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Molecular epidemiology and antigenic characteristics of measles virus in South Africa

Stephanie Kreis

A thesis submitted to the Faculty of Health Sciences
University of the Witwatersrand, Johannesburg
for the degree of Doctor of Philosophy

Johannesburg 1998

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Stephanie Kreis

on this 12 day of may, 1998.

PUBLICATIONS

1. Kreis S., Whistler, T. (1997). Rapid identification of measles virus strains by the heteroduplex mobility assay. Virus Research 47: 197-203.

2. Kreis, S., Vardas, E., Whistler, T. (1997). Sequence analysis of the nucleocapsid gene of measles virus isolates from South Africa identifies a new genotype. J Gen Virol. 78: 1581-1587.

3. Vardas, E., Andre, M., Kreis, S., Zachariades, N., Schoub, B. (1997). Measles virus transmission from infected to immune individuals- implications for measles control and elimination. SAMJ 87: 1709.

4. Kreis, S., Schoub, B.D. (1998). Amplification of the carboxyl terminus of the measles virus nucleocapsid gene from stored sera and cerebrospinal fluids. J Med. Virol. In Press.

5. Kreis, S., Rota, J.S., Rota, P.A. (1998). Differentiation of measles virus isolates from eight different genotypes by the heteroduplex mobility assay. (In preparation).

PRESENTATIONS

1. Kreis, S., Vardas, E., Whistler, T. Sequence analysis of measles virus isolates from South Africa. African Congress for Virology. July 1995, Johannesburg, South Africa.

2. Kreis, S., Whistler, T. Strain analysis of recent measles virus isolates from South Africa comparing heteroduplex mobility assay and sequencing data. 15th Annual Meeting of the American Society for Virology, July 1996, Ontario, Canada.

3. Kreis, S. Measles virus isolation for molecular epidemiological studies. Measles Elimination in Southern Africa Meeting, Dept of Health and WHO. December 1997, Pretoria, South Africa.

4. Kreis, S., Newton, B., Rota, J.S., Bellini, W.J., and Rota, P.A. Heteroduplex mapping of measles virus as a genotyping tool. Atlanta, 1998.

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1. INTRODUCTION AND LITERATURE REVIEW

Measles virus (MV) is a member of the genus Morbillivirus within the family Paramyxoviridae of RNA viruses. Other members of the same genus include canine distemper virus (CDV), rinderpest virus (RV) in cattle, peste des petits ruminants virus (PPRV), phocine distemper virus (PDV) in seals, and porpoise and dolphin morbilliviruses. Recently, another morbillivirus was isolated from an outbreak of fatal respiratory disease in horses and humans in Australia (Murray,K. et al., 1995). Measles virus and other morbilliviruses lack neuramidase activity but cause the formation of intranuclear inclusion bodies, which distinguishes them from other paramyxoviruses (Griffin, D.E. and Bellini, W.J., 1996).
Measles virus is a highly contagious and important human pathogen causing disease in the nonimmune individual. The monotypic nature of the virus, the life-long immunity after infection, the lack of an animal reservoir, and the fact that a very effective live-attenuated vaccine is available, make it theoretically possible to eradicate measles (Rima, B.K., et al., 1995b). However, the virus continues to be a major cause of morbidity and mortality in children and infants in the developing world.

1.1 History

Measles appears to be a relatively new disease of humans. The Greek physician Hippocrates (460-377 BC) did not mention the disease in his annals (Gastel, B., 1973). The explanation lies in the epidemiology of the virus; a human population of several hundred thousand is required to provide a sufficient number of new susceptibles to sustain the continued circulation of the virus (Gastel, B., 1973; Black, F.L., 1966). Populations of this size did not exist until the development of urban centers in the Middle East, India and China late in the third millennium BC, which provided population densities high enough to maintain transmission of the virus (McNeill, W.H., 1976).
The first written description of measles is attributed to Abu Becr, a tenth century Persian physician. Abu Becr distinguished measles from smallpox, which he considered a less severe disease (Katz, S.L. and Enders, J.E., 1965). Thomas Sydenham first delivered an astute description of a measles virus outbreak in London in 1670, pointing out the increased severity of the disease in adults and the danger of pulmonary complications (Sydenham, T., 1922). The studies by Peter Panum, a young Danish physician, about an isolated epidemic on the Farao Islands in 1846, greatly increased our knowledge about measles. He noted the highly contagious

nature of the virus with an attack rate of close to 100% in the nonimmune individual, the 14-day incubation period and further documented that infection conferred life-long immunity (Panum, P.L., 1938). In 1905, Hektoen demonstrated the transmission of measles by transfer of blood from patients in the acute stage of disease to susceptible volunteers (Hektoen, L., 1905). The viral etiology of measles was confirmed in 1911 when Anderson and Goldberger reproduced the disease in monkeys that were infected with filtered respiratory tract material from acutely infected patients (Goldberger, J. and Anderson, J.F., 1911). Significant success was reported by Enders and Peebles in 1954 when they were able to isolate the virus from human and rhesus monkey kidney cells and described the cytopathic effects of MV in tissue culture (Enders, J.F. and Peebles, T.C., 1954). This led directly to the development of other virus isolation procedures, infectivity assays, the measurement of neutralising antibodies (Enders, J.F., 1962) and a live virus vaccine (Enders, J.F. et al., 1960).

1.2 The virus

The MV RNA genome is enclosed in a lipid-containing envelope derived from the host cell. The pleomorphic virions have a diameter varying in size from 100 to 250 nm. The envelope carries two kinds of surface projections (peplomers) with distinct morphology, which are between 9 and 15 nm in length. They consist of the viral transmembrane hemagglutinin (H) glycoproteins which have a conical shape and the fusion (F) glycoproteins peplomers that are dumbbell-shaped (Oxman, M.N., 1997). Beneath the layer of lipid molecules forming the envelope, is the matrix or membrane (M) protein in contact with the inner layer of the envelope. The envelope surrounds the helical nucleocapsid (17-21 nm in diameter, 1 nm in length) that consists of primary nucleocapsid (N) protein surrounding the genomic RNA (Örvell, C., 1990). The phosphoprotein (P) and the large protein (L) are bound to the RNA and are also part of the nucleocapsid, which is packed within the envelope in the form a symmetrical coil (Griffin, D.E. and Bellini, W.J., 1996).

1.2.1 The genome and the structural proteins

(A) The genome:

The viral genome consists of linear single-stranded RNA which is of negative polarity. The RNA contains approximately 15 900 ribonucleotides with a molecular mass of close to 4.5 $x10^6$ daltons (Horikami, S.M. and Moyer, S.A., 1995a). The 3'

end of the genome begins with an untranslated leader sequence of 53-55 nucleotides (nt) in length. The 5' end contains a 40 nt sequence which shows a high degree of complementarity to the 3' leader sequence, theoretically allowing for the formation of a stable panhandle structure. The function of these sequences has not been clearly defined but they may represent sites for binding of the RNA polymerase (Örvell, C., 1990). The MV genome encodes six structural proteins and two further nonstructural proteins that are encoded by the P gene. The gene order is 3' N, P (plus C and V), M, F, H, and L (large protein) 5' (Richardson, C.D. et al., 1985). A schematic diagram of the genome is shown in Fig. 1.1. Transcription of the genome is more efficient at the 3' end with a transcriptional gradient from N to L (Ray, J. and Fujinami, R.S., 1987) and this is a major determinant of the relative abundance of the proteins. The intergenic sequence contains a conserved CUU trinucleotide, except for the intergenic region between the H and L gene which displays a CGU trinucleotide (Crowley, J.C. et al., 1988). The intergenic sequence is followed by a conserved sequence of 11 nucleotides before the start of the next gene.

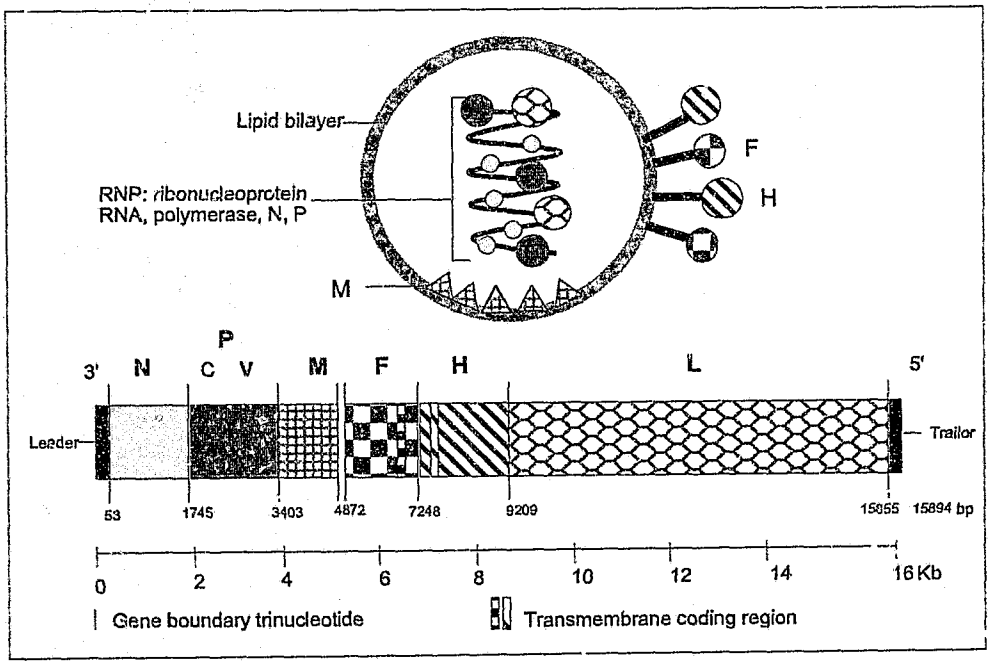


Fig. 1.1 Schematic diagram of the measles virus genome

(B) The proteins:

A summary of measles virus proteins and their suggested functions is given in Table 1.1. Functions are described in more detail below.

Table 1.1: Measles virus structural proteins

Protein	No. of amino acids	Mol. weight x10^3	Location in the *virion*	Function
Nucleocapsid (N)	525	60	nucleocapsid	binds to full-lengths (+) and (-) RNA to form nucleocapsids.Binding to nascent RNA switches from transcription to replication. Most abundant protein, phosphorylated.
Phosphoprotein (P)	507	72	nucleocapsid	forms complex with N and L. Probably involved in transcription and replication; phosphorylated.
C	186	21	not present	downregulates transcription?
V	298	40	not present	Virion assembly and budding; inhibits transcription?
Large (L)	2213	200	nucleocapsid	catalytic component of viral polymerase; RNA transcription and replication; forms complex with P.
Matrix (M)	335	37	inner membrane	Virion assembly and budding; binding to nucleocapsid inhibits transcription?
Fusion (F)	553	60	transmembrane surface	Virus entry, membrane fusion, and hemolysis in association with H. Inactive precursor (F_0) cleaved to active disulfide-linked F_1 (41 kd) and F_2 (18 Kd) subunits.
Hemagglutinin (H)	617	80	transmembrane surface	binds to CD46 receptor, hemagglutination activity, induces membrane fusion together with F, glycosylated, disulfide-linked dimer.

Adapted from Oxman, M.N., (1997).

N protein:

The N protein is the dominant internal component and the most abundant of all of the MV-encoded proteins. It is synthesized on free ribosomes, folded into the cytoplasm, and phosphorylated on serine and threonine residues (Robbins, S.J. and Bussell, R.H., 1979; Gombart, A.F. et al., 1995). The P protein regulates the efficiency with which N assembles into nucleocapsids (Spehner, D. et al., 1997). In MV-infected cells, N surrounds viral genomic and messenger RNAs (mRNAs) that possess the leader sequence to form nucleocapsid structures which are the template for both mRNA transcription and RNA replication (Horikami, S.M. and Moyer, S.A., 1995a). Together with P and L, the nucleocapsid structure forms the ribonucleoprotein complex (Ray, J. and Fujinami, R.S., 1987). It has recently been shown that two independent binding sites for P are located within the variable carboxyl-terminus and the conserved middle domain, respectively (Liston, P. et al., 1997). However, another study demonstrated that the amino-terminal amino acids (aa) 4-188 and aa 304-373 located in the middle domain of the N protein are required for the formation of soluble N-P complexes (Bankamp, B. et al., 1996). Both the conserved amino-terminus and the variable carboxyl-terminus contain T-Helper epitopes in vaccinated and naturally infected individuals and in a mice model (Giraudon, P. et al., 1991; Buckland, R. et al., 1989; Hickman, C.J. et al., 1997). The highest variability within the MV genome lies within the carboxyl-terminal 125 aa (Baczko, K., et al., 1992; Rima, B.K. et al., 1995a; Rota, P.A. et al., 1994; Taylor, M.J. et al., 1991) that are located on the outside of the nucleocapsid. Fig. 1.2 shows a diagram illustrating the regions of variability, P binding sites within the N protein. Sequence analysis of the carboxyl-terminal 150 aa of the N gene has been used extensively to discriminate between different groups of MV wild-type (wt) isolates and for the differentiation of wt and vaccine strains (Kreis, S., et al., 1997; Rima, B.K. et al., 1995a; Rota, J.S. et al., 1994; Rota, J.S. et al., 1998; Rota, P.A. et al., 1994; Taylor, M.J. et al., 1991).

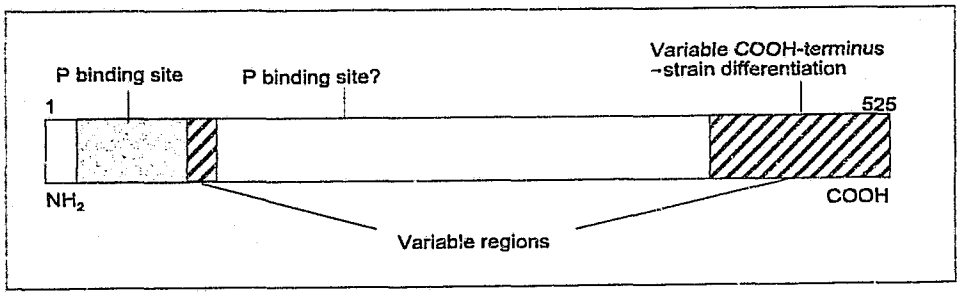


Fig. 1.2 Schematic diagram of the measles virus nucleocapsid gene. Adapted from Griffin, D.E. and Bellini, W.J., 1996.

P, C, and V proteins:

The phosphorylated P protein (Das, T. et al., 1995) is abundant in infected cells, but only small amounts of the protein are present in the virion. P together with the L protein form the RNA-dependent RNA polymerase (Liston, P. et al., 1995; Liston, P. and Briedis, D., 1995). Both proteins are associated with the nucleocapsid to form the ribonucleoprotein (RNP) (Horikami, S.M. et al., 1994). P also binds to individual molecules of the N protein in the cytoplasm of the infected cell to form soluble N-P complexes that are required for RNA encapsidation. The domains on P that are important for interaction with N have been mapped to the carboxyl-terminal 100 aa and the extreme amino-terminal residues (Harty, R.N. and Palese, P., 1995).

Apart from P, MV expresses at least two more proteins C, and V from the P gene. An alternative methionine translation initiation codon directs synthesis of the C protein (Bellini, W.J. et al., 1985) in the overlapping +1 reading frame. The insertion of an untemplated G at position 751 by the RNA polymerase during transcription, a mechanism called mRNA editing, generates the V protein, which shares the amino-terminal 231 aa with the P protein. The 68 carboxyl-terminal aa are cysteine rich and unique to V (Wardrop, E.A. and Briedis, D.J., 1991). RNA editing is an additional function of the RNA polymerase, presumably of the L protein, as was also proposed for Sendai virus and other paramyxoviruses (Vidal, S., et al., 1990a and 1990b). The functions of V and C have not been clearly defined. However, both proteins might play a role in MV RNA synthesis and regulation of genome replication (Horikami, S.M. and Moyer, S.A., 1995a). Another recent study has shown that both, V and C are interacting with cellular proteins but not with any of the MV specific proteins (Liston, P. et al., 1995).

L protein:

The L protein together with P forms the virus-encoded RNA-dependent RNA polymerase, as mentioned before. L contains six regions that are highly conserved among the RNA polymerases of negative-stranded RNA viruses (Chandrika, R. et al., 1995). L is believed to contain the majority of the catalytic activities of the RNA polymerase (Horikami, S.M. et al., 1994). The L protein is present in small quantities in association with viral nucleocapsids in infected cells and in measles virions.

M protein:

The M protein, a basic protein with several conserved hydrophilic domains, is the second most abundant protein in the measles virion (Oxman, M.N., 1997). M forms a continuous layer on the inner surface of the envelope in infected cells and probably interacts with progeny nucleocapsids and with the cytoplasmic tails of the H and/or F proteins to mediate virion maturation and budding (Tyrrell, D.L. and Ehrnst, A.,

1979; Wild, T.F. and Buckland, R., 1995). A study by Hirano, A. et al. (1992) showed that only M protein synthesized during lytic infection was found to associate with the nucleocapsid and the plasma membrane, whereas M synthesized in persistently infected cells was soluble in the cytoplasm. When bound to the nucleocapsid, M inhibits transcription of MV mRNA (Suryanarayana, K. et al., 1994).

F protein:

The F protein is directly involved in the fusion of viral and target cell membranes required for penetration of the virus. F is synthesized as a biologically inactive precursor (F_0) which is activated through cleavage by cellular Furin, a subtilisin-related protease in the trans-Golgi to give the active form, a disulfide-linked heterodimer, F1-F2 (Wild, T.F. et al., 1994). Both viral glycoproteins, F and H, are cotranslationally inserted into the endoplasmic reticulum and transported to the cell surface via the Golgi apparatus, and during this process undergo various modifications. Sato, T.A. et al. (1988) have demonstrated that glycosylation is essential for the proteolytic cleavage of F_0 and its transport to the cell surface.

The F1 subunit is derived form the carboxyl-terminus of F_0, is anchored in the viral envelope and has a cytoplasmic tail, of which the terminal 14 aa are highly conserved among morbilliviruses (Oxman, M.N., 1997). The amino-terminus of F1 is also conserved and contains a region of 25 hydrophobic aa, a region which is believed to interact with the host cell membrane to induce fusion (Richardson, C. et al., 1986). The F2 subunit contains all of the potential N-glycosylation sites.

H protein:

The H protein is a type II transmembrane glycoprotein (Alkhatib, G. and Briedis, D.J., 1986; Wild, T.F. and Buckland, R., 1995), which is located on the surface of infected cells in close proximity to the F protein. The primary function of the H protein is to bind to the cellular receptor CD46 on host cells. A second essential function of H is to interact with F to mediate fusion of the virion envelope with the host cell membrane, which facilitates the entry of the nucleocapsid into the cell (Malvoisin, E. and Wild, T.F., 1993). H also accounts for the capacity of MV to agglutinate red blood cells from a variety of monkeys, but not from humans by binding to the simian homolog of CD46 (Gerlier, D. et al., 1995). Furthermore, H has recently been shown to be responsible for cell tropism (Stern, L.B. et al., 1995). The H protein elicits a strong immune response and life-long immunity, which follows acute infection and this can in part be attributed to neutralizing antibodies against H (Norrby, E., and Oxman, M.N., 1990).

After synthesis and glycosylation in the rough endoplasmic reticulum, disulfide-linked dimers are formed and transported to the Golgi for further oligosaccharide

modification and then to the cell membrane, where they form H peplomers. The specific details of glycosylation of H proteins have been described by Ogura, H. et al. (1991). Isolated H peplomers are homotetramers and have a conical shape (Ogura, H., et al.,1991).The carboxyl-terminal domain is located on the extracellular side of the infected cell, a single hydrophobic domain close to the amino-terminus represents the transmembrane region and is conserved between different paramyxoviruses (Wild, T.F. and Buckland, R., 1995). H has 13 conserved cystein residues that participate in the intra- and intermolecular disulfide bonding (Hu, A. and Norrby, E., 1995). The H protein has 5 potential N-linked glycosylation sites between aa 168 and 238, the last of which is heterogeneous and not actually used (Hu, A. et al., 1994b; Wild, T.F. and Buckland, B., 1995). The optional glycosylation at 1 of these 5 sites is thought to be responsible for the 2 different sizes of H proteins often observed on polyacrylamide gels (Griffin, D.E. and Bellini, W.J., 1996). Glycosylation is essential for dimerization, proper folding of the protein, and antigenicity (Hu, A. et al., 1994b). Several antigenic determinants have been identified (Hu, A. et al., 1993; Hummel, K.B. and Bellini, W.J., 1995; Liebert, U.G. et al., 1994; Sheshberadaran, H. and Norrby, E., 1986). A recent study by Schlender, J. et al. (1996) showed that the coexpression of H and F induced immune suppression *in vitro* by interaction with the cell surface of peripheral blood mononuclear cells (PBMCs). Some CD46 binding sites on the H protein have been suggested and are shown in Fig. 1.3 (Saito, H. et al., 1994; Shibahara, K. et al., 1994).

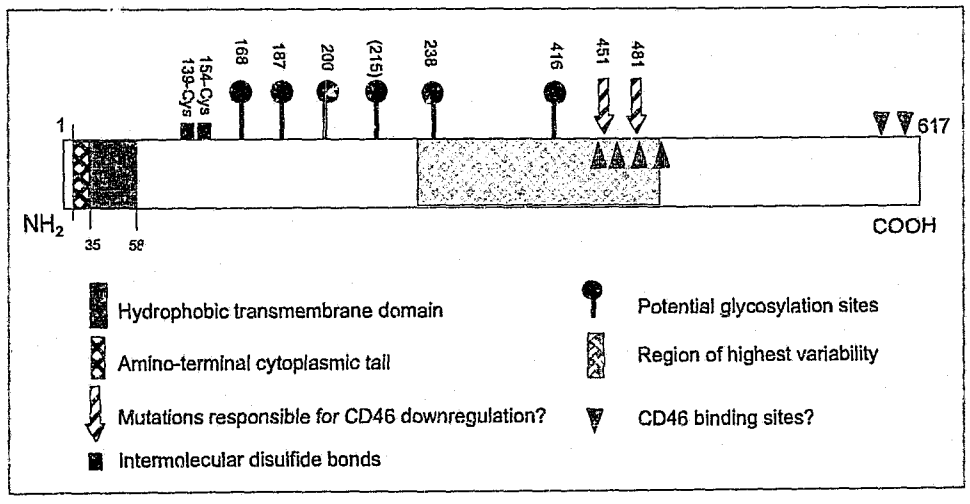


Fig. 1.3 Schematic diagram of the measles virus hemagglutinin gene.

