across Africa, it also became possible to identify WT virus circulating in neighbouring countries and the African region, as potential and actual sources of introduction into South Africa.

In this study, RNA was extracted from more than 2500 specimens (serum, urine, throat swabs, OF, DBS, virus isolates) collected in more than 30 African countries during the period 2002-2011. The extracted RNA was tested for the presence of MV RNA, specifically for the 3'-terminus of the N-gene, by RT-PCR and hemi-nested PCR, following which the positive samples were sequenced and the genotypes determined by phylogenetic analysis of the N-450 sequences. Amplification and sequencing of the entire H-gene was only attempted as a confirmatory method for a small subset of specimens when genotype B2, thought to be inactive, was detected.

2.0 MATERIALS AND METHODS

2.1 Specimens

2.1.1 Specimen types

For the purposes of case-based measles surveillance, the recommended specimen for serological confirmation is serum while the specimen for virological characterisation is a throat swab or urine. Within the period covered by this project, the WHO recommended throat swabs as the preferred specimen because virus is detectable for a longer period and it is easier to collect from young children. Many African countries therefore collected throat swabs from SMC whereas South Africa continued to collect urine specimens. The specimens received from different countries for the duration of this project consequently varied and included throat swabs, urine, viral isolates, DBS, serum, OF and even PCR products. The latter were sequenced directly whereas the other listed specimens required an RNA extraction step and PCR amplification.

The South African national guidelines for measles surveillance request that blood and urine specimens be collected from SMC and sent to the laboratory along with the completed case investigation form (CIF). In reality, fewer than 50% of the specimens that were received fulfilled this dual specimen requirement and fewer still had a CIF. Thus it was often necessary to test serum samples for the presence of MV as no urine was available.

All national laboratories (NL) that are accredited by the WHO and belong to the LabNet need to ensure that their measles-IgM serological test-results are accurate, because they are diagnostic results. Thus the WHO has an external quality assurance (EQA) program in place where the national laboratories (NL) send, on a quarterly basis, a selection of the serum specimens they have tested, to their regional reference lab (RRL) for retesting. Results are compared and any necessary corrective action is taken by the NL. The NICD measles laboratories (serology, virus isolation and molecular) function as a RRL for the NL of the southern African countries. These EQA serum samples are often the only specimens that are available for genotypic analysis of the MV strain(s)

circulating in those countries, and therefore all EQA sera that had either a positive or indeterminate for measles-IgM were tested by RT-PCR and hemi-nested PCR for the presence of MV RNA (N-gene). The amplicons of PCR-positive specimens were then sequenced and the genotypes determined by phylogenetic analysis.

There are two other measles-RRLs in Africa: one located at the Pasteur Institute, Abidjan, Côte d'Ivoire (serving the countries in West and Central Africa) and the other at the Uganda Virus Research Institute, Entebbe, Uganda (serving the countries in East Africa). The NLs in these blocks send throat swabs/urine/OF/DBS from SMC for virus isolation, in addition to the serum EQA specimens, to their respective RRL. These RRLs have molecular and virus isolation laboratories but no sequencing capacity at present, and therefore forward virus isolates, PCR-positive specimens or clinical specimens to the NICD for molecular testing and characterization.

2.1.2 Specimen numbers and country of origin

The country of origin, the number of specimens tested, as well as the number of sequences and genotypes obtained from these specimens, per year for the period 2002-2011, is presented in Table 2.1. In summary, 1402 sequences from 2655 specimens (52.8% PCR-positivity rate) from 33 countries, were generated during this study.

Table 2.1 Numbers of sequences and genotypes obtained are shown per year per country. The total number specimens tested and sequences obtained are also indicated per year of this study.

COUNTRY NAME	YEAR									
	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Algeria					9xB3-1					
Angola		3xB2		1xB2; 3xB3-1	6xB2; 1xB3-1	1xB2		11xB2; 1xB3-1	1xB2	5xB2; 2xB3-1
Benin				1xB3-1	14xB3-1	13xB3-1	25xB3-1	17xB3-1	11xB3-1	1xB3-1
Botswana			1xD2; 1xD4	1xB3-1; 3xD4	1xB3-1			5xB3-1	1xB2; 5xB3-1	5xB3-1
Burkina Faso					4xB3-1		5xB3-1	15xB3-1		
Burundi								1xB3-1		
Cameroon				2xB3-1			10xB3-1	19xB3-1		
Chad						5xB3-1				
Cote d'Ivoire		3xB3-2	3xB3-1; 8xB3-2	4xB3-1; 2xB3-2	2xB3-1			7xB3-1	23xB3-1	46xB3-1
Comores				3xD4						
DRCongo				10xB2	13xB2	18xB2	1xB3-1			
Equatorial Guinea							3xB3-1			
Ethiopia					4xB3-1		4xB3-1			
Kenya				7xB3-1; 3xD4	1xB2; 102xB3-1; 1xD4					17xB3-1
Lesotho			10xD2	1xD2				10xB3-1	8xB3-1	
Liberia									5xB3-1	
Malawi				2xD2				5xB3-1	20xB3-1	2xB3-1
Mali					3xB3-1	1xB3-1		2xB3-1		
Mauritania									2xB3-1	
Mozambique		3xD2; 1xD4							7xB3-1	
Namibia	1xA		2xD2					4xB2	31xB2; 2xB3-1	15xB2; 12xB3-1
Niger							17xB3-1	5xB3-1	4xB3-1	8xB3-1
Nigeria								7xB3-1	69xB3-1	88xB3-1
Rwanda				2xB2					15xB3-1	5xB3-1
Senegal								10xB3-1		
Sierra Leone									16xB3-1	
South Africa	14xB2	1xA; 68xD2; 3xD4	116xD2	33xD2	3xB2; 9xB3-1; 15xD4	4xB3-1	1xD8	91xB3-1; 1xD8	26xB3-1; 1xD4	5xA; 15xB3-1
Swaziland								2xB3-1		
Tanzania					1xB3-1					1xB3-1
Togo							1xB3-1	4xB3-1	10xB3-1	3xB3-1
Uganda					17xB3-1	2xB3-1		2xB3-1		
Zambia				1xB3-1; 2xD4	6xB3-1; 4xD4	2xB2; 3xB3-1		2xB2	6xB3-1	
Zimbabwe			5xD2; 1xD4		2xD4			19xB3-1	10xB3-1	
Total Number of sequences	15	82	147	81	218	49	67	240	273	230
Number of specimens tested	23	279	205	133	331	243	186	528	301	426

2.2 Extraction of RNA

The extraction protocol is provided in Appendix B. All extractions were performed in a laboratory or area reserved for specimen processing and extraction i.e. free from PCR products. The cellular fractions of viral isolates, urine specimens, throat swabs or oral fluid from serologically-confirmed measles cases were obtained by centrifugation of 1.5 ml of specimen (or less if the specimen volume was limited) for two min at 2300 *g.* RNA was extracted from the pellet using the spin-column procedure of the QIAamp Viral RNA Mini kit according to the manufacturer's protocol (Qiagen, Germany). Essentially this involved the addition of a guanidine thiocyanate lysis buffer (containing carrier RNA to improve recovery) to the sample to lyse any cells or virions and also inactivate RNases. The nucleic acids (both DNA and RNA) were then recovered from the mini columns containing a silica-gel membrane by elution in 50 µl of TE buffer (10 mM TRIS, 1 mM EDTA buffer) instead of the kit elution solution AVE (RNase-free water plus a preservative). TE buffer was used in the elution step to ensure that the RNA would be more stable for long-term storage. Every extraction run contained at least one negative extraction control to monitor possible cross-contamination in the extraction process; if RNA was extracted from many samples, then one negative extraction control was included for every 10-12 specimens.

2.3 Primers, RT-PCR and hemi-nested PCR (N-gene)

N-gene primer positions were numbered according to the sequence of the Edmonston strain of MV (Appendix C). Following extraction of the RNA, a one-step RT-PCR was performed using the Titan One Tube RT-PCR System (Roche, Germany) with a minor modification of the manufacturer's protocol – a single master mix instead of two, was routinely prepared (Appendix D). Previously described primers MV60 and MV63 were initially used to amplify a 599 base pair (bp) fragment from the 3'- region of the measles N gene from RNA extracted from clinical specimens or viral isolates (Katz, 2002). However, it was found that this RT-PCR was not sensitive enough for many of the clinical specimens, especially serum and DBS, and so primer N16 was designed upstream of MV60 (Appendix C) to allow a hemi-nested PCR method to be implemented.

All specimens were therefore tested first by a one-step RT-PCR of 30 cycles using primers MV16-MV63 to generate a 641 bp fragment (Appendix D). The master mix was prepared and aliquotted in an area/laboratory that was not exposed to specimens or PCR products – the "clean" area. Aliquots of the extracted RNA (10 µl) were added to appropriately labelled tubes containing 40 µl of reaction mix in the extraction area. Every run included a positive MV-RNA control and a negative control (nuclease-free water) to monitor the RT-PCR reagents. Specimens that tested negative or very weakly-positive relative to the positive control in the RT-PCR run were subjected to a hemi-nested PCR (Appendix E) using the primers MV60-MV63 to generate the 599 bp fragment suitable for sequencing.

The hemi-nested PCR master mix was also prepared and aliquotted in the "clean" area. The tubes containing the RT-PCR products were pulse-centrifuged before 2 µl of the RT-PCR products were transferred to the tubes containing the hemi-nested reaction mix in the area/lab reserved for performing nested PCR. The extraction controls and negative RT-PCR controls were treated the same way as the specimens, whereas the positive RT-PCR controls were first diluted to 100 µl before transfer of 2 µl to the hemi-nested reaction mix.

2.4 Comparison of detection of MV RNA from urine *versus* serum

Although South African health facilities are requested to collect a urine or throat swab in addition to clotted blood from suspected measles cases, there is poor compliance and blood is often the only specimen submitted. Serum is also the only specimen provided for the EQA serological rechecking programme. Therefore, RNA was extracted from a selection of both specimen types, to compare whether or not MV RNA could be detected equally successfully in the RT-PCR method that had been developed. Figure 2.1a clearly shows that serum samples were uniformly negative for MV RNA after the standard N-gene RT-PCR, whereas urines from measles cases contained detectable levels of MV RNA. As this RT-PCR was not sensitive enough to detect measles virus in serum, a hemi-nested PCR method was developed to increase the sensitivity. When the RT-PCR-negative specimens were tested using this hemi-nested PCR, many of these

specimens were found to be positive (Figure 3.1b). This strategy was therefore routinely implemented for testing of specimens from suspected measles cases.

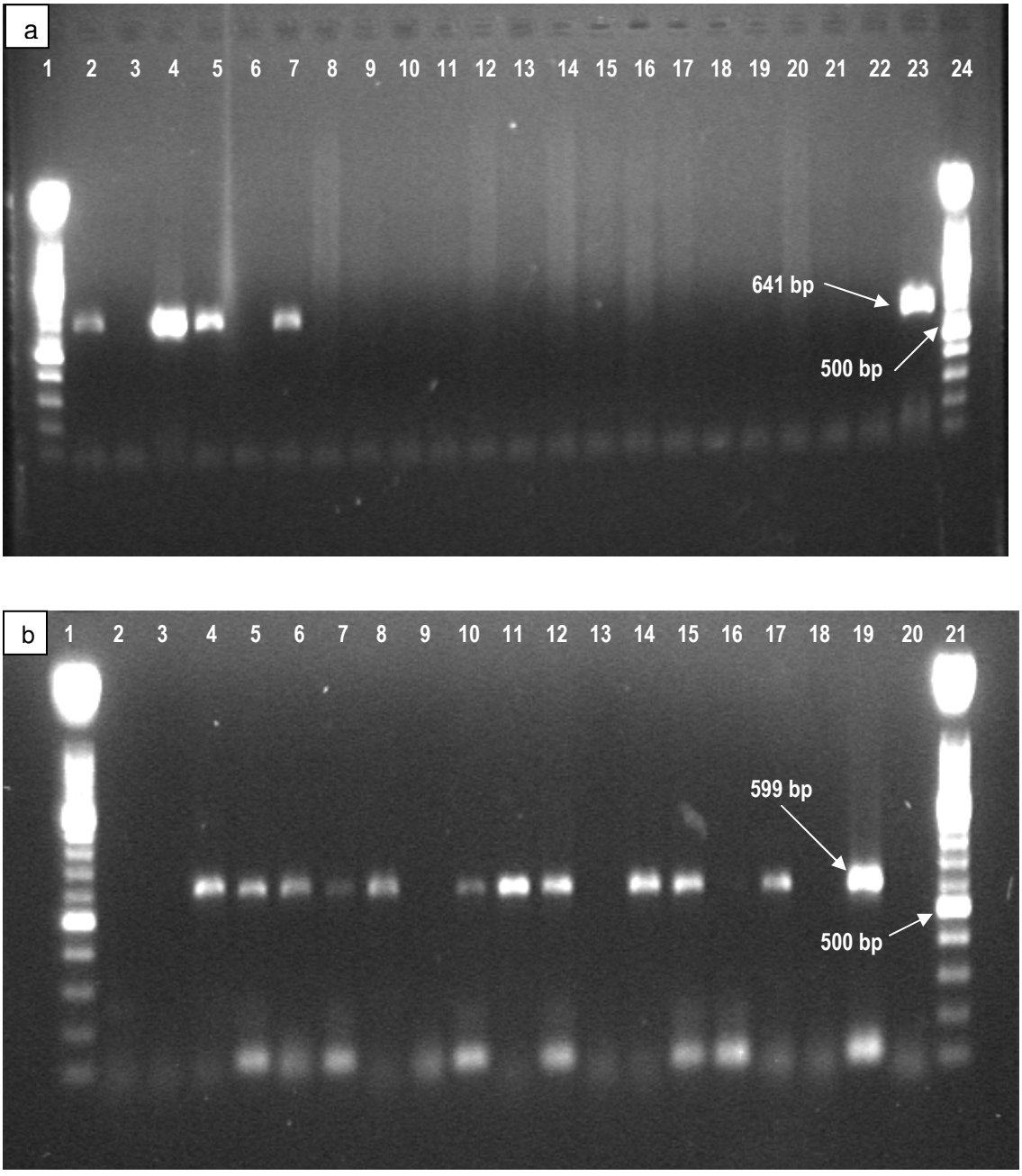

Figure 2.1 Comparison of results obtained using urine versus serum (a) N-gene RT-PCR. Lanes 1 and 24: molecular weight marker 100 bp ladder; Lanes 2-7 urine specimens; Lanes 8-21 serum specimens; lane 22: negative control; Lane 23: positive control. (b) hemi-nested PCR of the RT PCR-negative specimens shown in (a). Lanes 1 and 21: molecular weight marker 100 bp ladder; Lanes 2 and 3: urine specimens; Lanes 4-17 serum specimens; Lane 18: nested negative control; Lane 19: nested positive control; Lane 20: negative control for the nested reagents.

2.5 Sensitivity of the N-gene RT-PCR and hemi-nested PCR

As the intention was to use the N-gene RT-PCR and hemi-nested PCR methods to detect MV genome routinely in clinical specimens, it was necessary to determine the sensitivity of these methods to demonstrate that they were suitably sensitive for this purpose. Therefore serial dilutions of a positive control of known copy number were subjected to the RT-PCR and hemi-nested PCR. The RT-PCR products were subjected to electrophoresis through 1% (w/v) agarose gels containing ethidium bromide, visualised by UV-transillumination, in order to compare the results of the RT-PCR using the original primer set (N16/MV63) and the newly designed CDC primer set (MeV214/MeV216). This revealed a sensitivity of 10^6 cp/µl and 10^4 cp/µl respectively. Thus the new primers showed a 100-fold increased sensitivity relative to primer set N16/MV63 by RT-PCR (Figure 2.2). These new CDC primers will be of great benefit to the LabNet where nested PCR is not routinely implemented.

However, the sensitivity of both primer sets was the same after a hemi-nested PCR (N16/MV63-MV60/MV63) or nested PCR (MeV214/MeV216-MV60/MV63) i.e.100 cp/µl using MV60/MV63 as the nested primers for both sets of RT-PCRs (Figure 2.3). As the methodology on which this study is based was developed using the N16/MV63 (RT-PCR) and MV60/MV63 (hemi-nested PCR) primer sets, it is reassuring that the sensitivity of detecting MV in clinical specimens surpasses that of current LabNet recommended RT-PCR routine screening assays.

Figures 2.3a and 2.3b also illustrate the size difference in the amplicons produced using the CDC synthetic RNA positive control containing a 220 bp insert, relative to the amplicon size of the positive control used in this study (vaccine). As amplicons from clinical specimens would be the same size as the vaccine-derived amplicons, use of the synthetic RNA would highlight any contamination by the positive control in the nested PCR.

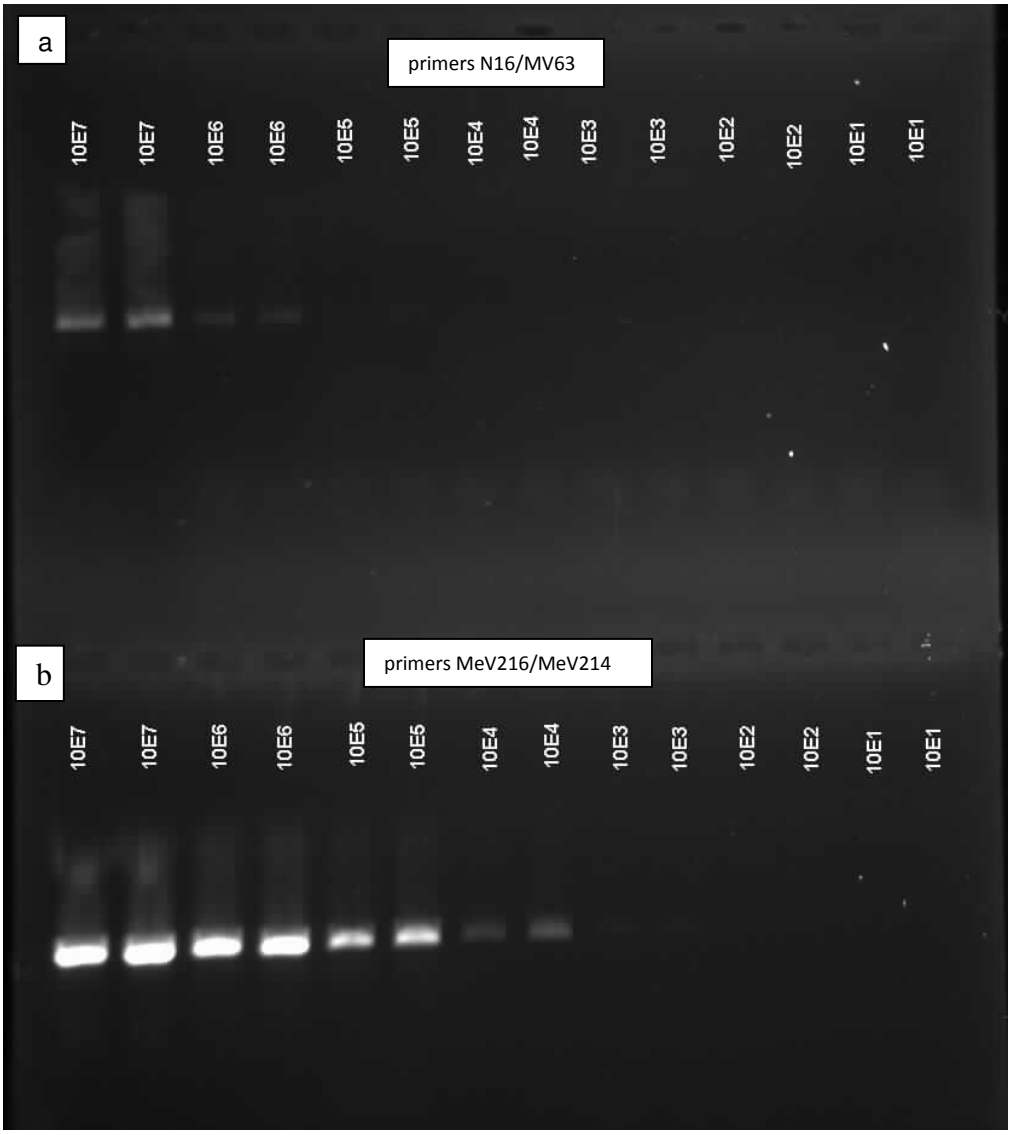

Figure 2.2 Gel analyses of the N-gene RT-PCRs of ten-fold serial dilutions of the synthetic RNA positive control (provided by CDC) using primer sets (a) N16/MV63 and (b) MeV216/MeV214.

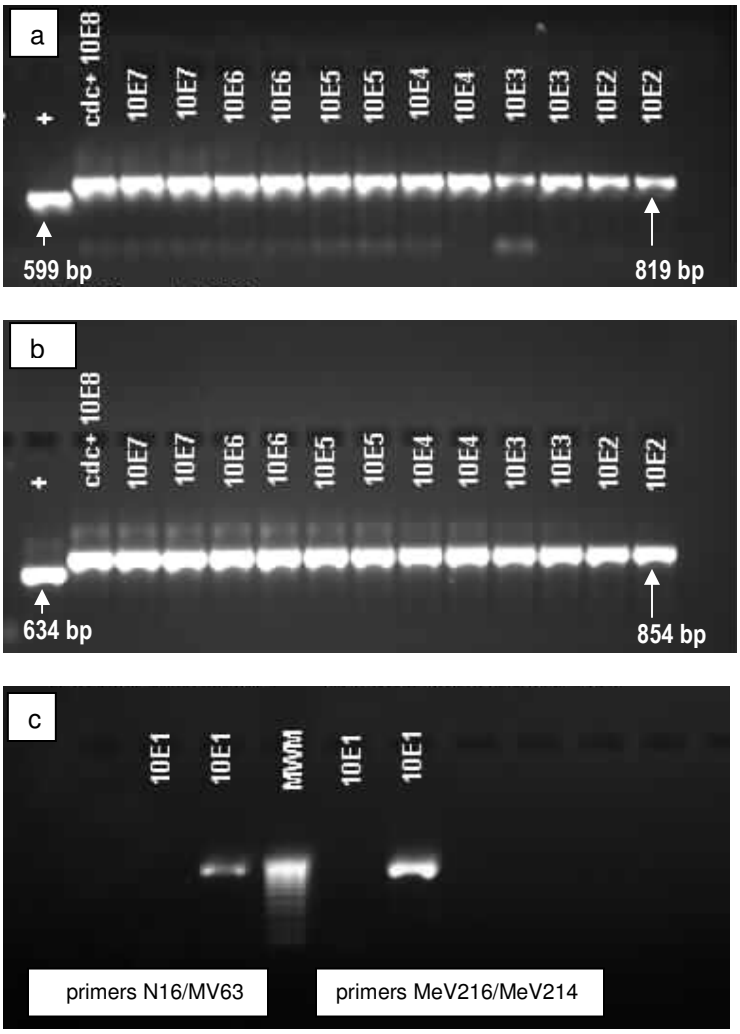

Figure 2.3 Gel analyses of the nested PCR products generated with MV60/MV63 using the RT-PCR products obtained with (a) N16/MV63; the vaccine RNA control yields a 599 bp product, while the synthetic RNA control yields an 819 bp product (b) MeV214/MeV216; the vaccine RNA control yields a 634 bp product, while the synthetic RNA control yields an 854 bp product and (c) final dilution of both sets of primers (insufficient lanes on gel apparatus).

2.6 Primers, RT-PCR and hemi-nested PCR (H-gene)

H-gene primer positions were numbered according to the sequence of the Edmonston strain of MV (Appendix C). 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) with a minor modification of the manufacturer's protocol – a single master mix instead of two, was routinely prepared. Previously described primers MVH-IF and MVH-IR were initially used to amplify a 2036 bp fragment spanning the entire H-gene, from clinical specimens (Katz *et al*., 2002).

However, it was found that this RT-PCR was not sensitive enough for most of the clinical specimens, especially because it is a one-step RT-PCR and the fragment is large. Therefore an overlapping pair of nested fragments, using a selected combination of primers (Kreis & Schoub, 1998; Chibo *et al*., 2000) was designed for a hemi-nested PCR (Appendix C).

Selected specimens were therefore tested first by a one-step RT-PCR of 30 cycles using primers MVH-IF and MVH-IR to generate a 2036 bp fragment (Appendix F). The master mix was prepared and aliquotted in a laboratory that was not exposed to specimens or PCR products – the "clean" area. Aliquots of the extracted RNA (10 µl) were added to appropriately labelled tubes containing 40 µl of reaction mix in the extraction area. Every run included a positive MV-RNA control and a negative control (nuclease-free water) to monitor the RT-PCR reagents. Specimens that tested negative or very weakly-positive relative to the positive control in the RT-PCR run were subjected to hemi-nested PCRs to generate two overlapping fragments using the primers MVH-IF and H7 (1080 bp) and H4 and MVF-IR (1227 bp) to generate the two fragments suitable for sequencing (Appendix G). Four primers were used to sequence each fragment, the two PCR primers plus two internal primers (Appendix C and J).

The hemi-nested PCR master mix was also prepared and aliquotted in the "clean" area. The tubes containing the RT-PCR products were pulse-centrifuged before 2 µl of the RT-PCR products were transferred to the tubes containing the hemi-nested reaction mix in the laboratory reserved for performing nested PCR. The extraction controls and negative RT-PCR controls were treated the same way as the specimens, whereas the positive RT-PCR controls were first diluted to 100 µl before transfer of 2 µl to the hemi-nested reaction mix.

Amplification and sequencing of the entire H-gene was only attempted as a confirmatory method for a small subset of specimens when genotype B2, thought to be inactive, was detected.

2.7 MV positive control for RT-PCR

The ideal positive control for the RT-PCR should have a sequence that differs from circulating strains of MV, so that it is possible to determine whether the sample sequence is "real" or the result of a PCR contamination with the positive control. Within the last year, the measles section at the CDC, Atlanta, USA, generated an RNA control by inserting a 220 bp fragment into the PCR product generated with primers MV60-MV63 and then cloned it into a transcription plasmid. The resultant transcribed RNA was distributed to molecular laboratories in the WHO measles lab network for use as a RT-PCR positive control; the expected amplicon size using primers MV60 and MV63 is 819 bp. The lyophilised, synthetic RNA positive control was resuspended in 100 µl of water, to obtain a master stock solution containing10^9 copies/µl (cp/µl), which was stored as 10 µl aliquots. A working stock of 10^8 cp/µl was prepared by addition of 90 µl of water to a master stock aliquot, and 1 µl was used as the RNA template for the positive control. However, this synthetic RNA control was not available for most of the time frame of this project.

The RNA that was routinely used as an RT-PCR positive control for this project was extracted from vials of measles vaccine obtained from the NICD clinic (Rouvax, Pasteur Mérieux, France). Each single-dose vial contained not less than 1000 $TCID_{50}$ live attenuated measles virus (Schwarz strain) cultivated in chick embryo tissue. The lyophilised contents were reconstituted in 140 µl nuclease-free water and transferred to a tube of AVL for RNA extraction according to the protocol presented in Appendix B. The RNA was eluted in 100 µl TE buffer and stored in 10 µl aliquots at -70 ºC to minimize degradation from multiple freeze-thaw cycles. Each aliquot was sufficient for 6 RT-PCR runs, using 1.5 µl RNA for the positive control; the difference in volume (8.5 µl) in the positive control tube of the RT-PCR was made up using nuclease-free water so that the final volume in the tube was 50 µl as per the RT-PCR protocol in Appendix D. Whenever it was necessary to set up the hemi-nested PCR, the RT-PCR positive control products were first diluted to 100 µl with nuclease-free water before transfer of 2 µl to the hemi-nested reaction mix (to avoid an unnecessarily strong hemi-nested PCR positive).

The use of the vaccine strain of MV as the positive control was functional but not ideal because when this strain was identified in a specimen, the possibility of contamination

could not be excluded. If there was a case-history of recent vaccination, it helped to clarify the result, but this information was not always available.

2.8 Negative controls for RNA extraction, RT-PCR and hemi-nested PCR

Nuclease-free water was used for the extraction control; every extraction run contained at least one negative extraction control to monitor possible cross-contamination in the extraction process; if RNA was extracted from many samples, then one negative extraction control was included for every 10-12 specimens.

Every RT-PCR run included a negative control (10 µl nuclease-free water) to monitor the RT-PCR reagents and the negative extraction control to monitor the extraction process. Similarly, every hemi-nested PCR run included a negative control (2 µl nuclease-free water) to monitor the hemi-nested PCR reagents; in addition, the negative control from each RT-PCR run was also subjected to the hemi-nested PCR for the batch of specimens included in the run.

2.9 Agarose gel electrophoresis

To visualise the RT-PCR and hemi-nested PCR results, 4 µl of the PCR products were mixed with a drop of 10x loading buffer containing a marker dye (bromophenol blue), loaded into the wells of 1% (w/v) agarose gels containing 0.2 µg/ml ethidium bromide in 0.5x TRIS-Borate-EDTA (TBE) buffer and resolved by electrophoresis at 100V in 0.5x TBE buffer until the dye marker had migrated approximately 75% of the length of the gel (Appendix H). Bands were visualized by UV-transillumination and the photos were digitally captured using a gel documentation system (G box or earlier version, Syngene). A molecular weight marker (100 bp ladder) was included in every run (Roche and BioLine).

2.10 Determination of the sensitivity of the RT-PCR and hemi-nested PCR

During the initial phase of this study, the sensitivity of the N-gene and H-gene RT-PCR was estimated using a positive control (RNA extracted from a vial of single dose Rouvax measles vaccine (registration number T/30.1/688) containing not less than 1000 $TCID_{50}$ of live attenuated MV, Schwarz strain, cultivated in chick embryo tissue). The extracted RNA was eluted in 100 µl TE buffer, thus representing 10 $TCID_{50}$ per µl. In the original method for the one-step RT-PCR using MV60 and MV63 (N-gene), and MVH-IF and MVH-IR (H-gene), the sensitivity was estimated to be 10 $TCID_{50}$ using the above-mentioned positive control. However, the caveat for this determination, was that the exact amount of virus in the vaccine was not stipulated (merely 'not less than' 1000 $TCID_{50}$).

New N-gene primers (MeV214, MeV216), described in Appendix C, were recently designed by the CDC, and distributed to the LabNet together with the new, synthetic N-gene RNA positive control (described above). These new primers were tested in parallel with the existing primer set (N16 and MV63) for the RT-PCR, followed by a nested or hemi-nested PCR using MV60 and MV63, testing duplicate serial dilutions of the synthetic RNA positive control. The dilution range spanned 10^8 to 10^1 RNA cp/µl.

2.11 Preparation of PCR products for sequencing

Specimens that were positive on either the RT-PCR or hemi-nested PCR were processed in one of two ways: if there were no non-specific bands present, the reaction product was recovered directly using the Wizard SV Gel and PCR clean-up system (Promega, USA) as detailed in Appendix I. If multiple bands were present, the entire reaction mix was loaded onto 1% (w/v) agarose gels with large wells to accommodate the required volume and again electrophesed at 100V for the desired length of time (this varied according to the size of the gel). The correctly-sized bands were excised from the gel using scalpel blades under long-wave UV-transillumination and placed into marked microfuge tubes and weighed, and the correct volume of membrane-binding buffer corresponding to the weight of the gel-slice was added (10 µl of membrane binding buffer per 10 mg of gel-slice). The tubes were placed in a heating block set at 56 ℃ to

melt the agarose, mixed by inversion every few minutes and the amplicons were then recovered as described in Appendix I. The purified amplicons were eluted in 50 µl of TE buffer.

The concentration of these purified PCR products was initially estimated by running a 4 µl aliquot of the product on a 1% (w/v) agarose gel and estimating the intensity of the band relative to a band of known concentration in the 100 bp ladder used as the molecular weight marker. In the course of the time frame of this project, a Nanodrop spectrophotometer (Thermo-Scientific, USA) became available – this instrument measures the absorbance spectrum of samples over a range of wavelengths (options selected by the operator) using only 1-2 µl of the samples and calculates the concentration of the DNA in the purified PCR products. Aliquots of purified amplicons were diluted to 3 ng/µl prior to setting up the sequencing reactions.

2.12 Sequencing reactions

The amplified regions of the N and H genes were sequenced using the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, USA), using 15 ng of the amplicon as template and 10 pmol of the sequencing primer according to the method described in Appendix J. Unincorporated BigDye® terminators were removed from the reactions by various methods over the duration of the project. Initially gel filtration using Centri-Sep columns (Princeton Separations, USA) was used. This was replaced by a precipitation method using 0.3 M sodium acetate and 50% ethanol (final concentrations) to allow for higher specimen throughput. Although this method was excellent for 96-well plate formats, small fragments were poorly recovered. Finally, BDX beads were used (BigDye® XTerminator Kit, Applied Biosystems, USA). This kit is designed to remove cycle-sequencing reaction components such as unincorporated dye terminators and dNTPs, and thereby prevent co-injection of these impurities with the dye-labelled products of the cycle-sequencing reaction. BigDye® XTerminator solutions are added directly to completed cycle-sequenced reactions. During the vortexing step the BDX beads capture and immobilize unwanted components which are subsequently collected in the bottom of the reaction plate by a brief centrifugation step. The purified dye-labelled products of the cycle-sequencing reactions remain in the supernatant and are then

directly injected from the supernatant into the DNA sequencer capillaries. The kit comprises two reagents; the suspension containing the BDX beads, which capture unincorporated dye-terminators and free salts, and SAM™ solution which improves the performance of the BDX beads and stabilises the samples after purification. This BigDye® XTerminator method, although expensive, gave excellent recovery of all labelled fragments and was not labour intensive (Appendix J). The labelled fragments were resolved by electrophoresis through a 16-capillary array ABI 3130XL genetic analyser (Applied Biosystems, USA).

2.13 Sequence editing and phylogenetic analysis

The sequences were assembled and edited using Sequencher software version 4.1.4 (Gene Codes Corporation, USA). Phylogenetic analyses were performed using the MEGA3.1 software package (Kumar *et al*., 2004) by comparison with WHO reference sequences (Appendix K). There are no insertions or deletions in the protein coding regions that were sequenced and thus all edited sequences were of the same length.

2.14 Naming of sequences

Sequences submitted to GenBank were named according to the current WHO recommendations for nomenclature at the time of submission (WHO, 1998; WHO, 2001a; WHO, 2001b; WHO, 2003; WHO, 2012), hence some sequences may have a city name, but if this information was not available, then either name of the health district or province was used. As discussed in the introduction (1.10 Nomenclature), sequence names should include the epidemiological week of onset of rash. However, as this information was frequently missing, either the date of specimen collection or even the date of sample receipt in the laboratory was used. As viral RNA can be generally detected only up to five days after onset of rash, the collection date would only differ from the onset date by approximately one week. In addition, the country was initially specified by the EPID code, but the recommendations changed during the course of this study to the ISO3 codes (Appendix A). As some of the earlier sequences were published and submitted to GenBank with the EPID code format, these could not be changed

retrospectively, and thus there is some variability in the naming of sequences in this study. The laboratory identification number was used for specimens where no information was available from submitting countries.

3.0 RESULTS AND DISCUSSION

3.1 Genotype B2

3.1.1 Outbreak in Cape Town, South Africa, 2002

In early November 2002, three young men, aged between 22 and 26 years, presented to Victoria Hospital in Cape Town with bronchopneumonia and a measles-like rash. All three were Angolan citizens who had recently arrived in South Africa and were residing in an informal settlement in Hout Bay where there is a large Angolan community. Measles was confirmed in all three cases by the presence of specific anti-measles IgM antibodies and the Department of Health was notified. An outbreak response team was mobilized and the contacts of each case were given measles vaccine as post-exposure prophylaxis. Surveillance was increased in the area, and blood and urine specimens were taken from patients who fitted the case definition of measles.

A total of 26 measles cases were serologically confirmed in the following six weeks (3 November -17 December 2002), mostly from Hout Bay, but with sporadic cases from the peninsula in the latter part of the outbreak. Only the samples from IgM-positive individuals were investigated further. As measles antibody is not always detectable during the first few days of the illness, this strategy probably underestimated the true number of cases. The majority of cases (20/26, 77%) were young adults aged from 16-30 years. Most of the adult cases from the informal settlement were not South African citizens and had no vaccination documents. Three of the patients had received measles vaccine as post exposure prophylaxis, but still developed infection with the wild-type virus. Notably, a doctor who had treated two of the initial cases also developed measles, despite having received measles vaccine as a child. Only six of the cases were children, and of these, two were under nine months of age (first dose of measles vaccine is administered at nine months of age in South Africa) and were therefore un-immunized. Two children were 10-15 months of age; one had received one dose of vaccine and the other was un-immunized. The remaining two children were aged 4-6 years and were fully vaccinated. Urine specimens from the confirmed cases were sent to the NICD for viral culture and genotypic analysis.

3.1.2 Luanda, Angola

In week 13 of March 2003, active measles surveillance in Angola resulted in the detection of an outbreak in Luanda, and four specimens from confirmed cases were sent to the NICD for genotypic analysis.

3.1.3 Specimens

Urine specimens from serologically-confirmed cases were received from measles outbreaks in Cape Town, South Africa (November 2002) and Luanda, Angola (March 2003). Unfortunately, there were considerable delays in transport which resulted in loss of refrigeration and consequently microbial overgrowth, and thus no virus isolations were possible from the urine specimens from either of the outbreaks. Despite the poor condition of the specimens, 14 of 22 specimens from Cape Town (64%) and three of four specimens from Luanda (75%) still contained amplifiable material, although some of the N-gene amplification reactions were very weak. The H-gene could only be amplified from specimens that contained higher levels of viral RNA (as estimated by the strength of the N-gene amplicons), because the sensitivity of the H-gene RT-PCR was lower than that of the N-gene RT-PCR.

3.1.4 Analysis of N-450 sequences

Comparison of the nucleotide sequences from the Cape Town outbreak to reference sequences of all known genotypes revealed that the outbreak was caused by MV genotype B2 (Figure 3.1). The N-gene sequences obtained from specimens collected during the six week period of the outbreak were identical, indicative of circulation of a single strain of MV. The Cape Town sequences differed from the reference B2 N-gene sequence by seven nucleotides (1.6%) and the predicted translation of the sequence resulted in two AA substitutions relative to the B2 reference sequence (Figure 3.2).

The genotype B2 virus amplified from the Luandan specimens was very closely related to the Cape Town virus and differed by only two nucleotides in the sequenced fragment

which is predicted to result in a single AA substitution relative to the Cape Town sequence (Figures 3.1 and 3.2). The Luandan sequences differed from the B2 reference N-gene sequence by nine nucleotides (2%) and by three AA in the predicted translated sequence.

Although most of the point mutations occurred in the third codon position (and were therefore silent), these mutations were preserved through the chains of transmission, and were often characteristic of the genotype and of the B clade. Predicted translation of the Cape Town and Luanda viral N-gene sequences (Figure 3.2) revealed that the nucleoprotein carboxyl-termini differed from each other by only one AA and from the reference B2 sequence by two and three AA (p-distance 1.3% and 2%) respectively. The locations of these coding changes were not unique: the change from tyrosine to a histidine residue at position 77 (Y77H, numbering according to Figure 3.2) has been noted previously (reference strain genotype B3-1) while the G96S change in the Angolan sequence has been detected in most genotypes within clades D and H (Appendix L). Although both new B2 sequences had a novel S76R substitution, variability at this locus has also been noted previously (e.g. asparagine and isoleucine in reference genotypes H1 and E respectively). This notation of AA changes at a particular site (vaccine strain to WT), does not imply directional change as vaccine is not a progenitor of WT, rather the opposite is true, and the vaccine strain is being used merely as the consensus sequence.

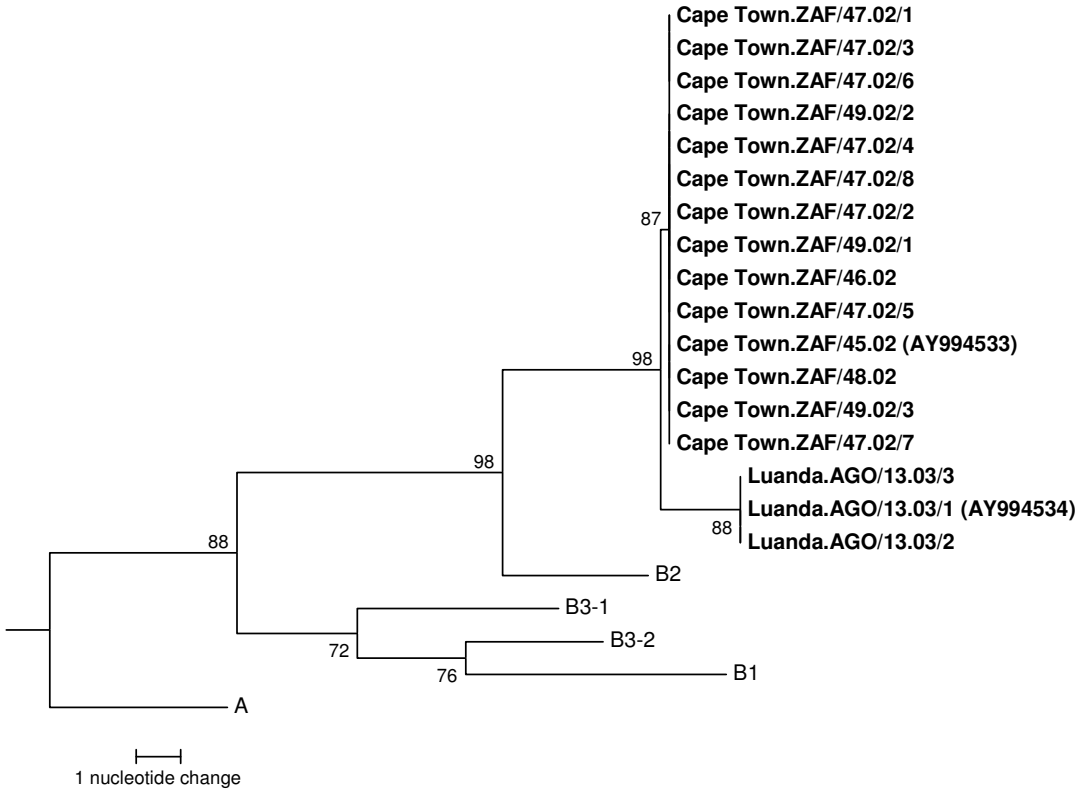

Figure 3.1 Phylogenetic analysis of the partially-sequenced N genes of MV from specimens collected during the outbreaks in South Africa and Angola (in bold font). The N-450 sequences were compared to the WHO reference sequences; the names of the reference strains have been deleted for clarity (WHO, 2003). The unrooted neighbour-joining tree (number of nucleotide changes algorithm) was generated by bootstrap analysis (1000 replicates) using MEGA3.1 software. Only bootstrap frequencies greater than 70% are shown. GenBank accession numbers are indicated in brackets after the sequence names.

```
A                        KVSSTLASEL GITAEDARLV SEIAMHTTED KISRAVGPRQ AQVSFLHGDQ  [ 50]
B1                       .......... .......... .......A.. R......... ..........  [ 50]
B3-1                     .......... .......... .......... R......... ..........  [ 50]
B3-2                     .......... .......... .......... R......... ..........  [ 50]
B2 (Libreville.GAB/84)   .......... .......... .......... R......... ..........  [ 50]
MVs/Cape Town.SOA/45.02  .......... .......... .......... R......... ..........  [ 50]
MVs/Luanda.ANG/13.03/1   .......... .......... .......... R......... ..........  [ 50]

A                        SENELPRLGG KEDRRVKQSR GEARESYRET GPSRASDARA AHLPTGTPLD  [100]
B1                       .......... .......... ...G...... .......... ..P.......  [100]
B3-1                     .......... .......... ...G..H... .......... ..P.......  [100]
B3-2                     .......... ........G. ...G...... .......... ..P.......  [100]
B2 (Libreville.GAB/84)   .......... .......... ...G...... .......... T.........  [100]
MVs/Cape Town.SOA/45.02  .......... .......... ...G.RH... .......... T.........  [100]
MVs/Luanda.ANG/13.03/1   .......... .......... ...G.RH... .......... T....S....  [100]

A                        IDTASESSQD PQDSRRSADA LLRLQAMAGI SEEQGSDTDT PIVYNDRNLL  [150]
B1                       ......F... .......... .......... .......... .R.....D..  [150]
B3-1                     ......F... .......... .......... ....D..... .R.....D..  [150]
B3-2                     ......F... .......... .......... .......... .R...G.D..  [150]
B2 (Libreville.GAB/84)   ...V....L. .......... .......... .......... .R.....D..  [150]
MVs/Cape Town.SOA/45.02  ...V....L. .......... .......... .......... .R.....D..  [150]
MVs/Luanda.ANG/13.03/1   ...V....L. .......... .......... .......... .R.....D..  [150]
```

Figure 3.2 Alignment of the predicted amino acid sequences of the variable carboxyl-terminal region of the nucleoprotein of the outbreak B2 viruses with the B-clade reference strains by comparison to reference strain genotype A.

3.1.5 Sequence analysis of the entire H-gene

The complete H-gene sequences of the newly-detected B2 viruses were very closely related to each other and to the B2 reference strain (Figure 3.3). Although the N-gene sequences of all 14 Cape Town specimens were identical, a single nucleotide mutation was detected in the H-gene of one of six specimens positive for H-gene amplification. This mutation was silent despite being a first codon position transversion, because the codons AGA and CGA both encode arginine. Interestingly, the single Angolan H-sequence had the identical mutation, but at a different position. Comparison of the Cape Town sequences to the Luandan sequence revealed changes at only six of the 1854 sites (0.3%); the predicted translation resulted in a single substitution – the Angolan haemagglutinin contained a proline at residue 244 (Table 3.1). Although both recent groups differed from the B2 reference strain H-gene by 31 nucleotides (1.7%), some of the mutations were not at shared positions. Nevertheless, translation of the nt-sequences predicted that the Cape Town and Luanda H proteins differed from the B2 reference protein by seven (1.1%) and eight (1.3%) AA, respectively (Table 3.1). Whereas the 2002-2003 B2 viruses retained all the B2-like characteristics in the N-450 window and evolved some additional changes, only seven of the B2-characteristic AA residues were retained in H (residues 82, 225, 241, 328, 389, 445, 481), three were lost (residues 298, 318, 372), and four or five additional changes evolved (residues 9, 244,

286, 479, 493) (Table 3.1). The positions at which two of these B2-like residues were lost (V298A, S372T) resulted in loss of variation at these sites when compared to all other reference strains. Some of the AA substitutions were at novel sites (244P, 479K, 493G) whereas others represented variability at previously reported residues (9D seen previously in genotype H2 reference strain, and 286S noted in G1 reference strain as lysine).