Apart from the functions outlined above, sequence analysis of the H gene has been used for determination of MV strain variations (Rima, B.K. et al., 1997; Rota, J.S. et al., 1992 and 1996). In addition to the variable carboxyl-terminus of the N gene, the H gene sequences have also proved suitable for differentiation between different genetic groups of wild-type (wt) and vaccine MVs. Sequence analysis of the H gene for molecular epidemiological purposes is discussed in greater detail in chapter 3.2.

1.2.2 Replication of measles virus

As described above, the first step in the MV infectious cycle is the adsorption of the H protein to the cell surface via it's receptor. The fusion of the viral envelope with the plasma membrane requires both the H and F proteins (Wild, T.F. and Buckland, R., 1995). Upon fusion the viral nucleocapsid is released into the cytoplasm, where the reproductive cycle takes place. Fig. 1.4 shows a simplified diagram of the infectious cycle.

The activity of the viral RNA-dependent RNA polymerase results in primary transcripts, the mRNAs. The helical, encapsidated nucleocapsid and not free genomic RNA serves as the template for all RNA synthesis (Oxman, M.N., 1997).

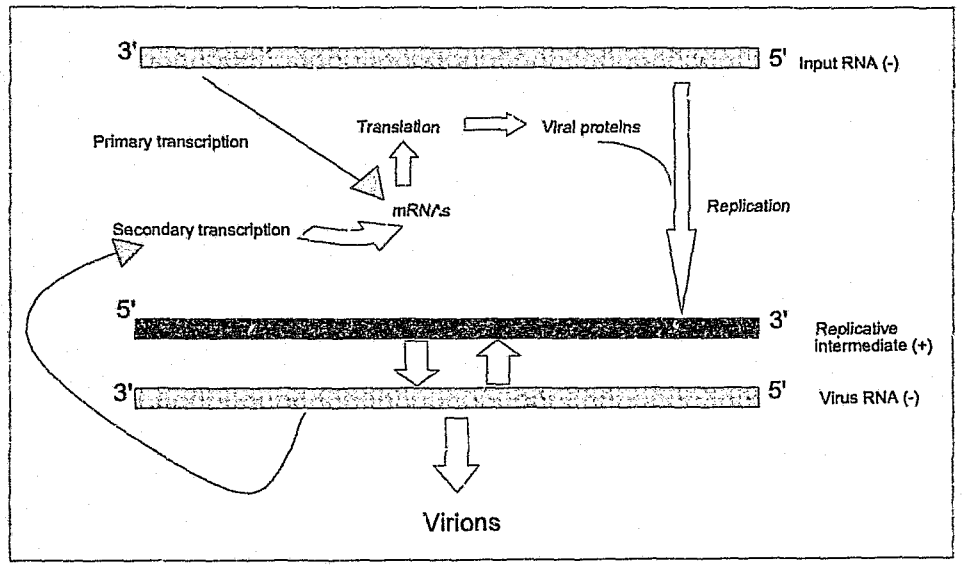


Fig. 1.4 Infectious cycle of measles virus.

The mRNA is monocistronic, unencapsidated, capped and methylated at the 5' end and polyadenylated at the 3' end similar to cellular mRNAs. The mRNAs are translated by the cellular translation machinery (Norrby, E. and Oxman, M.N., 1990). The accumulation of a critical level of N protein is necessary for the subsequent switch from transcription to genome replication, since replicative RNA synthesis is coupled to its encapsidation by N. The (-) strand nucleocapsid serves as a template for the synthesis of (+) strand replicative intermediates which in turn serves as a template for the synthesis of progeny nucleocapsids containing the (-) strand genome RNA. The newly synthesised (-) strand nucleocapsids are templates for secondary transcription, amplifying the viral mRNAs and proteins in the infected cell, as well as for budding of progeny virus (Horikami, S.M. and Moyer, S.A., 1995a; Kingsbury, D.W., 1990).

1.3 The cellular receptor

The cellular receptor for MV has recently been identified by two independent groups as the membrane cofactor protein (MCP) or CD46, which is ubiquitously present on human and monkey cells (Naniche, D. et al., 1993; Dörig, R.E. et al., 1993). Naniche, D. et al. (1993) purified the proteins that were immunoprecipitated with mAbs and identified them as CD46 by microsequencing. Dörig, R.E. et al. (1993) showed that chromosome 1 confers the binding phenotype to previously unsusceptible rodent cells and that anti-CD46 mAb blocked infection. The only surface protein on chromosome 1 is CD46, which made it a plausible candidate. Further evidence that CD46 acts as a MV receptor was provided by both groups when they demonstrated that previously insusceptible murine cells could productively be infected with MV when they expressed CD46. Moreover, the species-specific expression and tissue distribution of CD46 correlates well with susceptibility to MV infection (Gerlier, D. et al., 1995). The primary viral protein to interact with CD46 is the H protein (Buchholz, C.J. et al., 1997; Nussbaum, O. et al., 1995). The normal function of CD46 is to protect the cell from complement lysis (Liszewski, M.K. and Atkinson, J.P., 1992). During complement activation, C3b and C4b (components of the C3 convertases) can form non-specific covalent linkages to the host cell. Factor I, a serine protease in plasma, inactivates membrane-bound C3b/C4b associated with CD46. This process plays an important role in discriminating self from non-self tissue and helps to prevent destruction of the host cell by the membrane attack complex (MAC) (Dörig, R.E. et al., 1994). Fig. 1.5 shows diagrammatically the structure of CD46, which can be found on most primate cells. The extracellular domain of the mature protein

consists of 4 short consensus repeats (SCR1-4) (Devaux, P. and Gerlier, D., 1997) and 1-3 Ser-Thr-Pro rich domains (STP) (Iwata, K. et al., 1994). The transmembrane region consists of 24 hydrophobic aa. One of 2 cytoplasmic domains is alternatively spliced to form the carboxyl-terminus (Dörig, R.E. et al., 1994). The binding sites for MV and for C3 have been shown to be distinct on CD46 (Manchester, M. et al., 1995). CD46 is highly glycosylated and is expressed in most cells in isotypic forms, which range in size between 51 and 68 kDa (Buchholz, C.J. et al., 1996; Gerlier, D. et al., 1994). The STP domains, shown in Fig. 1.5 contain 5-10 O-linked sugars.

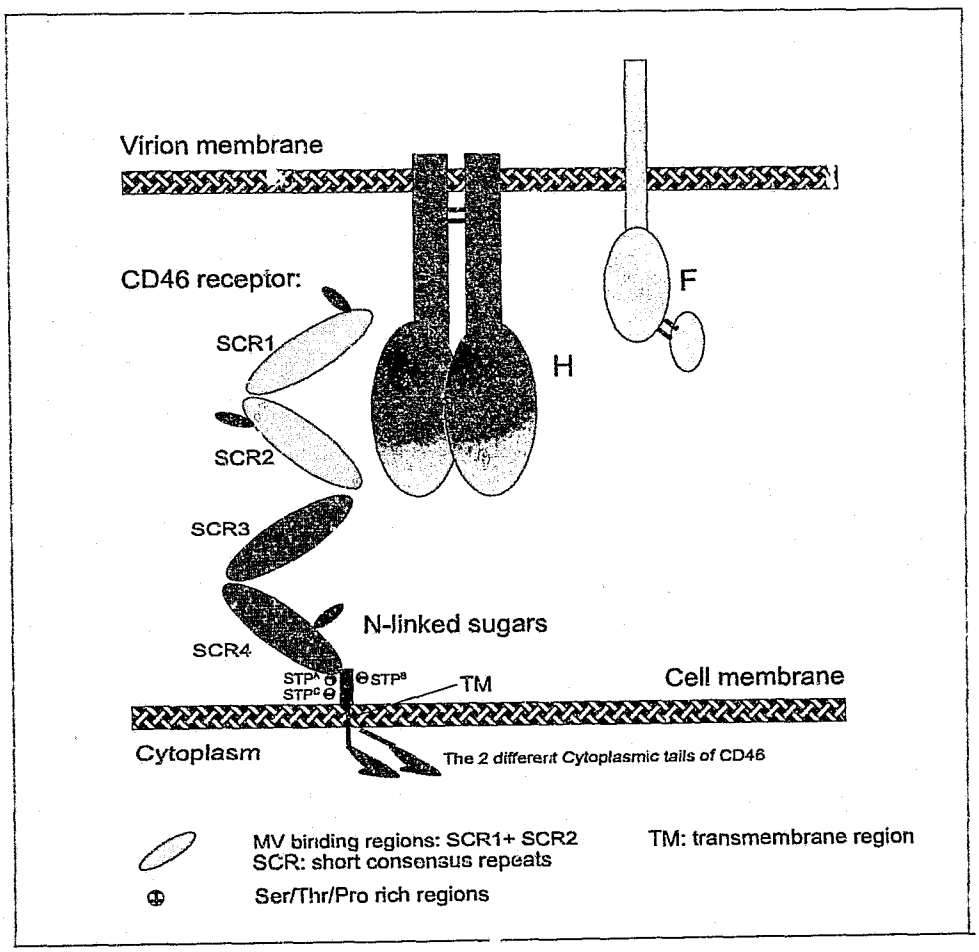


Fig. 1.5 Model of the measles virus receptor CD46 with virus binding regions. Adapted from Manchester, M. et al., 1997.

Several studies have shown that MV differentially downregulates CD46 (Hirano, A. et al., 1996; Schneider-Schaulies, J., et al., 1995; Schnorr, J.-J. et al., 1995; Krantic, S. et al., 1995). Two independent groups have mapped the aa in the H protein, that might be responsible for the downregulation of CD46 to residues 451 and 481 (Lecouturier, V. et al., 1996; Bartz, R. et al., 1996).
CD46 has been firmly established as the high-affinity receptor for MV. However, it remains unclear whether CD46 is the sole MV receptor. Other viruses, for example HIV-1 utilize additional co-receptors in order to transport viral nucleocapsid into the cell (Fantini, J. et al., 1993). It seems plausible that a co-receptor could interact with the F protein to facilitate penetration of the virus into the host cell (Dörig, R.E. et al., 1994). Dunster, L.M. et al. (1994 and 1995) suggested that moesin, a human membrane organising extension spike protein, also acts as a receptor for MV. However, several recent studies have shown that the role of moesin as a MV receptor is still inconclusive (Devaux, P. and Gerlier, D., 1997; Doi, Y. et al., 1998). Buckland, R. and Wild, T.F. (1997) recently reviewed numerous results that have been described on CD46 receptor usage and raised doubt as to whether CD46 is the receptor for MV wt strains that have been propagated in either B95a cells or human B-lymphocytes.

1.4 Measles virus strain variations

Measles virus is considered to be monotypic, antigenically stable and only one serotype has been described so far. However, diversity on nucleotide and amino acid level as well as analytical differences between proteins from different MV strains have been described (Rima, B.K. et al., 1995a and 1995b). Antigenic differences between strains affect functions such as temperature sensitivity (Bergholz, C.M. et al., 1975; Haspel., M.V. et al., 1975; Rager-Zisman, B. et al., 1984), plaque forming characteristics (Rapp, F., et al., 1964; Gould, E.A., et al., 1976; Carrigan, D., et al., 1986), the ability to downregulate CD46 (as above), epitopic variations in most proteins (Giraudon, P. et al., 1988; Sheshberadaran, H. et al., 1983; Tamin, A., et al., 1994) and H protein specific variations that are discussed below.

1.4.1 Differences of measles virus proteins

Differences in the apparent sizes of MV proteins H, P, and M on SDS-PAGE have been reported but none of these differences could be linked to a specific phenotype

(Rima, B.K. et al., 1995b). The mobility and genetic features of the M protein have been studied by many groups since the variability of M was thought to play a significant role in the pathogenesis of subacute sclerosing panencephalitis (SSPE) (Wechsler, S.L. et al., 1979; Cattaneo, R. et al., 1986, 1988 and 1989). However, the diversity found between different M proteins and/or genes was subsequently shown not to be characteristic for SSPE (Rima, B.K. et al., 1995b). The P protein has a molecular mass of 51 kDa (Bellini, W.J. et al., 1985) but the apparent size by SDS-PAGE approaches 70 kDa. Phosphorylation is thought to play a major role in these differences in size. Various antigenically different H proteins have been described that show defective hemagglutination activity (Giraudon, P. and Wild, T.F., 1985; Saito, H., et al., 1992 and 1994), salt-dependency of hemagglutination (Shirodaria, P.V. et al., 1976), differential recognition by H protein-specific mAb (Tamin, A., et al., 1994; Trudgett, A., et al., 1981), and differences in migration of H proteins by SDS-PAGE, that could be due to additional potential glycosylation sites (Rota, J.S., et al., 1992; Sakata, H. et al., 1993).

1.4.2 Genetic variations

Over the past 10 years more emphasis has been placed on the investigation of genetic variability between MV strains. So far, 8 to 9 different genetic groups have been described (Rima, B.K. et al., 1995a, Bellini, W.J. and Rota, P.A., 1998, Kreis, S., et al., 1997; Jin, L. et al., 1997; Katayama, Y. et al., 1997; Outlaw, M.C. and Pringle, C.R., 1995). However, it is likely that more distinct genetic groups will be identified as molecular epidemiological data will become available from more countries, especially in Africa and Asia. To date, there is no generally accepted consensus as to how to define MV genotypes, how to define genetic subgroups and subsequently how to name the different genetic groups. In this thesis, terms like genetic group or genetic lineage will be used rather then the term genotype. Genetic groups will be described using the numbering code (Rota, P.A., et al., 1992; Rota, J.S. et al., 1998) and the letter code used by other groups will be given in brackets (Kreis, S., et al., 1997; Rima, B.K., et al., 1995a) .

Nucleotide as well as amino acid changes occur in almost every region of the MV genome but the degree of variation varies considerably between the different structural genes (Rozenblatt, S. et al., 1985). The highest variability is seen in the carboxyl-terminal 450 nt of the N gene and in parts of the H gene. These regions have therefore been used extensively to differentiate and characterise different genetic groups of MV (Rima, B.K. et al., 1995a, Rota, P.A. et al., 1994 and 1995a, Rota, J.S. et al., 1996; Kreis, S., et al., 1997). Nucleotide variation in the carboxyl-

terminus of the N gene can reach up to 12% whereas H genes can vary up to 7% between different lineages (Bellini, W.J. and Rota, P.A., 1998). The genetic groups that have been established by phylogenetic comparison of the carboxyl-terminal N gene sequences and partial or full coding sequences of the H genes compare well to each other and groupings are mostly identical comparing the different genes. Group 1 (A) represents vaccine and vaccine-like strains that are characterised by relatively high stability over the decades and this is also the earliest genetic group of MV known. The prototype strain is the Edmonston wild-type (wt) strain which was isolated in 1954. The group 1 (A) viruses must have had widespread distribution in the pre-vaccine era since strains belonging to this group were isolated during the 1950s and the 1960s in countries as distant as Russia, USA, Finland, Japan, and Romania (Bellini, W.J. and Rota, P.A., 1998). The genetic variations of MV N and H genes between different viral strains will be discussed further in the result chapters of this thesis. Some of the genetic groups that have been described appear to be extinct or are circulating in such small numbers that they have evaded detection by routine surveillance systems. These groups include isolates mainly from Europe that have not been re-isolated in the past 10-15 years (Bellini, W.J. and Rota, P.A., 1998).

1.4.3 Geographic distribution of MV genetic groups

Several groups of wt MV have been shown to have world-wide circulation (Bellini, W.J., and Rota, P.A., 1998) whereas others seem to be extinct. Some genetic groups can be assigned to certain countries. However, increased travelling contributes to the exportation and importation of these geographically restricted strains from and into other continents or countries (Rota, J.S. et al., 1996 and 1998; Rota, P.A. et al., 1995a). The vaccine and vaccine-like sequences in group 1 (A) were described above. Bellini, W.J. and Rota, P.A. (1998) have very recently described and updated the genetic groups of MV that are currently circulating and their geographic distribution. Strains that have not been isolated for 10 to 15 years were excluded in this update since they are likely to be extinct. Their findings are summarised below. Group 2 (D2) viruses circulated in USA until 1993, when transmission of MV was interrupted by successful vaccination programs (Rota, J.S. et al., 1996). Other recent isolates that fall in that group were made in Guam and Japan (Yamaguchi, S. et al., 1997). Group 3 (D4) includes viruses from Europe, USA, Palau and Thailand. Group 4 (D3) and 5 (C2) appear to be the predominant genotypes circulating in many parts of Europe. African isolates form Cameroon and Gabon (B1 and B2) and a strain from Gambia (H) as well as other isolates that were originated in African countries are

found in group 6. MV strains from South Africa and a Canadian isolate made in 1988 form group 7 (I). Analysis of H gene sequences revealed that recent strains from Pakistan also group with group 7 viruses. Group 8 consists of very distinct isolates made in China in the 1990s.

1.5 Pathogenesis and Pathology

1.5.1 Clinical manifestations

(A) Classic measles

The highly contagious MV is transmitted via large droplets of respiratory tract secretions that are inhaled upon face to face contact (Black, F.L., 1989; Bloch, A.B. et al., 1985; Goldberger, J. and Anderson, J.F., 1911). The disease has a incubation period of 8-12 days. Clinical symptoms that appear first are fever, malaise, and anorexia followed by one or more of the following: cough, coryza, and conjunctivitis. This prodromal phase of measles corresponds to the development of an enanthem, the Koplik spots that become visible on the buccal mucosa and generally appear 2 days before the exanthematous rash. The rash is maculopapular and appears first on the face and then spreads to the trunk and extremities. The rash generally lasts for up to 4 days (Katz, M. and Enders, J.E., 1965).

Primary replication of the virus takes place in the epithelial cells of the respiratory tract (Katz, S.L. and Enders, J.E., 1965). Further replication occurs in the lymph nodes and from there the virus enters the blood stream in infected leucocytes, producing the primary viremia. Lymphoid tissues, such as tonsils, adenoids, lymph nodes, thymus, spleen, appendix, Peyer's patches, and submucosal lymphoid tissue in the respiratory and the gastrointestinal tracts become major sites for viral replication during primary viremia (Oxman, M.N., 1997). The continued replication of MV at these sites results in secondary viremia that begins 5-7 days after infection. The virus then spreads to the skin, conjunctivae, genital mucosa, kidneys, liver, and oropharynx (Moench, T.R. et al., 1988; Katz, S.L. and Enders, J.E., 1965; Forthal, D.N. et al., 1992). At this stage more than 5% of leucocytes may be infected. The predominant cell infected in the peripheral blood is the monocyte (Esolen, L.M. et al., 1993). This expanded replication of MV together with the host immune response is responsible for the prodromal signs and symptoms (Oxman, M.N., 1997). In uncomplicated measles, fever reaches a peak of 39°C to 40°C followed by a rapid deferescence on the third or fourth day of the rash which marks the beginning of recovery (Kempe, C.H. and Fulginiti, V.A, 1965).

Factors that may cause a more severe disease and predict a poor outcome are: poor antibody response, lymphopenia, compromised immune system, other illnesses, malnutrition, poor socioeconomic conditions, low serum retinol levels, and extremes of age (Frieden, T.R. et al., 1992; Kaplan, L.J. et al., 1992; Griffin, D.E. and Bellini, W.J., 1996).

(B) Atypical and modified measles

Modified measles:

Modified measles is milder and generally shorter than classic measles and is seen in partially immunised patients that have received immune serum globulin after exposure to measles and in children with residual maternal antibodies (Krugamn, S. et al., 1985).

Atypical measles:

In contrast, atypical measles has been reported to be more severe than classic measles with high fever, pneumonia, myalgia, headache, dyspnea, and abdominal pain. Atypical measles has been observed in recipients of formalin-inactivated measles vaccine who were subsequently exposed to wild-type MV (Frey, H.M. and Krugman, S., 1981). This inactivated vaccine was administered between 1963 and 1970 and is not in use any longer (Markowitz, L.E. and Katz.S.L., 1994). The formalin (or Tween-ether) inactivation altered the antigenic properties of F and patients failed to raise antibodies to F.

(C) Neurological manifestations

Postinfectious encaphalomyelitis

Uncomplicated measles is frequently accompanied by cerebrospinal fluid pleocystis and electroencephalographic abnormalities, but there is no evidence that the brain parenchyma is directly infected (Moench, T.R. et al., 1988; Oxman, M.N., 1997). Postinfectious encaphalomyelitis, an autoimmune demyelinating disease is detected in 1 in a 1000 cases of measles and usually occurs within 1 or 2 weeks after onset of rash. The disease is characterised by an abrupt onset of high fever and obtundation, frequently accompanied by seizures and neurological signs (Griffin, D.E. and Bellini, W.J., 1996).

Measles inclusion body encephalitis (MIBE):

This progressive, generally fatal neurological manifestation of measles virus occurs only in immunocompromised individuals some months after infection (Kaplan, L.J., 1992). The disease usually presents with refractory myoclonic seizures and altered mental status and generally progresses within 6 to 8 weeks to coma and death. Survivors have severe neurological sequelae (Oxman, M.N., 1997).