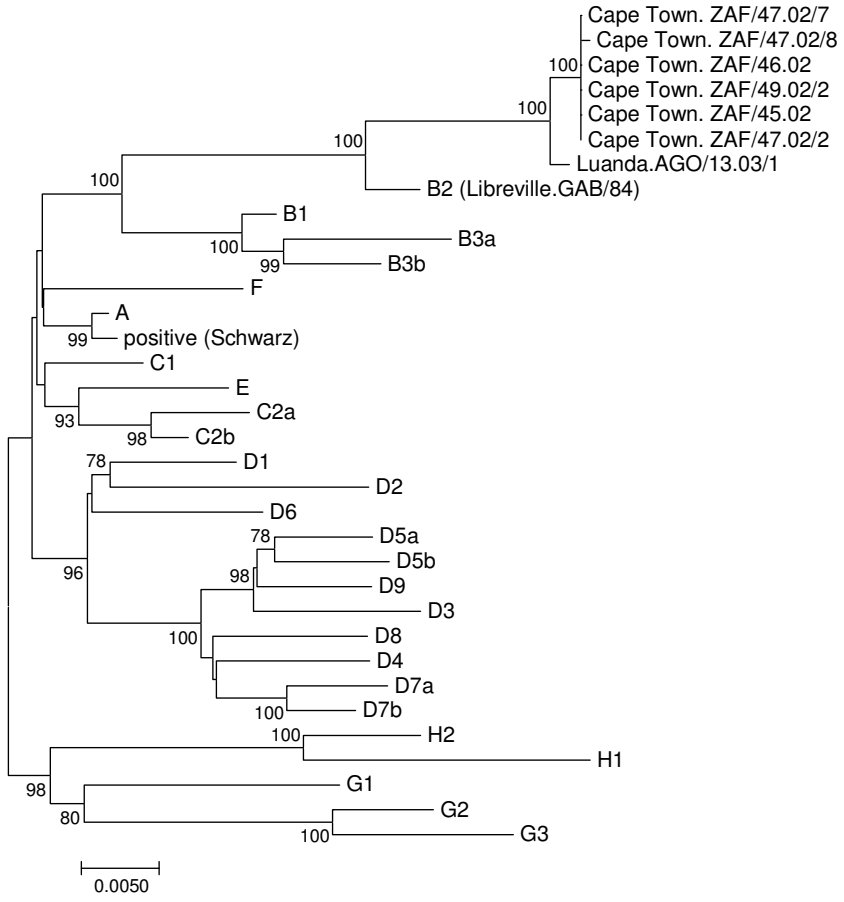

Figure 3.3 Phylogenetic analysis of the complete H-gene sequences of MV from the Cape Town and Luanda outbreaks. The unrooted neighbour-joining tree (Kimura 2-parameter algorithm) was generated by bootstrap analysis (1000 replicates). Only bootstrap frequencies higher than 70% are shown. The scale bar indicates nucleotide substitutions per site.

Table 3.1 Comparison of the predicted haemagglutinin amino acid sequences of the recent B2 viruses with the B-clade reference strains in relation to reference strain genotype A.

strain	Substitution at various AA residues in the haemagglutinin gene																												
	9	82	191	211	225	240	241	244	283	285	286	298	303	309	318	328	346	348	372	389	445	448	471	479	481	493	546	608
reference strain A	N	N	P	S	Q	S	S	S	D	S	N	A	E	P	S	K	I	R	T	K	K	H	E	S	N	D	G	V
reference strain B1	.	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
reference strain B3-1	.	.	.	G	.	N	.	.	G	G	.	.	D	L	.	.	V	.	.	.	.	R	.	.	.	.	S	A
reference strain B3-2	.	.	T	G	.	.	.	.	.	G	.	.	.	.	.	.	V	K	.	.	.	R	A	.	.	.	S	A
reference strain B2	.	K	.	.	L	.	N	.	.	.	.	V	.	.	G	R	.	.	S	Q	R	.	.	.	S	.	S	.
Cape Town.SOA/45.02	D	K	.	.	L	.	N	.	.	.	S	.	.	.	.	R	.	.	.	Q	R	.	.	K	S	G	S	.
Cape Town.SOA/47.02/8	D	K	.	.	L	.	N	.	.	.	S	.	.	.	.	R	.	.	.	Q	R	.	.	K	S	G	S	.
Luanda.ANG/13.03/1	D	K	.	.	L	.	N	P	.	.	S	.	.	.	.	R	.	.	.	Q	R	.	.	K	S	G	S	.

The complete H gene was sequenced. A dot (.) indicates predicted homology with the genotype A reference strain.

3.1.6 Evidence of continued circulation of genotype B2 viruses

As part of the routine surveillance activities in South Africa, genotype B2 was detected in three sporadic (not outbreak-related) measles cases in 2006: Gauteng.ZAF/25.06, Gauteng.ZAF/35.06 and Limpopo.ZAF/40.06 (Figure 3.4). These represented different strains of B2 compared to the outbreak described above. The first of the sporadic strains (Gauteng.ZAF/25.06) differed from the B2 reference strain by three nucleotides and from the Cape Town and Luanda outbreak strains by six and eight nucleotides respectively (Table 3.2). The latter two sporadic strains differed by one additional nucleotide in each comparison i.e. differed from the B2 reference strain by four nucleotides and from the Cape Town and Luanda outbreak strains by seven and nine nucleotides respectively.

Additional strains of genotype B2 were detected in EQA specimens sent to NICD for serological confirmation, and in specimens sent specifically for molecular analysis and genotyping. The 45 sequences shown in Figure 3.4 were selected from the available 115 genotype B2 sequences to avoid duplications and to enhance clarity, and therefore only the first available sequence for a specific country or year has been included. In 2005, genotype B2 was identified in Rwanda and the DRC. The following year (2006) related strains continued to circulate in the DRC and were also detected in Angola, Kenya and Zambia. However, detection of genotype B2 in the latter three countries, should not necessarily be interpreted as circulation of this genotype, given the limited number of samples received and the fact that the selection criteria for EQA serological rechecking (random selection of specimens with IgM positive, negative, indeterminate serology results) differ from those for molecular epidemiology (selection from clustered cases which are also geographically representative). In subsequent years there was still evidence of genotype B2 circulating in the DRC in 2004-2006 (Kremer, 2010) and in 2007 (this study), as well as in Angola and Namibia from 2009–2011.

A phylogenetic tree comparing the predicted amino acid sequences of these more recent genotype B2 strains was constructed (Figure 3.5) using only 32 unique N-gene nucleotide sequences from Figure 3.4. The 2009-2011 variants from Angola and Namibia form a distinct cluster in both nucleotide and amino acid trees (Figures 3.4, 3.5). The AA alignment shown in Figure 3.6 depicts only 22 of the 32 strains that had unique predicted AA changes. Although some of these predicted AA changes appear random (positions 27, 36, 42, 47, 60, 95, 96, 101, 114, 128), other changes appear fixed within outbreaks (positions 26, 41, 53, 76, 77, 89, 92, 113). However, the data is

probably skewed by a sampling bias as suitable specimens are more likely to be collected during an outbreak rather than from sporadic cases of measles; also, bigger health facilities or clinics are more able to collect appropriate specimens for molecular epidemiology. Therefore repetitions of the same change are more likely to be apparent. Positions 31 and 142 characterise the difference between all WT strains and reference strain A, from which virtually all vaccines were derived. The presence of a R74G residue appears to specify a clade B strain. It is therefore most unusual that Kindu.COD/11.06/2 has retained an R residue at this position, yet has all the other clade B and genotype B2 features. The original chromatograms have been rechecked to ensure that this is not an error: the codon in question is a CGG (translated as arginine, R), whereas all other B2 strains sequenced in this study, have a GGG nucleotide triplet (translated as glycine, G).

By comparison with all measles virus reference strains, some of the AA substitutions were at novel sites (T26N, R89I, D113A) whereas others represented variability at previously reported residues (41S seen previously in reference strains of genotypes C2a and C2b as 41S; 53T seen previously in reference strains of genotypes C2b and D7b as 53S; 92R seen previously in reference strain of genotype G2 as 92D). By comparison with only the genotype B2 reference strain, three strains (Kinshasa COD/41.05/1; Jamba.AGO/8.11 and Windhoek.NAM/52.10) were found to lack the 104V. The phylogenetic cluster of 2009-2011 Angolan and Namibian sequences (Figures 3.4 and 3.5) share two AA residues (T26N, H92R) not present in previous B2 variants described in this study. The sequence from Romford.GBR, obtained from GenBank, has a number of unusual changes, which are probably related to the sample origin (brain biopsy from a case of SSPE). Every other B2 sequence obtained from acute specimens has a 109L residue, whereas the Romford.GBR sequence has a 109P residue.

The most distantly related viruses in this group are represented by Luanda.AGO/13.03/1 and Windhoek.NAM/52.10 (Table 3.2), which differ by 13 nucleotides and 7 AA in the predicted translated sequence. This equates to a nucleotide difference of 2.9% and a predicted AA difference of 4.7% over a seven year period. Luanda.AGO/13.03/1 remains the most distant strain from the genotype B2 reference strain, with a difference of nine nucleotides (2%) but only three predicted AA (2%) as some of the nucleotide changes were silent. Windhoek.NAM/52.10 and MVsRomford.GBR/18.08 both differed from the reference strain by four AA, despite differing by eight and seven nucleotides respectively (Table 3.2).

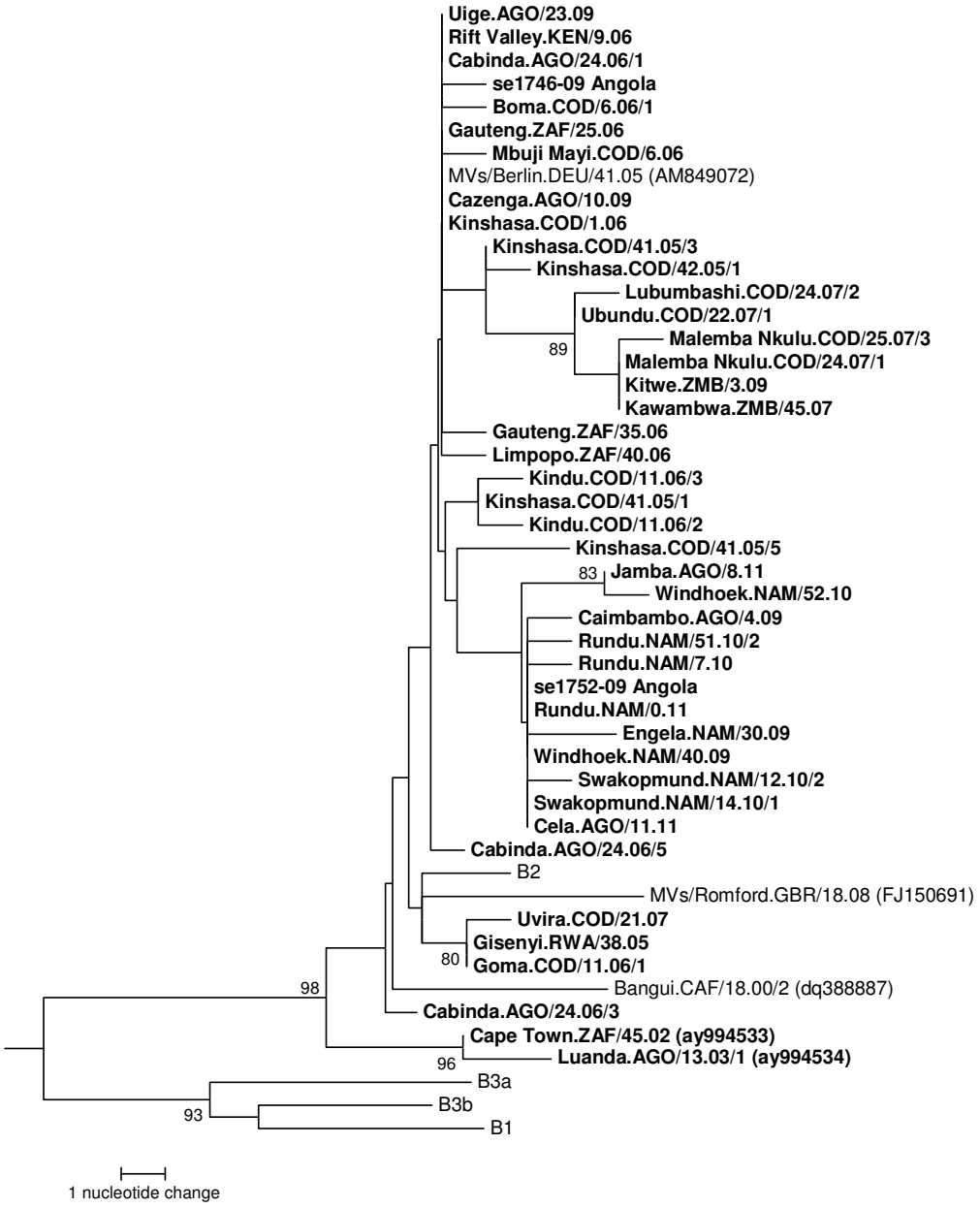

Figure 3.4 Phylogenetic analysis of the N-450 sequences of MV genotype B2 from specimens collected subsequent to the 2002 South African outbreak. Sequences from this study are shown in bold font. The unrooted neighbour-joining tree was generated by bootstrap analysis (1000 replicates) using MEGA 3.1 software. Only bootstrap frequencies greater than 70% are shown.

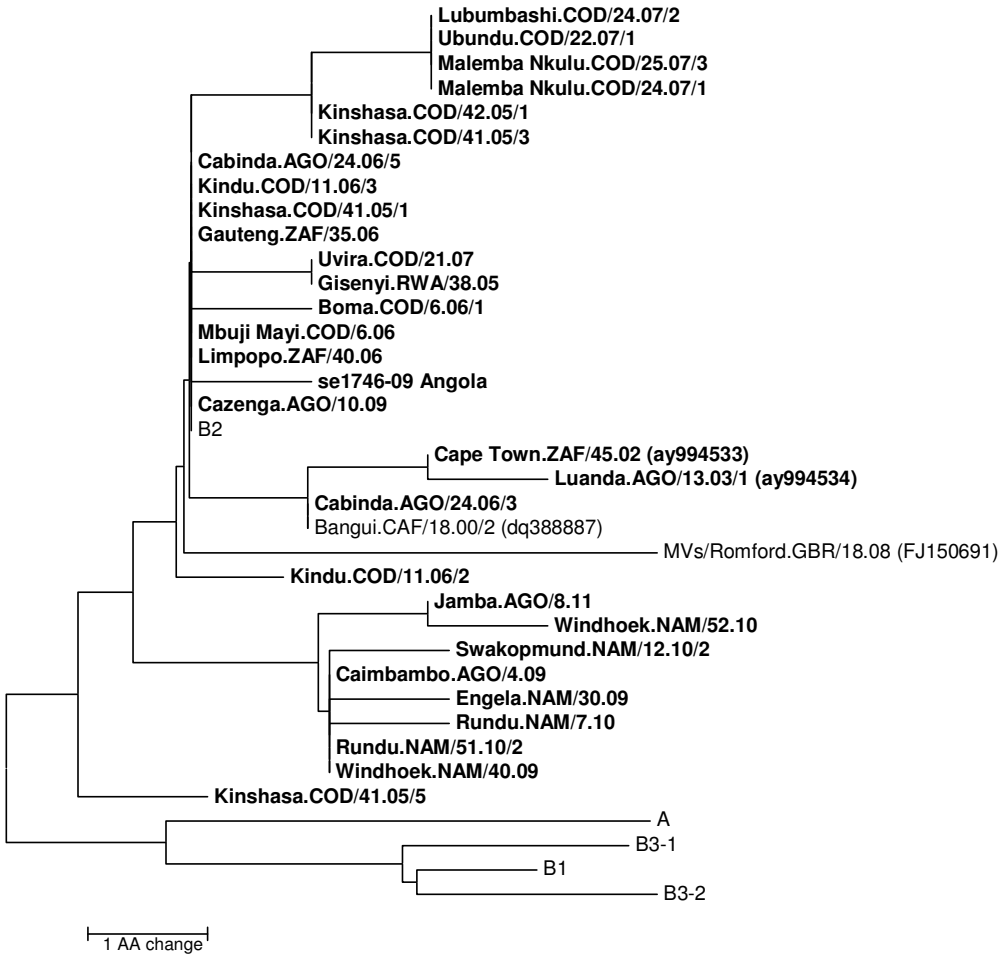

Figure 3.5 Phylogenetic analysis of predicted amino acid translation of selected genotype B2 sequences based on the unique N-450 sequences. Sequences from this study are shown in bold font.

```
A                       KVSSTLASEL GITAEDARLV SEIAMHTTED KISRAVGPRQ AQVSFLHGDQ  [ 50]
B1                      .......... .......... .......A.. R......... .........  [ 50]
B3-1(Ibadan.NGA/97/1)   .......... .......... .......... R......... .........  [ 50]
B3-2(New York.USA/94)   .......... .......... .......... R......... .........  [ 50]
B2 (Libreville.GAB/84)  .......... .......... .......... R......... .........  [ 50]
Cape Town.ZAF/45.02     .......... .......... .......... R......... .........  [ 50]
Luanda.AGO/13.03/1      .......... .......... .......... R......... .........  [ 50]
Cabinda.AGO/24.06/3     .......... .......... .......... R......... .........  [ 50]
se1746-09 Angola        .......... .......... .......... R......... .........  [ 50]
Limpopo.ZAF/40.06       .......... .......... .......... R......... .........  [ 50]
Boma.COD/6.06/1         .......... .......... .......... R......... .........  [ 50]
Gisenyi.RWA/38.05       .......... .......... .......... R......... .........  [ 50]
MVs/Romford.GBR/18.08   .......... .......... ......A... R....A.... .........  [ 50]
Gauteng.ZAF/35.06       .......... .......... .......... R......... .........  [ 50]
Kinshasa.COD/41.05/3    .......... .......... .......... R......... S........  [ 50]
Ubundu.COD/22.07/1      .......... .......... .......... R......... S........  [ 50]
Bangui.CAF/18.00/2      .......... .......... .......... R......... .........  [ 50]
Kindu.COD/11.06/2       .......... .......... .......... R......... .........  [ 50]
Kinshasa.COD/41.05/1    .......... .......... .......... R......... .........  [ 50]
Kinshasa.COD/41.05/5    .......... .......... .......... R......... .........  [ 50]
Jamba.AGO/8.11          .......... .......... .....N.... R......... .........  [ 50]
Swakopmund.NAM/12.10/2  .......... .......... .....N.... R......... .........  [ 50]
Windhoek.NAM/52.10      .......... .......... .....N.... R......... .H.......  [ 50]
Caimbambo.AGO/4.09      .......... .......... .....N.... R......... .........  [ 50]
Engela.NAM/30.09        .......... .......... .....N.... R......... ......L...  [ 50]
Rundu.NAM/7.10          .......... .......... .....N.... R......... .........  [ 50]
Windhoek.NAM/40.09      .......... .......... .....N.... R......... .........  [ 50]


A                       SENELPRLGG KEDRRVKQSR GEARESYRET GPSRASDARA AHLPTGTPLD  [100]
B1                      .......... .......... ...G...... .......... ..P.......  [100]
B3-1(Ibadan.NGA/97/1)   .......... .......... ...G..H... .......... ..P.......  [100]
B3-2(New York.USA/94)   .......... ........G. ...G...... .......... ..P.......  [100]
B2 (Libreville.GAB/84)  .......... .......... ...G...... .......... T.........  [100]
Cape Town.ZAF/45.02     .......... .......... ...G.RH... .......... T.........  [100]
Luanda.AGO/13.03/1      .......... .......... ...G.RH... .......... T....S....  [100]
Cabinda.AGO/24.06/3     .......... .......... ...G..H... .......... T.........  [100]
se1746-09 Angola        .......... .......... ...G...... .......... T.........  [100]
Limpopo.ZAF/40.06       .......... .......... ...G...... .......... T.........  [100]
Boma.COD/6.06/1         .......... .......... ...G...... .......... T.........  [100]
Gisenyi.RWA/38.05       .......... .......... ...G...... ........I. T.........  [100]
MVs/Romford.GBR/18.08   .......... .......... ...G...... .......... T.........  [100]
Gauteng.ZAF/35.06       .......... .......... ...G...... .......... T.........  [100]
Kinshasa.COD/41.05/3    .......... .......... ...G...... .......... T.........  [100]
Ubundu.COD/22.07/1      ..T....... .......... ...G...... .......... T.........  [100]
Bangui.CAF/18.00/2      .......... .......... ...G..H... .......... T.........  [100]
Kindu.COD/11.06/2       .......... .......... .......... .......... T.........  [100]
Kinshasa.COD/41.05/1    .......... .......... ...G...... .......... T.........  [100]
Kinshasa.COD/41.05/5    .......... .......... ...G...... .......... T.........  [100]
Jamba.AGO/8.11          .......... .......... ...G...... .......... TR........  [100]
Swakopmund.NAM/12.10/2  .......... .......... ...G...... .......... TR..N.....  [100]
Windhoek.NAM/52.10      .......... .......... ...G...... .......... TR........  [100]
Caimbambo.AGO/4.09      .......... .......... ...G...... .......... TR........  [100]
Engela.NAM/30.09        .......... .......... ...G...... .......... TR........  [100]
Rundu.NAM/7.10          .........A .......... ...G...... .......... TR........  [100]
Windhoek.NAM/40.09      .......... .......... ...G...... .......... TR........  [100]
```

Figure 3.6 continued overleaf

```
A                       IDTASESSQD PQDSRRSADA LLRLQAMAGI SEEQGSDTDT PIVYNDRNLL  [150]
B1                      ......F... .......... .......... .......... .R.....D..  [150]
B3-1(Ibadan.NGA/97/1)   ......F... .......... .......... ....D..... .R.....D..  [150]
B3-2(New York.USA/94)   ......F... .......... .......... .......... .R...G.D..  [150]
B2 (Libreville.GAB/84)  ...V....L. .......... .......... .......... .R.....D..  [150]
Cape Town.ZAF/45.02     ...V....L. .......... .......... .......... .R.....D..  [150]
Luanda.AGO/13.03/1      ...V....L. .......... .......... .......... .R.....D..  [150]
Cabinda.AGO/24.06/3     ...V....L. .......... .......... .......... .R.....D..  [150]
se1746-09 Angola        ...V....L. ..A....... .......... .......... .R.....D..  [150]
```

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Limpopo.ZAF/40.06          ...V....L. .......... .......... .......... .R.....D..  [150]
Boma.COD/6.06/1            ...V....L. ...N...... .......... .......... .R.....D..  [150]
Gisenyi.RWA/38.05          ...V....L. .......... .......... .......... .R.....D..  [150]
MVs/Romford.GBR/18.08      ...V....P. .......... .......V.. .......... .R.....D..  [150]
Gauteng.ZAF/35.06          ...V....L. .......... .......... .......... .R.....D..  [150]
Kinshasa.COD/41.05/3       ...V....L. .......... .......... .......... .R.....D..  [150]
Ubundu.COD/22.07/1         ...V....L. .......... .......... .......... .R.....D..  [150]
Bangui.CAF/18.00/2         ...V....L. .......... .......... .......... .R.....D..  [150]
Kindu.COD/11.06/2          ...V....L. .......... .......... .......... .R.....D..  [150]
Kinshasa.COD/41.05/1       ...V....L. .......... .......... .......... .R.....D..  [150]
Kinshasa.COD/41.05/5       T.......L. .......... .......... .......... .R.....D..  [150]
Jamba.AGO/8.11             ........L. .......... .......... .......... .R.....D..  [150]
Swakopmund.NAM/12.10/2     ...V....L. .......... .......... .......... .R.....D..  [150]
Windhoek.NAM/52.10         ........L. .......... .......... .......... .R.....D..  [150]
Caimbambo.AGO/4.09         ...V....L. .......... .......... .......... .R.....D..  [150]
Engela.NAM/30.09           ...V....L. .......... .......... .......... .R.....D..  [150]
Rundu.NAM/7.10             ...V....L. .......... .......... .......... .R.....D..  [150]
Windhoek.NAM/40.09         ...V....L. .......... .......... .......... .R.....D..  [150]
```

Figure 3.6 Alignment of the predicted amino acid sequences of the variable carboxyl-terminal region of the nucleoprotein of all unique B2 viruses with the B-clade reference strains by comparison to reference strain genotype A.

Table 3.2 Amino acid and nucleotide distance matrix of selected genotype B2 N-gene sequences.

AA distances (number of changes)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	B2_Libreville.GAB/84		0	1	1	0	0	1	1	0	1	0	0	1	1	1	0	2	2	2	3	2	3	2	2	2	3	3	1	2	4	2	4	3
2	Gauteng.ZAF/25.06	3		1	1	0	0	1	1	0	1	0	0	1	1	1	0	2	2	2	3	2	3	2	2	2	3	3	1	2	4	2	4	3
3	Gisenyi.RWA/38.05	3	2		2	1	1	2	0	1	2	1	1	2	2	2	1	3	3	3	4	3	4	3	3	3	4	4	2	3	5	3	5	4
4	se1746-09_Angola	4	1	3		1	1	2	2	1	2	1	1	2	2	2	1	3	3	3	4	3	4	3	3	3	4	4	2	3	5	3	5	4
5	Limpopo.ZAF/40.06	4	1	3	2		0	1	1	0	1	0	0	1	1	1	0	2	2	2	3	2	3	2	2	2	3	3	1	2	4	2	4	3
6	Mbuji Mayi.COD/6.06	4	1	3	2	2		1	1	0	1	0	0	1	1	1	0	2	2	2	3	2	3	2	2	2	3	3	1	2	4	2	4	3
7	Boma.COD/6.06/1	4	1	3	2	2	2		2	1	2	1	1	2	2	2	1	3	3	3	4	3	4	3	3	3	4	4	2	3	5	3	5	4
8	Uvira.COD/21.07	4	3	1	4	4	4	4		1	2	1	1	2	2	2	1	3	3	3	4	3	4	3	3	3	4	4	2	3	5	3	5	4
9	Gauteng.ZAF/35.06	4	1	3	2	2	2	2	4		1	0	0	1	1	1	0	2	2	2	3	2	3	2	2	2	3	3	1	2	4	2	4	3
10	Kinshasa.COD/41.05/3	4	1	3	2	2	2	2	4	2		1	1	2	0	2	1	3	1	3	4	3	4	3	1	1	4	4	2	3	5	1	5	4
11	Cabinda.AGO/24.06/5	4	1	3	2	2	2	2	4	2	2		0	1	1	1	0	2	2	2	3	2	3	2	2	2	3	3	1	2	4	2	4	3
12	Kinshasa.COD/41.05/1	4	1	3	2	2	2	2	4	2	2	2		1	1	1	0	2	2	2	3	2	3	2	2	2	3	3	1	2	4	2	4	3
13	Cabinda.AGO/24.06/3	5	2	4	3	3	3	3	5	3	3	3	1		2	2	1	3	3	3	4	3	4	3	3	3	4	4	0	1	5	3	5	2
14	Kinshasa.COD/42.05/1	5	2	4	3	3	3	3	5	3	1	3	3	4		2	1	3	1	3	4	3	4	3	1	1	4	4	2	3	5	1	5	4
15	Kindu.COD/11.06/2	5	2	4	3	3	3	3	5	3	3	3	1	2	4		1	3	3	3	4	3	4	3	3	3	4	4	2	3	5	3	5	4
16	Kindu.COD/11.06/3	5	2	4	3	3	3	3	5	3	3	3	1	2	4	2		2	2	2	3	2	3	2	2	2	3	3	1	2	4	2	4	3
17	Windhoek.NAM/40.09	5	2	4	3	3	3	3	5	3	3	3	3	4	4	4	4		4	4	1	0	1	0	4	4	1	1	3	4	6	4	2	5
18	Ubundu.COD/22.07/1	6	3	5	4	4	4	4	6	4	2	4	4	5	3	5	5	5		4	5	4	5	4	0	0	5	5	3	4	6	0	6	5
19	Kinshasa.COD/41.05/5	6	3	5	4	4	4	4	6	4	4	4	4	5	5	5	5	5	6		5	4	5	4	4	4	5	3	3	4	6	4	4	5
20	Swakopmund.NAM/12.10/2	6	3	5	4	4	4	4	6	4	4	4	4	5	5	5	5	1	6	6		1	2	1	5	5	2	2	4	5	7	5	3	6
21	Caimbambo.AGO/4.09	6	3	5	4	4	4	4	6	4	4	4	4	5	5	5	5	1	6	6	2		1	0	4	4	1	1	3	4	6	4	2	5
22	Rundu.NAM/7.10	6	3	5	4	4	4	4	6	4	4	4	4	5	5	5	5	1	6	6	2	2		1	5	5	2	2	4	5	7	5	3	6
23	Rundu.NAM/51.10/2	6	3	5	4	4	4	4	6	4	4	4	4	5	5	5	5	1	6	6	2	2	2		4	4	1	1	3	4	6	4	2	5
24	Cape Town.ZAF/45.02	7	6	6	7	7	7	7	7	7	7	7	5	4	8	6	6	8	9	9	9	9	9	9		0	5	5	3	4	6	0	6	5
25	MVs/Romford.GBR/18.08	7	6	6	7	7	7	7	7	7	7	7	7	8	8	8	8	8	9	9	9	9	9	9	10		5	5	3	4	6	0	6	5
26	MalembaNkulu.COD/24.07/1	7	4	6	5	5	5	5	7	5	3	5	5	6	4	6	6	6	1	7	7	7	7	7	10	10		2	4	5	7	5	3	6
27	Lubumbashi.COD/24.07/2	7	4	6	5	5	5	5	7	5	3	5	5	6	4	6	6	6	1	7	7	7	7	7	10	10	2		4	5	7	5	1	6
28	Bangui.CAF/18.00/2	7	6	6	7	7	7	7	7	7	7	5	7	6	8	8	8	8	9	9	9	9	9	9	8	10	10	10		1	5	3	5	2
29	Jamba.AGO/8.11	7	6	6	5	5	5	5	7	5	5	5	3	4	6	4	4	2	7	5	3	3	3	3	8	10	8	8	10		6	4	6	1
30	Engela.NAM/30.09	7	4	6	5	5	5	5	7	5	5	5	5	6	6	6	6	2	7	7	3	3	3	3	10	10	8	8	10	4		6	8	7
31	MalembaNkulu.COD/25.07/3	8	5	7	6	6	6	6	8	6	4	6	6	7	5	7	7	7	2	8	8	8	8	8	11	11	1	3	11	9	9		6	5
32	Windhoek.NAM/52.10	8	5	7	6	6	6	6	8	6	6	6	4	5	7	5	5	3	8	6	4	4	4	4	9	11	9	9	11	1	5	10		7
33	Luanda.AGO/13.03/1	9	8	8	9	9	9	9	9	9	9	9	7	6	10	8	8	10	11	11	11	11	11	11	2	12	12	12	10	10	12	13	11	

Nucleotide distance (number of changes)

3.2 Genotype D2

3.2.1 Southern African outbreak 2003-2005

Towards the end of May 2003, there was a suspected measles outbreak in Maputo, Mozambique, and ten sera and matched urines for three of the specimens, were sent to NICD for laboratory confirmation and genotyping. Genotype D2 was identified. In August 2003, the identical strain of D2 virus was detected in specimens collected from measles cases in Mpumalanga and Gauteng, the outbreak then spread to the Western Cape and KwaZulu-Natal in 2004, and by 2005, hundreds of cases had been reported from the Eastern Cape. Of the affected provinces, Gauteng had the most cases (775/1676, representing 46% of the laboratory-confirmed cases from July 2003 to November 2005). A national measles mass campaign was conducted in August-September 2004, targeting children younger than five years of age, in an attempt to interrupt transmission of the WT virus by decreasing the number of susceptibles. In addition, to prevent nosocomial spread, vaccination of children between six months and 15 years of age on admission to a health facility or institution, was advocated. Despite these interventions, the outbreak continued for another year.

RNA was extracted from 191 South African specimens (one cerebrospinal fluid, six throat or nasal swabs, 25 sera, 159 urines) from IgM-positive measles cases; of these 110 specimens (57%) were positive by PCR and phylogenetic analysis of the sequences revealed that the two and a half year-long outbreak was caused by a single strain of measles virus, characterized as genotype D2 (Figure 3.7). Molecular characterization of the viruses obtained at the start of the outbreak established that they were identical to the strain that had been circulating in Mozambique in 2003, and that these outbreaks were therefore virologically linked. Standard epidemiological field investigations also indicated that the outbreak had started in Mpumalanga in Mozambican citizens employed on farms along the shared border, thus confirming that the source of the virus was Mozambique. As a result of this extended outbreak, measles was once again considered endemic in South Africa. The definition of endemic versus sustained transmission is somewhat imprecise but implies transmission for longer than a year. Although genotype D2 had been shown to circulate endemically in South Africa prior to 2000, the lack of viral diversity seen in the 2003-2005 outbreak suggests an epidemic pattern of transmission (clonal) rather than an endemic pattern.

It was found that the outbreak strain of genotype D2 that was circulating in South Africa and Mozambique in 2003-2005 was also present in Lesotho, Namibia, Zimbabwe, Botswana, Cote d'Ivoire and Malawi variously from 2001-2005 (Figure 3.7).

From the nucleotide data (Figure 3.7) it appears that the 2003-2005 outbreak was caused by the introduction of a single strain of genotype D2 into South Africa from Mozambique, with subsequent clonal spread. However, as there were many chains of transmission, there were some nucleotide changes over the two and a half year period, but these did not all translate into AA changes (Figure 3.8). The AA tree (Figure 3.8) indicates that one of the South African sequences (North West.ZAF/44.03), as well as the sequences from Malawi (Chiradzulu.MWI/27.05) and the DRC (Sakania.COD/16.05/1) are likely to have been introduced from Zambia (Lusaka.ZAM/19.01), as opposed to the other outbreak strains which were clearly derived from Mozambique.

The sequence South Australia.AUS/20.03 downloaded from GenBank, is an outlier, differing by 17 nucleotides (3.8%) and eight predicted AA from the genotype D2 reference strain (Table 3.3). This percentage is greater than the maximum allowed within a genotype, although it still groups most clearly within the D2 genotype (Figure 3.7). It is not possible to speculate about the reason for this variation, as there is no available epidemiological information about the source of this specimen.

These genotype D2 viruses show very few changes relative to the reference strain from 1988 (Figure 3.9). This is remarkable for an RNA virus, given that the RNA polymerase lacks proof-reading functionality. The early endemic D2s (prior to 2001) had a 96S (serine), whilst later strains all had a 96N (asparagine). Furthermore, strains from the 2003-2005 outbreak were characterized by 107L (leucine) in contrast to an earlier serine residue. The consequence of replacing serine (an amino acid with a polar, neutral side-chain) with leucine (which has a hydrophobic aliphatic side-chain) probably has little functional relevance.

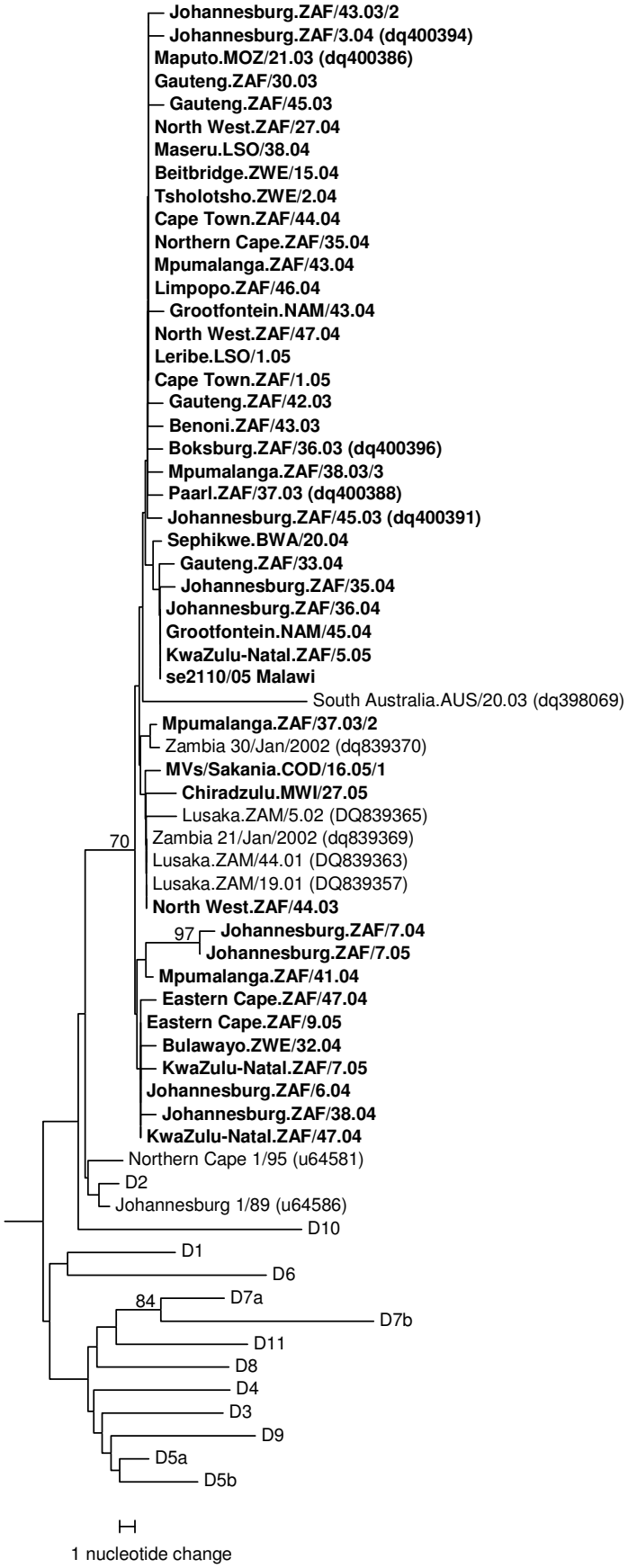

Figure 3.7 Phylogenetic analysis of the N-450 sequences of MV genotype D2 from the 2003-2005 South African outbreak, from EQA submissions from the southern block countries and the WHO reference sequences, shown only as designated genotype for clarity (WHO, 2003). Sequences from this study are shown in bold font. The unrooted minimum evolution tree was generated by bootstrap analysis (1000 replicates) using MEGA 3.1 software. Only bootstrap frequencies greater than 70% are shown.

.

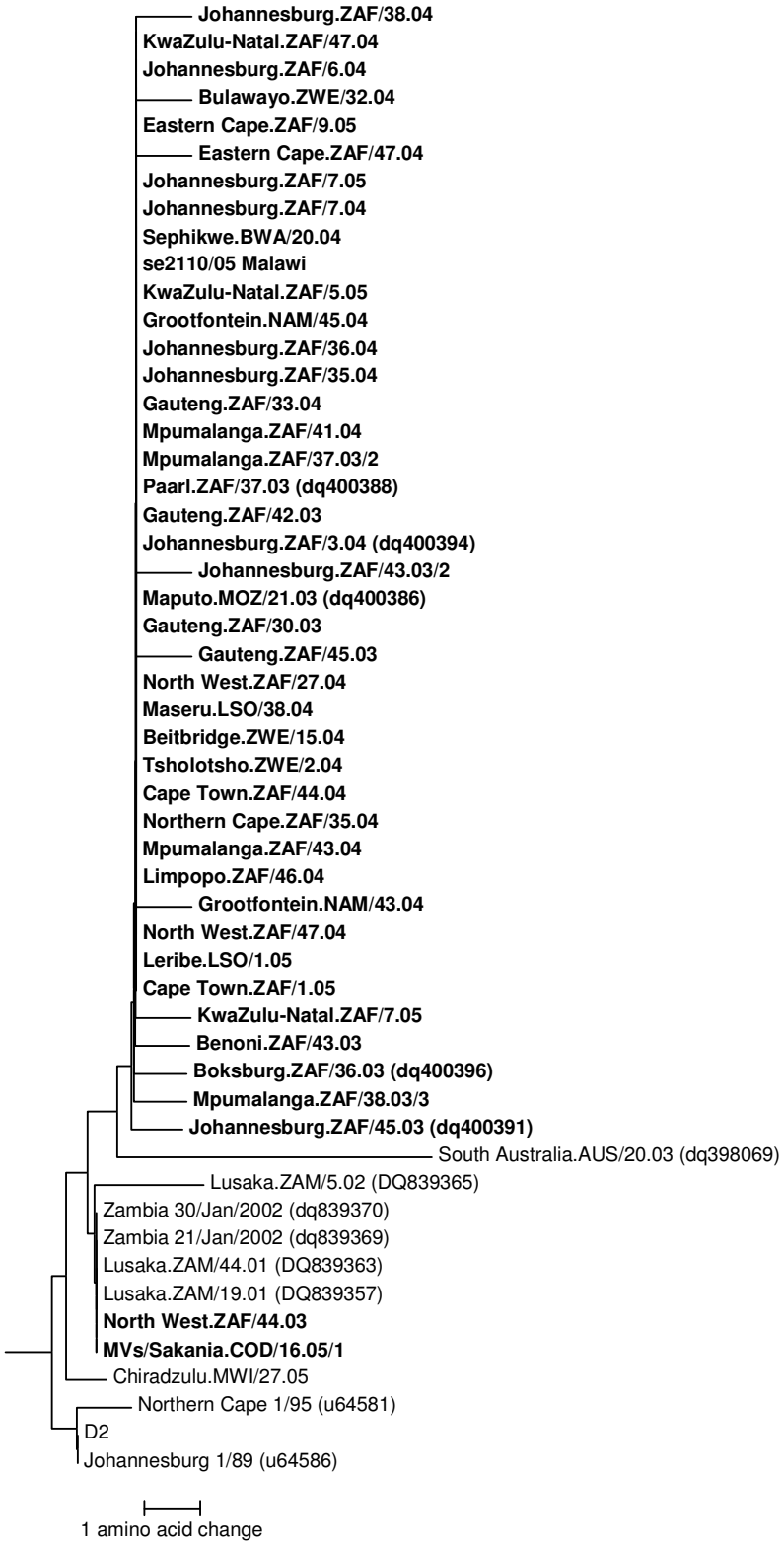

Figure 3.8 Minimum evolution amino acid tree using predicted translations of the nucleotide sequences of genotype D2 MV depicted in Figure 3.7 above. Sequences in bold font are from this study.

Table 3.3 Amino acid and nucleotide distance matrix of selected genotype D2 N-gene sequences.

AA distances (number of changes)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	D2		0	1	1	1	1	2	2	3	3	3	3	3	3	2	3	2	3	3	3	3	3	8
2	Jhb 1/89	2		1	1	1	1	2	2	3	3	3	3	3	3	2	3	2	3	3	3	3	3	8
3	NC 1/95	4	4		2	2	2	3	3	2	4	4	4	4	4	3	4	3	4	4	4	4	4	9
4	Lusaka.ZAM/19.01	5	5	5		0	0	1	1	2	2	2	2	2	2	1	2	1	2	2	2	2	2	7
5	North West.ZAF/44.03	5	5	5	0		0	1	1	2	2	2	2	2	2	1	2	1	2	2	2	2	2	7
6	Sakanaia.COD/16.05/1	6	6	6	1	1		1	1	2	2	2	2	2	2	1	2	1	2	2	2	2	2	7
7	Gauteng.ZAF/30.03	6	6	6	1	1	2		0	1	1	1	1	1	1	0	1	2	3	1	1	1	1	6
8	Maputo.MOZ/21.03	6	6	6	1	1	2	0		1	1	1	1	1	1	0	1	2	3	1	1	1	1	6
9	Benoni.ZAF/43.04	7	7	5	2	2	3	1	1		2	2	2	2	2	1	2	3	4	2	2	2	2	7
10	Grootfontein.NAM/43.04	7	7	7	2	2	3	1	1	2		2	2	2	2	1	2	3	4	2	2	2	2	7
11	Gauteng.ZAF/45.03	7	7	7	2	2	3	1	1	2	2		2	2	2	1	2	3	4	2	2	2	2	7
12	Johannesburg.ZAF/43.03/2	7	7	7	2	2	3	1	1	2	2	2		2	2	1	2	3	4	2	2	2	2	7
13	Mpumalanga.ZAF/38.03/3	7	7	7	2	2	3	1	1	2	2	2	2		2	1	2	3	4	2	2	2	2	7
14	Boksburg.ZAF/36.03	7	7	7	2	2	3	1	1	2	2	2	2	2		1	2	3	4	2	2	2	2	7
15	Paarl.ZAF/37.03	7	7	7	2	2	3	1	1	2	2	2	2	2	2		1	2	3	1	1	1	1	6
16	Johannesburg.ZAF/45.03	7	7	7	2	2	3	3	3	2	2	2	2	2	2	2		3	4	2	2	2	2	7
17	Chiradzulu.MWI/27.05	7	7	7	2	2	3	3	3	4	4	4	4	4	4	4	4		3	3	3	3	3	8
18	Lusaka.ZAM/5.02	7	7	7	2	2	3	3	3	4	4	4	4	4	4	4	4	4		4	4	4	4	9
19	Eastern Cape.ZAF/47.04	8	8	8	3	3	4	2	2	3	3	3	3	3	3	3	3	5	5		2	2	2	7
20	Bulawayo.ZWE/32.04	8	8	8	3	3	4	2	2	3	3	3	3	3	3	3	3	5	5	2		2	2	7
21	Kwazulu-Natal.ZAF/7.05	8	8	8	3	3	4	2	2	3	3	3	3	3	3	3	3	5	5	2	2		2	7
22	Joahnnesburg.ZAF/38.04	8	8	8	3	3	4	2	2	3	3	3	3	3	3	3	3	5	5	2	2	2		7
23	South Australia.AUS/20.03	17	17	17	12	12	13	11	11	12	12	12	12	12	12	12	12	14	14	13	13	13	13	

Nucleotide distances (number of changes)

```
D2                          KVSSTLASEL GITAEDARLV SEIAMHTTED RISRAVGPRQ AQVSFLHGDQ  [ 50]
NC 1/95                     .......... .......... .......... .......... ..........  [ 50]
Lusaka.ZAM/5.02_            .......... .......... .......... .......... ..........  [ 50]
Lusaka.ZAM/19.01_           .......... .......... .......... .......... ..........  [ 50]
Chiradzulu.MWI/27.05        .......... .......... .......... .......... ..........  [ 50]
Maputo.MOZ/21.03            .......... .......... .......... .......... ..........  [ 50]
Boksburg.ZAF/36.03          .......... .......... .......... .......... ..........  [ 50]
Benoni.ZAF/43.03            .......... .......... .......... .......... ..........  [ 50]
Johannesburg.ZAF/38.04      .......T.. .......... .......... .......... ..........  [ 50]
Grootfontein.NAM/43.04      ........F. .......... .......... .......... ..........  [ 50]
Gauteng.ZAF/45.03           .......... .....N.... .......... .......... ..........  [ 50]
Johannesburg.ZAF/43.03/2    .......... .......... .......... .......... ..........  [ 50]
Mpumalanga.ZAF/38.03/3      .......... .......... .......... .......... ..........  [ 50]
Johannesburg.ZAF/45.03      .......... .......... .......... .......... ..........  [ 50]
South Australia.AUS/20.03.. .......... .......... .......... .......... ...A...R.S  [ 50]
Eastern Cape.ZAF/47.04      .......... .......... .......... .......... ..........  [ 50]
Bulawayo.ZWE/32.04          .......... .......... .......... .......... ..........  [ 50]
KwaZulu-Natal.ZAF/7.05      .......... .......... .......... .......... ..........  [ 50]