Subacute sclerosing panencephalitis (SSPE):
Another fatal form of measles encephalitis, SSPE, occurs in 1 in 300 000 cases generally 6 to 10 years after measles infection (Oxman, M.N., 1997; Fontaine, R.E. and Martin, A.S., 1978). However, South African studies have shown that SSPE can occur as early as 2 years after acute infection (Schoub, B.D. et al., 1992; Vardas, E., personal communication). Early symptoms of this persistent infection of the CNS are personality changes, and deteriorating intellectual performance (Carter, M.J. and ter Meulen, V., 1987). Later, myoclonic jerks appear with characteristic electroencephalographic changes. Death occurs in most cases within 1 or 2 years after onset of illness (Oxman, M.N., 1997). Titres of antibody to MV are typically elevated in both the serum and CSF of SSPE patients (Connolly, J.H., et al., 1967; Chiodi, F. et al., 1986).The virus present in SSPE patients is defective and cannot be isolated by conventional methods. The virus carries multiple mutations throughout the genome, especially in the M gene but also in the F and H gene (Rima, B.K. et al., 1995b; Schneider-Schaulies, S. et al., 1995). The pathogenesis of SSPE remains poorly understood.

Measles virus has been shown to be able to persist *in vitro* and *in vivo*. For studies of persistence *in vitro*, a number of cell lines have been established using neuronal cells, lymphoid, glial, and epithelial cells (Rager-Zisman, B. et al., 1984; Gould, E.A. and Linton, P.E., 1975). Apart from persistence in SSPE patients there is some evidence that MV can persist outside the CNS (Brown, H.R. et al., 1989; Katayama, Y. et al., 1998).
MV has also been suggested to play a role in diseases like Paget's disease, Crohn's disease, chronic active hepatitis, and multiple sclerosis but for none has a definite role for MV been established (Griffin, D.E. and Bellini, W.J. et al., 1996).

(D) Complications
In developed countries approximately 10% of measles cases are associated with complications, whereas developing countries have complication rates as high as 80% (Clements, C.J. and Cutts, F.T., 1994). Pneumonia occurs in 1-7% of cases and is commonly caused by secondary bacterial or other viral infections (Morton, R. and Mee, J., 1986). Other frequent complications include otitis media, laryngotracheo-bronchitis, childhood blindness, diarrhea and encephalitis, as mentioned above (Beckford, A.P. et al., 1985; Reddy, V. et al., 1986; Katz, M. and Enders, J.E., 1965).

1.5.2 Diagnosis

(A) Clinical Diagnosis

In countries with endemic circulation, MV is usually well diagnosed by assessment of the clinical signs (fever, rash, Koplik's spots, cough, or coryza, or conjunctivitis). However, in developed countries where measles has been reduced to sporadic outbreaks, clinical diagnosis has a higher error rate. In these cases, laboratory confirmation becomes increasingly important.

(B) Virus isolation

MV can be isolated from a variety of samples including, urine, nasopharyngeal swabs, throat swabs, PBMCs, and conjunctival swabs (Gresser, I. and Katz, S.L., 1960; Rota, P.A. et al., 1995b, Kobune, F. et al., 1990; Oxman, M .N., 1997; O'Neill, H.J. et al., 1996). The most sensitive cell line described so far is the adherent derivate (B95a) of the EBV-transformed marmoset B-lymphoblastoid cell line B95-8 (Kobune, F. et al., 1990). Other cell lines that have been used in the past, mostly derived from primate kidneys, are Vero and CV-1 (Forthal, D.N. et al., 1993). Successful isolation is likely when specimens are collected during the febrile phase of the illness and decreases with every day after the rash has disappeared. Several passages are often required before MV specific CPE appears. CPE in all susceptible cell lines is characterised by multinucleated giant cells (syncytia) and eosinophilic intranuclear and intracytoplasmic inclusion bodies (Salmi, A.A., 1995).

(C) Serological diagnosis

There are a variety of serological tests available that have been used to determine and/or titrate MV antigens and/or antibodies to it. Tests measuring antibodies are neutralization tests, that are not widely used any longer since they require MV cell culture and are labour intensive; the hemagglutinin inhibition test (HI), which primarily detects antibodies to H by inhibition of agglutination of monkey red blood cells, and the complement fixation test that has been used in the past to determine immunity against MV. The complement fixation test mainly detects antibodies to the N protein (Norrby, E. and Gollmar, Y., 1972).The plaque reduction neutralization assay (PRN) still is considered the standard against which other tests are measured because it has been shown to be far more sensitive than the tests mentioned above (Chen, R.T. et al., 1990). The most commonly used test systems today are the commercially available ELISA kits. ELISA kits that measure IgM, IgG, and IgA antibodies to all MV proteins are easy to use and can provide rapid and reliable results (Helfand, R.F. et al., 1996) . In primary infection in the normal host, detectable antibodies to MV appear 1 to 3 days after onset of rash and remain detectable for

years (Krugman, S. et al., 1985). Detection of IgM antibodies, the first isotype to be synthesised, is indicative for an acute primary infection.

(D) Nucleic acid detection and immunofluorescence

In immunocompromised patients that do not have a detectable antibody response or for diagnosis of CNS disease, where virus cannot easily be isolated, RT-PCR for detection of nucleic acids (Nakayama, T. et al., 1995; Matsuzono, Y. et al., 1994; Rota, P.A. et al., 1994; Baram, T.Z. et al., 1994) and direct immunofluorescent staining of MV antigens in tissue samples are important diagnostic tools (Minnich, L.L. et al., 1991). Furthermore, these techniques can detect antigen and/or nucleic acids later in the disease when infectious virus can no longer be isolated (Oxman, M.N., 1997).

1.6 Immune responses to measles virus

Specific host immune responses to MV are essential for clearance of the virus , for recovery, and for the establishment of long-term immunity against measles. The early non-specific responses that occur during the prodromal phase of the illness and before the rash develops, include activation of natural killer cells (NK) and the increased production of interferon-α and-β (IFN), which contribute to the control of MV replication before the onset of more specific immune responses.

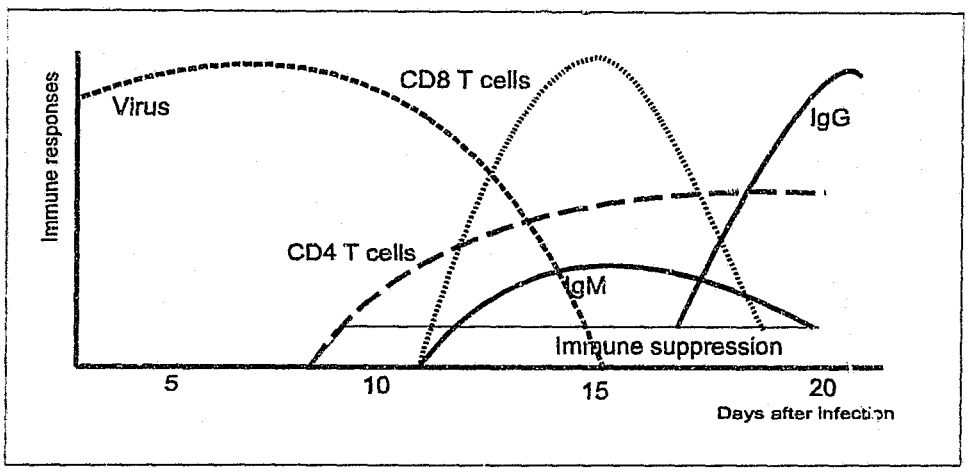


Fig. 1.6 Immune responses to measles virus infection.
Adapted from Griffin, D.E. and Bellini, W.J., 1996.

Generally, there is a clear activation of the immune system resulting in the mobilisation of various defence mechanisms during measles. Paradoxically, at the same time, a profound immune suppression can be observed, evidenced by impaired delayed-type hypersensitivity skin test (DTH) to recall antigens and by reduced humoral and cellular responses to new antigens (Beckford, A.P. et al., 1985; von Pirquet, C., 1908; Griffin, D.E. et al., 1994). Fig. 1.6 summarises diagrammatically the main cellular and humoral immune responses to measles infection that appear after onset of disease.

(A) Humoral immune response

The first antibody to be detected at the onset of rash is the isotype IgM followed by IgG (Fig. 1.6), mainly of the IgG1 and IgG4 subclasses. An IgM response is absent on re-vaccination of those previously immunised and therefore serves as a valuable marker to distinguish primary infection or vaccination from previously infected individuals or secondary vaccination (Erdman, D.D. et al., 1993; Linnemann, C.C. et al., 1973). IgG1 antibodies are efficiently transported across the placenta and thus provide the newborn with specific protection against measles. Maternally derived antibodies can persist for up to 12 months but generally fade to protect the child from infection after 9 months of age (De Serres, G. et al., 1997). Maternal antibodies derived from mothers that had a natural infection seem to persist longer and in higher titres than those from vaccinated mothers (De Serres, G. et al., 1995; Brugha, R. et al., 1996). The MV proteins N, H, and F act as major antigens that give rise to a strong immune response.

The N protein, the most abundant of all MV proteins, acts as a potent antigen. The first and most abundant antibodies produced are in fact against N (Griffin, D.E. et al., 1995) and the presence of N antibodies is a good indicator for previous infection and immunity (Krugman, S. et al., 1985).

Neutralising antibodies to H, which are measured by HI and neutralisation tests, appear to play a major role in preventing re-infection. Thus, measuring the presence and titre of these antibodies is a good indicator for assessing susceptibility and response to immunisation (Oxman, M.N., 1997).

Antibodies to F most likely prevent fusion of the cell membrane with the viral envelope and therefore limit the spread of MV to uninfected cells (Malvoisin, E. and Wild, T.F. , 1990). F-specific antibodies can be measured by inhibition of MV induced hemolysis of monkey erythrocytes (HLI) (Griffin, D.E. et al., 1995).

MV-specific antibodies persist for decades and in most cases for life and protect from re-infection with the virus. They may also play a critical role in recovery from acute disease and can protect from initial infection (Griffin, D.E. and Bellini, W.J., 1996). Recently, Katayama, Y. et al. (1998) have detected viral antigens in autopsied

organs, such as liver spleen, kidney, and lung, which may be an indicator that MV antigens can persist in the absence of viral replication. However, the presence of MV antigens in healthy individuals may have to be confirmed by further studies.

(B) Cellular immune response

MV has a dual effect on cell-mediated immunity (CMI) of the infected individual. It depresses temporarily the pre-existing T-cell reactivity to non-MV antigens and simultaneously activates T-cell driven CMI (Salmi, A.A., 1995). Markers for the activation of CMI are the MV-induced elevation of plasma levels of CD4, CD8 (Fig. 1.6), IL-2 receptor, β2-microglobulin (Griffin, D.E. and Ward, B.J., 1993), the T cell products IL-12, IL-4, and IFN-γ (Griffin, D.E. et al., 1990). Although present earlier in infection, as evidenced by infiltration of sites of viral replication by lymphocytes and macrophages, T cell activation first becomes apparent during the prodrome, when plasma levels of IL-2 and IFN-γ are increased and proliferating T- and B-lymphocytes can be detected in peripheral blood (Griffin, D.E. et al., 1989). CD8 cytotoxic T-lymphocytes recognize viral antigens after the viral proteins have been processed to peptides and presented on the surface of infected cells complexed with major histocompatibility complex (MHC) class I molecules (Griffin, D.E. et al., 1995). IFN-γ, an important product of CD8 cytotoxic T cells, is elevated during measles infection (Griffin, D.E. et al., 1990). CD4 T cells are activated as well during the rash and remain elevated for several weeks (Griffin, D.E. and Ward, B.J., 1993).

The immune suppression caused by MV is thought to be responsible for increased susceptibility to secondary infections and therefore contributes to the morbidity and mortality caused by the virus. A typical marker for immune suppression is the decreased number of circulating T cells during the acute phase of infection (Griffin, D.E. et al., 1995). Several studies have recently been undertaken to explain the mechanisms involved in MV-associated immune suppression. Karp, C.L. et al. (1996) have shown that MV monocytes specifically down-regulate IL12. IL12 is important for the generation of a normal CMI, it induces IFN-γ from T- and NK cells, it is required for the development of a T-helper 1 (Th1) response, necessary for DTH responses and acts as an enhancer of NK cell cytotoxicity. Monocytes and macrophages are thought to be the main IL12-producing cells in vivo. Esolen, L.M. et al. (1993) have shown that MV efficiently infects monocytes. The direct interaction of MV with its receptor, CD46, might play a critical role in the MV-induced downregulation of monocyte production of IL12 (Karp, C.L. et al., 1996).

Three recent studies have suggested further mechanisms by which MV could cause immune suppression (Fugier-Vivier, I. et al., 1997; Grosjean, I. et al., 1997, Schnorr, J.-J. et al., 1997). All 3 groups showed that MV efficiently infects dendritic cells, which are important antigen-presenting cells. Fugier-Vivier, I. et al. (1997) demonstrated that CD40-activated dendritic cells (DC) decrease their capacity to

produce IL12, and T cells are unable to proliferate in response to MV-infected DC stimulation. In contrast, it was demonstrated by others that IL12 production was enhanced after MV infection of DCs (Schnorr, J.-J. et al., 1997). Whereas MV replication in DC was low, the replication could be markedly boosted by DC-T cell interaction. In MV pulsed DC-T cell co-cultures, massive apoptosis of both T cells and DC was observed. The authors concluded that DC are a major target of MV. Grosjean, I. et al. (1997) suggested that carriage of MV by DC may facilitate virus spreading to secondary lymphoid organs. DCs are strategically located in the lung, gut, liver, and skin, and are an important components of the protective immunity against a broad spectrum of antigens and microorganisms (Bhardwaij, N., 1997). Thus, DC may represent a reservoir for MV infection and a means of transport for the virus to lymphoid cells in draining lymph nodes (Bhardwaij, N., 1997).

1.6.1 Immunisation

A live-attenuated MV vaccine was first licenced in 1963 and it remains one of the most cost-effective public health tools available. The Edmonston B strain, the first live-attenuated MV vaccine strain, was often administered simultaneously with gamma globulin (Krugman, S. et al., 1983). This strain was attenuated by numerous passages through a variety of cells (primary renal cells→ primary amnion cells→ chick embryos→ chick embryo fibroblasts) (Enders, J.F. et al., 1960 and 1962). Further passage in chick embryo fibroblasts resulted in the Schwarz vaccine strain, which was licenced in 1965 and remains the most widely used MV vaccine. Other vaccine strains like CAM-70 and Len-16 have been attenuated from wt isolates other than the Edmonston strain (Rota, J.S. et al., 1994). Attenuated vaccine strains differ from wt MV strains considerably, affecting pathogenicity in primates, temperature sensitivity, downregulation of CD46, and nucleotide sequences (Rota, J.S. et al., 1994; Rima, B.K. et al., 1995a; Gellin, B.G. and Katz, F.T., 1994a and 1994b; Markowitz, L.E. and Cutts, F.T., 1994; Krugman, S. et al., 1983; Schneider-Schaulies, J. et al., 1995). However, the specific markers that confer attenuation have not been identified yet. Measles vaccine is available in monovalent formulation (MR) but is most commonly administered subcutaneously in trivalent formulations (MMR) together with attenuated mumps and rubella vaccines at a standard dose of 10^3 to 10^4 plaque forming units (CDC, 1994).

The immune response to the live-attenuated vaccine is similar to that induced by natural infection. However, levels of MV specific antibodies and CMI generated after vaccination seem to be somewhat lower compared with the levels after wt infection; furthermore, vaccine-induced antibodies appear to wane earlier (Christenson, B. and

Bottiger, M., 1994; Ward, B.J. et al., 1995; Wu, V.H. et al., 1993; Pedersen, R. et al., 1986).

The recommended age of immunisation has varied from 6 to 15 months (Aaby, P. and Clements, C.J., 1989). In infants 6 months of age, the presence of maternal antibodies is more likely than in older children (Markowitz, L.E. et al., 1990). The presence of maternal antibodies has been shown to markedly reduce the seroconversion rates and vaccine efficacy (Cutts, F.T. and Markowitz, L.E., 1994; Clements, C.J. and Cutts, F.T., 1995) and antibodies of vaccinated mothers decline earlier than in mothers that had a natural infection (De Serres, G. et al., 1997). The recommended age of MV immunisation is determined by a balance between the optimum age for seroconversion and the probability of acquiring measles before that age (Aaby, P. et al., 1996; Griffin, D.E. and Bellini, W.J., 1996). In South Africa, and in many other countries where measles remains prevalent, the vaccination schedule requires that routine immunisations are given at 9 and 15 months of age (Epidemiological Comments, 1994-1997; Schoub, B.D. et al., 1990).

Advances in the fields of molecular genetics and immunology have provided possibilities for development of innovative measles vaccines. The main objective of a new vaccine would be to generate a product for use in the young infant in the presence of maternal antibodies (Katz, S.L. and Gellin, B.G., 1994; Norrby, E., 1995; Osterhaus, A.D.M.E. et al., 1994). Four different principles are currently under investigation. The classic replicating attenuated MV, replicating vectors containing 1 or more MV genes and non-replicating immunogens including 1 or more selected components, and DNA-based immunisations (Norrby, E., 1995; Brinckmann, U.G. et al., 1991; Pedersen, I.R. et al., 1992; Fooks, A.R. et al., 1996; Hathaway, L.J. et al., 1995 and 1998; Wild, T.F. et al., 1992; Obeid, O.E. et al., 1993; Beauverger, et al., 1993; Yang, K. et al., 1997; Cardoso, A.I. et al., 1996).

Primary vaccine failure due to failure to maintain the cold chain remains a serious problem in the developing world and secondary vaccine failures, which may only affect few vaccine recipients, are contributing to the continued problems to control and interrupt MV transmission in many countries.

1.7 Epidemiology

The WHO estimates that 45 million MV infections occur world-wide annually with more than 1 million MV related deaths (Weekly Epidemiological Record, WHO, 1994). Measles is one of the most contagious diseases of humans (Clements, C.J. and Cutts, F.T., 1995). It is estimated that 90% of household exposures of

susceptible individuals lead to infection (Gellin, B.E. and Katz, S.L., 1994). The absence of an animal reservoir and significant persistent infections, respectively, requires a continuous supply of susceptibles in order to maintain transmission of the virus. Mathematical models have shown that a population size of 250 000 to 300 000 is needed to establish measles as an endemic disease (Black, F.L., 1966). The main pool of susceptible individuals is formed by young infants when the maternal antibodies disappear and before receiving routine vaccination (Oxman, M.N., 1997). The frequency of MV infections is highest in the winter months and transmission is most efficient upon direct contact, the inhalation of droplets of infected respiratory tract secretions, with an infected individual (Griffin, D.E. and Bellini, W.J., 1996). It has been estimated that in countries with a high viral burden, vaccine coverage exceeding 95% is needed to effectively interrupt transmission of MV and to prevent outbreaks (Anderson, R.M. and May, R.M., 1990). However, numerous outbreaks of the disease have been reported in highly vaccinated communities (Gustafson, T.L. et al., 1987; Chen, R.T. et al., 1989). Of great concern in most developing countries, where MV remains endemic, are the infections in children less than 9 months of age (Aaby, P. and Clements, C.J., 1989). Routine vaccination in most of the developing world is carried out at 9 months of age. The maternal antibodies, however, have been shown to decrease earlier when the mother was vaccinated instead of having acquired immunity through natural infection (De Serres, G. et al., 1997; Altintas, D.U. et al., 1996; Brugha, R. et al., 1996). The age specific attack rates thus largely depend on the age at which maternal antibodies are lost. Side effects from vaccination at 6 months of age have been reported to be more severe than at 9 or more months of age. Immunisation at 6 months of age should therefore only be carried out in high-risk areas (Weekly Epidemiological Record, WHO, 1994; Aaby, P. and Clements, C.J., 1989).

1.7.1 Molecular epidemiology

Molecular epidemiological studies on MV have increased markedly over the past 5 years. Investigations of strains differences and the determination of genotypes that are circulating in a country over time have made significant contribution towards worldwide control and elimination efforts. Only the profound knowledge of the genetic groups present in a region or country can subsequently allow for the identification of imported strains and patterns of viral transmission (Kew, O. and Nathanson, N., 1995). This is an important means for assessing the success of local vaccination and MV control programs. In view of enhanced efforts that are being made worldwide to eliminate and eventually eradicate MV, molecular epidemiological

investigation will become even more important. The increase in worldwide travel may contribute to the importation of geographically restricted virus groups into countries that have previously succeeded in interrupting transmission of indigenous MV within their borders (Bellini, W.J. and Rota, P.A., 1998; Rota, J.S. et al., 1996). For example, the ongoing investigations of strain differences in the USA have enabled them to show that transmission of indigenous MV was interrupted after 1992 and that the sporadic cases that occurred after 1992/93 were caused by importations from various countries (Rota, J.S. et al., 1996 and 1998; Bellini, W.J. and Rota, P.A., 1998). Considering the success that especially the USA had with molecular epidemiological studies, South Africa decided in 1994 to enroll in similar investigations to support the local MV control efforts. The molecular epidemiology of MV in South Africa is a major topic of this study and will be discussed in greater detail in the result chapters.

1.7.2 Epidemiology of measles virus in South Africa

Information about the annual number of measles cases, the age-specific attack rate, case fatality rates (CFR), vaccination coverage and other classical epidemiological information has been collected in South Africa by the Department of Health for several years. However, the Epidemiological Comments published monthly are only considering notified cases. Routine vaccination against measles was introduced in South Africa in 1975 and in 1979 measles was first made a notifiable disease. The actual number of cases is estimated to be 3 to 5 times higher than the notified figures.

The South African Department of Health has launched the first mass vaccination campaign in 1990, which was followed, in 1991, by a reduced number of cases and measles related deaths. However, this was followed by a major epidemic in 1992 that for the first time affected both infants and preschool children as well as older children and adolescents, most of whom had been vaccinated (Schoub, B.D., 1993 and 1994a; Coetzee, N. et al., 1994). A survey conducted in 1994 revealed that vaccine coverage approached 76% for the whole of South Africa varying between 66% and 89% between different provinces. In 1996 and 1997, South Africa has again completed 2 mass vaccination campaigns in order to reduce the incidence of measles. In these 2 campaigns, different provinces have applied different vaccination strategies, such as targeting different age groups. Fig. 1.7 shows a map of South Africa with the 9 provinces and the vaccine coverages achieved for each province. The total coverage achieved in 1996 and 1997 mass vaccination campaigns was 91% and 79%, respectively.