D2                          SENELPGLGG KEDRRVKQSR GEARESYRET GSSRTSDARA AHLPTSTPLD  [100]
NC 1/95                     .......... .......... .D........ .......... ..........  [100]
Lusaka.ZAM/5.02             .......... .......... .......... .......... T....N....  [100]
Lusaka.ZAM/19.01            .......... .......... .......... .......... .....N....  [100]
Chiradzulu.MWI/27.05        .......... .......... .......... ....I..... .....N....  [100]
Maputo.MOZ/21.03            .......... .......... .......... .......... .....N....  [100]
Boksburg.ZAF/36.03          .......... .......... ..S....... .......... .....N....  [100]
Benoni.ZAF/43.03            .......... .......... .D........ .......... .....N....  [100]
Johannesburg.ZAF/38.04      .......... .......... .......... .......... .....N....  [100]
Grootfontein.NAM/43.04      .......... .......... .......... .......... .....N....  [100]
Gauteng.ZAF/45.03           .......... .......... .......... .......... .....N....  [100]
Johannesburg.ZAF/43.03/2    .......... .......... .......... .......... .....N....  [100]
Mpumalanga.ZAF/38.03/3      .......... .......... .......... R......... .....N....  [100]
Johannesburg.ZAF/45.03      .......... .......... ......H... .......... .....N....  [100]
South Australia.AUS/20.03.....GR... ...T...... .......... .......... .....N....  [100]
Eastern Cape.ZAF/47.04      .......... .......... .......... .......... .....N....  [100]
Bulawayo.ZWE/32.04          .......... .......... .......... .......... .....N....  [100]
KwaZulu-Natal.ZAF/7.05      .......... .......... .......... .........S .....N....  [100]

D2                          IDTASESSQD PQDSRRSADA LLRLQAMAGI SEEQGSDTDT PRVYNDRDLL  [150]
NC 1/95                     .......... .......... .......... .......... ..........  [150]
Lusaka.ZAM/5.02             .......... .......... .......... ........E. ..........  [150]
Lusaka.ZAM/19.01            .......... .......... .......... .......... ..........  [150]
Chiradzulu.MWI/27.05        .......... .......... .......... .......... ..........  [150]
Maputo.MOZ/21.03            ......L... .......... .......... .......... ..........  [150]
Boksburg.ZAF/36.03          ......L... .......... .......... .......... ..........  [150]
Benoni.ZAF/43.03            ......L... .......... .......... .......... ..........  [150]
Johannesburg.ZAF/38.04      ......L... .......... .......... .......... ..........  [150]
Grootfontein.NAM/43.04      ......L... .......... .......... .......... ..........  [150]
Gauteng.ZAF/45.03           ......L... .......... .......... .......... ..........  [150]
Johannesburg.ZAF/43.03/2    ......L... .......... ......I... .......... ..........  [150]
Mpumalanga.ZAF/38.03/3      ......L... .......... .......... .......... ..........  [150]
Johannesburg.ZAF/45.03      ......L... .......... .......... .......... ..........  [150]
South Australia.AUS/20.03.....L... .......... .......... .......... ..........  [150]
Eastern Cape.ZAF/47.04      T.....L... .......... .......... .......... ..........  [150]
Bulawayo.ZWE/32.04          ......L... ...N...... .......... .......... ..........  [150]
KwaZulu-Natal.ZAF/7.05      ......L... .......... .......... .......... ..........  [150]
```

Figure 3.9 Alignment of selected predicted amino acid translations of the N-gene genotype D2 sequence data.

3.3 Genotype D4

3.3.1 Sporadic cases

Two unrelated genotype D4 viruses were detected in 2003, during the genotype D2 outbreak. One of these viruses was obtained from the Bushbuckridge area in Limpopo province (Bushbuckridge.ZAF/29.03); the other unrelated genotype D4 virus was identified in Gauteng in September 2003 in a Mozambican child who had traveled from Beira to Boksburg during or just prior to showing symptoms of a rash-like illness (Beira.MOZ/38.03). It therefore appears that at least two genotypes of measles virus (D2 and D4) were circulating in Mozambique during 2003.

Although no further cases of transmission of MV genotype D4 were identified in Gauteng, there were two cases in the Northern Cape during October-November 2003 (represented by Northern Cape.ZAF/44.03 in subsequent figures). This D4 genotype, introduced into RSA from Mozambique, was very closely related to the measles virus that had been circulating endemically in Kenya in 2002 (Figure 3.10). Identical or very closely related MV D4 strains were also identified in specimens from Zimbabwe and Botswana in 2004 and in the Comoros in 2005. The latter outbreak was initially masked by an ongoing Chikungunya outbreak, which delayed identification of the correct aetiology. The direct source of the imported genotype D4 MV into the Comoros Islands is unclear, as there were three additional AA changes (Figures 3.11 and 3.12) relative to the Mozambican D4 strain. Nevertheless the sequences from the Comoros share the 92Y residue common to Group 2 (also referred to as ‘’Montreal-like’’ by Mbugua *et al*. in 2003), and therefore any of the countries in this cluster could have been the source of the virus.

South Africa experienced a large measles outbreak from 2009-2011, which was caused by a single strain of genotype B3-1 (not described in this study, as the N-450 data could not differentiate between the many identical sequences and thus will require a different experimental approach). A single case of genotype D4 was identified in 2010, immediately prior to the FIFA World Cup competition. The virus was isolated from a French journalist and was identical to the strain causing the extensive D4 outbreak in France (Figures 3.10 and 3.11). This French strain differed by only one nucleotide and one AA from the infamous Enfield strain, which was spread across Europe by travelers, nomadic people of Irish origin (Muscat, 2011).

3.3.2 Outbreak 2006

During 2006, 81 out of the 6600 sera (1.2%) which were submitted for routine national rash surveillance in South Africa, were found to be measles IgM-positive. Not all 81 specimens were tested for the presence of measles virus genome

(insufficient volume, duplicate specimens, specimens not found), and 27 of 61 (44.3%) were PCR-positive. Fifteen of these were from the Mafikeng area of North West Province and clearly represented a measles outbreak – a single strain of virus, genotype D4, was identified. This same strain was circulating in Botswana, Zimbabwe and Zambia in 2005-6 (Figure 3.10) and it is thus likely to have been introduced into South Africa from one of these countries (Botswana being the most likely when considering the geographical location of Mafikeng). Of the remaining PCR-positive specimens from South Africa, three were sporadic cases of genotype B2 (refer to section 3.1.6) and nine were cases of genotype B3-1 (not discussed further as phylogenetic analysis of the N-450 sequence could not discriminate between these and later B3-1 sequences).

In the midst of the very large genotype B3-1 outbreak during 2005–2006 in Kenya (Rota, 2006), a small proportion of measles cases were caused by genotype D4 viruses. These were unrelated to the D4 strains that circulated in Kenya in 2002, which clustered in Groups 2 (Montreal-like) and 4 (Johannesburg-like). The 2005-2006 sequences were identical or very closely related to strains in India and Ethiopia in the Group 1 (India-like) cluster (Figures 3.10 and 3.11). This terminology of naming the four previously assigned groups (Mbugua *et al*., 2003), has been used here for ease of comparison of African strains. However, global detection and phylogenetic analysis of recent genotype D4 strains no longer supports the separation into four distinct groups (WHO, 2012).

With reference to Figure 3.12, very few AA changes occur within the first 50 residues of the predicted sequence window. Most of the nucleotide changes were silent, resulting in an AA tree (Figure 3.11) differentiated by very few changes, thus Group 1 (India-like) is characterised by an isoleucine (I) at residue 95; Group 2 (Montreal-like) by a tyrosine (Y) at residue 92; Group 3 (Ethiopia-like) by a threonine (T) at residue 85; and Group 4 (Johannesburg-like) by an alanine (A) at residue 80. Additional AA changes evolved within outbreaks (for example, the group represented by North West.ZAF/31.06, all had 59S and 91T residues; the Enfield, Reims and Johannesburg.ZAF/20.10 cluster within the India-like group, all have a valine at residue 101).

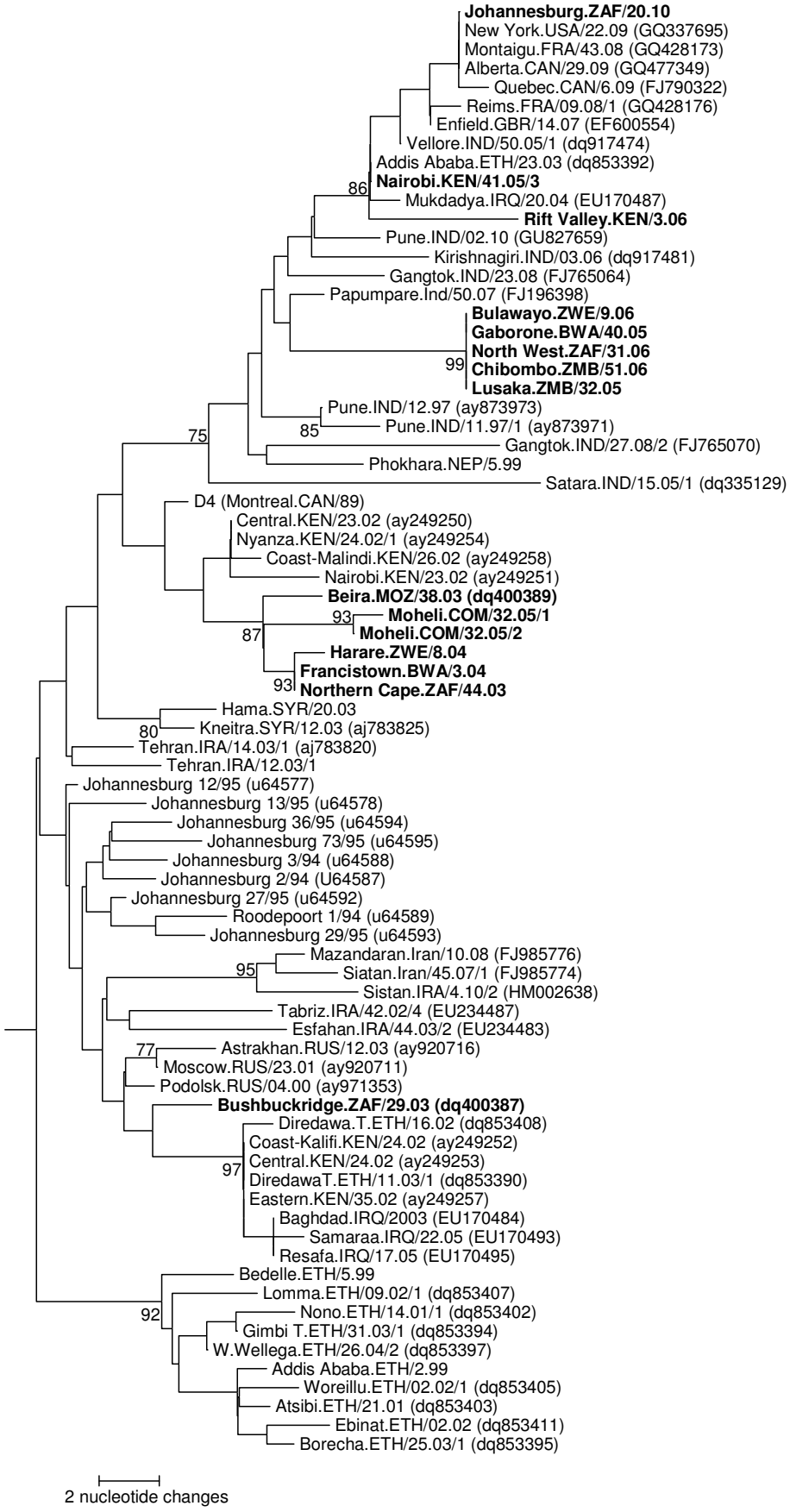

Figure 3.10 Phylogenetic analysis of selected N-450 sequences of genotype D4 MV. The unrooted neighbour-joining tree was generated by bootstrap analysis (1000 replicates) using MEGA 3.1 software. Only bootstrap frequencies greater than 70% are shown. Sequences from this study are shown in bold.

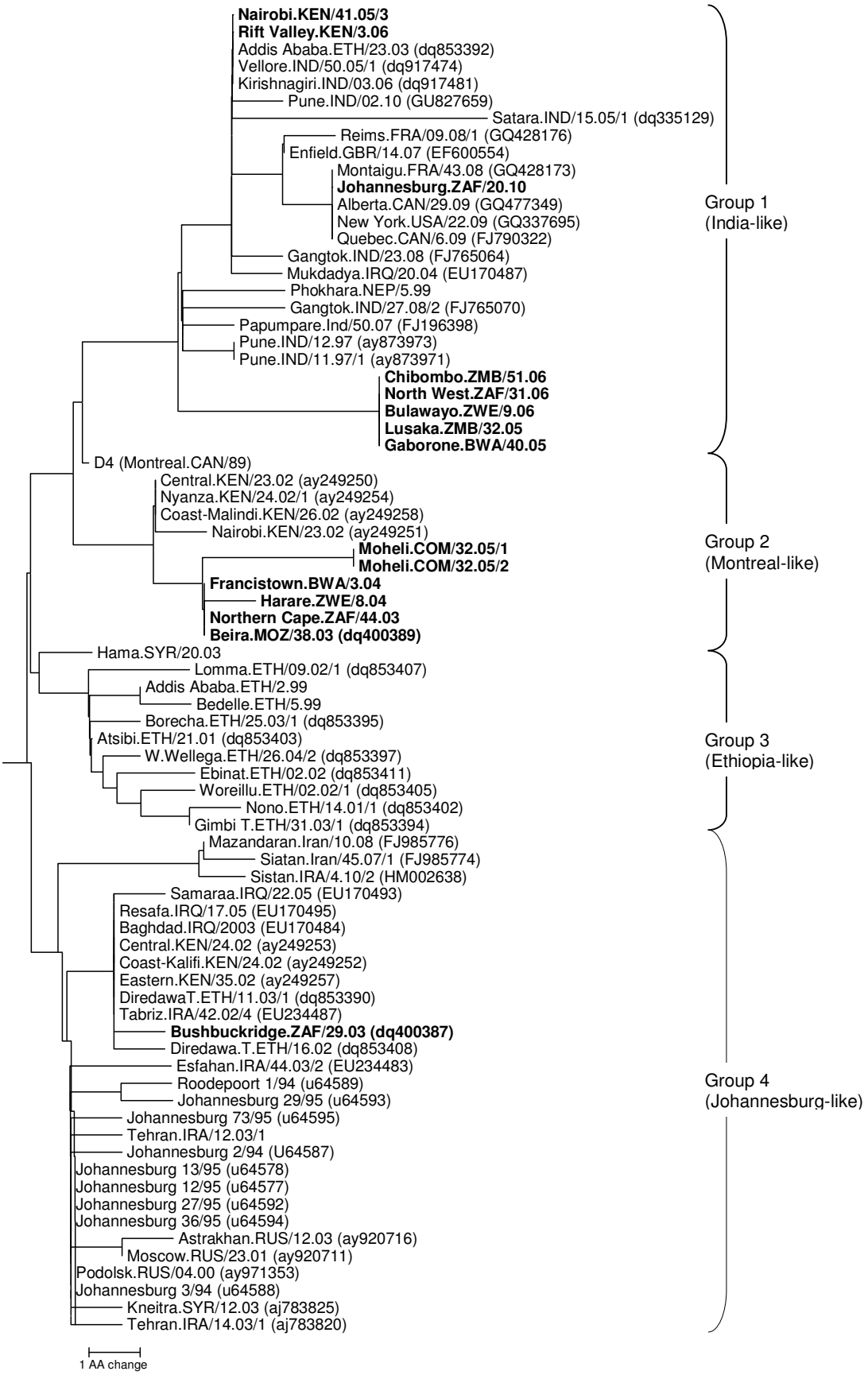

Figure 3.11 Phylogenetic analysis of selected predicted amino acid sequences of the 150 carboxyl-terminal residues of the N-gene of genotype D4 strains. Sequences from this study are shown in bold.