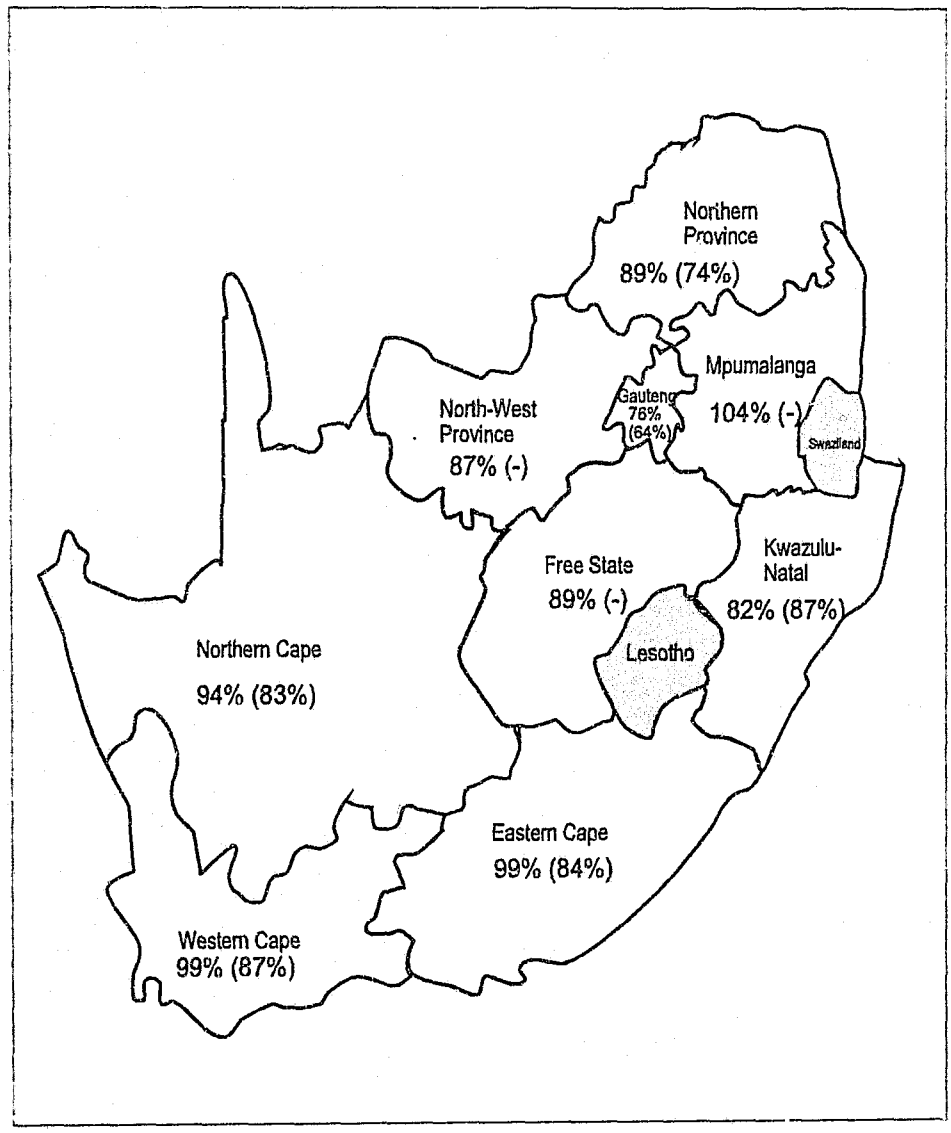


Fig.1.7 South Africa with nine official provinces.
The vaccine coverage achieved in the 1996 mass vaccination campaign is indicated by the first number below the name of the provinces. The 1997 coverage results are shown in brackets. Three provinces did not take part in the 1997 campaign (indicated by a dash).

The average number of notified measles cases and related deaths in South Africa over the past decade averaged 15 000 and 300 respectively and this is certainly a vast underestimate of actual cases (Naidoo, S. and Meyers, K., 1994). The number of notified cases in South Africa from 1988 until 1996 is shown in Fig. 1.8. As mentioned above the number of cases dropped to below 5000 in 1991 after the first MV control programs were employed in 1990. This was followed by a substantial outbreak in 1992 with notified case numbers approaching 24 000. An all time low of ~3000 cases was achieved in 1994. Cases notified for 1997 were not available but numbers are believed to have dropped markedly after the 1996/97 MV mass vaccination campaigns.

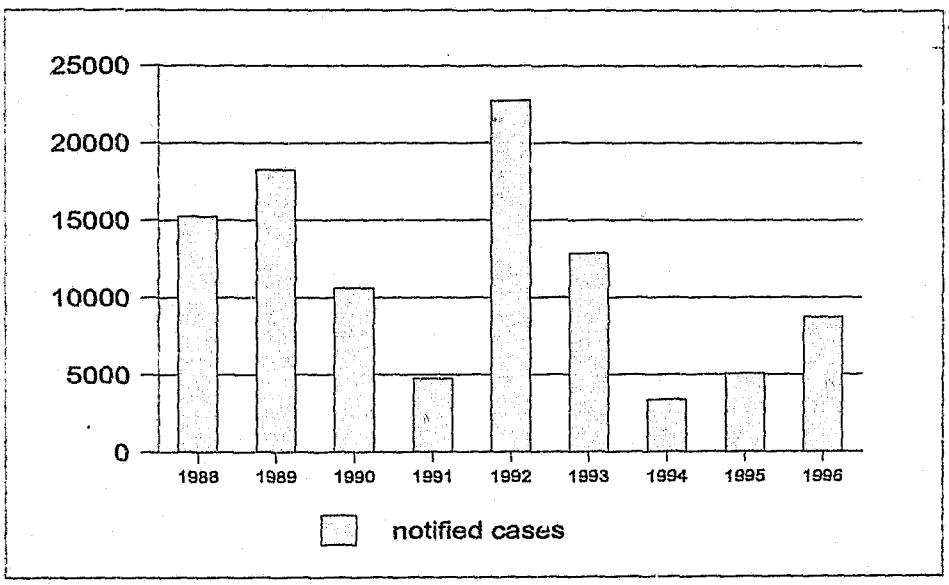
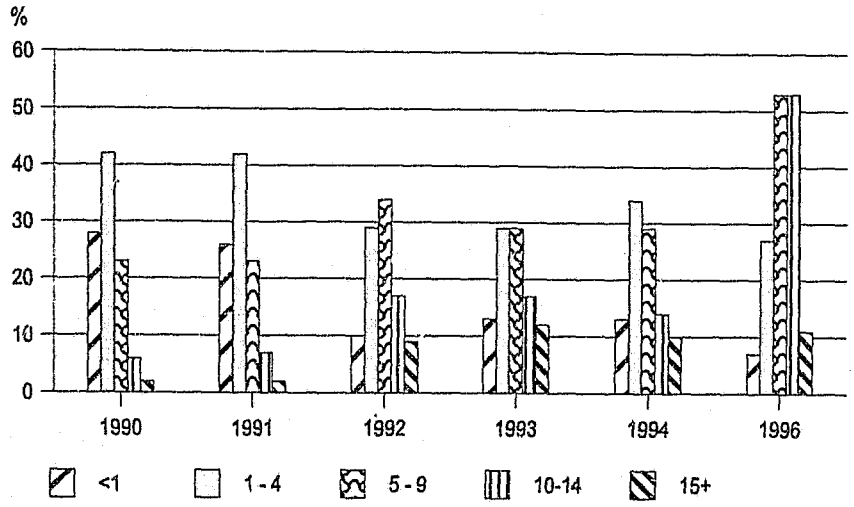


Fig. 1.8 Number of notified cases of measles infections in South Africa between 1988 and 1996.

Fig. 1.9 summarizes the age specific attack rates in South Africa from 1990 until 1996. A clear trend towards increased cases in children 5 years of age and older can be seen from 1992/93 onwards.



1996:double bar reflects age group 5-14; individual data for the age groups were not available. Data for 1997 were only available for the first half of the year with 422 cases notified from January to July '97.

Fig. 1.9 Age distribution of measles cases in South Africa between 1990 and 1996.

Cases in children under one year of age have decreased from 28% in 1990 to 8% in 1996. This reflects the global trend of measles cases shifting towards older children and young adults as vaccine coverage improves (Schoub, B.D.,1994a and 1994b; Schoub, B.D. and Martin, D.J., 1993; Padayachee, N., 1990).

1.8 Objectives of this study

The objectives of this study were:

1. To establish how many different viral groups of MV are circulating in South Africa (by sequence and phylogenetic analysis of the MV hemagglutinin (H) and nucleocapsid (N) genes).
2. To compare phylogenetic results for N and H genes and to compare South African MV strains to strains isolated elsewhere.
3. To determine whether genetic drift occurs over a period of 4 to 5 years in certain genetic groups.
4. To investigate the geographic distribution of genetic groups in South Africa.
5. To establish rapid, simple, and cost-effective pre-screening techniques that allow for reproducible genetic screening of multiple samples at a time.
6. To study basic antigenic properties of South African MV H proteins.
7. To analyse whether South African wild-type MVs downregulate the MV receptor CD46 and to establish whether wild-type and vaccine, or vaccine-like strains show differential downregulation patterns.

To date, very little is known about the molecular epidemiology of MV in African countries. Africa, especially sub-saharan countries still account for most measles cases and measles related deaths worldwide. Some information is available about strains that have circulated in Cameroon, Gabon and the Gambia some years ago (Outalw, M.C. et al., 1997; Rota, P.A. et al., 1994). MV remains to be a major health burden in Africa; improved vaccination strategies resulting in high enough vaccine coverages will certainly help to reduce measles infections. However, the knowledge of genetic groups circulating in a given country is the only way to identify imported from indigenous cases, and will therefore be a crucial tool in assessing the success of local vaccination programs. Continued serological and molecular epidemiological surveillance are important means for countries that are attempting to reduce the spread of the virus and eventually interrupt transmission of MV. Furthermore, the endemic presence of the virus in most African countries can serve as a continued source of importation of MV into countries that have already succeeded in interrupting transmission of the virus within their borders.

South Africa has enrolled in improved MV control strategies in 1990, when the first mass vaccination campaign was launched. In 1996 and 1997 2 further mass

vaccination campaigns followed in addition to continued attempts to improve routine vaccine coverage. Nevertheless, up until now MV circulated endemically causing unacceptably high numbers of measles cases. Now that vaccination programs seem to be showing the first effects of greatly reducing the numbers of cases, serological diagnosis will gain in importance as doctors see less cases and become unsure of the clinical signs of a measles infection. Molecular epidemiological studies will certainly play a vital role in assisting in the determination of how successful local elimination campaigns have been and will be. The knowledge of MV strains circulating in other southern African countries will become important for South Africa, as travelling across the borders increases and the risk for importation of MV from neighbouring countries is high.

In this study, 97 MV specimens collected between 1978 and 1997 were analysed in order to establish the molecular epidemiology of MV in South Africa. Of these samples, 44 were analysed by sequence analysis of the carboxyl-terminus of the N gene and/or the full coding sequence of the H gene. Furthermore, all specimens were pre-screened and analysed by HMA.

The genetic changes over time were compared and assessed for the specimens analysed. Despite efforts to include representative isolates from all 9 provinces, specimens from only 4 provinces were obtained. However, efforts will continue to collect MV specimens from all regions within the country and to further include specimens from neighbouring countries or other African nations. Sequences from southern African MV strains were compared to those obtained from the rest of the world.

The large number of specimens collected in southern African countries demanded the use of a rapid and reliable technique which would allow for genetic pre-screening of MV strains. A potential test system had to be easy to perform and require only standard laboratory equipment. Furthermore, a pre-screening technique had to be rapid, delivering results on multiple PCR products within a short period of time, and should preferably be more cost-effective when compared to techniques such as sequencing. Finally, pre-screening results had to be reproducible and to compare well with sequencing data obtained for the same specimens. Such a technique would be particularly useful for developing countries where only basic laboratory facilities are available and the cost for molecular epidemiological testing needs to be kept to a minimum.

The analysis of the antigenic properties of South African hemagglutinin (H) proteins was performed to establish whether the typical nt and aa changes seen in South

African H genes could change the migration patterns of the proteins and whether different antigenic epitopes exist on H when comparing South African MV strains to those described previously.

Finally, the differential downregulation of CD46 by South African wild-type versus vaccine-like strains was investigated by FACS analysis. Different titres were used as an input to establish whether varying concentrations of the virus would show dose-dependent effects on the downregulation. Further, strains grown in B95a and Vero cells were compared for their ability to alter the expression of CD46 on the host-cell surface.

2. MATERIALS AND METHODS

2.1 Virus isolation and specimens

2.1.1 Virus isolation

B95a is an EBV-transformed marmoset B-lymphoblastoid cell line and has been shown to be highly sensitive to measles virus (MV) (Kobune, F. et al., 1990). B95a monolayer cultures were used to isolate MV from either throat swab (TS) or urine specimens. Specimens were collected from patients meeting the clinical criteria for a measles infection, which are fever and maculopapular rash and one of the following: cough, coryza or conjunctivitis. TS's were transported to the laboratory in viral transport medium (VTM), containing 1% bovine plasma albumin in Dulbecco buffer. Upon arrival the VTM was spun at 13 000 rpm (20 800 xg) in an Eppendorf centrifuge 5415 C (Eppendorf, Hamburg, Germany) for 5 min. The pellet was resuspended in 1 ml of EMEM (Earle's minimum essential medium) supplemented with 2% fetal calf serum (FCS) and inoculated onto a 80-90% confluent monolayer of B95a cells in a T25 tissue culture flask. The virus was left to adsorb for 1 h at 37°C in a humidified atmosphere with 5% CO_2. After adsorption the virus inoculum was removed and stored at -70°C to allow for further virus isolation attempts if necessary. Six ml of maintenance medium (EMEM + 2% FCS) were added, cells again incubated at 37°C and observed daily for typical measles virus cytopathic effects (CPE), such as cell fusion and syncytia formation. When CPE approached 80-90%, cells were scraped off and centrifuged for 5 min at 1000 rpm in a GLC-4 Sorvall centrifuge (Du Pont). The pellet was either used for extraction of RNA (see 2.2.1) or for preparation of viral stocks by resuspending the pellet in an appropriate volume of EMEM + 5% FCS. Viral stocks were stored in aliquots at -70°C.

Urine specimens were treated as above with a minor modification. Upon arrival the urine was spun (5 min, 1000 rpm) and the pellet was washed once with PBS. The urinary pellet was then resuspended in 1 ml of EMEM + 2% FCS supplemented with Fungizone and an antibiotic cocktail (Penicillin 200U/ml, Streptomycin 0.2 mg/ml) and then inoculated onto B95a cells and treated as above.

2.1.2 Specimens

Apart from TS and urine specimens, several sera and cerebrospinal fluids (CSF) were analysed. For recent sera and CSF collected after 1990, IgM and IgG status was determined using the Enzygnost Anti-Measles virus IgM/IgG ELISA (Behring, Marburg, Germany) test according to the manufacturer's instructions. Samples collected before 1990 had unknown IgM status but were collected during clinically confirmed measles outbreaks within South Africa.
Between 1994 and 1998 a total of 143 specimens were processed for virus isolation. The majority of isolates were obtained from throat swab samples. A total of 100 measles virus isolates were prepared, 97 of which were further characterised. Table 2.1 summarizes information on all 97 measles specimens a selection for which sequencing or other results are presented in the results chapters. Strains marked with "(V)" were initially isolated on Vero cells by the diagnostic section at our Institute and viral stocks were stored at -70°C. All isolates made on Vero cells were further adapted to grow in B95a cells.

Table 2.1: Specimens analysed in this study.

strain*1	sex	age	race	immuniz. status	specimen
SA289/78	-*	-	-	-	serum
SA168/78	-	-	-	-	serum
SA280/78	-	-	-	-	CSF
SA361/78	-	-	-	-	serum
Jhb18/84	-	-	-	-	serum
Jhb1/86 (V)	F	-	-	-	TS*2
Jhb1/88 (V)	M	2	E*3	-	TS
Jhb2/88 (V)	-	-	-	-	-
Jhb1/89 (V)	F	1	B*3	-	TS
Jhb2/89 (V)	-	-	-	-	-
Jhb1/90	-	-	-	-	TS
Rdpt1/94	M	12y	E	no	urine
Jhb1/94	M	15y	B	yes	TS
Jhb2/94	M	21y	E	-	TS
Jhb3/94	F	7m	B	no	TS
NC1/95	M	3y	B	yes	TS

Table 2.1: continued

strain	sex	age	race	immuniz. status	specimen
Pta1/95	F	9m	E	no	TS
Mdbg1/95*1	M	9y	E	yes	TS
Mdbg2/95	M	11y	E	yes	TS
Mdbg4/95	M	2y	E	yes	TS
Jhb2/95	M	20y	B	no	TS
Jhb12/95	M	23y	B	yes	TS
Jhb13/95	F	6y	B	-	TS
Jhb14/95	M	20y	B	-	TS
Jhb20/95	F	10m	B	no	TS
Jhb22/95	F	8y	B	no	TS
Jhb23/95	F	6y	B	no	TS
Jhb24/95	M	4y	B	yes	TS
Jhb27/95	M	2y	B	yes	TS
Jhb28/95	F	3y	B	no	TS
Jhb29/95	M	1y	B	no	TS
Jhb30/95	M	6y	B	yes	TS
Jhb32/95	F	2y	B	-	TS
Jhb35/95	F	3y	B	no	TS
Jhb36/95	M	19y	B	-	TS
Jhb38/95	M	5y	B	-	TS
Jhb41/95	M	3y	B	no	TS
Jhb43/95	M	6y	B	-	TS
Jhb45/95	-	-	-	-	TS
Jhb49/95	M	7y	B	-	TS
Jhb50/95	F	4y	B	-	TS
Jhb51/95	F	6y	B	-	TS
Jhb54/95	M	3y	B	-	TS
Jhb55/95	M	5y	B	yes	TS
Jhb56/95	M	9y	E	-	TS
Jhb57/95	F	3y	B	-	TS
Jhb58/95	M	3y	B	-	TS
Jhb59/95	M	10y	B	-	TS
Jhb61/95	M	6y	B	-	TS
Mrd64/95*1	M	7y	B	-	TS
Jhb65/95	F	4y	B	-	TS
Jhb66/95	M	3y	B	-	TS
Jhb67/95	M	9y	B	-	TS
Jhb68/95	F	3y	B	-	TS
Jhb70/95	M	1y	B	no	TS
Jhb71/95	F	1y	B	-	TS
Jhb72/95	F	5y	B	-	TS
Jhb73/95	F	7y	B	-	TS
Jhb74/95	F	21y	B	-	TS
Jhb75/95	M	21y	B	-	TS
Jhb76/95	F	9y	B	-	TS
Jhb77/95	M	18m	C	-	TS
Jhb78/95	M	5y	B	-	TS
Jhb79/95	M	5y	B	-	TS
Natal1/96	M	7y	-	-	TS
Natal4/96	M	9y	-	-	TS

Table 2.1: continued

strain	sex	age	race	immuniz. status	specimen
Natal7/96	F	23y	-	-	TS
Natal8/96	M	18m	-	-	TS
Natal9/96	M	3y	-	no	TS
Natal10/96	F	9m	-	yes	TS
Natal12/96	-	-	-	-	TS
T3/96 (Jhb)	M	25y	E	yes	Urine
T5/96 (Jhb)	F	56y	E	no	Urine
Jhb5/96	F	9m	B	no	TS
Jhb6/96	M	10y	B	-	TS
Jhb14/96	M	1y	B	no	TS
Jhb18/96	F	15y	B	-	TS
Jhb23/96	M	19m	B	yes	TS
Jhb24/96	M	8m	B	no	TS
Jhb25/96	F	10m	E	-	TS
Jhb26/96	M	-	E	yes	TS
Jhb28/96	F	18m	B	-	TS
Jhb29/96	M	7y	B	-	TS
Jhb32/96	F	8y	B	-	TS
Jhb33/96	F	11y	E	-	TS
Jhb37/96	F	23y	E	no	TS
Jhb45/96	M	18y	E	-	TS
Jhb47/96	M	1y	B	-	TS
Jhb50/96	M	7y	B	-	TS
Jhb51/96	M	7m	B	no	TS
SSPEs/97PE	M	5y	B	-	serum
SSPEc/97PE	M	5y	B	-	CSF
Dbn1/97		-	-	-	serum
Wdk28/97	M	-	-	-	serum
Wdk91/97					serum
Jhb4/97	F	6y	B	-	TS
Jhb5/97	F	22y	B	-	TS

* unknown

*1 abbreviations indicate origin of specimen/ year of collection:
- SA: South Africa
- Sow: Soweto, Gauteng Province
- Jhb: Johannesburg, Gauteng Province
- Rdpt: Rodepoort, Gauteng Province
- NC: Newcastle, Natal Province
- Mdbg: Middleburg, Mpumalanga Province
- PE: Port Elizabeth, Eastern Cape Province
- Pta: Pretoria, Gauteng Province
- Mrd: Midrand, Gauteng Province
- Natal: Ubanbo, Natal Province
- Dbn: Durban, Natal Provinve
- Wdk: Windhoek, Namibia

*2 TS: Throat Swab

*3 E: European, B: Black, C: Coloured

2.2 Extraction of RNA

Nucleic acids were extracted either from virus-infected B95a cells or directly from clinical samples (serum and CSF).