```
D4 (Montreal.CAN/89)    KVSSTLASEL GITAEDARLV SEIAMHTTED RISRAVGPRQ AQVSFIHGDQ
Atsibi.ETH/21.01        .......... .......... .......... .......... .......... [ 50]
Hama.SYR/20.03          .......... .......... .......... .......... .......... [ 50]
Esfahan.IRA/44.03/2     .......... .......... .......... .......... .......... [ 50]
Johannesburg 2/94       .......... .......... .......... .......... .......... [ 50]
Johannesburg 73/95      .......... .......... .......... .......... .......... [ 50]
Johannesburg 3/94       .......... .......... .......... .......... .......... [ 50]
Mazandaran.Iran/10.08   .......... .......... .......... .......... .......... [ 50]
Tehran.IRA/12.03/1      .......... .......... .......... .......... .......... [ 50]
Bushbuckridge.ZAF/29.03 .......... .......... .......... .......... .......... [ 50]
Coast-Kalifi.KEN/24.02  .......... .......... .......... .......... .......... [ 50]
Diredawa.T.ETH/16.02    .......... .......... .......... .......... .......... [ 50]
Samaraa.IRQ/22.05       .......... .......... .......... .......... .......... [ 50]
Harare.ZWE/8.04         .......... .......... .......... .......... .......... [ 50]
Northern Cape.ZAF/44.03 .......... .......... .......... .......... .......... [ 50]
Moheli.COM/32.05/1      .......... .......... .......... .......... .......... [ 50]
Nairobi.KEN/23.02       .......... .......... .......... ....T..... .......... [ 50]
Central.KEN/23.02       .......... .......... .......... .......... .......... [ 50]
Pune.IND/11.97/1        .......... .......... .......... .......... .......... [ 50]
North West.ZAF/31.06    .......... .......... .......... .......... .......... [ 50]
Satara.IND/15.05/1      .......C.. .M........ ..L..... .M........ .......... [ 50]
Addis Ababa.ETH/23.03   .......... .......... .......... .......... .......... [ 50]
Nairobi.KEN/41.05/3     .......... .......... .......... .......... .......... [ 50]
Johannesburg.ZAF/20.10  .......... .......... .......... .......... .......... [ 50]
Reims.FRA/09.08/1       .......... .......... .......... .......... .......... [ 50]
Enfield.GBR/14.07       .......... .......... .......... .......... .......... [ 50]
D4 (Montreal.CAN/89)    SENELPGLGG KEDRRVKQSR GEARESYRET GSSRASDARA AHLPTSTPLD [100]
Atsibi.ETH/21.01        .......... .......... .......... ....T..... .......... [100]
Hama.SYR/20.03          .......... .......... .......... .......... .......... [100]
Esfahan.IRA/44.03/2     .......... .......... .........A E......... ....I..... [100]
Johannesburg 2/94       .......... .......... .........A .......... .......... [100]
Johannesburg 73/95      .......... .......... .........A .......... .......... [100]
Johannesburg 3/94       .......... .......... .........A .......... .......... [100]
Mazandaran.Iran/10.08   .......... .......... .........A .......... VP........ [100]
Tehran.IRA/12.03/1      .......... .......... .........A ..G....... .......... [100]
Bushbuckridge.ZAF/29.03 .......... .......... .........A .........S ........P. [100]
Coast-Kalifi.KEN/24.02  .......... .......... .........A .......... ........P. [100]
Diredawa.T.ETH/16.02    .......... .......... .........A .P........ ........P. [100]
Samaraa.IRQ/22.05       .......... .......... .G.......A .......... ........P. [100]
Harare.ZWE/8.04         .......... .......... ......H... ......G... .Y......P. [100]
Northern Cape.ZAF/44.03 .......... .......... ......H... .......... .Y......P. [100]
Moheli.COM/32.05/1      .......... .......... ...K..H... R..I...... .Y......P. [100]
Nairobi.KEN/23.02       .......... .......... .......... .......... .Y......P. [100]
Central.KEN/23.02       .......... .......... .......... .......... .Y......P. [100]
Pune.IND/11.97/1        .......... .......... .......... .......... ....I..S.. [100]
North West.ZAF/31.06    .........S .......... ......H... .......... T...I..... [100]
Satara.IND/15.05/1      .......... ........G. .......... .......... ....I..... [100]
Addis Ababa.ETH/23.03   .......... ........G. .......... .......... ....I..... [100]
Nairobi.KEN/41.05/3     .......... ........G. .......... .......... ....I..... [100]
Johannesburg.ZAF/20.10  .......... ........G. .......... .......V.. ....I..... [100]
Reims.FRA/09.08/1       .......... ........G. .......... .......... ....I..... [100]
Enfield.GBR/14.07       .......... ........G. .......... .......... ....I..... [100]
D4 (Montreal.CAN/89)     IDTASESGQD PQDSRRSADA LLRLQAMAGI LEEQGSDTDI PRVYNDKDLL [150]
Atsibi.ETH/21.01)        .......... .......... .......... .......... ......R... [150]
Hama.SYR/20.03           .......... .......... .......... .......... .....AR... [150]
Esfahan.IRA/44.03/2      .......... .......... .......... .......... ......R... [150]
Johannesburg 2/94        ..........E......... .......... .......... ......R... [150]
Johannesburg 73/95       ..........L......... .......... .......... ......R... [150]
Johannesburg 3/94        .......... .......... .......... .......... ......R... [150]
Mazandaran.Iran/10.08    .......... .......... .......... .......... S.....R... [150]
Tehran.IRA/12.03/1       .......... .......... .......... .......... ......R... [150]
Bushbuckridge.ZAF/29.03  .......... .......... .......... .......... ......R... [150]
Coast-Kalifi.KEN/24.02   .......... .......... .......... .......... ......R... [150]
Diredawa.T.ETH/16.02     .......... .......... .......... .......... ......R... [150]
Samaraa.IRQ/22.05)       .......... .......... .......... .......... ......R... [150]
Harare.ZWE/8.04          .......... .......... .......... .......... .......... [150]
Northern Cape.ZAF/44.03  .......... .......... .......... .......... .......... [150]
Moheli.COM/32.05/1       .......... .......... .......... .......... .......... [150]
Nairobi.KEN/23.02        .......... .......... .......... .......... .......... [150]
Central.KEN/23.02        .......... .......... .......... .......... .......... [150]
Pune.IND/11.97/1         .......... .......... .......... .......... S......... [150]
North West.ZAF/31.06     ..........L......... .......... .......... S......... [150]
Satara.IND/15.05/1       .......... .......... .......... ..G....... S......... [150]
Addis Ababa.ETH/23.03    .......... .......... .......... .......... S......... [150]
Nairobi.KEN/41.05/3      .......... .......... .......... .......... S......... [150]
Johannesburg.ZAF/20.10   V......... .......... .......... .......... S......... [150]
Reims.FRA/09.08/1)       V......... .......... .......E.. .......... S......... [150]
Enfield.GBR/14.07        V......... .......... .......... .......... S......... [150]
```

Figure 3.12 Alignment of selected predicted amino acid translation of the genotype D4 sequence data.

Table 3.4 Amino acid and nucleotide distance matrix for selected genotype D4 group 1 N-gene sequences.

AA distances (number of changes)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	D4 (Montreal.CAN/89)		4	3	3	3	3	3	4	4	4	3	3	4	6	6	6	6	6	5	5	5	5	5	5	4	3	8
2	Phokhara.NEP/5.99	7		3	3	3	3	3	4	4	4	3	3	4	6	6	6	6	6	5	5	5	5	5	5	4	3	8
3	Pune.IND/12.97	7	6		2	2	0	2	3	3	3	2	2	3	5	5	5	5	5	4	4	4	4	4	4	3	2	7
4	Papumpare.IND/50.07	7	6	4		2	2	2	3	3	3	2	2	3	5	5	5	5	5	4	4	4	4	4	4	3	2	7
5	Addis Abab.ETH/23.03	9	8	6	4		2	0	1	1	1	0	0	1	5	5	5	5	5	2	2	2	2	2	2	3	0	5
6	Pune.IND/11.97/1	9	8	2	6	8		2	3	3	3	2	2	3	5	5	5	5	5	4	4	4	4	4	4	3	2	7
7	Nairobi.KEN/41.05/3	9	8	6	4	0	8		1	1	1	0	0	1	5	5	5	5	5	2	2	2	2	2	2	3	0	5
8	Gangtok.IND/23.08	9	8	6	6	6	8	6		2	2	1	1	2	6	6	6	6	6	3	3	3	3	3	3	4	1	6
9	Pune.IND/02.10	9	8	6	4	4	8	4	6		2	1	1	2	6	6	6	6	6	3	3	3	3	3	3	4	1	6
10	Mukdadya.IRQ/20.04	10	9	7	5	1	9	1	7	5		1	1	2	6	6	6	6	6	3	3	3	3	3	3	4	1	6
11	Vellore.IND/50.05/1	10	9	7	5	1	9	1	7	5	2		0	1	5	5	5	5	5	2	2	2	2	2	2	3	0	5
12	Kirishnagiri.IND/03.06	11	10	8	6	6	8	6	8	6	7	7		1	5	5	5	5	5	2	2	2	2	2	2	3	0	5
13	Enfield.GBR/14.07	11	10	8	6	2	10	2	8	6	3	1	8		6	6	6	6	6	1	1	1	1	1	1	4	1	6
14	Chibombo.ZMB/51.06	12	11	9	7	9	11	9	11	9	10	10	11	11		0	0	0	0	7	7	7	7	7	7	6	5	10
15	North West.ZAF/31.06	12	11	9	7	9	11	9	11	9	10	10	11	11	0		0	0	0	7	7	7	7	7	7	6	5	10
16	Bulawayo.ZWE/9.06	12	11	9	7	9	11	9	11	9	10	10	11	11	0	0		0	0	7	7	7	7	7	7	6	5	10
17	Lusaka.ZMB/32.05	12	11	9	7	9	11	9	11	9	10	10	11	11	0	0	0		0	7	7	7	7	7	7	6	5	10
18	Gaborone.BWA/40.05	12	11	9	7	9	11	9	11	9	10	10	11	11	0	0	0	0		7	7	7	7	7	7	6	5	10
19	Montaigu.FRA/43.08	12	11	9	7	3	11	3	9	7	4	2	9	1	12	12	12	12	12		0	0	0	2	0	5	2	7
20	Johannesburg.ZAF/20.10	12	11	9	7	3	11	3	9	7	4	2	9	1	12	12	12	12	12	0		0	0	2	0	5	2	7
21	Alberta.CAN/29.09	12	11	9	7	3	11	3	9	7	4	2	9	1	12	12	12	12	12	0	0		0	2	0	5	2	7
22	New York.USA/22.09	12	11	9	7	3	11	3	9	7	4	2	9	1	12	12	12	12	12	0	0	0		2	0	5	2	7
23	Reims.FRA/09.08/1	12	11	9	7	3	11	3	9	7	4	2	9	1	12	12	12	12	12	2	2	2	2		2	5	2	7
24	Quebec.CAN/6.09	13	12	10	8	4	12	4	10	8	5	3	10	2	13	13	13	13	13	1	1	1	1	3		5	2	7
25	Gangtok.IND/27.08/2	14	11	11	11	13	13	13	13	13	14	14	15	15	16	16	16	16	16	16	16	16	16	16	17		3	8
26	Rift Valley.KEN/3.06	14	13	11	9	5	13	5	11	9	6	6	11	7	14	14	14	14	14	8	8	8	8	8	9	17		5
27	Satara.IND/15.05/1	18	17	15	13	13	17	13	15	13	14	14	15	15	18	18	18	18	18	16	16	16	16	16	17	22	18	

Nucleotide distances (number of changes)

The Group 1 strains had an intragroup difference of six to seventeen nucleotides (mean difference of nine) and an AA range from three to eight (mean difference of four residues). The difference between individual sequences of Group 1 and the genotype D4 reference strain, ranged from seven to eighteen nucleotides (1.6%-4.0%) and four to eight AA residues (2.7%-5.3%), with a mean difference of 11 nucleotides (2.4%) and four AA residues (2.7%) (Table 3.4).

As Group 2 includes the reference strain, it is not unexpected that the difference between this group and the Montreal.CAN/89 strain ranges from two to nine nucleotides (0.4-2%) with a mean difference of six nucleotides (1.3%), and ranges from two to six AA residues (1.3- 4%) with a mean difference of three AA (2%). Excluding the reference strain, the Group 2 intragroup mean distances are five nucleotides (range of zero to seven) and two AA residues (range zero to four). Actual values are presented in Table 3.5.

Table 3.5 Amino acid and nucleotide distance matrix for selected genotype D4 group 2 N-gene sequences.

AA distances (number of changes)

		1	2	3	4	5	6	7	8	9	10	11
1	D4 (Montreal.CAN/89)		2	2	2	3	3	3	4	3	6	6
2	Central.KEN/23.02	2		0	0	1	1	1	2	1	4	4
3	Nyanza.KEN/24.02/1	2	0		0	1	1	1	2	1	4	4
4	Coast Malinidi.KEN/26.02	3	1	1		1	1	1	2	1	4	4
5	Nairobi.KEN/23.02	5	3	3	4		2	2	3	2	5	5
6	Francistown.BWA/3.04	6	4	4	5	7		0	1	0	3	3
7	Northern Cape.ZAF/44.03	6	4	4	5	7	0		1	0	3	3
8	Harare.ZWE/8.04	7	5	5	6	8	1	1		1	4	4
9	Beira.MOZ/38.03	7	5	5	6	8	3	3	4		3	3
10	Moheli.COM/32.05/2	8	6	6	7	9	4	4	5	5		0
11	Moheli.COM/32.05/1	9	7	7	8	8	5	5	6	6	1	

Nucleotide distances (number of changes)

Since none of the sequences described in this study belonged to Group 3, no detailed analysis was performed. Relative to the genotype D4 reference strain, the mean nucleotide difference of the group was eight (range five to fourteen) and the mean AA residue difference was three (range three to five).

The sequences comprising Group 4 differed from the genotype D4 reference strain by six to seventeen nucleotides (1.3-3.8%) and two to six AA residues (1.3-4%) with a mean distance of 12 nucleotides (2.7%) and three AA (2%). The intragroup

distances ranged from three to eleven nucleotides (0.7- 2.4%) and zero to four AA residues (mean of eight nucleotides and two AA respectively). Actual values are presented in Table 3.6.

Table 3.6 Amino acid and nucleotide distance matrix for selected genotype D4 group 3 N-gene sequences.

AA distances (number of changes)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	D4 (Montreal.CAN/89)		2	3	3	3	3	4	4	2	4	3	4	3	5	4
2	Hama.SUR/20.03	5		3	3	3	3	4	4	2	4	3	4	3	5	4
3	Kneitra.SYR/12.03	6	3		2	2	4	5	5	3	5	4	5	4	6	5
4	Tehran.IRA/14.03/1	6	7	6		2	4	5	5	3	5	4	5	4	6	5
5	Tehran.IRA/12.03/1	7	8	7	5		4	5	5	3	5	4	5	4	6	5
6	W.Wellega.ETH/26.04/2	10	9	10	10	11		1	3	1	3	2	3	2	2	3
7	Gimbi T.ETH/31.03/1	11	10	11	11	12	1		4	2	4	3	2	3	1	3
8	Bedelle.ETH/5.99	11	12	13	11	12	5	6		2	4	1	4	3	5	4
9	Atsibib.ETH/21.01	12	11	12	12	13	4	5	7		2	1	2	1	3	2
10	Lomma.ETH/09.02/1	12	11	12	12	13	4	5	5	6		3	4	3	5	4
11	Addis Ababa.ETH/2.99	12	11	12	12	13	4	5	5	2	6		3	2	4	3
12	Woreillu.ETH/02.02/1	13	12	13	13	14	5	4	8	3	7	3		3	3	3
13	Borecha.ETH/25.03/1	13	12	13	13	14	5	6	8	3	7	3	4		4	3
14	Nono.ETH/14.01/1	13	12	13	11	14	3	2	8	7	7	7	6	8		4
15	Ebinat.ETH/02.02	14	13	14	14	15	6	7	9	4	8	4	5	3	9	

Nucleotide distances (number of changes)

Table 3.7 Amino acid and nucleotide distance matrix for selected genotype D4 group 4 N-gene sequences.

Amino Acid Distances (Number of changes)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	D4 (Montreal.CAN/89)		2	3	2	2	2	3	3	2	4	2	4	4	3	4	4	3	3	3	3	3	4	3	4	5	6	6
2	Johannesburg 12/95	6		1	0	0	0	1	1	0	2	0	2	2	1	2	2	1	1	1	1	1	2	1	2	3	4	4
3	Johannesburg 2/94	8	3		1	1	1	2	2	1	3	1	3	3	2	3	3	2	2	2	2	2	3	2	3	4	5	5
4	Johannesburg 13/95	9	3	6		0	0	1	1	0	2	0	2	2	1	2	2	1	1	1	1	1	2	1	2	3	4	4
5	Johannesburg 27/95	9	3	4	4		0	1	1	0	2	0	2	2	1	2	2	1	1	1	1	1	2	1	2	3	4	4
6	Johannesburg 36/95	9	3	4	6	4		1	1	0	2	0	2	2	1	2	2	1	1	1	1	1	2	1	2	3	4	4
7	Moscow.RUS/23.01	10	4	5	5	3	5		2	1	3	1	3	1	2	3	3	2	2	2	2	2	3	2	3	4	5	5
8	Johannesburg 73/95	10	4	5	7	5	5	6		1	3	1	3	3	2	3	3	2	2	2	2	2	3	2	3	4	5	5
9	Podolsk.RUS/04.00	10	4	5	5	3	5	2	6		2	0	2	2	1	2	2	1	1	1	1	1	2	1	2	3	4	4
10	Johannesburg 29/95	10	5	6	6	4	8	7	9	7		2	4	4	3	4	4	3	3	3	3	3	4	3	4	5	6	6
11	Johannesburg 3/94	10	5	4	6	4	4	5	5	5	6		2	2	1	2	2	1	1	1	1	1	2	1	2	3	4	4
12	Bushbuckridge.ZAF/29.	12	6	7	7	5	7	4	8	4	9	7		4	1	4	4	1	1	1	1	1	2	1	2	5	6	6
13	Astrakhan.RUS/12.03	12	6	7	7	5	7	2	8	4	9	7	6		3	4	4	3	3	3	3	3	4	3	4	5	6	6
14	Tabriz.IRA/42.02/4	12	6	9	9	9	9	8	10	8	11	11	8	10		3	3	0	0	0	0	0	1	0	1	4	5	5
15	Roodepoort 1/94	12	7	6	8	4	8	7	9	7	4	6	9	9	13		4	3	3	3	3	3	4	3	4	5	6	6
16	Esfahan.IRA/44.03/2	12	8	10	9	9	11	8	12	8	10	10	10	10	10	12		3	3	3	3	3	4	3	4	5	6	6
17	DiredawaT.ETH/11.03.1	13	7	8	8	6	8	5	9	5	10	8	5	7	9	10	11		0	0	0	0	1	0	1	4	5	5
18	Coast Kalifi.KEN/24.02	13	7	8	8	6	8	5	9	5	10	8	5	7	9	10	11	0		0	0	0	1	0	1	4	5	5
19	Eastern.KEN/35.02	13	7	8	8	6	8	5	9	5	10	8	5	7	9	10	11	0	0		0	0	1	0	1	4	5	5
20	Central.KEN/24.02	13	7	8	8	6	8	5	9	5	10	8	5	7	9	10	11	0	0	0		0	1	0	1	4	5	5
21	Resafa.IRQ/17.05	14	8	9	9	7	9	6	10	6	11	9	6	8	10	11	12	1	1	1	1		1	0	1	4	5	5
22	Driedawa.T.ETH/16.02	14	8	9	9	7	9	6	10	6	11	9	6	8	10	11	12	1	1	1	1	2		1	2	5	6	6
23	Baghdad.IRQ/2003	14	8	9	9	7	9	6	10	6	11	9	6	8	10	11	12	1	1	1	1	0	2		1	4	5	5
24	Samaraa.IRQ/22.05	15	9	10	10	8	10	7	11	7	12	10	7	9	11	12	13	2	2	2	2	1	3	1		5	6	6
25	Mazandaran.Iran/10.08	15	9	10	10	8	10	9	11	7	12	10	11	11	13	12	11	12	12	12	12	13	13	13	14		1	1
26	Siatan.Iran/45.07/1	16	10	11	11	9	11	10	12	8	13	11	12	12	14	13	14	13	13	13	13	14	14	14	15	3		2
27	Sistan.IRA/4.10/2	17	11	12	12	10	12	11	13	11	14	12	13	13	15	14	13	14	14	14	14	15	15	15	16	4	7	

Nucleotide Distances (Number of changes)

3.4 Genotype D8

3.4.1 Sporadic cases

During 2008, 4777 specimens collected from cases of rash and fever for suspected measles case-based surveillance, were tested for measles and rubella IgM antibodies. Of these specimens, 42 were from patients with onset of symptoms in 2007. Of the remaining 4735 specimens the largest number, 1025 (21.6%) were from KwaZulu-Natal, followed by 867 (18.3%) from Gauteng. Blood and urine specimens were received from 63% of cases, blood-only from 32% and urine-only from 5%. Of the 4796 blood specimens 40 (0.8%) were positive for measles IgM antibodies, and 2160 (45%) for rubella IgM antibodies. Of the 40 patients with positive measles IgM results, the majority were from Gauteng (12 cases), and the Eastern Cape (7 cases). Ages of patients with positive measles results ranged from 9 months to 46 years (median 3 years). Urine specimens accompanied 22 of the 40 specimens (55%) with positive measles IgM results. Nine of the measles IgM-positive patients had dual measles IgM and rubella IgM positive results, and seven of these were found to be rubella PCR positive. Measles was confirmed by PCR on urine in only one patient (Carltonville.ZAF/16.08; Figure 3.13). The virus was identified as genotype D8, suggesting importation, although no epidemiological information was available, and the patient had no history of travel. However, the sequence was identical to Indian sequences from 2006 and 2007, and to a 2008 Canadian sequence (Figures 3.13, 3.14). It differed by one nucleotide from the genotype D8 reference strain at position 442. However, the D8 reference strain is atypical of the reference strains, as it has an AAT at position 442 which translates to an asparagine (148N), while all other reference strains have a GAT which translates to an aspartic acid (148D) residue (Appendix L).

The second genotype D8 sequence (Pretoria.ZAF/19.09), was identified in a patient with history of travel to India. The nucleotide sequence was identical to Amstelveen.NLD/08.09 (Figure 3.13) but the predicted AA translation was identical to a number of strains from the USA and India (Figure 3.14). The exact source of the virus is unclear, as a detailed travel itinerary was not obtained.

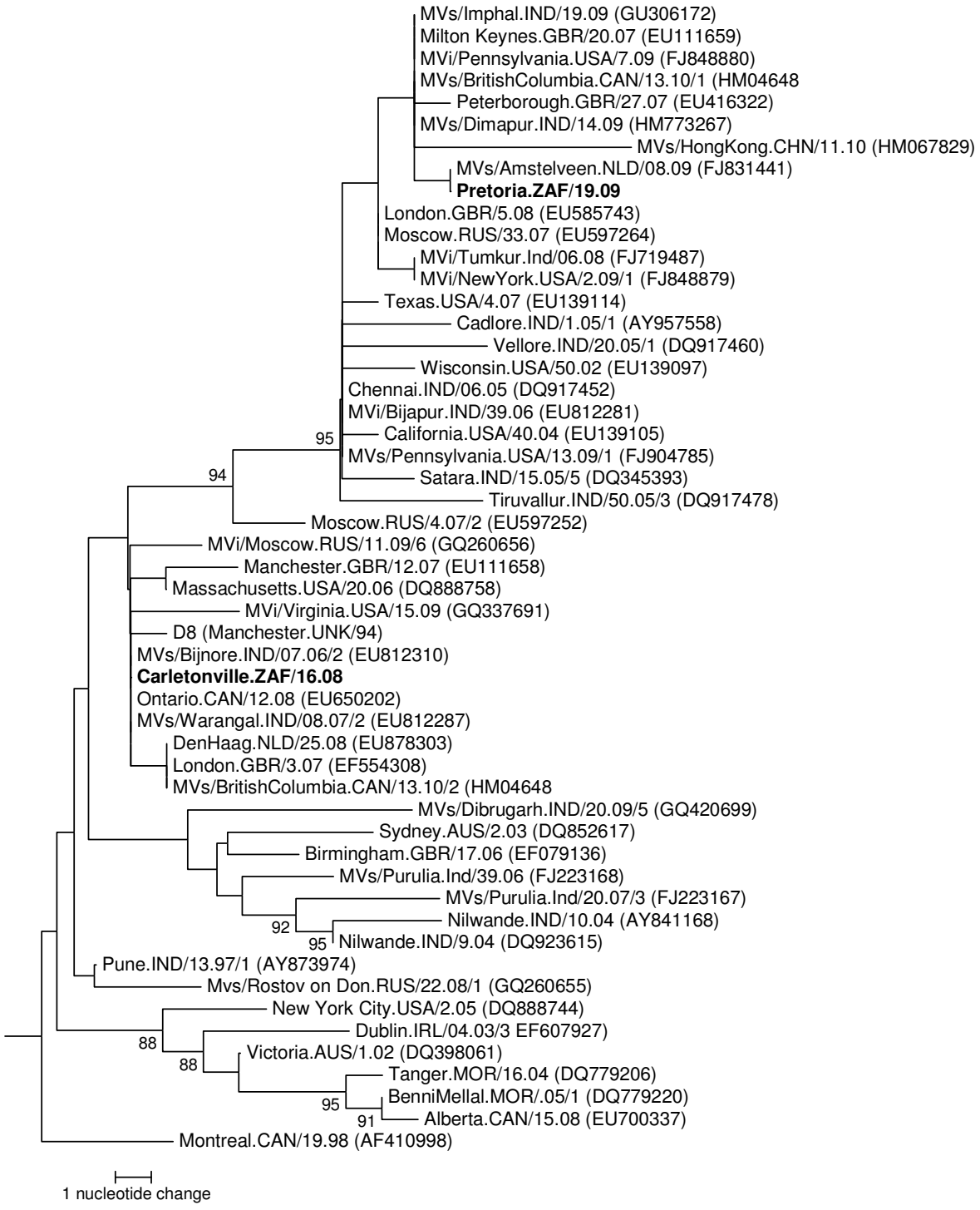

Figure 3.13 Phylogenetic analysis of selected N-450 sequences of genotype D8 MV. The unrooted neighbour-joining tree was generated by bootstrap analysis (1000 replicates) using MEGA 3.1 software. Only bootstrap frequencies greater than 70% are shown. Sequences from this study are shown in bold.

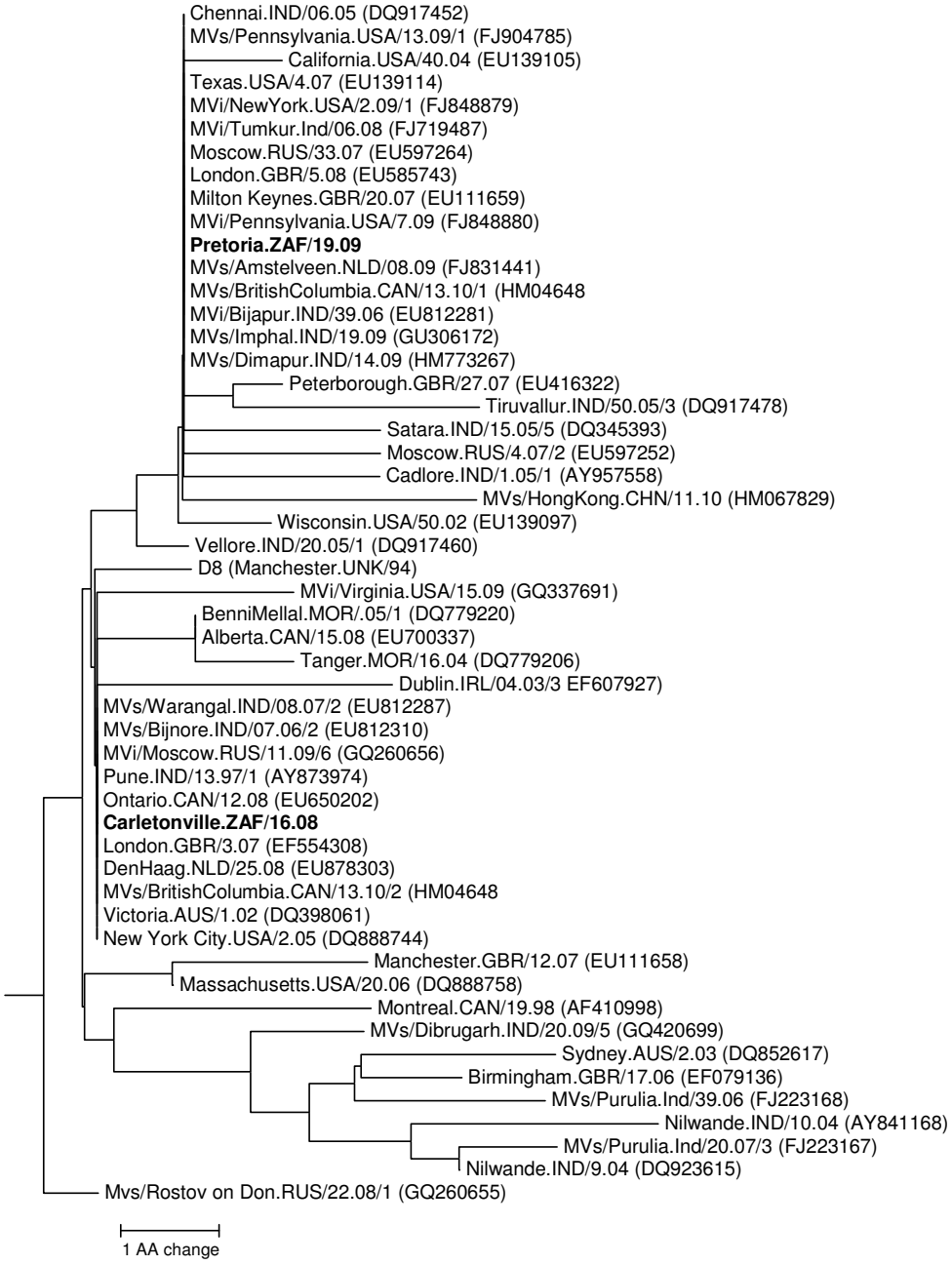

Figure 3.14 Phylogenetic analysis of selected predicted amino acid sequences of the 150 carboxyl-terminal residues of the N-gene of genotype D8 strains. Sequences from this study are shown in bold.

4. CONCLUDING REMARKS

The primary objective of this study was to provide molecular characterisation of measles virus strains in South Africa in order to determine whether or not interruption and elimination of endemic strains had been achieved. The development of a very sensitive hemi-nested PCR, with a detection limit of 100 cp/µl, was crucial to this achievement as the majority of available specimens were sera. Serum is not widely used for viral characterisation as the viral load is generally too low. Therefore, a hemi-nested PCR was developed for this study. This method has permitted amplification and subsequent sequencing of MV from a large proportion of serum specimens, and is a hundred-fold more sensitive than the RT-PCR method recently developed by the CDC and shared with the WHO measles LabNet. The recommendation of the WHO measles LabNet for molecular characterisation of MV is collection of a throat swab, oral fluid or urine at the same time as the blood sample (serum) collection.

The molecular data generated by this study clearly indicates that since 2002, all South African outbreaks occurred as a result of introduction of WT viruses, with either localised transmission (the 2002 genotype B2 outbreak, the genotype D4 outbreak in 2006) or widespread, sustained transmission (the genotype D2 outbreak 2003-2005) or sporadic cases (genotypes B2, D4, D8 in 2006, 2003 and 2010, 2008 and 2009, respectively). Since national rash-based surveillance has improved over the past decade, and the endemic strains (genotypes D2 and D4) have not been detected since the late 1990s, it must be inferred that these strains no longer circulate in South Africa. The epidemic outbreaks of genotypes D2 (2003-2005) and D4 (2006) were caused by strains unrelated to the endemic D2 and D4 genotypes. Thus this molecular epidemiology project demonstrated that although the measles control programme in South Africa has interrupted endemic transmission, the population immunity is not sufficiently high to prevent measles outbreaks. This should be addressed by improving routine EPI vaccine coverage nationally, paying particular attention to districts and sub-districts with poor coverage (the ‘Reach Every District’ RED approach advocated by the WHO) and ensuring that planned SIAs are not delayed since this permits the accumulation of sufficent susceptibles to allow sustained, prolonged transmission of virus.

With the caveat that surveillance gaps exist in Africa, the data generated by this study and the NICD-RRL, suggest that endemic circulation has been interrupted in

South Africa, Uganda, Kenya and the DRC. Interruption of endemic strain transmission in many other African countries may have occurred as a result of the implementation of measles control programmes, but this could not be demonstrated since baseline data did not exist. As genotype B1 was last detected in 1983 in Cameroon, and genotypes D2 and D10 in 2006 in Southern Africa and Uganda respectively, it is possible that these genotypes are no longer in circulation in Africa. On a global scale, there have been no reports of genotypes C1, D1, E, F and G1 since 1995, and genotypes D3, G2 and H2 since 2006 (WHO, 2012). As genotype B2 remained undetected for 18 years in Africa before demonstration of active circulation (Smit *et al*, 2005), it is probably judicious to refer to undetected genotypes as 'inactive' rather than 'eliminated'.

One of the outbreaks described in this study (genotype D2, 2003-2005), as well as two outbreaks not described here (genotype B3-1 2006-2007, 2009-2011; data from NICD-RRL), highlighted the problem that arises when strains with limited genetic diversity are circulating within a large geographical area. Phylogenetic analyses based on hundreds of very closely related N-450 sequences (identical or differing by only one or two nucleotides) obtained from South African and other African outbreak specimens, resulted in lineages forming vertical straight lines. This did not allow for determination of: links between outbreaks on the continent; origin of the outbreak strain; transmission pathways or re-introductions of closely related strains; or any temporal or geographic interpretation. Thus the genotype B3-1 N-450 data was not included in this dissertation, as it lacked epidemiological meaning. As the N-450 region is the most variable region of the MV genome, it was originally chosen as the sequencing window at a time when there was a large global diversity of circulating endemic strains. However, as a result of global measles vaccination programmes, many endemic strains and genotypes have been eliminated (WHO, 2012), thereby reducing diversity and thus N-450 alone is frequently not sufficiently variable to discriminate between sequences in large outbreaks. There is therefore a need to expand the length of the sequencing window to include other regions of the MV genome, to generate more phylogentically meaningful sites. This approach was recently used to discriminate between identical N-450 outbreak sequences of genotypes D4 in Europe, D6a in the Russian Federation and D6b in Germany and Belarus (Kessler *et al*, 2011). It involved sequencing of the entire P- and H-genes, and phylogenetic analyses of the separate and concatenated N-, P- and H- sequence data, which was able to resolve the data in terms of temporal and spatial variables

such as virus transmission patterns, multiple introductions, identification of chains of transmission and links between outbreaks.

Despite the short-comings of N-450 in differentiating the African B3-1 outbreaks, the data generated by this study and the NICD-RRL enabled the identification of Kenya (Ethiopian and Somalian refugees) as the source of virus in measles cases detected in the Netherlands, Mexico, United States of America and Canada during 2005 (Rota *et al*., 2006). Standard epidemiological investigations were unable to link the sporadic cases in these four countries with the origin of the virus. The identical strain was detected in Ethiopian and Ugandan specimens collected in 2006 (Lemma *et al*., 2008; Baliraine *et al*., 2011).

Similarly, sharing of genotype B2 data from this study, with the measles laboratory group in Luxembourg, contributed to a more comprehensive understanding of the circulating viruses in the DRC (Kremer *et al*., 2010).

Molecular surveillance of measles virus in Africa has been sub-optimal to date because the regional goal set in 2001 was measles control and only seven African countries had committed to achieving elimination (Botswana, Lesotho, Malawi, Namibia, South Africa, Swaziland, and Zimbabwe). However, in September 2011, the committee for the WHO African region set a 2020 measles elimination target, which will require routine molecular surveillance. Therefore future studies will benefit from more comprehensive specimen collection in order to meet the more stringent criteria of elimination. Although the data from this study is fragmented due to the limited specimen selection from the African region, this study has generated measles genotype data from at least thirty African countries, and more than 1300 sequences were submitted to global databases.

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6.0 APPENDICES

Appendix A.

Listing of WHO "EPID" Country codes, ISO3 Country codes and English name

EPID	ISO3	Name	EPID	ISO3	Name	EPID	ISO3	Name
AFG	AFG	Afghanistan	GRA	GRD	Grenada	PAK	PAK	Pakistan
ALB	ALB	Albania	GRE	GRC	Greece	PAN	PAN	Panama
ALG	DZA	Algeria	GUA	GLP	Guadeloupe	PAR	PRY	Paraguay
AMS	ASM	American Samoa	GUB	GNB	Guinea-Bissau	PER	PER	Peru
AND	AND	Andorra	GUI	GIN	Guinea	PHL	PHL	Philippines
ANG	AGO	Angola	GUM	UM	Guam	PNG	PNG	Papua New Guinea
ANI	ATG	Antigua & Barbuda	GUT	GTM	Guatemala	POL	POL	Poland
ANU	AIA	Anguilla	GUY	GUY	Guyana	POR	PRT	Portugal
ARG	ARG	Argentina	HAI	HTI	Haiti	PUR	PRI	Puerto Rico
ARM	ARM	Armenia	HON	HND	Honduras	QAT	QAT	Qatar
AUS	AUS	Australia	HUN	HUN	Hungary	REU	REU	Reunion
AUT	AUT	Austria	ICE	ISL	Iceland	ROM	ROU	Romania
AZE	AZE	Azerbaijan	IND	IND	India	RUS	RUS	Russian Federation
BAA	BHR	Bahrain	INO	IDN	Indonesia	RWA	RWA	Rwanda
BAH	BHS	Bahamas	IRA	IRN	Iran	SAA	SAU	Saudi Arabia
BAN	BGD	Bangladesh	IRE	IRL	Ireland	SAH	SHN	Saint Helena
BAR	BRB	Barbados	IRQ	IRQ	Iraq	SAL	LCA	Saint Lucia
BEL	BEL	Belgium	ISR	ISR	Israel	SAV	VCT	Saint Vincent& The Grenadines
BEN	BEN	Benin	ITA	ITA	Italy	SCN	KNA	Saint Kitts & Nevis
BER	BMU	Bermuda	IVC	CIV	Côte d'Ivoire	SEN	SEN	Senegal
BFA	BFA	Burkina Faso	JAM	JAM	Jamaica	SEY	SYC	Seychelles
BHU	BTN	Bhutan	JOR	JOR	Jordan	SIL	SLE	Sierra Leone
BIH	BIH	Bosnia&Herzegovina	JPN	JPN	Japan	SIN	SGP	Singapore
BLA	PLW	Palau	KAZ	KAZ	Kazakhstan	SMA	WSM	Samoa
BLR	BLR	Belarus	KEN	KEN	Kenya	SMR	SMR	San Marino
BLZ	BLZ	Belize	KGZ	KGZ	Kyrgyzstan	SOA	ZAF	South Africa
BOL	BOL	Bolivia	KIR	KIR	Kiribati	SOL	SLB	Solomon Islands
BOT	BWA	Botswana	KOR	KOR	Republic of Korea	SOM	SOM	Somalia
BRA	BRA	Brazil	KRD	PRK	DPRKorea	SPA	ESP	Spain
BRU	BRN	Brunei	KUW	KWT	Kuwait	SRL	LKA	Sri Lanka
BUL	BGR	Bulgaria	LAO	LAO	Laos	STP	STP	Sao Tome & Principe
BUU	BDI	Burundi	LEB	LBN	Lebanon	SUD	SDN	Sudan
CAE	CMR	Cameroon	LES	LSO	Lesotho	SUR	SUR	Suriname
CAF	CAF	CAR	LIB	LBR	Liberia	SVK	SVK	Slovakia
CAM	KHM	Cambodia	LIY	LBY	Libya	SVN	SVN	Slovenia
CAN	CAN	Canada	LTV	LTU	Lithuania	SWE	SWE	Sweden
CAV	CPV	Cape Verde	LUX	LUX	Luxembourg	SWI	CHE	Switzerland
CAY	CYM	Cayman Islands	LVA	LVA	Latvia	SWZ	SWZ	Swaziland
CHA	TCD	Chad	MAA	MYS	Malaysia	SYR	SYR	Syria
CHI	CHL	Chile	MAD	MDG	Madagascar	TAN	TZA	Tanzania
CHN	CHN	China	MAI	MLI	Mali	TCA	TCA	Turks&Caicos Islands
CNG	COG	Congo	MAL	MWI	Malawi	THA	THA	Thailand
COD	COD	DRCongo	MAR	MTQ	Martinique	TJK	TJK	Tajikistan
COK	COK	Cook Islands	MAS	MUS	Mauritius	TKM	TKM	Turkmenistan
COL	COL	Colombia	MAT	MLT	Malta	TMP	TMP	Timor-Leste
COM	COM	Comoros	MAU	MRT	Mauritania	TOG	TGO	Togo
COR	CRI	Costa Rica	MAV	MDV	Maldives	TOK	TKL	Tokelau
CRO	HRV	Croatia	MDA	MDA	Republic of Moldova	TON	TON	Tonga
CUB	CUB	Cuba	MEX	MEX	Mexico	TRT	TTO	Trinidad & Tobago
CYP	CYP	Cyprus	MIC	FSM	Micronesia	TUN	TUN	Tunisia
CZH	CZE	Czech Republic	MKD	MKD	Macedonia	TUR	TUR	Turkey
DEN	DNK	Denmark	MMR	MMR	Myanmar	TUV	TUV	Tuvalu
DEU	DEU	Germany	MOG	MNG	Mongolia	UAE	ARE	United Arab Emirates
DJI	DJI	Djibouti	MON	MCO	Monaco	UGA	UGA	Uganda
DOM	DMA	Dominica	MOR	MAR	Morocco	UKR	UKR	Ukraine
DOR	DOM	Dominican Republic	MOT	MSR	Montserrat	UNK	GBR	United Kingdom
ECU	ECU	Ecuador	MOZ	MOZ	Mozambique	UNR	UN	Relief&Works Authority For Palestine Refugees in the Near East (UNRWA)
EGY	EGY	Egypt	MSI	MHL	Marshall Islands	URU	URY	Uruguay
ELS	SLV	El Salvador	NAM	NAM	Namibia	USA	USA	United States of America
EQG	GNQ	Equatorial Guinea	NEA	ANT	NetherlandsAntilles	UZB	UZB	Uzbekistan
ERI	ERI	Eritrea	NEC	NCL	New Caledonia	VAN	VUT	Vanuatu
EST	EST	Estonia	NEP	NPL	Nepal	VEN	VEN	Venezuela
ETH	ETH	Ethiopia	NET	NLD	Netherlands	VIB	VGB	British Virgin Islands
FIJ	FJI	Fiji	NEZ	NZL	New Zealand	VTN	VNM	Viet Nam
FIN	FIN	Finland	NIC	NIC	Nicaragua	VUS	VIR	US Virgin Islands
FRA	FRA	France	NIE	NGA	Nigeria	WAF	WLF	Wallis & Futuna Islands
FRG	GUF	French Guiana	NIG	NER	Niger	WBG	PSE	West Bank and Gaza

EPID	ISO3	Name	EPID	ISO3	Name	EPID	ISO3	Name
FRP	PYF	French Polynesia	NIU	NIU	Niue	YEM	YEM	Yemen
GAB	GAB	Gabon	NMI	MNP	CN Mariana Islands	YUG	SCG	Serbia & Montenegro
GAM	GMB	Gambia	NOR	NOR	Norway	ZAM	ZMB	Zambia
GHA	GHA	Ghana	NRU	NRU	Nauru	ZIM	ZWE	Zimbabwe
GOR	GEO	Georgia	OMA	OMN	Oman			

Countries in red are those from which specimens were received for this study.

Appendix B.

Extraction of MV RNA from urine, virus isolates, throat swabs, oral fluid and serum

Switch the heating block on, set to 56 °C.
Use a variable-speed micofuge (Eppendorf 5415C)

Reagents/materials required:
QIAamp viral RNA mini kit (Qiagen), cat # 52904 containing buffers AVL, AW1, AW2 and AVE
Autoclaved aerosol resistant tips (ART) and microfuge tubes
Ethanol, 96%
TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA)

If opening a new kit:
i) Check that there is no precipitate in the AVL buffer (if there is, then heat the bottle at 56 °C for 5 min, swirling intermittently to get precipitate to dissolve).
ii) Once everything is in solution, add 1 ml of the AVL buffer to the tube containing carrier RNA, pipette up and down several times to ensure dissolution, then transfer the entire volume into the remainder of the AVL buffer. Rinse the carrier RNA tube with another small aliquot of AVL and transfer back into AVL bottle and mix thoroughly. Dispense 560 µl aliquots of this AVL+carrier RNA buffer into autoclaved 1.5 ml microfuge tubes, label a box with the date and store at 4 °C (stable for 6 months). These aliquots are referred to as "a tube of AVL" in the protocol below.
iii) Add 25 ml ethanol to wash buffer AW1, mix thoroughly.
iv) Add 30 ml ethanol to wash buffer AW2, mix thoroughly.
Before use, take required number of tubes of AVL out of fridge and warm at 56 °C for ≤5 min, shake intermittently. When the precipitate has dissolved, remove from heating block.
Include one negative extraction control for every 10-12 specimens.

Protocol:

1. Add 140 µl of specimen (or nuclease-free water for the extraction control) to a tube of AVL, mix, leave at RT for at least 10 min.
    - For specimens containing cells (urine, virus isolates, TS and OF: first transfer 1.5 ml specimen, or less if volume is limited, into a microfuge tube and centrifuge for 2 min at 2300 *g* to pellet cellular material. Aspirate 140 µl of the supernatant, discard remainder of supernatant (pour off), resuspend the pellet in the reserved 140 µl and transfer 140 µl into a tube of AVL.
    - For serum, transfer 140 µl directly into a tube of AVL.
2. Briefly centrifuge all tubes to remove drops from the inside of the lid.
3. Add 560 µl ethanol to each tube of lysed specimen, mix thoroughly, pulse-centrifuge to remove drops from lid.
4. Transfer 610 µl from each tube to a marked spin column. Use a fresh tip for each transfer and close the cap before touching next specimen.
5. Centrifuge at 5200 *g* for 1 min. Transfer the spin column to a clean collection tube and discard the used tube.
6. Transfer the remainder of the lysed specimen plus ethanol to the appropriately marked spin column.
7. Repeat step 5.
8. Add 500 µl of buffer AW1 to each spin column. Centrifuge at 5200 *g* for 1 min then transfer column to a fresh collection tube and discard used tube.
9. Add 500 µl of buffer AW2 and centrifuge at full speed for 3 min. Discard collection tube and transfer the column to a marked microfuge tube.
10. Add 60 µl of TE buffer to each column to elute the RNA. Use a fresh tip for each column. Incubate at RT for at least 1min. Centrifuge at 5200 *g* for 1 min.
11. Discard the column, and place the tube containing the RNA on ice if setting up the RT-PCR next, or at -70 °C for storage until required.

Appendix C.

Primer Information

Table C1 Details of the N-gene primers used in the project. Numbering is according to the Edmonston strain of MV (Genbank accession number U01987).

Primer	Direction: Purpose	Location	Primer Sequence (5' – 3')
N16	Forward: RT-PCR	959-988	TTCAGAACAAGTTCAGTGCAGGATCATACC
MV60	Forward: hemi-nested PCR; sequencing	1001-1024	GCTATGCCATGGGAGTAGGAGTGG
MV63	Reverse: RT-PCR; hemi-nested PCR; sequencing	1579-1599	CTGGCCCTCGGCCTCTCGCAC
MV61	Forward: sequencing	1177-1200	CTTGTTTCAGAGATTGCAATGCAT
MeV 216	Forward: RT-PCR (CDC); sequencing	997-1016	TGGAGCTATGCCATGGGAGT
MeV 214	Reverse: RT-PCR (CDC); sequencing	1611-1630	TAACAATGATGGAAGGTAGG