2.2.1 Extraction of RNA from virus-infected cells

This technique has been described by Chomczynski, P. and Sacchi, N. (1987) and was successfully applied to extraction of total RNA from B95a cells (2.1) infected with MV obtained from TS or urine specimens. The method utilizes the strong protein denaturing abilities of guanidinium thiocyanate (GTC) together with the simultaneous inactivation of ribonucleases (RNase). Cells are lysed and denatured, almost instantaneously, in a 4M solution of GTC containing the mild detergent sarkosyl. The method then takes advantage of the observation that RNA can be separated from DNA and proteins because of its greater density. RNA can be extracted under acidic conditions because it remains soluble in a 4M GTC solution (pH 4) in the presence of a phenol/chloroform organic phase, whereas most proteins and smaller DNA fragments (50 bp to 10 kbp) are in the organic phase and larger DNA fragments and some proteins remain at the interphase. The fragmentation of DNA during homogenization helps to remove DNA from the H_2O-soluble phase. All solutions were prepared using RNase-free chemicals and RNase-free H_2O.

Method and solutions:

GTC solution: 4M guanidinium thiocyanate
25mM sodium citrate (pH 7.0)
0.5% sodium sarkosyl
0.1M 2-mercaptoethanol, added just before use

B95a cells showing CPE typical of MV were scraped off and centrifuged at 1500 rpm for 5 min. The resulting cell pellet was resuspended in 100 µl of PBS and added to 400 µl of GTC solution. Cells were homogenized in this solution by pipetting them up and down 10-20 times. To obtain an acidic pH, 50 µl of a 2M sodium acetate solution (pH 4.0) was added and mixed by inversion of the 1.5 ml Eppendorf tube. RNA was extracted by addition of 500 µl of H_2O-saturated Phenol and 100 µl of Chloroform and was chilled on ice for 15 min. Phases were separated by centrifugation 13 000 rpm for 30 min at 4°C. The aqueous phase was then transferred to a fresh tube and precipitated with 600 µl of ice cold isopropanol for at least 1 h at -20°C. The nucleic acids were centrifuged as above and then

resuspended in 150 µl of GTC solution and 150µl of chilled isopropanol and placed at -20°C for another hour. Centrifugation was once more performed as above and the resulting pellet was washed 3x with 75% EtOH. The nucleic acid pellet was dried at 37°C for 15-30 min and then resuspended in 20 µl of RNase-free H_2O.

Commercial kits for extraction of RNA:

Alternatives to the above method of RNA extraction which was used for the majority of MV isolates were the High Pure RNA Isolation Kit (Boehringer Mannheim, Germany) or the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). Both were used when RNA was needed within a shorter period of time. The kits utilise microspin technology and achieved the extraction of high yields of RNA within 15-30 min. The BM kit is optimized for RNA to bind to a glass fibre fleece whereas the Qiagen kit uses a silica gel membrane for the selective binding of RNA. Both kits were used according to the manufacturer's instructions.

2.2.2 Extraction of RNA from clinical samples

Several methods including the techniques described under 2.2.1 were tested for the efficient extraction of RNA from clinical samples such as serum and CSF. Of these, the method described below proved to be most efficient with regard to the quality, yield of extracted RNA and speed of isolation.

Method and solutions:

GITC solution: 4M Guanidinium isothiocyanate, dissolved in dH_2O
25 mM sodium citrate, pH 7.0
0.5% lauroyl sarcosine
0.1 M 2-mercaptoethanol

200 µl of serum or CSF was thawed on ice and added to 600 µl of chilled GITC solution. After incubation on ice for 5 min, a 1/10 volume of 3M sodium acetate, pH 5.0 was added and RNA was extracted with 500 µl of water-saturated phenol and 100 µl of chloroform. Nucleic acids were precipitated with isopropanol at -20°C for a minimum of 1 hour. Centrifugation at 13 000 rpm for 10 min resulted in a pellet that was then washed 3 times with 70% ethanol. The extracted nucleic acids were dried at 56°C for 3 min and at 37°C for 5 min. RNA was resuspended in 12 µl of nuclease-free water and used directly for RT-PCR as described in section 2.4.3.

2.3 RT-PCR

Two different RT-PCR protocols were used to amplifiy MV-specific nucleic acids. The first method reverse transcribes viral RNA into cDNA using MV-specific primers and was used for RNA extracted from MV-infected B95a cells. The second approach used random hexanucleotide primers for transcription of RNA extracted directly from clinical samples (sera and CSF's).

2.3.1 Primers for RT-PCR

The primers used for amplification of either the MV H, or partial N genes are summarised in Table 2.2. Primers were designed using the DNASIS for Windows software package (Hitachi, version 2.5).

Table 2.2: Measles virus-specific PCR primers

primer	position	size	sequence
N1→*[1]	86-103	333	5' CATTACATCAGGATCCGG 3'
N2←[1]	402-419		5' GTATTGGTCCGCCTCATC 3'
N5→	1030-1053	587	5' GCTATGCCATGGGAGTAGGAGTGG 3'
N8←	1599-1617		5' GGCCTCTCGCACCTAGTCTAG 3'
N16→	988-1017	375	5' TTCAGAACAAGTTCAGTGCAGGATCATACC 3'
N17←	1332-1363		5' CCTCGACTCTGTTTGACCCTCCTATCTTCC 3'
N18→	1235-1264	382	5' TGAGGACAAGATCAGTAGAGCCGGTTGGACC 3'
N8←	as above		as above
H1→[2]	-10-18	1874	5' GATCGAATTCATGTCACCACAACG 3'
H2←[2]	1837-1864		5' GATCGGATCCCTATCTGCGATTGG 3'

* N: nucleocapsid gene, H: hemagglutinin gene, F: fusion gene
→ :forward orientation, ←: reverse orientation

1 as described by Rota, P.A., et al., 1995.

2 as described by Rota, J.S., et al., 1994.

2.3.2 RT-PCR of MV isolates

Method and solutions:

10x PCR buffer: 500mM KCl
100mM Tris-HCl (pH 8.3)
15mM $MgCl_2$

dNTP mix: dATP, dTTP, dCTP, dGTP at a concentration of 10mM each

All solutions, buffers, and enzymes were obtained from Boehringer Mannheim unless stated otherwise.

Reverse transcription (RT):

For reverse transcription of RNA into cDNA, 3µl of RNA (2.2.1) was combined with 1 µl of each forward and reverse primer (20 µM), 2 µl of a 10x PCR buffer, 8 µl of dNTP mix, and an additional 4 µl of 25 mM $MgCl_2$. Depending on the fragment to be amplified, primer pairs used were either N1-N2, N5-N8, or H1-H2 (Table 2.2). For annealing of the primer to the RNA, the RT mix was heated for 90 sec at 95°C, then cooled at room temperature for 5 min and placed on ice for a further 5 min. To the chilled RT mix, 1 µl of AMV-RT (10 U) and 1 µl of RNasin (20 U) were added and incubated for 50 min at 42°C. The RT enzyme was inactivated by heating the mixture for 5 min at 95 °C.

PCR:

PCR was performed by adding an additional 4 µl of each forward and reverse primer (as above) to 20µl of cDNA reaction mix, as well as 8 µl of the 10x PCR buffer described above, 5U of Taq polymerase and dH_2O to a final volume of 100 µl. Cycling conditions were as follows: 95°C for 1 min, 30 cycles of 95 °C for 1 min, 50°C for 2 min, and 72°C for 2 min. PCR products were visualized on a 1% agarose gel (2.3.4).

2.3.3 RT-PCR of clinical samples

Method and solutions:

5x RT buffer: 250 mM Tris-HCl (pH 8.5)
40 mM $MgCl_2$
150 mM KCl
5 mM DTT

Reverse transcription (RT):
To 12 µl of extracted nucleic acids (2.2.2), 0.2 µg of random hexamer (N_6) primers (Boehringer Mannheim) were added and incubated at 70°C for 10 min and then chilled on ice for 5 min. To the annealed primer-template mix, 4 µl of 5x RT buffer, 1 µl of dNTP mix and an additional 2 µl of 0.1M DTT were added. After incubation for 10 min at 25°C and for 2 min at 42°C, reverse transcription was initiated by the addition of 20 U of AMV-RT. Incubation was continued for 50 min followed by the denaturation of the AMV-RT for 5 min at 85°C.

PCR:
3-5 µl of cDNA were added to a PCR master mix containing 10 µl of 10x PCR buffer, 3 µl of 25 mM $MgCl_2$, 3 µl of dNTPmix, 60 pmol of each forward and reverse primer, and 5 U of Taq polymerase in a total volume of 100 µl. MV-specific primer pairs N16-N17 and N18-N8 (Table 2.2) amplified two overlapping fragments of the carboxyl-terminus of the N gene of 375 bp and 384 bp length, respectively. PCR cycling conditions were as follows: 95°C for 1 min followed by 30 cycles of 94°C for 50 sec, 50°C for 1 min, and 72°C for 1min with an additional 1 sec per cycle and a final incubation for 5 min at 72°C.10 µl of PCR products were visualized on 1.5% agarose gels. Where first-round PCR products were poorly visible, a second round PCR was performed using the above mentioned primers and conditions, and 5-10 µl of the first-round product as template.

2.3.4 Agarose gel electophoresis

Depending on the size of the fragments to be analysed, 10 µl of PCR products were electrophoresed on 1-2.5% agarose gels containing ethidium bromide at a concentration of 1µg/ml. Gels were run in 1x TBE (Tris-boric acid-buffer) at 100V for 1-2 h. DNA bands were visualized on a UVP UV transilluminator (UVP, Life Science, California, USA) and their size estimated by comparison with DNA molecular weight markers (Boehringer Mannheim).

2.4 Sequence analysis

Sequence analysis of the short fragments (456 bp) of the carboxyl-terminus of the MV N gene was performed manually, directly on PCR-amplified fragments. Sequences of the entire H gene covering 1874 bp were determined using 2 different

automated sequencing apparatuses. Sequencing primers are summarised in Table 2.3.

2.4.1 Sequencing primers

Table 2.3: Sequencing primers

primer	position	sequence
N5→*	1030-1053	5' GCTATGCCATGGGAGTAGGAGTGG 3'
N8←	1617-1599	5' GGCCTCTCGCACCTAGTCTAG 3'
N6→	1206-1230	5' CTTGTTTCAGAGATTGCAATGCAT 3'
N17←	1363-1323	5' CCTCGACTCTGTTTGACCCTCCTATCTTCC 3'
N7→	1478-1502	5' AGATCCGCAGGACAGTCGAAGGTC 3'
H1	-10-18	5' GATCGAATTCATGTCACCACAACG 3'
H2	1864-1837	5' GATCGGATCCCTATCTGCGATTGG 3'
H3→	399-423	5' AGAGATCTCACTTGGTGTATCCAAC 3'
H4→	753-774	5' GTACCGAGTGTTTGAAGTAGG 3'
H5←	1145-1167	5' TCCAACAGGCGTGTAAGGGTAA 3'
H6→	998-1024	5' GCAATCCTGGGTCCCCTTATCAACGG 3'
H7←	1023-995	5' CGTTGATAAGGGGACCCAGGATTGCATG 3'

* N: nucleocapsid gene, H: hemagglutinin gene
→: forward orientation, ←: reverse orientation

2.4.2 Manual sequencing

Method and solutions:

GTG electrophoresis buffer: 1.78M Tris
0.58M Taurine
10mM EDTA

Manual sequencing of the carboxyl-terminal 456 bp of MV N genes was performed directly on PCR products. 5 µl of PCR products were pre-treated enzymatically with 1 µl of Exonuclease I, which removes residual single-stranded primers and any single-stranded DNA products produced during the PCR reaction. Shrimp alkaline phosphatase (1 µl) was added to remove remaining dNTPs. Annealing of the sequencing primer to the template, labelling reactions using [^{35}S]-dATP (ICN,

California, USA) and the termination reactions were set up using the Sequenase PCR Product Sequencing Kit (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's instructions. DNA bands were separated during denaturing gel electrophoresis on an 8% polyacrylamide gel electrophoresed at 2000 V for 2-6h. The gel mixture as well as the electrophoresis buffer contained glycerol tolerant gel buffer (GTG). Following electrophoresis, gels where fixed in 5% acetic acid and 15% methanol to remove urea. The gel was vacuum-dried at 80°C for 1h and then exposed to Hyperfilm ßmax (Amersham) autoradiographic film for 12- 36 h.

2.4.3 Automated Sequencing

Sequences of the entire hemagglutinin gene (1874 bp) were determined by automated sequencing on either an ABI PRISM 377 DNA sequencer (Perkin Elmer, New Jersey, USA) or on an ALFexpress sequencer (Pharmacia Biotech, Uppsala, Sweden).

ABI 377 sequencing:
PCR products were purified using CentriStep columns (Princeton Separations, NJ, USA). Purified PCR products were cycle sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Roche Molecular Systems, NJ, USA) according to the manufacturer's instructions.
ALFexpress sequencing:
The ALFexpress sequencer was used for sequence analysis of cloned H genes (2.5). Cloned products were sequenced with unlabelled primers using the Cy5 AutoRead Sequencing kit with the Cy5-dATP Labelling Mix (Pharmacia) according to the manufacturer's instructions.

2.5 Molecular cloning of measles virus H genes

MV H genes were amplified using primers H1 and H2 (Table 2.2). Primer H1 contained a unique recognition site for the restriction enzyme EcoRI and H2 contained a unique BamHI site. PCR products were purified using the Wizard PCR Preps DNA purification kit (Promega, Madison, USA). 10 µl of the resultant PCR product was double-digested with EcoRI and BamHI for 3h at 37°C in a total volume of 20 µl. H genes were ligated into the Bluescript II SK+/- plasmid (Stratagene,

California, USA), pre-digested with the same restriction enzymes and treated with calf intestine alkaline phosphatase (Boehringer Mannheim) to prevent self annealing of the vector by dephosphorylation of the 5' phosphorylated ends. Alternatively, unpurified PCR amplified H genes were cloned directly into the pre-digested plasmid pMOS*Blue* T-vector (Amersham). Transformation into provided competent cells was carried out as outlined in the instructions accompanying the cloning kits. LB plates were coated with 35 µl of X-Gal (50 mg/ml) and 20 µl of IPTG (100 mM) 1 h prior to use. Recombinants were blue/white-screened according to standard procedures and as described in the kit manual. White colonies were picked from LB agar plates containing ampicillin (50 µg/ml) and tetracycline (15 µg/ml). White colonies were then grown over night (o/N) in 2 ml LB medium and plasmid minipreparations were performed using the Wizard Miniprep kit (Promega). Plasmid DNA's were checked for the presence of the right insert by double-digestion with BamHI and EcoRI or by PCR amplification of the H gene insert. Minipreparations containing the MV insert were inoculated into larger volumes of LB medium and plasmid DNA was isolated using the QIAGEN Plasmid Maxi kit (Qiagen).Cloned H genes were stored at -70°C and used mainly for sequence analysis on the ALFexpress automated sequencer (2.4.3).

2.6 Genetic pre-screening of measles virus strains

The large number of MV isolates that required genetic analysis, demanded the use of a rapid and reliable pre-screening technique. For this purpose, restriction fragment length polymorphism (RFLP) and the heteroduplex mobility assay (HMA) were evaluated for use in pre-screening analyses of MV isolates.

2.6.1 Heteroduplex mobility assay

For HMA analysis, equal quantities of 2 different PCR-amplified DNA fragments from a reference and a test sample were mixed together in a 10 µl volume, denatured at 95°C for 5 min and reannealed by slow cooling in a 90°C water bath which was left to cool to room temperature over a period of 45-60 min. The Schwarz vaccine strain (Rimevax, Smith Kline & Beecham, T501) served as a reference for heteroduplex formation with the South African strains to be analysed. Heteroduplexes were resolved by non-denaturing gel electrophoresis. Separation of heteroduplexes was performed at 370V for 5.5 h on a 16 cm gel at constant temperature (40°C) using a 1% mutation detection enhancement (MDE) gel matrix (FMC Bio Products,

Rockland, USA) in 0.6x TBE-buffer (60 mM Tris, 50 mM boric acid, 1.2 mM EDTA), 15% urea and 2% glycerol. MDE gels were stained for a minimum of 1 h with SYBR-Green (Molecular Probes Europe BV, Leiden, Netherlands).

2.6.2 Restriction Fragment Length Polymorphism

Several restriction enzymes were selected for RFLP analysis of MV carboxyl-terminal N genes, which were amplified as described under 2.3.2. A restriction enzyme table and map was constructed for representatives of the 3 South African genetic groups with the WDNASIS program (Hitachi, version 2.5).

Method:

The carboxyl-terminus of MV N genes was amplified using primers N5 and N8 (Table 2.2). PCR products were purified with the QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. 5-10 µl of purified PCR product was then digested with 1 µl (10 U) of restriction enzyme, 2 µl of an appropriate 10x incubation buffer (Promega, Madison, USA) in a total volume of 20 µl for 3 h at 37°C. Fragments were separated on a 2.5% agarose gel as described under 2.3.4.

2.7 Antigenic analysis of measles virus H proteins

The antigenic properties of MV H proteins were analysed using two different approaches, the radioimmunoprecipitation assay and a Western Blot analysis.

2.7.1 Radioimmunoprecipitation assay (RIPA)

Method and solutions:

starvation medium: methionine-free EMEM
10% dialyzed FCS
10% normal EMEM

NET-BSA: 150 mM NaCl
5 mM EDTA

50 mM Tris-HCl (pH 7.4)
0.5% Nonidet P40
1 µg/ml BSA

Protein G Agarose: Protein G Agarose was washed 3x in NET-BSA prior to use. The final solution was diluted 1:1 in NET-BSA.

Sample buffer: 1M Tris-HCl, pH 6.8
10% SDS
15% Glycerol
10% 2-Mercaptoethanol
0.01% Bromophenol Blue

Radiolabelling of cells with ^{35}S-methionine:

Viral stocks were inoculated onto B95a monolayer cells in T25 flasks as described in 2.1 and were incubated until CPE reached ~ 90%. The maintenance medium (EMEM + 2% FCS) was then removed and the cells washed once with methionine-free EMEM. Cells were starved of methionine for 1h at 37°C by the addition of 2 ml of starvation medium. The addition of 10% normal EMEM to this medium aided in the completion of protein synthesis and post transcriptional modifications. The medium was aspirated and 2 ml of fresh starvation medium together with 100 µCi of ^{35}S-methionine (ICN, California, USA) was added and the cells were left o/N at 37°C. Cells were scraped off gently and pelleted at 2500 rpm for 5 min. The supernatant was carefully removed with a thin pasteur pipette and the pellet was washed 1x in chilled PBS.

Immunoprecipitation of H proteins:

The cell pellet was resuspended in 1 ml of NET-BSA and was kept on ice for 20 min. Cells were pelleted at 13 000 rpm for 5 min and the supernatant (= cytoplasmic fraction) transferred to a new tube and was either used immediately for precipitation or was stored at -20°C. Anti-H monoclonal antibodies (diluted in NET-BSA, to yield a 1:100 diluton in the final volume) were added to the lysates and left to bind to the antigen for 45 min on ice. The α-H monoclonal antibodies, CV2, CV4, CV5, V17, B2, and 366 were kindly provided by Dr. P. Rota (CDC, Atlanta, USA). 30 µl of Protein G agarose (Boehringer Mannheim) was added and incubated for 2h on ice. Immune complexes were then centrifuged at 2500 rpm for 5 min at 4°C and washed 3x in NET-BSA buffer and 1x in cold PBS. The precipitated antigen/antibody complexes were resuspended in 50 µl sample buffer and boiled for 5 min. Cell debris was spun

down at 13 000 rpm for 5 min (4°C) and the supernatant recovered into a fresh tube and stored at -70°C. Prior to loading onto polyacrylamide gels, samples were again boiled for 5 min and centrifuged as above.

SDS-PAGE:
Method and solutions:

10% resolving gel:		4%stacking gel:
26.7 ml	Acrylamide/Bisacrylamide (30:0.8)	2 ml
30 ml	1M Tris-HCl, pH 8.8	1.9 ml (pH 6.8)
0.8 ml	10% SDS	150 µl
-	80% glycerol	1 ml
18.5 ml	dH_2O	9.25 ml
4 ml	1.5% APS	700 µl
20 µl	TEMED	20 µl

10x Laemmli buffer:	0.25M Tris-HCl, pH 8.3
	1M Glycine
	0.1% SDS

20-30 µl of sample buffer containing the immuno-precipitated H proteins were loaded on a polyacrylamide gel and run at 50V or for 6-8h at 150V in 1x Laemmli buffer. Gels were fixed for 15-30 min in 45% MetOH and 15% acetic acid, soaked briefly in H_2O and then dried for 1h at 80°C. The dried gel was exposed to a βmax Hyperfilm (Amersham) for 2-3 days.

2.7.2 Western Blot

Protein extracts:
Virus stocks were inoculated onto B95a monolayers as described above (2.7.1). When CPE reached ~ 90%, cells were scraped off and pelleted for 5 min at 2500 rpm. The cell pellet was resuspended in 50 µl of 2% SDS in 0.1M Tris-HCl (pH 6.8) and passed 20x through a syringe (23 gauge) to homogenize the cells. 5 µl of the protein extract was diluted in 0.1M NaOH to measure the protein concentration. Absorptions were determined at 280 nm on the Spectronic 1001 Spectrophotometer

(Bausch&Lomb). The amount of cell suspension to yield 15 µg of protein was calculated using the following formula: (0.8/E) x 8 = µl cell extract per lane. E= extinction.

SDS-PAGE:

Media and solutions:

Transfer buffer (pH 8.3):	25 mM Tris 192 mM glycine 0.25% SDS 20% methanol
TBS buffer (pH 7.5):	50 mM Tris 150 mM sodium chloride
TBST buffer:	TBS buffer + 0.1% Tween 20

A 10% resolving gel with a 4% stacking gel was run as described above (2.7.1). After electrophoresis was completed, the gel was equilibrated for 10 min in transfer buffer. A PVDF membrane (Boehringer Mannheim) was soaked in MetOH for a few seconds and then submerged in transfer buffer for 5-10 min. Proteins were transferred onto the PVDF membrane in a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Biorad, California, USA) for 1h at 18 V according to the manufacturer's instructions. To visualize protein bands, the membrane was reversibly stained in Ponceau S solution (Sigma, St. Louis, USA) and destained in H_2O. The protein molecular weight marker (Gibco BRL) lane was cut off with a scalpel, stained in amido black solution (Sigma) for 2 min and then destained (in 10% acetic acid and 15% isopropanol) for 1h. Antibody-specific staining of the remaining membrane was performed using the BM Chromogenic Western Blotting Kit (Boehringer Mannheim) according to accompanying instructions. Briefly, the membrane was washed twice in TBS and non-specific binding of antibody was blocked for 30 min in 1% blocking solution. The membrane was then incubated with the primary monoclonal antibody diluted 1:1000 in blocking solution. Following incubation for 30 min the membranes were washed 4x in TBST buffer and secondary antibody-conjugate (Anti-mouse Ig/anti-rabbit IgG-AP) added for 30 min at a concentration of 800 mU/ml. Membranes were then washed as before. Finally, the colour reaction was initiated by the addition of 12 ml of chromogenic substrate for every 10x10 cm membrane and developed for 10-30 min.

2.8 FACS analysis

Method and solutions:

wash buffer: 0.5% BSA
0.01% NaN_3
in PBS

cell fixative: 1.5% formaldehyde
2% BSA

B95a or Vero cells were grown in 6- or 12-well plates and monolayers (90% confluent) were infected with different dilutions or titres (2.9) of MV stocks. When CPE reached 50-90%, cells were gently scraped and pelleted for 5 min at 1500 rpm. After complete removal of the SN, the cell pellet was washed with 500 µl of wash buffer. Cells were then resuspended in 100 µl of wash buffer and divided into 2 Eppendorf tubes (50 µl each). One aliquot of cells was incubated with 2.5 µl FITC-labelled mouse-anti-human-CD46 antibody, clone 122-2 (Dako, Denmark); the second one was incubated with a control FITC-conjugated mouse anti-human IgG1 antibody (Dako, Denmark). Antibody-cell suspensions were incubated on ice for 30 min, pelleted and resuspended in 200 µl of cell fixative. Fluorescence was analysed on a FACSort Flow cytometer with a 488 nm argon laser (Becton Dickinson, California, USA) and the Cellquest™ (version 1.0) software.

2.9 Plaque reduction assay

To determine the titre of MV stocks, a plaque reduction assay was performed. This technique has been used to titrate viruses on various cell lines including Vero cells. The method below summarises the titration of MV on B95a cells. Timing in this assay proved to be critical since B95a cells tended to round up and lift off the culture dish after the 3rd day post infection (p.i.) under the agarose overlay. The identical protocol, however, worked well for Vero cells.

Method and solutions:

Overlay medium: 0.5 volume of 2% Sea Plaque Agarose, autoclaved (FMC, Rockland, USA)
0.5 volume of 2x Hanks minimum essential medium (H-MEM)
5% FCS

Vero or B95a were seeded into 6-well plates at 2.5 x10^5 and 3 x10^5 cells per ml, respectively, and were incubated at 37°C o/N. Monolayers were washed once with EMEM + 2% FCS and then infected in triplicate with 10-fold serial dilutions of viral stocks. Dilutions were made in Leibowtiz medium + 5% FCS. Viruses were adsorbed for 1h at 37°C in a moist chamber. The virus inoculum was then aspirated and 3ml of overlay medium added to each well. Plates were kept at 4°C for 15 min for the agarose to set, then inverted and incubated at 37°C in a moist chamber until plaques became visible. Cells were then fixed with 3 ml of 5% formaldehyde in PBS per well and left at room temperature for 2-4 h. Fixative and agarose were removed and cells were stained for a minimum of 3 h with 0.1% crystal violet in 50% EtOH. Plates were dried at room temperature and plaques were enumerated.

2.10 Infection inhibition assay

B95a cells were grown in 6-well plates until they reached 80-90% confluence. Monolayers were then washed with PBS and incubated with or without mAb against CD46, clone J4/48 (10 µg/ 200 µl per well) (Biodesign International, Kennebunk, ME) at 4°C for 45-60 min. Viral stocks were added and further processed as described under 2.8.

2.11 Computer software

All polaroid photographs or gel images printed with the UVP white/ UV transilluminator (UVP) were scanned with the ScanAce III (Pacific Image Electronics) scanner and processed using the Adobe Photoshop software package (version 4.0).

The Phylip inference package, version 3.5c (Felsenstein, J., 1985 and 1989) was used for phylogenetic analysis of sequence data. Large data sets had to be divided into smaller sets in order to perform bootstrap analysis. Results obtained with the Phylip program were very similar to those obtained with the ClustalW program (Thompson et al., 1994).

Sequences were aligned using the WDNASIS for Windows (Hitachi Software Engeneering, version 2.5). This program was also used for generation of restriction enzyme maps and for design of PCR primers.

3. RESULTS

CHAPTER 3.1 MOLECULAR EPIDEMIOLOGY OF SOUTH AFRICAN MEASLES VIRUS STRAINS - ANALYSIS OF THE NUCLEOCAPSID GENES

3.1.1 Introduction

The main objectives of this study were (i) to determine the sequence variation among South African (SA) MV strains, (ii) to establish how many distinct genetic groups are currently circulating in South Africa, (iii) how SA genetic groups compare to those circulating elsewhere, and (iv) whether a significant genetic drift in SA MV isolates could be seen over a period of 4 years.

The majority of published sequences have been obtained from MV strains originating in Europe and the USA. Depending on the criteria used for allocation of MVs into separate genotypes, 7 to 8 different genotypes have been described to date (Rima, B.K. et al., 1995b). Several of these appear to be geographically restricted and still circulating, whereas others seem to be extinct (Rima, B.K. et al., 1995a; Rota, P.A. et al., 1994). The carboxyl-terminus of the MV N gene has been widely used for genetic characterisation and differentiation of wt, vaccine-like and vaccine strains. Most sequences published include the COOH-terminal 456 nt or 151 aa. This region of the genome has the highest variability and nt divergence of up to 12% has been reported between the most distant genetic groups (Bellini, W.J. and Rota, P.A., 1998). However, although a RNA virus, MV is relatively stable and nt variation for this region averages around 7-8%.

Molecular surveillance of MV strains in outbreak situations has enabled the identification of transmission patterns and importation of viruses into countries, as was demonstrated for a virus strain from the United Kingdom associated with a measles outbreak in the USA in 1989/90 (Rota, P.A. et al., 1994). However, the real geographical distribution and the question of how many lineages exist in the world can only be determined by including more sequences from Asia, Australia, and Africa. Until tis study, the only sequences available from the African continent were derived from isolates made in Gabon (1983), Cameroon (1984) (Giraudon, P. et al., 1988; Rota, P.A. et al., 1994) and Gambia (1994) (Rota, J.S. et al., 1996; Outlaw,

M.C. et al., 1997), which showed little relation to any of the previously described strains.

The endemic occurrence of MV in South Africa offers an opportunity to study a greater number of isolates and to monitor the evolution of the virus under circumstances of relatively low vaccine coverage, a condition which can still be found in some parts of the country (Dept. of Health, South Africa, 1995).

Similar studies that have described genetic variations of MV strains are summarised under 1.4.2 and 1.4.3. This was the first study to investigate the molecular epidemiology of MV in a southern African country. Africa still accounts for the majority of annual measles infections worldwide. The knowledge of the genetic properties of the virus circulating under endemic conditions in Africa can make valuable contributions towards the goal of eradication of MV in southern Africa and worldwide. Furthermore, molecular epidemiological surveillance plays an important role in assessing the success of national or local immunization programs. Since South Africa is moving forward toward better control and final elimination of the virus from within it's borders, the knowledge of indigenous genetic groups will enable us to identify the presence of imported viruses.

3.1.2 Analysis of N gene sequences of measles virus strains

(A) Analysis of recent measles virus isolates

To establish the molecular epidemiology of MV in SA, carboxyl-termini of the N genes of 42 MVs have been sequenced and phylogenetically analysed. Thirty-three of the 42 strains were isolated in tissue culture (2.1.1). The remaining 9 sequences were obtained by directly analysing clinical samples such as sera and CSF's, the results of which are presented in 3.1.2 (B).

MV specimens (urine or throat swab) were isolated on B95a cells as described in 2.1.1, total RNA was extracted (2.2.1) and carboxyl-termini of the N genes were amplified (2.3.2) using primers N5 and N8 (Table 2.2.1). Fig. 3.1 shows the PCR amplification products of selected MV strains. The amplification of the carboxyl-terminus of the N gene resulted in a 589 bp fragment. The figure also shows PCR products of the full H gene (1874 bp) which will be discussed further in chapter 3.2.

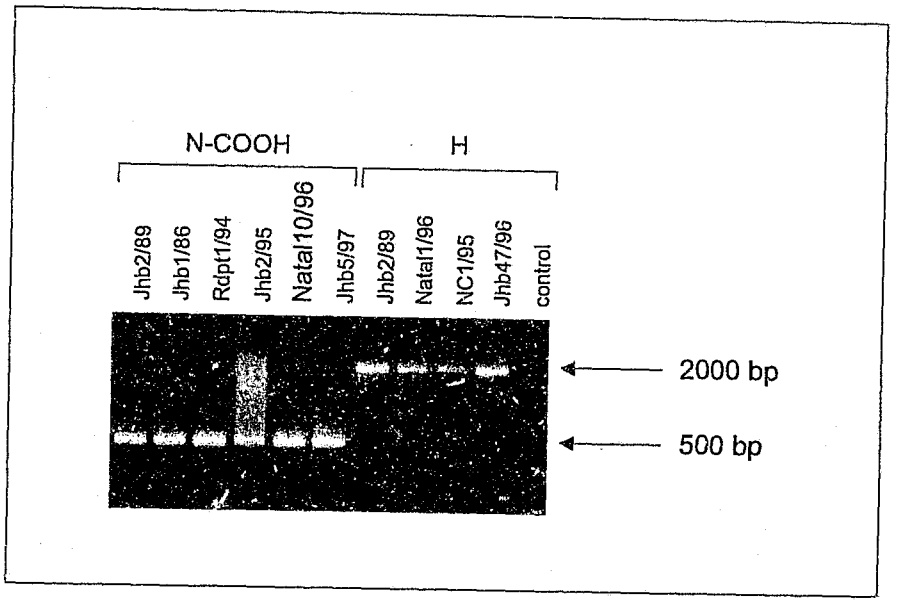


Fig. 3.1 PCR amplification products of the carboxyl-terminus of the N gene and the full H gene for a selection of South African MV strains.

(B) Regional origins of MV strains analysed for this study

Fig. 3.2 shows a map of South Africa with the 9 official provinces. Provinces from which specimens were obtained for this study are highlighted. Despite substantial effort to include collection of specimens from all 9 provinces, to date only 4 provinces have participated in the molecular epidemiological measles surveillance program. The majority of specimens were collected in Gauteng, the province in which Johannesburg is located. The number of specimens analysed from regions other than Gauteng totalled 15. Efforts will continue to include sampling of MV specimens from more provinces and from neighbouring countries. General information on all specimens analysed was summarised in Table 2.1.

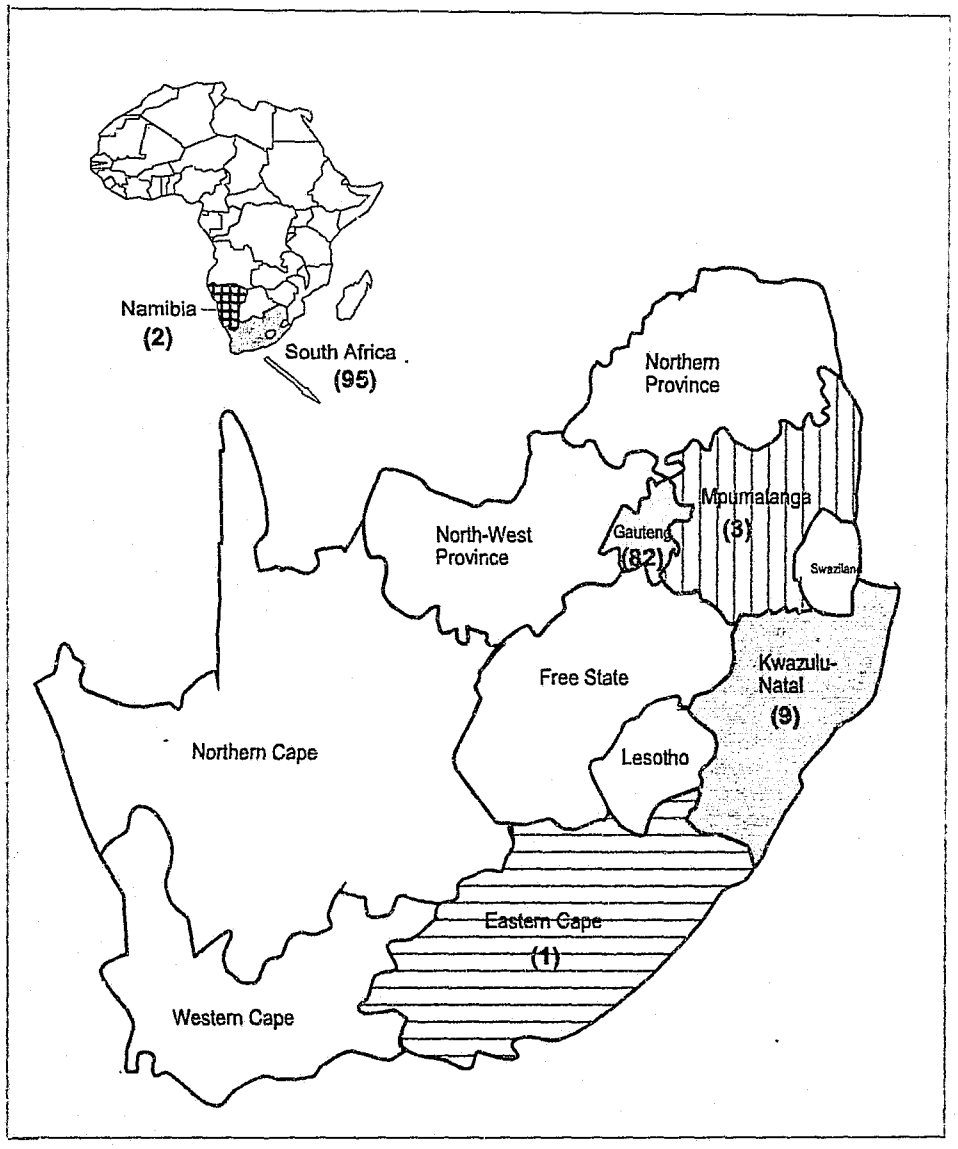


Fig. 3.2 Map of South Africa showing the regional origin of specimens analysed for this study. Provinces from which specimens where obtained for this study are highlighted and the number of strains analysed for each province is indicated in brackets.

3.1.3 Analysis of stored sera and cerebrospinal fluids for molecular epidemiological studies

The analysis of stored sera for retrospective molecular epidemiological studies provided a powerful tool to investigate strain variation in measles viruses that had circulated in South Africa up to 20 years ago. For this purpose, a rapid and simple method for extraction of RNA from stored sera and cerebrospinal fluids (CSF) was developed (2.2.2 and 2.3.3) (Kreis, S. and Schoub, B.D. et al., 1998).
When used on sera and CSF's that had been frozen for as long as 20 years, this method proved to be more efficient than established techniques. The extracted RNA was reverse transcribed into cDNA by using hexamer random primers. The PCR amplification of the carboxyl-terminus of the nucleocapsid gene (N) was divided into two overlapping fragments of 375 and 384 bp length, covering the entire region of interest. Fig. 3.3 shows amplification products for a selection of analysed sera and CSF's. Information on clinical samples is provided in Table 3.1.

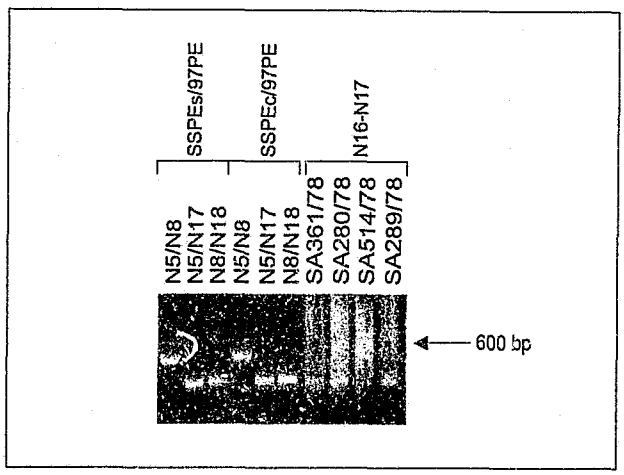


Fig. 3.3 PCR amplification products from selected sera and CSF's.
The 375 bp fragment resulting from amplification with primers N16 and N17 is shown for 4 specimens collected in 1978. All 3 primer pairs (N5/N8; N5/N17; N8/N18) that were alternatively used to amplify the carboxyl-terminus of the N gene are shown for a serum (SSPEs/97PE) and a CSF sample (SSPEc/97PE) from the same SSPE patient.

Fourteen sera and 3 CSF's collected in 1978 during clinically confirmed measles outbreaks in South Africa were selected for extraction of RNA and subsequent amplification of the carboxyl-terminus of the measles virus N gene. Of the 17 specimens analysed 4 (3 sera and 1 CSF) (24%) resulted in PCR products that were suitable for direct sequencing analysis (Table 3.1).

Table 3.1 Clinical samples used in this study

name	source	IgM status	origin	group
SA 168/78	serum	?	Soweto, SA[1]	II[3]
SA 280/78	CSF	?	Soweto, SA	III
SA 289/78	serum	?	?, SA	III
SA 361/78	serum	?	SA	III
Dbn 1/97	serum	+	Durban, SA	III
Wdk 28/97	CSF	+	Windhoek, Namibia	III
Wdk 91/97	serum	+	Windhoek, Namibia	III
SSPE/97PE[2]	CSF	IgG+	Port Elizabeth, SA	III
Jhb1/90	serum	+	Johannesburg, SA	III

[1] SA: South Africa; [2] analysis of a serum sample taken from the same patient (SSPE/97PE) revealed identical sequences compared to the CSF specimen. [3] indicates the SA genetic group to which specimens belonged determined by sequence and/or HMA analysis.

Out of a total of 8 specimens collected in 1997 in Windhoek, Durban and Port Elizabeth, MV-specific DNA could be amplified from 6 (75%). These specimens were included in the study for molecular epidemiological analysis of MV strains collected in regions other than Johannesburg. As clinical samples suitable for virus isolation were not available from these locations it appeared useful to obtain sequence data from RNA extracted from sera or CSF's.

Where sufficient quantities of serum (4/14) were available, the RNA extraction method described 2.2.1 (Chomczynski, P. and Sacchi, N., 1987) as well as the RT-PCR conditions described under 2.3.2 were performed in parallel with the method described above. Neither of these standard approaches yielded PCR products suitable for sequencing analysis (data not shown). The method for RNA extraction and RT-PCR of the carboxyl-terminal 450 bp of the N gene described here proved to be rapid, relatively simple, and yielded PCR products that could be used directly for sequencing analysis.

3.1.4 Phylogenetic analysis of measles virus N gene sequences

(A) Nomenclature of genetic groups

So far, no general consensus has been reached as to how to describe and designate MV genetic groups; lineages; genotypes and subgroups. Several studies have used different systems of designating individual MV strains and the genetic groups they belong to. We have previously described MV genetic groups in South Africa (Kreis, S. et al., 1997) and have used the letter code to describe MV genotypes or groups and subgroups. To acknowledge previously used nomenclature systems, both the letter and the number code will be applied here, either of which will be given in brackets. All MV strains analysed for this study show the year of their isolation behind the dash. The abbreviations of the names also indicate regional origin, i.e. Jhb12/95 would be a specimen that was taken in Johannesburg in 1995.

(B) Phylogenetic analysis of South African measles virus strains

Sequence information was obtained in most cases by manual sequence analysis (2.4.2) using sequencing primers N5, N6, N7, N8, N16, and N17 (Table 2.4.1). Alternatively, full N genes have been cloned for some strains (2.5) and were subsequently sequenced on the ALFexpress automated sequencer (2.4.3). Sequences were analysed phlyogenetically with the maximum likelihood program (DNAML) of the Phylip package. Files that contained more than 40 sequences could not be bootstrapped as a whole since the temporary files created by such analysis exceeded the capacity of the computers available. Files were therefore divided into smaller data sets of max. 40 species containing only few representatives of each genetic group and bootstrapped using the SEQBOOT program (Phylip). The

bootstrap intervals obtained for the main nodes ranged between ~80-100% (data not shown) and it was therefore assumed that the confidence interval of the genetic groups was satisfactory. Furthermore, each data set was run using different programs, such as Neighbor Joining, Kitsch, DNAPARS (parsimony) of the Phylip package or using the different phlyogeny program ClustalW. All trees generated showed identical groupings with only minor modifications on the inner nodes (data not shown). Fig.3.4 shows the phylogenetic analysis for South African MV strains sequenced so far. Three distinct genetic groups were evident, preliminarily designated I, II, and III. The comparison of these SA groups to previously described lineages is described below (3.1.5). South African viruses of group I and II formed part of previously published genetic groups whereas group III appeared to form a distinct and new group.

Group I viruses:

The 4 isolates Jhb2/88, Jhb2/89, Jhb38/95, and Jhb25/96 proved to be closely related to vaccine strains such as the Edm wt strain and Schwarz (group I). Although detailed patients' histories and information about possible contact to vaccinees was not available, these strains reflect cases of a measles infection caused by a vaccine-like virus. The 2 recent isolates in this group (Jhb38/95, and Jhb25/96) were made from patients that have been hospitalised because of severe symptoms related to measles. The Jhb38/95 specimen was obtained from a 5 year old male and Jhb25/96 was obtained from a 10 month old female; immunisation histories were unknown for both patients. The nt and aa alignments for the 4 strains (Fig. 3.5 and 3.6) illustrate that, except for Jhb25/96, sequences were identical to the Schwarz vaccine strain. Jhb25/96 carried 2 distinct non-coding nt substitutions. The question as to whether these strains reflect re-isolated vaccine viruses or wt vaccine-like viruses will be discussed further below. The 4 isolates of this group were very homogenous with a maximum nt divergence of only 0.4%.