```
   1 atggccacac ttttaaggag cttagcattg ttcaaaagaa acaaggacaa accacccatt
  61 acatcaggat ccggtggagc catcagagga atcaaacaca ttattatagt accaatccct
 121 ggagattcct caattaccac tcgatccaga cttctggacc ggttggtcag gttaattgga
 181 aacccggatg tgagcgggcc caaactaaca ggggcactaa taggtatatt atccttattt
 241 gtggagtctc caggtcaatt gattcagagg atcaccgatg accctgacgt tagcataagg
 301 ctgttagagg ttgtccagag tgaccagtca caatctggcc ttaccttcgc atcaagaggt
 361 accaacatgg aggatgaggc ggaccaatac ttttcacatg atgatccaat tagtagtgat
 421 caatccaggt tcggatggtt cgagaacaag gaaatctcag atattgaagt gcaagaccct
 481 gagggattca acatgattct gggtaccatc ctagcccaaa tttgggtctt gctcgcaaag
 541 gcggttacgg ccccagacac ggcagctgat tcggagctaa gaaggtggat aaagtacacc
 601 caacaaagaa gggtagttgg tgaatttaga ttggagagaa aatggttgga tgtggtgagg
 661 aacaggattg ccgaggacct ctccttacgc cgattcatgg tcgctctaat cctggatatc
 721 aagagaacac ccggaaacaa acccaggatt gctgaaatga tatgtgacat tgatacatat
 781 atcgtagagg caggattagc cagttttatc ctgactatta agtttgggat agaaactatg
 841 tatcctgctc ttggactgca tgaatttgct ggtgagttat ccacacttga gtccttgatg
 901 aacctttacc agcaaatggg ggaaactgca ccctacatgg taatcctgga gaactcaatt
 961 cagaacaagt tcagtgcagg atcatacct ctgctctgga gctatgccat gggagtagga
1021 gtggaacttg aaaactccat gggaggtttg aactttggcc gatcttactt tgatccagca
1081 tattttagat tagggcaaga gatggtaagg aggtcagctg gaaaggtcag ttccacattg
1141 gcatctgaac tcggtatcac tgccgaggat gcaaggcttg tttcagagat tgcaatgcat
1201 actactgagg acaagatcag tagagcggtt ggacccagac aagcccaagt atcatttcta
1261 cacggtgatc aaagtgagaa tgagctaccg agattggggg gcaaggaaga taggagggtc
1321 aaacagagtc gaggagaagc cagggagagc tacagagaaa ccgggcccag cagagcaagt
1381 gatgcgagag ctgcccatct tccaaccggc acacccctag acattgacac tgcatcggag
1441 tccagccaag atccgcagga cagtcgaagg tcagctgacg ccctgcttag gctgcaagcc
1501 atggcaggaa tctcggaaga acaaggctca gacacggaca cccctatagt gtacaatgac
1561 agaaatcttc tagactaggt gcgagaggcc gagggccaga acaacatccg cctaccctcc
1621 atcattgtta taaaaaactt aggaaccagg tccacacagc cgccagccaa ccaaccatcc
```

Figure C1 Measles virus nucleoprotein gene sequence and primer map.

Table C2 Measles virus H-gene sequence and primer map. Numbering is according to the Edmonston strain of MV (Genbank accession number U03669).

Primer	Direction: Purpose	Location	Primer Sequence (5′ – 3′)
MVH-IF	Forward: RT-PCR; hemi-nested PCR fragment 1; sequencing fragment 1	-57 to -38	CCTCTGGCCGAACAATATCG
MVH-IR	Reverse: RT-PCR; hemi-nested PCR fragment 2; sequencing fragment 2	1960-1979	CAGATAGCGAGTCCATAACG
H7	Reverse: hemi-nested PCR fragment 1; sequencing fragment 1	996-1023	CGTTGATAAGGGGACCCAGGATTGCATG
H4	Forward: hemi-nested PCR fragment 2; sequencing fragment 2	753-773	GTACCGAGTGTTTGAAGTAGG
MVH-AR	Reverse: sequencing fragment 1	387-407	GATCTCTGAAGTCGTACTCC
H3	Forward: sequencing fragment 1	400-423	AGAGATCTCACTTGGTGTATCAAC
H5	Forward: sequencing fragment 2	1145-1166	TCCAACAGGCGTGTAAGGGTAA
MVH-ER	Reverse: sequencing fragment 2	1297-1316	GAACCGTGTGTGATCAATGG

Figure C2 Measles virus haemagglutinin gene sequence and primer map.

Appendix D.

RT-PCR for measles virus N-gene RNA:

Manipulations are to be performed in the pre-PCR (clean) room.
Work on ice.

Requirements:
Titan one tube RT-PCR system (Roche, cat # 1855476)
Forward primer N16 (TTC AGA ACA AGT TCA GTG CAG GAT CAT ACC), 20 pmol/µl
Reverse primer MV63 (CTG GCC CTC GGC CTC TCG CAC), 20 pmol/µl
RNase inhibitor, 40 U/µl (Roche)
Nuclease-free water

Pulse spin the reagents to get drops off the lid.
Set up a master mix using the volumes of reagents listed below (volumes stated are for 1 reaction), allow 0.5-1 reaction volume extra (e.g. if need 3 reactions, make up volume for 3.5 or 4 reactions).

Nuclease-free water	22.25 µl
5x Titan buffer	10 µl
100 mM DTT (dithiothreitol)	1 µl
25 mM $MgCl_2$ (Magnesium chloride)	1.5 µl
10 mM mix of dNTPs	1 µl
Titan enzyme mix	1 µl
RNase inhibitor, 40 U/ µl	0.25 µl
Measles primers: MV16 and MV63	0.75 µl each

Mix properly by inversion, pulse spin to get drops off the lid.
Aliquot 40 µl of master mix into 0.2 ml tubes labeled with specimen details.

Pack reagents away, and move to the template-addition area.
Add 10 µl of extracted RNA to correctly labeled tube (use nuclease-free water for the negative control).
For the positive control, add 1.5 µl of control RNA (make up volume difference with nuclease-free water).

Program on Applied Biosystems 2400 cyclers: MV-N
Program on Merck Eppendorf cycler: MV

Measles RT-PCR cycling conditions