Group II viruses:

A second group of South African wt MVs included 6 strains, 3 of which were isolated on Vero cells between 1986 and 1989 in Johannesburg. These stored viruses were adapted to B95a cell culture for this study. The 2 isolates from Natal and Johannesburg (NC1/95 and Jhb4/97, respectively) were the only recent strains that grouped with lineage II strains. Another virus, the sequence of which was obtained from a stored serum sample (SA168/78) (3.1.3) also proved to be related to this group. This indicates that group II wt viruses have been circulating in the country since 1978. Although not isolated frequently, these strains seem to represent a stable genetic group in SA that is either present in only small numbers, or it could be circulating in higher numbers in other provinces of South Africa or in

neighbouring countries that have not been included in the molecular epidemiological surveillance program yet. The nt variations of group II strains isolated between 1986 and 1995 approached 1.1% which reflects the high degree of relatedness of these viruses. However, nt variation between Jhb1/86 and Jhb4/97 was considerably higher (2.5%).

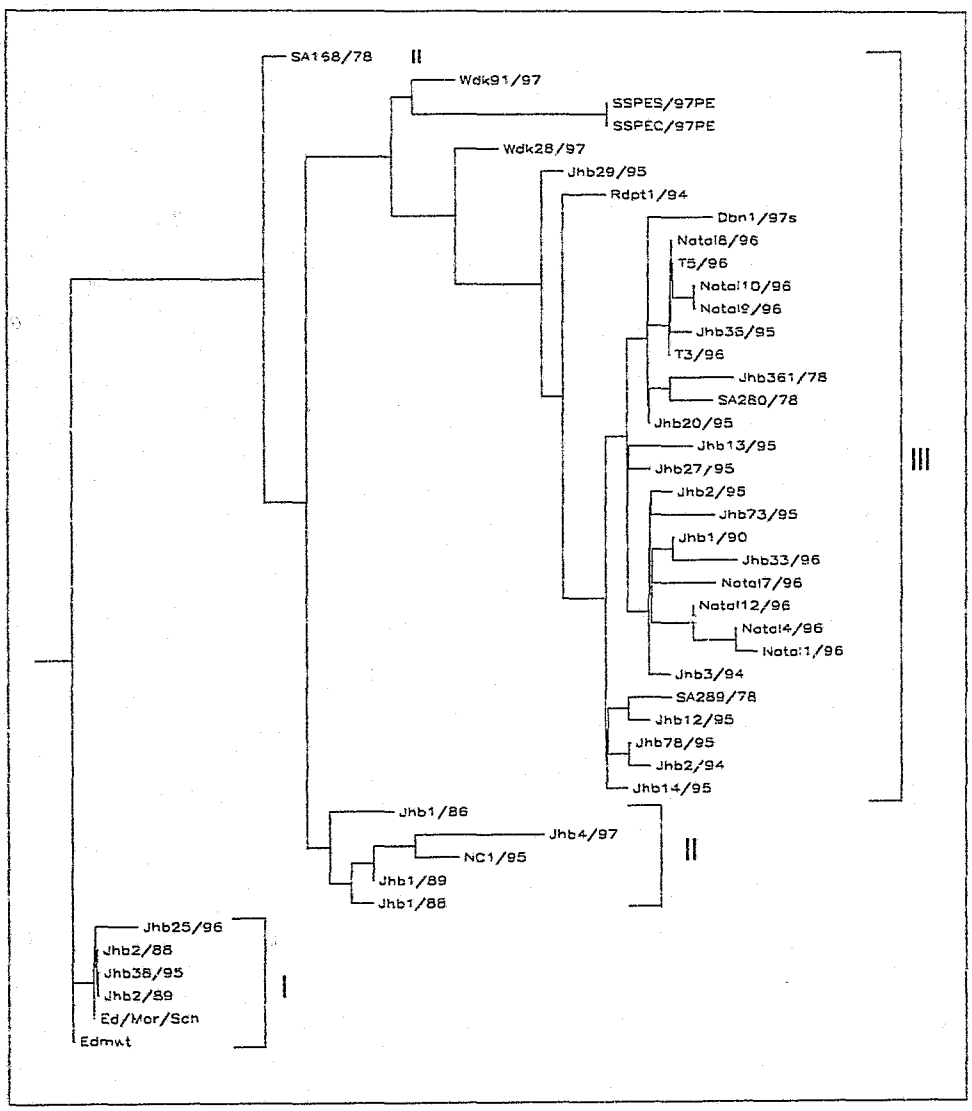


Fig. 3.4 Phylogenetic tree of South African measles virus N genes.
The dendrogram is unrooted und was generated with the DNAML program (Phylip, version 3.5).

The highest nt divergence of 2.7% was detected when comparing Jhb4/97 and Sa168/78. Variation rates of SA168/97 compared to the other 4 isolates of this group reached a maximum of 1.6%

Group III viruses:
The majority of wt MVs grouped in lineage III. This group seems to be the dominant genetic group in South Africa. Viruses of this lineage have been circulating in the country for at least 20 years, illustrated by the fact that the sequence of 3 serum samples from 1978 also formed part of group III. The comparison of South African N gene sequences with MV strains isolated elsewhere revealed that a Canadian virus isolated in 1989 in Montreal was closely related to the SA group III (Fig. 3.7). The variation on nt level for group III strains ranged between 0.1- 2.0%. However, when analysing the phylogenetic tree in Fig. 3.4, Wdk28/97 appears to be the most distinct virus within this group. This was confirmed by calculation of nt differences, which show values of 1.6- 2.9% between Wdk28/97 and other members of this lineage.

T3/96 and T5/96 represent sequences obtained from urine samples that were taken from a 26 year old male in Johannesburg (T3), who was acutely infected with measles and from his asymptomatic mother (T5). The mother had a history of natural measles in her childhood. MV was isolated from T5 in B95a tissue culture after 3 passages. The fact that virus was isolated from an asymptomatic person has, to our knowledge, not been reported before. Sequences from both the son and the mother proved to be identical, indicating that the virus isolated from the asymptomatic mother was indeed the virus that caused infection in the son. Further studies on these 2 samples are in progress.

(C) Nucleotide and amino acid alignments of South African measles virus strains

The alignment of the carboxyl-terminal 450 nt for some of the South African strains is illustrated in Fig.3.5. For reasons of simplicity, only a selection of SA MV strains is described, representing the 3 different genetic groups currently circulating in South Africa. The strains not shown in this figure carry very similar nt changes and have, apart from few scattered mutations, identical characteristic mutations to the ones presented here.

The 4 isolates in group I shared one nt substitution at position 313 (T→A), which

resulted in an aa substitution at position 105 (S→T), identical to the change seen in the Schwarz vaccine strain (Fig. 3.5). To firmly establish that this nt substitution is also present in the Schwarz vaccine strain that is used in South Africa, the sequence was determined again using virus from recent vaccine vials. The results indicated that the current Schwarz vaccine strain exhibited this same substitution, as was previously described (Rota, J.S. et al., 1994). The sequences of the carboxyl-terminal 450 nt are identical for the 3 strains Jhb2/88, Jhb2/89, Jhb38/95 and the Schwarz strain. However, analysis of the full H gene indicated that sequences of these SA isolates and the Schwarz vaccine strain are not completely identical, as will be discussed in chapter 3.2. Apart from the aa substitution described above, Jhb25/96 carried 2 more silent nt changes, that made this strain clearly different from Schwarz. This could be seen as an indicator that Jhb25/96 represents a vaccine-like strain rather than a re-isolated vaccine strain.

Group II wt strains carried several characteristic nt and aa changes (bold letters, Figs. 3.5 and 3.6). The mutations at positions 19 (G→A), 253 (G→A), and 321 (C→A) were distinctive for this group. However, the substitution at position 321 was not present in SA168/78. The second G→A substitution (nt 253) resulted in an aa replacement from alanine to threonine (A→T). This change was characteristic to only this small group of viruses and was not found in any other genetic group described to date. A double mutation or inversion at position 168, 169 (GA→AG) could be detected in both SA wt groups (II and III) and this appeared to mark a common mutation in almost all genotype D and I strains (groups 2,3,4, and 7).

The vast majority of recent MVs isolates from South Africa group within a distinct group (III, Fig.3.4). These isolates displayed more variation at nt and aa levels relative to the Schwarz vaccine strain than did group II virus strains, carrying on average 22 group-specific nt, 20 of which were non-synonymous and 11 which resulted in the replacement of 1 aa.

Fig. 3.5

```
                        10         20         30         40         50
SCH.SEQ            1 AAGGTCAGTT CCACATTGGC ATCTGAACTC GGTATCACTG CCGAGGATGC   5
JHB2-88.SEQ        1 .......... .......... .......... .......... ..........  50
JHB2-89.SEQ        1 .......... .......... .......... .......... ..........  50
JHB25-96.SEQ       1 .......... .......... .......... .......... ..........  50
JHB38-95.SEQ       1 .......... .......... .......... .......... ..........  50
JHB1-86.SEQ        1 .......... ....T..A.. .......... .......... ..........  50
JHB1-88.SEQ        1 .......... .......A.. .......... .......... ..........  50
JHB4-97.SEQ        1 .......... .......A.. .......... .......... ..........  50
SA168-78.SEQ       1 .......... .......A.. .......... .......... ..........  50
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Fig. 3.5, continued

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JHB3-94.SEQ          1 .......... .......... .......... .......... ..........  50
JHB12-95.SEQ         1 .......... .......... .......... .......... .T........  50
JHB2-96.SEQ          1 .......... .......... .......... .......... .T........  50
JHB33-96.SEQ         1 .......... .......... .......... .......... .T........  50
NATAL1.SEQ           1 .......... .......... .......... .......... ..........  50
NATAL10.SEQ          1 .......... .......... .......... .......... .T........  50
DBN1-97.SEQ          1 .......... .......... .......... .......... .T........  50
SSPES-97.SEQ         1 .......... .......... .......... .......... ..........  50
WDK28-97.SEQ         1 .......... .......... .......... .......... ..........  50
WDK91-97.SEQ         1 .......... .......... .......... .......... .T........  50
SA361-78.SEQ         1 .......... .......... .......... .......... ..........  50

                                60         70         80         90         100
SCH.SEQ             51 AAGGCTTGTT TCAGAGATTG CAATGCATAC TACTGAGGAC AAGATCAGTA  100
JHB2-88.SEQ         51 .......... .......... .......... .......... ..........  100
JHB2-89.SEQ         51 .......... .......... .......... .......... ..........  100
JHB25-96.SEQ        51 .......... .......... .......... .......... ..........  100
JHB38-95.SEQ        51 .......... .......... .......... .......... ..........  100
JHB1-86.SEQ         51 .......... .......... .......... .......... ..........  100
JHB1-88.SEQ         51 .......... .......... .......... .......... ..........  100
JHB4-97.SEQ         51 .......... .......... .......... .G........ .G........  100
SA168-78.SEQ        51 .......... .......... .......... .......... .G........  100
JHB3-94.SEQ         51 .......... .......... .......... .......... .G........  100
JHB12-95.SEQ        51 .......... .......... .......... .......... .G........  100
JHB2-96.SEQ         51 .......... .......... .......... .......... .G........  100
JHB33-96.SEQ        51 .......... .......... .......... .......... .G........  100
NATAL1.SEQ          51 .......... .......... .......... .......... ..........  100
NATAL10.SEQ         51 .......... .......... .......... .......... .G........  100
DBN1-97.SEQ         51 .......... .......C.. .......... .......... .G........  100
SSPES-97.SEQ        51 .......... .......... .......... .......... .G........  100
WDK28-97.SEQ        51 .......... .......... .......... .......... ..........  100
WDK91-97.SEQ        51 .......... .......... .......... .......... .G........  100
SA361-78.SEQ        51 .......... .......... .......... .......... ..........  100

                               110        120        130        140        150
SCH.SEQ            101 GAGCGGTTGG ACCCAGACAA GCCCAAGTAT CATTTCTACA CGGTGATCAA  150
JHB2-88.SEQ        101 .......... .......... .......... .......... ..........  150
JHB2-89.SEQ        101 .......... .......... .......... .......... ..........  150
JHB25-96.SEQ       101 .......... .......... .......... .......... ..........  150
JHB38-95.SEQ       101 .......... .......... .......... .......... ..........  150
JHB1-86.SEQ        101 .......... .......... .......... .......... ..........  150
JHB1-88.SEQ        101 .......... .......... .......... .......... ..........  150
JHB4-97.SEQ        101 .......... .......... ........G. .......... ..........  150
SA168-78.SEQ       101 .......... .......... .......... .......... ..........  150
JHB3-94.SEQ        101 .......... .......... ........G. .....A.... ..........  150
JHB12-95.SEQ       101 .......C.. .......... ........G. .....A.... ..........  150
JHB2-96.SEQ        101 .......C.. .......... ........G. .....A.... ..........  150
JHB33-96.SEQ       101 .......... .......... .......... .....A.... ..........  150
NATAL1.SEQ         101 .......... .......... .......... .....A.... ..........  150
NATAL10.SEQ        101 .......C.. .......... ........G. ....A.... ..........  150
DBN1-97.SEQ        101 .......C.. .......... .......... .....A.... ..........  150
SSPES-97.SEQ       101 .......C.. .......... .......... .......... ..........  150
WDK28-97.SEQ       101 .......... .......... .......... .......... ..........  150
WDK91-97.SEQ       101 .......C.. .......... .......... .......... ..........  150
SA361-78.SEQ       101 .......C.. .......... ........G. .....A.... ..........  150
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Fig. 3.5, continued

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                      160        170        180        190        200
SCH.SEQ          151 AGTGAGAATG AGCTACCGAG ATTGGGGGGC AAGGAAGATA GGAGGGTCAA 200
JHB2-88.SEQ      151 .......... .......... .......... .......... .......... 200
JHB2-89.SEQ      151 .......... .......... .......... .......... .......... 200
JHB25-96.SEQ     151 .......... .......... .......... .......... .......... 200
JHB38-95.SEQ     151 .......... .......... .......... .......... .......... 200
JHB1-86.SEQ      151 .......... .......AG. .......... .......... .......... 200
JHB1-88.SEQ      151 .......... .......AG. .......... .......... .......... 200
JHB4-97.SEQ      151 .......... .......AG. .......... .......... .......... 200
SA168-78.SEQ     151 .......... .......AG. .......... .......... .......... 200
JHB3-94.SEQ      151 .......... .......AG. .......... .......... .......... 200
JHB12-95.SEQ     151 .......... .......AG. .......... .......... .......... 200
JHB2-96.SEQ      151 .......... .......AG. .......... .......... .......... 200
JHB33-96.SEQ     151 .......... .......AG. .......... ........C. .......... 200
NATAL1.SEQ       151 .......... .......AG. .......... .......... .........G 200
NATAL10.SEQ      151 .......... .......AG. .......... .......... .......... 200
DBN1-97.SEQ      151 .......... .......AG. .......... .......... .......... 200
SSPES-97.SEQ     151 .......... .......AG. .......... ........C. .......... 200
WDK28-97.SEQ     151 .......... .......AG. .......... .......... .......... 200
WDK91-97.SEQ     151 .......... .......AG. .......... .......... .......... 200
SA361-78.SEQ     151 .......... .......AG. .......... .......... .......... 200

                      210        220        230        240        250
SCH.SEQ          201 ACAGAGTCGA GGAGAAGCCA GGGAGAGCTA CAGAGAAACC GGGCCCAGCA 250
JHB2-88.SEQ      201 .......... .......... .......... .......... .......... 250
JHB2-89.SEQ      201 .......... .......... .......... .......... .......... 250
JHB25-96.SEQ     201 .......... .......... .......... .......... .......... 250
JHB38-95.SEQ     201 .......... .......... .......... .......... .......... 250
JHB1-86.SEQ      201 .......... .......... .......... .......... ...T...... 250
JHB1-88.SEQ      201 .........G .......... .......... .......... ...T...... 250
JHB4-97.SEQ      201 .........G .......... .......... .......... ...T...... 250
SA168-78.SEQ     201 .........G .......... .......... .......... ...T...... 250
JHB3-94.SEQ      201 .........G .......... .......... .......G.. ...T...... 250
JHB12-95.SEQ     201 .........G .......... .......... .......G.. ...T...... 250
JHB2-96.SEQ      201 .......... .......... .......... .......G.. ...T...... 250
JHB33-96.SEQ     201 .........G .......... .......... .......G.. ...T...... 250
NATAL1.SEQ       201 .........G .......... .......... .......G.. ...T...... 250
NATAL10.SEQ      201 .........G .......... .......... .......G.. ...T...... 250
DBN1-97.SEQ      201 .........G .......... .......... .......G.. ...T...... 250
SSPES-97.SEQ     201 .........G .......... .......... .......... ...T...... 250
WDK28-97.SEQ     201 .........G .......... .......... .......... ...T...... 250
WDK91-97.SEQ     201 .........G .......... .......... .......G.. ...T. .... 250
SA361-78.SEQ     201 .........G .......... .......... .......G.. ...T...... 250

                      260        270        280        290        300
SCH.SEQ          251 GAGCAAGTGA TGCGAGAGCT GCCCATCTTC CAACCGGCAC ACCCCTAGAC 300
JHB2-88.SEQ      251 .......... .......... .......... .......... .......... 300
JHB2-89.SEQ      251 .......... .......... .......... .......... .......... 300
JHB25-96.SEQ     251 .......... .......... .......... .......... .......... 300
JHB38-95.SEQ     251 .......... .......... .......... .......... .......... 300
JHB1-86.SEQ      251 ..A....... .......... .......... .....A.... .......... 300
JHB1-88.SEQ      251 ..A....C.. .......... .......... .....A.... .......... 300
JHB4-97.SEQ      251 ..A....C.. .......... ....G..... .....AA... .......... 300
SA168-78.SEQ     251 ..A ..... .......... .......... .....A.... .......... 300
JHB3-94.SEQ      251 .......... .......... .......... .....A.... .......... 300
JHB12-95.SEQ     251 .......... .......... .......... .....A.... .......... 300
JHB2-96.SEQ      251 .......... .......... .......... .....A.... .......... 300
JHB33-96.SEQ     251 ......A... .......... .......... .....A.... .....C.... 300
NATAL1.SEQ       251 .......... .......... .......... .....A.... .......... 300
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Fig. 3.5, continued

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NATAL10.SEQ     251 .......... .......... .......... .....A.... .......... 300
DBN1-97.SEQ     251 .......... ...T...... .......... .....A.... .......... 300
SSPES-97.SEQ    251 .......... .......... .......C.. .....A...T G......... 300
WDK28-97.SEQ    251 .......... .......... .......... .....A.... .......... 300
WDK91-97.SEQ    251 .......... .......... .......... .....A.... .......... 300
SA361-78.SEQ    251 .......... .......... .......... .....A.... .......... 300

                        310        320        330        340        350
SCH.SEQ         301 AT.ACACTG CAACGGAGTC CAGCCAAGAT CCGCAGGACA GTCGAAGGTC 350
JHB2-88.SEQ     301 .......... .......... .......... .......... .......... 350
JHB2-89.SEQ     301 .......... .......... .......... .......... .......... 350
JHB25-96.SEQ    301 .......... .......... .......... .......... .......... 350
JHB38-95.SEQ    301 .......... .......... .......... .......... .......... 350
JHB1-86.SEQ     301 .......... ..T....... A......... .......... .......... 350
JHB1-88.SEQ     301 .......... ..T....... A......... .......... .......... 350
JHB4-97.SEQ     301 .......... ..T....... A......... ........T. .......... 350
SA168-78.SEQ    301 .......... ..T....... .......... .......... .......... 350
JHB3-94.SEQ     301 .......... ..T.A..... GG........ .......... .......... 350
JHB12-95.SEQ    301 .......... ..T.A..... .G........ .......... .......... 350
JHB2-96.SEQ     301 .......... ..T.A..... GG........ .......... .......... 350
JHB33-96.SEQ    301 .......... ..T.A..... GG........ .......... .......... 350
NATAL1.SEQ      301 .......... ..T.A..... GG........ .......... .......... 350
NATAL10.SEQ     301 .......... ..T.A..... GG........ .......... .......... 350
DBN1-97.SEQ     301 .......... ..T.A..... .G........ .......... .......... 350
SSPES-97.SEQ    301 .......... ..T....... AG........ .......... .......... 350
WDK28-97.SEQ    301 .......... ..T....... .......... .......... .......... 350
WDK91-97.SEQ    301 .......... ..T....... .......... .......... .......... 350
SA361-78.SEQ    301 .......... ..T.A..... GG........ .....A.... .......... 350

                        360        370        380        390        400
SCH.SEQ         351 AGCTGACGCC CTGCTTAGGC TGCAAGCCAT GGCAGGAATC TCGGAAGAAC 400
JHB2-88.SEQ     351 .......... .......... .......... .......... .......... 400
JHB2-89.SEQ     351 .......... .......... .......... .......... .......... 400
JHB25-96.SEQ    351 .......... .......... .......A.. .........T .......... 400
JHB38-95.SEQ    351 .......... .......... .......... .......... .......... 400
JHB1-86.SEQ     351 .......... .....C.... .......... .......... .......... 400
JHB1-88.SEQ     351 .......... .......... .......... .......... .......... 400
JHB4-97.SEQ     351 .......... .....C.... .......... .......... .......... 400
SA168-78.SEQ    351 .......... .....C.... .......A.. .......... .......... 400
JHB3-94.SEQ     351 .......... .....C...T ....G..... .......... .T........ 400
JHB12-95.SEQ    351 .......... .....C...T ....G..... .......... .T........ 400
JHB2-96.SEQ     351 .......... .....C...T ....G..... .......... .T........ 400
JHB33-96.SEQ    351 .......... .....C...T ....G..... .......... .T........ 400
NATAL1.SEQ      351 .......... .....C...T ....G..... .......... .T........ 400
NATAL10.SEQ     351 .......... .....C...T ....G..... .......... .T........ 400
DBN1-97.SEQ     351 .......... .....C...T ....G..... .......... .T........ 400
SSPES-97.SEQ    351 .......... .....C.... .......... .......... .T........ 400
WDK28-97.SEQ    351 .......... .....C...T ....G....A .......... .T........ 400
WDK91-97.SEQ    351 .......... .....C.... .......... .......... .T........ 400
SA361-78.SEQ    351 .......... .....C...T ....G..... .....T.... .T........ 400

                        410        420        430        440        450
SCH.SEQ         401 AAGGCTCAGA CACGGACACC CCTATAGTGT ACAATGACAG AAATCTTCTA 450
JHB2-88.SEQ     401 .......... .......... .......... .......... .......... 450
JHB2-89.SEQ     401 .......... .......... .......... .......... .......... 450
JHB25-96.SEQ    401 .......... .......... .......... .......... .......... 450
JHB38-95.SEQ    401 .......... .......... .......... .......... .......... 450
```

Fig. 3.5, continued

```
JHB1-86.SEQ       401 .......... .......... ....G..... .......... .G........ 450
JHB1-88.SEQ       401 .......... .......... ....G..... .......... .G........ 450
JHB4-97.SEQ       401 .......... ...A...... ....G..... .......... .......... 450
SA168-78.SEQ      401 .......... .......... ....G..... .......... .G........ 450
JHB3-94.SEQ       401 .......... ........T. ....GG.... .......... .G........ 450
JHB12-95.SEQ      401 .......... ........T. ....GG.... .T........ .G........ 450
JHB2-96.SEQ       401 .......... ........T. ....GG.... .......... .G........ 450
JHB33-96.SEQ      401 .......... ........T. ....GG.... .......... .G........ 450
NATAL1.SEQ        401 .......... ........TT ....GG.... .......... .G........ 450
NATAL10.SEQ       401 .......... ........T. ....GG.... .......... .......... 450
DBN1-97.SEQ       401 .......... ........T. ....GG.... .T........ .G........ 450
SSPES-97.SEQ      401 .......... .......... ....GG..A. .......... .G........ 450
WDK28-97.SEQ      401 .......... .......... ....GG.... .T........ .G........ 450
WDK91-97.SEQ      401 .......... .......... ....GG.... .......... .G........ 450
SA361-78.SEQ      401 .......... ........T. ....GG.... .T........ .G........
```

Fig. 3.5 Nucleotide alignment of selected measles virus strains representing 3 distinct viral groups. Nucleotides were aligned using the Wdnasis program, version 2.5 (Hitachi); a dot indicates same residue. SSPES-97= SSPEs/PE97. Nucleotide (nt) 1 of this alignment corresponds to nt 1152 of the full N gene.