```
50˚C 30 min
94˚C 2 min
94˚C 15 sec  ┐
50˚C 30 sec  ┘x30
70˚C 30 sec
72˚C 7 min
4˚C hold
```

The expected amplicon size is 641 bp.

Appendix E.

Hemi-nested PCR for measles N-gene:

Manipulations are to be performed in the pre-PCR (clean) room.
Work on ice.

Requirements:
Expand High Fidelity PCR system (Roche, cat # 1732650)
Forward primer MV60 (GCT ATG CCA TGG GAG TAG GAG TGG), 20 pmol/µl
Reverse primer MV63 (CTG GCC CTC GGC CTC TCG CAC), 20 pmol/µl

Set up a master mix using the volumes of reagents listed below (volumes stated are for 1 reaction), allow 0.5 or 1 reaction volume extra (e.g. if need 3 reactions, make up volume for 3.5 or 4 reactions). Briefly centrifuge all reagents to get any drops off the lids.

Nuclease-free water	41.25 µl
10x Expand buffer	5 µl
10mM mix of dNTPs	1 µl
Expand enzyme	0.25 µl
measles primers: MV60 and MV63	0.75 µl each

Mix properly by inversion, pulse spin to get drops off the lid.
Aliquot 48 µl of master mix into each tube.

Pack reagents away, and move to the area reserved for nested PCR.

Briefly centrifuge the tubes containing the RT-PCR product.
Transfer 2 µl of each RT-PCR product to the appropriately marked tube containing the prepared master mix.
For the positive control, add 50 µl nuclease-free water to the RT-PCR product, mix and transfer 2 µl to the appropriately marked tube containing the prepared master mix.
Briefly centrifuge the tubes and place into thermocycler.

Program on Applied Biosystems 2400 cyclers: nested-N
Program on Merck Eppendorf cycler: MVNnest

Measles hemi-nested PCR cycling conditions

```
94˚C 2 min
94˚C 30 sec ┐
50˚C 30 sec ├x35
72˚C 30 sec ┘
72˚C 7 min
4˚C hold
```

The expected amplicon size is 599 bp.

Appendix F.

RT-PCR for measles virus H-gene RNA:

Manipulations are to be performed in the pre-PCR (clean) room.
Work on ice.

Requirements:
Titan one tube RT-PCR system (Roche, cat # 1855476)
Forward primer MVH-IF 20 pmol/µl
Reverse primer MVH-IR 20 pmol/µl
RNase inhibitor, 40 U/µl (Roche)
Nuclease-free water

Pulse spin the reagents to get drops off the lid.
Set up a master mix using the volumes of reagents listed below (volumes stated are for 1 reaction), allow 0.5-1 reaction volume extra (e.g. if need 3 reactions, make up volume for 3.5 or 4 reactions).

Nuclease-free water	22.25 µl
5x Titan buffer	10 µl
100 mM DTT	1 µl
25 mM $MgCl_2$	1.5 µl
10 mM mix of dNTPs	1 µl
Titan enzyme mix	1 µl
RNase inhibitor, 40 U/ µl	0.25 µl
Measles primers: MVH-IF and MVH-IR	0.75 µl each

Mix properly by inversion, pulse spin to get drops off the lid.
Aliquot 40 µl of master mix into 0.2 ml tubes labeled with specimen details.

Pack reagents away, and move to the template-addition area.
Add 10 µl of extracted RNA to correctly labeled tube (use nuclease-free water for the negative control).
For the positive control, add 1.5 µl of control RNA (make up volume difference with nuclease-free water).

Program on Applied Biosystems 2400 cyclers: MV-H
Program on Merck Eppendorf cycler: MVH

Measles RT-PCR H-gene cycling conditions

```
47˚C 45 min
94˚C 2 min
94˚C 30 sec  ┐
47˚C 30 sec  ├x5
72˚C  2 min  ┘
94˚C 30 sec  ┐
50˚C 30 sec  ├x30
72˚C  2 min  ┘
72˚C  7 min
4˚C hold
```

The expected amplicon size is 2036 bp.

Appendix G.

Hemi-nested PCR for measles H-gene:

Manipulations are to be performed in the pre-PCR (clean) room.
Work on ice.

Requirements:
Expand High Fidelity PCR system (Roche, cat # 1732650)
Fragment 1 primers: MVH-IFand H7, 20 pmol/µl each
Fragment 2 primers: H4 and MVH-IR, 20 pmol/µl each

Set up a master mix using the volumes of reagents listed below (volumes stated are for 1 reaction), allow 0.5 or 1 reaction volume extra (e.g. if need 3 reactions, make up volume for 3.5 or 4 reactions). Briefly centrifuge all reagents to get any drops off the lids.

Nuclease-free water	41.25 µl
10x Expand buffer	5 µl
10mM mix of dNTPs	1 µl
Expand enzyme	0.25 µl
measles primers: fragment 1 or fragment 2	0.75 µl each

Mix properly by inversion, pulse spin to get drops off the lid.
Aliquot 48 µl of master mix into each tube.

Pack reagents away, and move to the area reserved for nested PCR.

Briefly centrifuge the tubes containing the RT-PCR product.
Transfer 2 µl of each RT-PCR product to the appropriately marked tube containing the prepared master mix.
For the positive control, add 50 µl nuclease-free water to the RT-PCR product, mix and transfer 2 µl to the appropriately marked tube containing the prepared master mix.
Briefly centrifuge the tubes and place into thermocycler.

Program on Applied Biosystems 2400 cyclers: nested-H
Program on Merck Eppendorf cycler: MVHnest

Measles hemi-nested PCR cycling conditions

```
94˚C 2 min
94˚C 30 sec ┐
50˚C 30 sec ├x30
72˚C 80 sec ┘
72˚C 7 min
4˚C hold
```

The expected amplicon sizes are 1080 bp for fragment 1 and 1227 bp for fragment 2.

Appendix H.

Agarose gel electrophoresis

Requirements:
Agarose LE (Merck)
Ethidium Bromide 10 mg/ml (GibcoBRL)
10x TBE buffer (Sigma)
Range of small-to-large gel apparatus and combs
10x loading buffer
Parafilm
100 bp ladder as the molecular weight marker (Roche and BioLine)

Recipe 10x loading buffer:

Glycerol	5 ml
10x TBE	0.5 ml
10% (w/v) suspension bromophenol blue	100 µl

Make up to 10 ml with water, aliquot, store at -20 °C

Procedure:
Using 10x TBE stock solution, prepare a 0.5x TBE working solution using distilled water as diluent.
To prepare 100 ml of 1% (w/v) agarose gel, weigh out 1g agarose, pour into a 250 ml Schott bottle, add 100 ml 0.5x TBE, put Schott bottle lid on loosely and microwave gently until the agarose dissolves, swirling intermittently. When bottle is cool enough to handle, add 2 µl of Ethidium Bromide 10 mg/ml (final concentration 0.2 µg/ml) and swirl gently.
Prepare gel casting apparatus or tape up ends of gel tray, place comb(s) in position.
Pour sufficient agarose solution into the tray to a thickness of 3-4 mm, check that the comb hasn't moved, wait 15 min for the gel to set.
Gently remove the comb(s) and transfer the gel with the tray to the electrophoresis tank and pour in enough 0.5x TBE to just cover the gel.
Load an appropriate amount of the molecular weight marker (mixed with loading buffer) into the first well.
Place a series of 1-2 µl drops of 10x loading buffer onto a piece of parafilm (as many drops as there are samples).
Then work systematically through the samples: add 4 µl PCR product to a drop of the loading buffer, mix by pipetting up and down, and transfer into the next empty well of the gel. Repeat for all the samples.
Place the tank cover over the apparatus and connect to the power supply: the samples must run from black to red (anode to cathode/minus to plus).
Run the gel at 100V until the bromophenol blue marker has moved about three-quarters of the length of the gel.
View bands with a UV-transilluminator and photograph the gel.
Label the lanes with sample ID
Check that the negative extraction control and the reagent control do not have bands of the expected size of measles amplicons. If there are bands present, discard the run and repeat the RT-PCR/hemi-nested PCR. If the negative controls are still positive, repeat the RNA extraction and PCRs.
Compare the size of the bands for each specimen with the positive control and the molecular weight marker.
If specimens are PCR-positive, purify the amplicons from the remaining reaction in preparation for sequencing (Appendix I).

Appendix I.

Purification of PCR products in preparation for sequencing

Requirements:
Heating block set at 56 °C
Ethanol 96%
Autoclaved 1.5 ml microfuge tubes
UV-transilluminator
Scalpel blades
Wizard SV Gel and PCR Clean-Up System (Promega, USA) Cat # A9282

If a new kit is being used, add the required amount of 96% ethanol to the membrane wash solution (375 ml for the 250 prep kit). Mark and date the bottle.

Direct clean-up of PCR reactions (if there were no non-specific bands present)
1. Add an equal volume of membrane binding solution to the PCR reaction, mix.
2. Insert SV minicolumn into a collection tube.
3. Transfer the prepared PCR product to the assembled minicolumn; incubate at room temperature for 1 minute.
4. Centrifuge at 16 000 *g* for 1 minute, discard the flowthrough. Reinsert the minicolumn into the collection tube
5. Add 700 µl membrane wash solution (containing ethanol). Repeat step 4.
6. Add 500 µl membrane wash solution (containing ethanol). Centrifuge at 16 000 *g* for 5 minutes, discard the flowthrough.
7. Transfer the column to a labeled 1.5 ml microfuge tube, add 50 µl TE buffer to the minicolumn, incubate at RT for 1 minute, centrifuge at 16 000 *g* for 1 minute, discard the minicolumn and store eluted DNA at 4 °C or -20 °C.

Clean-up of PCR products from gel (If multiple bands were present in PCR reaction)
1. Load the entire reaction mix (mixed with $1/10^{th}$ volume loading buffer) onto 1% agarose gel with large wells to accommodate the required volume, electrophese at 100V for the desired length of time.
2. Under long-UV transillumination, excise the correctly-sized bands from the gel using scalpel blades and place into marked microfuge tubes. Weigh the tube (tare against an empty tube).
3. Add 10 µl membrane binding solution per 10 mg of gel slice, place in a heating block set at 56 °C and vortex occasionally until the agarose is dissolved (< 5 min).
4. Recover the amplicons using the Wizard SV Gel and PCR Clean-Up System – proceed according to steps 2-7 above.

Appendix J.

Sequencing of measles PCR-positive specimens

PCR-products must have been purified to remove excess primers and non-specific products before embarking on sequencing procedure.
Determine the concentration of DNA in the purified PCR-products (use a Nanodrop spectrophotometer if available, or by estimation after gel electrophoresis of an aliquot of the purified PCR-products).
Dilute an aliquot of the purified PCR-products to 3 ng/µl just before setting up the sequencing reactions (prepare 50 µl, using nuclease-free water as diluent).

Requirements:
BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems)
Sequencing primers for N-450: stock of 100 pmol/µl or 20 pmol/µl

MV60	5'-GCT ATG CCA TGG GAG TAG GAG TGG-3'
MV63	5'-CTG GCC CTC GGC CTC TCG CAC-3'
MV61	5'-CTT GTT TCA GAG ATT GCA ATG CAT-3'

Sequencing primers for H-gene: stock of 100 pmol/µl or 20 pmol/µl:
Fragment 1 (generated with MVH-IF and H7)

MVH-IF	5'-CCTCTGGCCGAACAATATCG-3'
H7	5'-CGTTGATAAGGGGACCCAGGATTGCATG-3'
MVH-AR	5'-GATCTCTGAAGTCGTACTCC-3'
H3	5'-AGAGATCTCACTTGGTGTATCAAC-3'

Fragment 2 (generated with H4 and MVH-IR)

H4	5'-GTACCGAGTGTTTGAAGTAGG-3'
MVH-IR	5'-CAGATAGCGAGTCCATAACG-3'
H5	5'-TCCAACAGGCGTGTAAGGGTAA-3'
MVH-ER	5'-GAACCGTGTGTGACTAATGG-3'

When opening a new kit, first thaw the reaction mix (pink solution, which contains the enzyme, dNTPs and ddNTPs) and aliquot into 0.2ml tubes: 50 µl per tube. Store at -20 ℃. Aliquot the 5x reaction buffer and store at -20 ℃.

Method:
1. Dilute the sequencing primers to 10 pmol/ µl using nuclease-free water.
2. Prepare 50 µl of the PCR products to be sequenced, diluted to 3 ng/ µl with nuclease-free water.
3. Label 3 x 0.2 ml tubes for each specimen to be sequenced; one for each primer (or use a 96-well PCR plate if there are a lot of specimens to be sequenced).
4. Add 5 µl of the diluted PCR product (i.e. 15 ng) to the correctly-labeled tube/well of plate.
5. Prepare 3 master mixes of the following components (one for each primer): volumes are given for 1 reaction, make up mixes for required number of reactions plus 0.5 x reaction volumes extra

5x sequencing buffer	2 µl
primer (10 pmol/µl)	1 µl
Reaction mix	2 µl

6. Dispense 5 µl aliquots into the labeled tubes/wells containing the template to be sequenced (final volume is 10 µl).
7. Briefly centrifuge the tubes/plate and place into thermal cycler.

Measles N-gene cycle-sequencing program:

```
96˚C 10 sec
96˚C 10 sec  ┐
50˚C  6 sec  ┘x30
60˚C 2.5 min
4˚C hold
```

Measles H-gene cycle-sequencing program:

```
96˚C 10 sec
96˚C 10 sec ┐
50˚C  6 sec ┘x30
60˚C  4 min
4˚C hold
```

Program on Applied Biosystems 2400 cyclers: abi-seq
Program on Merck Eppendorf cycler: abi-seq

To remove unincorporated fluorescent ddNTPs:
If the sequencing reactions were performed in tubes, spin briefly and transfer the contents to a 96-well plate (keep a record of the loading order). If a 96-well plate was used, spin briefly. Prepare sufficient volume of BDX purification solution (BigDye® Xterminator Purification Kit, Applied Biosystems), as follows:

1. For one 10 µl sequencing reaction, combine 10 µl Xterminator solution (BDX beads) with 45 µl SAM™ solution. As the ABI 3130XL genetic analyzer has a 16 capillary array, the minimum volume prepared must be 880 µl (16 x 55 µl), but allow for at least 6 extra aliquots for pipetting variation due to the surfactant properties of SAM™ solution. As the beads settle quickly, ensure that the combined Xterminator-SAM solution is mixed immediately before transferring each 55 µl aliquot into each well (or each row, if using a multichannel pipette).
2. Seal the plate with strip-lids.
3. Place on a digital plate vortexer, secure the plate, and vortex for 30 minutes at 1800 rpm.
4. Transfer the plate to a plate centrifuge and spin for 2 min at 1000 *g*.
5. Carefully remove strip-lids and cover the plate with a sequencing plate septum.
6. If the bead pellet was disturbed during the previous step, re-centrifuge the plate.
7. Load the plate onto the ABI 3130XL genetic analyzer according to manufacturer's manual.

Appendix K.

List of WHO measles virus reference strains (WHO, 2012)

Genotype	Last Observed*	Reference Strain	GenBank H	Genbank N
A	2008	MVi/Edmonston-wt.USA/54	U03669	U01987
B1[a]	1983	MVi/Yaounde.CAE/12.83	AF079552	U01998
B2	2010	MVi/Libreville.GAB/84	L46753	U01994
B3	2011	MVi/New York.USA/94	L46752	L46753
		MVi/ Ibadan.NIE/97/1	AJ239133	AJ232203
C1[a]	1992	MVi/Tokyo.JPN/84/K	AY047365	AY043459
C2	2007	MVi/Maryland.USA/77	M81898	M89921
		MVi/Erlangen.DEU/90	Z80808	X84872
D1[a]	1986	MVi/Bristol.UNK	Z80805	D01005
D2	2005	MVi/Johannesburg.SOA/88/1	AF085498	U64582
D3	2004	MVi/Illinois.USA/89/1	M81895	U01977
D4	2011	MVi/Montreal.CAN/89	AF079554	U01976
D5	2010	MVi/Palau.BLA/93	L46757	L46758
		MVi/Bangkok.THA/93/1	AF009575	AF07955
D6	2007	MVi/New Jersey.USA/94/1	L46749	L46750
D7	2007	MVi/Victoria.AUS/16.85	AF247202	AF243450
		MVi/Illinois.USA/50.99	AY043461	AY037020
D8	2011	MVi/Manchester.UNK/30.94	U29285	AF280803
D9	2011	MVi/Victoria.AUS/12.99	AY127853	AF481485
D10	2005	MVi/Kampala.UGA/51.01/1	AY923213	Ay923185
D11	2011	MVi/Menglian.Yunnan.CHN/47.09	GU440576	GU440571
E[a]	1987	MVi/Goettingen.DEU/71	Z80797	X84879
F[a]	1994	MVs/Madrid.SPA/94 [SSPE]	Z80830	X84865
G1[a]	1983	MVi/Berkeley.USA/83	AF079553	U01974
G2	2004	MVi/Amsterdam.NET/49.97	AF171231	AF171232
G3	2011	MVi/Gresik.INO/17.02	AY184218	AY184217
H1	2011	MVi/Hunan/CHN/93/7	AF045201	AF045212
H2	2003	MVi/Beijing.CHN/94/1	AF045203	AF045217

Appendix L.

Alignment of the predicted 150 amino acids at the carboxyl-terminus of the nucleoprotein of the MV reference strains

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A                    KVSSTLASEL GITAEDARLV SEIAMHTTED KISRAVGPRQ AQVSFLHGDQ  [ 50]
B1                   .......... .......... .......A.. R......... .........  [ 50]
B2                   .......... .......... .......... R......... .........  [ 50]
B3-1                 .......... .......... .......... R......... .........  [ 50]
B3-2                 .......... .......... .......... R......... .........  [ 50]
C1                   .......... .......... .......... R......... .........  [ 50]
C2a                  .......... .......... .......... R......... S........  [ 50]
C2b                  .......... .......... .......... R......... S........  [ 50]
D1                   ........G. .......... .......... R......... .........  [ 50]
D2                   .......... .......... .......... R......... .........  [ 50]
D3                   .......... .......... .......... R......... .........  [ 50]
D4                   .......... .......... .......... R......... .....I....  [ 50]
D5a                  .......... .......... .......... R......... .........  [ 50]
D5b                  .......... .......... .......... R......... .........  [ 50]
D6                   .......... .......... .......... R......... .........  [ 50]
D7a                  .......... .......... .......... RT........ .........  [ 50]
D7b                  .......... .......... .......... RT........ .........  [ 50]
D8                   .......... .......... .......... RT........ .........  [ 50]
D9                   .......... .......... .......... R......... .........  [ 50]
D10                  .......... .......... .......... R......... .........  [ 50]
D11                  .......... .......... .......... RT........ .........  [ 50]
G1                   .......... .......... .......... R......... .........  [ 50]
G2                   .......... .......... .......... R......... .........  [ 50]
G3                   .......... .......... .......... RT........ .........  [ 50]
H1                   .......... .......... .......... RT........ .........  [ 50]
H2                   .......... .......... .......... R......... .........  [ 50]
E                    .......... .......... .......... R......... .........  [ 50]
F                    R......... .......... .......... R......... .........  [ 50]


A                    SENELPRLGG KEDRRVKQSR GEARESYRET GPSRASDARA AHLPTGTPLD  [100]
B1                   .......... .......... ...G...... .......... ..P.......  [100]
B2                   .......... .......... ...G...... .......... T.........  [100]
B3-1                 .......... .......... ...G..H... .......... ..P.......  [100]
B3-2                 .......... ........G. ...G...... .......... ..P.......  [100]
C1                   .......W.. ...M...... .......... .......... .....D....  [100]
C2a                  N......W.. ...M...... .......... R......... T.P..D....  [100]
C2b                  N.S....W.. ...M...... .......... .......... ..P..D....  [100]
D1                   ......G... .......... ........D. .S........ .....S....  [100]
D2                   ......G... .......... .......... .S..T..... .....S....  [100]
D3                   ......G... .......... .......... .S........ ..P..SM...  [100]
D4                   ......G... .......... .......... .S........ .....S....  [100]
D5a                  ......G... .......... .......... .S........ .........  [100]
D5b                  ......G... .......... .......... .S.....E.. .....S....  [100]
D6                   ......G... .....A.... ....D..... .S........ .....S....  [100]
D7a                  ......G... ........G. .......... RS........ .....S..Q.  [100]
D7b                  .GS...G... R.....G... .......... .S........ .....S....  [100]
D8                   ......G... ......R... ......N... .S..L..... .....S....  [100]
D9                   ......G... .......... .........A .S...G.E.. .....S....  [100]
D10                  ......G..S .......... ...M..H... .SG.T..... V....S....  [100]
D11                  ......G... .....A.... ..S.....D. .S.....V.. .....N....  [100]
G1                   ......G... .......... ..T....... .....G.... .......L..  [100]
G2                   ......G... ...K...... .......... .H...N.... .D.......  [100]
G3                   ......G... ...KK..... .......... .....N.... .........  [100]
H1                   ......G..S .......... ..T..NS... ....S..... .....S..P.  [100]
H2                   ......G..V .......... ......P... ..N.T..V.V .....S..P.  [100]
E                    .......W.. .....A.... .....I.... .......... .........  [100]
F                    .......... .....I...Q .......... .......... .........  [100]
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A                   IDTASESSQD PQDSRRSADA LLRLQAMAGI SEEQGSDTDT PIVYNDRNLL  [150]
B1                  ......F... .......... .......... .......... .R.....D..  [150]
B2                  ...V....L. .......... .......... .......... .R.....D..  [150]
B3-1                ......F... .......... .......... ....D..... .R.....D..  [150]
B3-2                ......F... .......... .......... .......... .R...G.D..  [150]
C1                  ......F... ........E. .......... .......... .R.....D..  [150]
C2a                 .......... .......... .......... .......... .R.....D..  [150]
C2b                 .......... .......R.. .......... .......... .R.....D..  [150]
D1                  .......... .......... .......... .......... .R.....D..  [150]
D2                  .......... .......... .......... .......... .R.....D..  [150]
D3                  .......G.. .......... .......... L......... .R.....D..  [150]
D4                  .......G.. .......... .......... L.........I .R....KD..  [150]
D5a                 .......G.. .......... .......... L......... .R.....D..  [150]
D5b                 .......G.. .......... .......... L......... .R....SD..  [150]
D6                  ......T... L......... .......... .......... .R.....D.P  [150]
D7a                 .......G.. .......... .......... L......... .R.....D..  [150]
D7b                 .......G.. L......... .......... L......... .R.....D..  [150]
D8                  .......G.. .......... .......... L......... .R........  [150]
D9                  .......G.. .......... .......... L......... .R.....D..  [150]
D10                 ........R. ........E. .......... .......... .R.....D..  [150]
D11                 .......G.. .......... .......... L......... .R.....D..  [150]
G1                  .......... .......... .......... .......M.. LR.....D..  [150]
G2                  ......F... .......... .......... P......M.. .R.....D..  [150]
G3                  ......F... .......... .......... .......M.. .R.....D..  [150]
H1                  ......Y... .......... .......... L......... .R.....D..  [150]
H2                  ......Y... .......... .......... L...S..... .R.....D..  [150]
E                   .......... Q...Q..... .......... .......... .R.....D..  [150]
F                   .......... .L......E. .......... .......... .R.....D..  [150]
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