The nt substitutions at positions 136 (C→A), 237 (A→G), and 419 (C→T) resulted in distinct aa changes (L→I, T→A, and T→I, respectively) that were only seen in group III. The Canadian isolate Can (1989) is the only strain described so far that shares the mutations at position 136 and 419 with SA group III, as was described previously (Kreis, S. et al., 1997). As indicated in Fig. 3.6, aa replacements at positions 57 (R→G), 81 (P→S), and 96 (G→S) were common to both South African wt groups (II and III) and other genotype D viruses from America and Europe (genetic groups 2,3, and 4). Two substitutions at positions 108 (S→G) and 131 (S→L) were seen in SA group III and subgroups D2 (2) and D4 (3), which represent mainly American strains isolated between 1989 and 1994 (Fig. 3.8). SSPEs/PE97 and Wdk28/97 carried some unique aa changes that were not shared by any other viruses described in Fig.3.5 and 3.6, indicating that these 2 viruses were distinct from the common SA groups.

Fig. 3.6

```
                                  10         20         30         40         50
SCH.AMI              1 KVSSTLASEL GITAEDARLV SEIAMHTTED KISRAVGPRQ AQVSFLHGDQ      50
JHB2-88.AMI          1 .......... .......... .......... .......... ..........      50
JHB25-96.AMI         1 .......... .......... .......... .......... ..........      50
JHB38-95.AMI         1 .......... .......... .......... .......... ..........      50
JHB2-89.AMI          1 .......... .......... .......... .......... ..........      50
JHB1-86.AMI          1 .......... .......... .......... .......... ..........      50
JHB1-88.AMI          1 .......... .......... .......... R......... ..........      50
JHB4-97.AMI          1 .......... .......... .......A.. R......... ..........      50
SA168-78.AMI         1 .......... .......... .......... R......... ..........      50
JHB3-94.AMI          1 .......... .......... .......... R......... .....I....      50
JHB12-95.AMI         1 .......... .......... .......... R......... .....I....      50
JHB2-96.AMI          1 .......... .......... .......... R......... .....I....      50
JHB33-96.AMI         1 .......... .......... .......... R......... .....I....      50
NATAL1.AMI           1 .......... .......... .......... .......... .....I....      50
NATAL10.AMI          1 .......... .......... .......... R......... .....I....      50
DBN1-97.AMI          1 .......... .......... ..T....... R......... .....I....      50
SSPES-97.AMI         1 .......... .......... .......... R......... ..........      50
WDK28-97.AMI         1 .......... .......... .......... .......... ..........      50
WDK91-97.AMI         1 .......... .......... .......... R......... ..........      50
SA361-78.AMI         1 .......... .......... .......... .......... .....I....      50

                                  60         70         80         90        100
SCH.AMI             51 SENELPRLGG KEDRRVKQSR GEARESYRET GPSRASDARA AHLPTGTPLD     100
JHB2-88.AMI         51 .......... .......... .......... .......... ..........     100
JHB25-96.AMI        51 .......... .......... .......... .......... ..........     100
JHB38-95.AMI        51 .......... .......... .......... .......... ..........     100
JHB2-89.AMI         51 .......... .......... .......... .......... ..........     100
JHB1-86.AMI         51 ......G... .......... .......... .S..T..... .....S....     100
JHB1-88.AMI         51 ......G... .......... .......... .S..T..... .....S....     100
JHB4-97.AMI         51 ......G... .......... .......... .S..T..... .R...N....     100
SA168-78.AMI        51 .......... .......... .......... .S..T..... .....S....     100
JHB3-94.AMI         51 ......G... .......... .........A .S........ .....S....     100
JHB12-95.AMI        51 ......G... .......... .........A .S........ .....S....     100
JHB2-96.AMI         51 ......G... .......... .........A .S........ .....S....     100
JHB33-96.AMI        51 ......G... .......... .........A .S...N.... .....S..P.     100
NATAL1.AMI          51 ......G... ......R... .........A .S........ .....S....     100
NATAL10.AMI         51 ......G... .......... .........A .S........ .....S....     100
JHB1-97.AMI         51 ......G... .......... .........A .S........ .....S....     100
SSPES-97.AMI        51 ......G... .......... .......... .S........ ..P..SM...     100
WDK28-97.AMI        51 ......G... .......... .......... .S........ .....S....     100
WDK91-97.AMI        51 ......G... .......... .........A .S........ .....S....     100
SA361-78.AMI        51 ......G... .......... .........A .S........ .....S....     100

                                 110        120        130        140        150
SCH.AMI            101 IDTASESSQD PQDSRRSADA LLRLQAMAGI SEEQGSDTDT PIVYNDRNLL     150
JHB2-88.AMI        101 ....T..... .......... .......... .......... ..........     150
JHB25-96.AMI       101 ....T..... .......... .......... .......... ..........     150
JHB38-95.AMI       101 ....T..... .......... .......... .......... ..........     150
JHB2-89.AMI        101 ....T..... .......... .......... .......... ..........     150
JHB1-86.AMI        101 .......... .......... .......... .......... .R.....D..     150
JHB1-88.AMI        101 .......... .......... .......... .......... .R.....D..     150
JHB4-97.AMI        101 .......... .......... .......... .......... .R........     150
SA168-78.AMI       101 .......... .......... .......... .......... .R.....D..     150
JHB3-94.AMI        101 .......G.. .......... .......... L........I .R.....D..     150
JHB12-95.AMI       101 .......G.. .......... .......... L........I .R.....D..     150
JHB2-96.AMI        101 .......G.. .......... .......... L........I .R.....D..     150
JHB33-96.AMI       101 .......G.. .......... .......... L........I .R.....D..     150
NATAL1.AMI         101 .......G.. .......... .......... L........I .R.....D..     150
NATAL10.AMI        101 .......G.. .......... .......... L........I .R........     150
```

Fig. 3.6, continued

```
DBN1-97.AMI      101 .......... .......... .......... L........I .R.....D..  150
SSPES-97.AMI     101 .......G.. .......... .......... L......... .R.....D..  150
WDK28-97.AMI     101 .......... .......... ......K... L......... .R.....D..  150
WDK91-97.AMI     101 .......... .......... .......... L......... .R.....D..  150
SA361-78.AMI     101 .......G.. .......... ........V. L........I .R.....D..  150
```

Fig. 3.6 Alignment of predicted amino acids for a selection of South African strains relative to the Schwarz vaccine strain. Aa were aligned using the Wdnasis software package, version 2.5. SSPES-97= SSPEs/PE97. Aa 1 corresponds to residue 375 of the full N gene.

The 2 sequences that were obtained from Namibian serum samples (Wdk28/97 and Wdk91/97) strains, as well as the SSPE CSF sample (SSPEc/PE97) did not display the mutation at position 46 (L→I), which was characteristic for SA group III strains. Although quite different from group III viruses, upon phylogenetic analysis Wdk28/97 appears to be still part of this lineage (Figs. 3.4 and 3.7). The sequence that was derived from a 5 year old SSPE patient in Port Elizabeth (Eastern Cape province) carried 2 distinct aa changes (L→P, position 93 and T→S, position 97) that have not been detected in other MV strains from southern Africa. These changes were also detected in group 2 strains (D2), which represent strains from the USA (Fig. 3.8). It is not known whether the SSPE patient had travelled to other countries, but this strain clearly represents the American lineage of viruses that caused the re-emergence of MV from 1989 to 1991 in the USA. Viruses belonging to this group have also been isolated in Japan, the Phillippines, and Micronesia (Bellini, W.J. and Rota, P.A., 1998).

In summary, 3 distinct genetic groups have been found to exist in SA when analysing the carboxyl-terminus of the N gene. Four strains had vaccine or vaccine-like sequences and therefore formed part of genetic group 1 (A). Six strains, the earliest of which was a virus from 1978, grouped within group D (groups 2,3, and 4) and appeared to form an individual subgroup. The majority of analysed specimens proved to be part of a distinct genetic group I (7). The earliest strains in this group were circulating in South Africa in 1978 and this group continues to predominate in the country at present. Another strain that was very similar to group I or 7 viruses was isolated in Montreal, Canada in 1989.

3.1.5 Phylogenetic analysis of South African measles virus strains compared to previously described strains

Fig. 3.7 shows the phylogenetic analysis of a selection of SA strains and strains described previously representing all genetic groups known to date. This tree also includes viruses that are assumed to be extinct (groups C1, E, F, and G) since they have not been re-isolated for more than 10 years. However, for reasons of completeness they were included in this dendrogram. A list of MV strains shown in this tree, their origin, year of isolation and a brief description is given in Appendix B.

The genetic group 1 (A) represents a variety of vaccine and vaccine-like sequences including the 4 SA strains described above.

Group B (6) represents a few African strains from Gabon, Cameroon, Gambia, and Kenya. No significant relatedness between these strains and the southern African isolates was evident. Group C2 (5) contains sequences of MV from numerous European countries such as Germany, Spain, and the U.K. and appears to form a common genetic group in Europe. Group D2 (2) contains US American isolates and the SSPE case from SA. This genetic group was responsible for the resurgence of measles in the USA between 1989 and 1991. There has been no indigenous transmission of this particular lineage in the USA since 1993 and this has been shown by continued molecular epidemiological studies (Bellini, W.J. and Rota, P.A., 1998). Group 3 (D4), in some studies, is divided into 2 subgroups, 3A and 3B. Only group 3A representing isolates from the USA and the Namibian strain Wdk91/97 is presented here. At present, no further molecular epidemiological information is available concerning Namibian MV genetic groups; therefore it cannot be speculated on as to whether this may be a common lineage in this country.

Most of the recently isolated MVs from northern America, Europe and the strains from South Africa described here grouped within genotypes D and I (groups 2,3,4, and 7). These viruses share 3 characteristic amino acid substitutions (positions 431, 456 and 470), which may indicate a common evolutionary relationship for the 2 genetic groups. It appears that D and I represent the dominant genotypes currently circulating in many parts of the world.

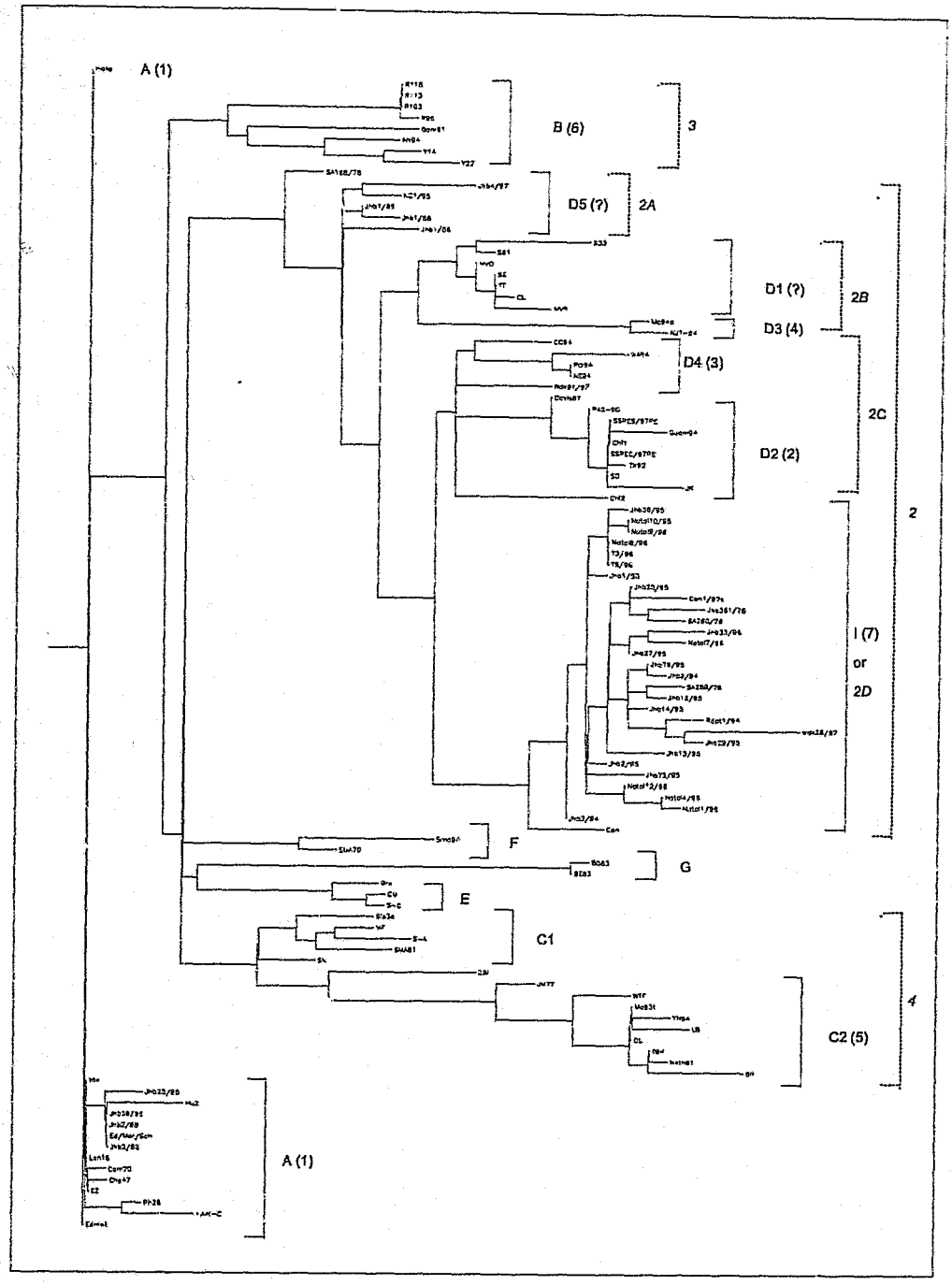

Fig. 3.7 Phylogenetic analysis of South African measles virus strains compared to strains described previously. The dendrogram is unrooted and was generated with the DNAML program. Group designations are indicated behind the brackets. Dotted brackets and italic numbers represent suggested group designations as discussed below.

The viruses belonging to group D (2,3, and 4) were divided into 5 subgroups (Fig. 3.7). However, all subgroup D and the group I (7) viruses from SA and Canada could also be interpreted to form 1 genotype since they all branch off from the same central node and this "genotype" could then logically be divided into 4 subgroups, each of which branches off from one of the 4 inner nodes. Suggested alternative designations are shown in italics with brackets in dotted lines (Fig. 3.7). To describe group D (2,3, and 4) and group I (7) together as 1 lineage or "genotype" would also be supported by distance analysis of nt and aa divergence (Table 3.2) which further shows that these groups appear to be related to each other.

(A) Analysis of consensus sequences

Consensus sequences for viral groups that are currently circulating were generated with the WDNAsis program, the GCG program and partly by manually determining shared nt and aa changes within genetic groups. Fig. 3.8 shows an alignment of the predicted aa for current lineages. Groups that have not been re-isolated for more than 10 years were not included in this analysis.

Fig. 3.8

```
                      10         20         30         40         50
CON-A              1 KVSSTLASEL GITAEDARLV SEIAMHTTED KISRAVGPRQ AQVSFLHGDQ  50
CON-B1             1 .......... .......... .......... R......... ..........  50
CON-B2             1 .......... .......... .......... R......... ..........  50
CON-C2             1 .......... .......... .......... R......... S.........  50
CON-D2             1 .......... .......... .......... R......... ..........  50
CON-D3             1 .......... .......... .......... R......... ..........  50
CON-D4             1 .......... .......... .......... R......... ..........  50
CON-D5             1 .......... .......... .......... R......... ..........  50
CON-F              1 .......... .......... .......... R......... ..........  50
CON-H              1 .......... .......... .......... R...V..... ..........  50
CON-I              1 .......... .......... .......... R......... .....I....  50

                      60         70         80         90        100
CON-A             51 SENELPRLGG KEDRRVKQSR GEARESYRET GPSRASDARA AHLPTGTPLD 100
CON-B1            51 .......... .......... ...G...... .......... ..P.......  100
CON-B2            51 .......... .......... ...G...... .......... T.........  100
CON-C2            51 N.S....W.. ...M...... .......... .......... ..P..D....  100
CON-D2            51 ......G... .......... .......... .S........ ..P..SM...  100
CON-D3            51 ......G... .....A.... .......... .S........ .....T....  100
CON-D4            51 ......G... .......... .......... .S.....E.. .....S....  100
CON-D5            51 ......G... .......... .......... .S..T..... .....S....  100
CON-F             51 .......... .....I...Q .......... .......... ..........  100
CON-H             51 .......... .......... E..G..F..I .......... .......S..  100
CON-I             51 ......G... .......... .........A .S........ .....S....  100
```

Fig. 3.8, continued

```
                          110        120        130        140        150
CON-A          101 IDTASESSQD PQDSRRSADA LLRLQAMAGI SEEQGSDTDT PIVYNDRNLL    150
CON-B1         101 .......F.. .......... .......... .......... .R.....D..    150
CON-B2         101 ...V....L. .......... .......... .......... .R.....D..    150
CON-C2         101 .......... .......... .......... .......... .R.....D..    150
CON-D2         101 .......G.. .......... .......... L......... .R.....D..    150
CON-D3         101 ......T... L......... .......... .......... .R.....D..    150
CON-D4         101 .......G.. .......... .......... L......... .R....SD..    150
CON-D5         101 .......... .......... .......... .......... .R.....D..    150
CON-F          101 .......... .L......E. .......... .......... .R.....D..    150
CON-H          101 .......... .......... .......V.. .......... .R.....D..    150
CON-I          101 .......G.. .......... .......... L........I .R.....D..    150
```

Fig. 3.8 Alignment of predicted amino acids for consensus sequences representing 11 viral groups or subgroups. A= group 1; B1 and B2= group 6; C2= group 5; D2= group 2; D3= group 4; D4= group 3; D5 (SA strains, bold) = group ?; F= ?; H= group 6; I (SA strains+ Can89, bold) = group 7; con= consensus sequence.

All wt groups that have been described to date share 3 aa substitutions at positions 31 (K→R), 142 (I→R), and 148 (N→D) as is shown in Fig. 3.8. The subgroups within genotype D (D2-D5, or groups 2,3, and 4), as well as the predominant genetic group in SA (I or 7) have, apart from the changes mentioned above, 3 additional aa substitutions in common (aa 57, 82, and 96) which proved that these genetic groups, although circulating in continents and countries far apart from each other, are indeed related to each other. The aa substitution at position 459 (A→T), characteristic for D5 viruses, was only shared by an isolate made in Cameroon (Y22) in 1983. Besides these common mutations, Fig. 3.8 also demonstrates the relative divergence between the different genetic groups, each of which carries changes that were group specific.

Con-H is not a real consensus sequence, because it only represents 1 isolate (Gam 91) form Gambia (1991). This strain is related to other African isolates such as the Gabon and Cameroon strains of group 6 (B1 and B2). However, the alignment of the consensus sequences shows that this strain carried 6 unique substitutions that were not shared by any other group of MVs and was therefore given an individual group designation (Kreis, S. et al., 1997).

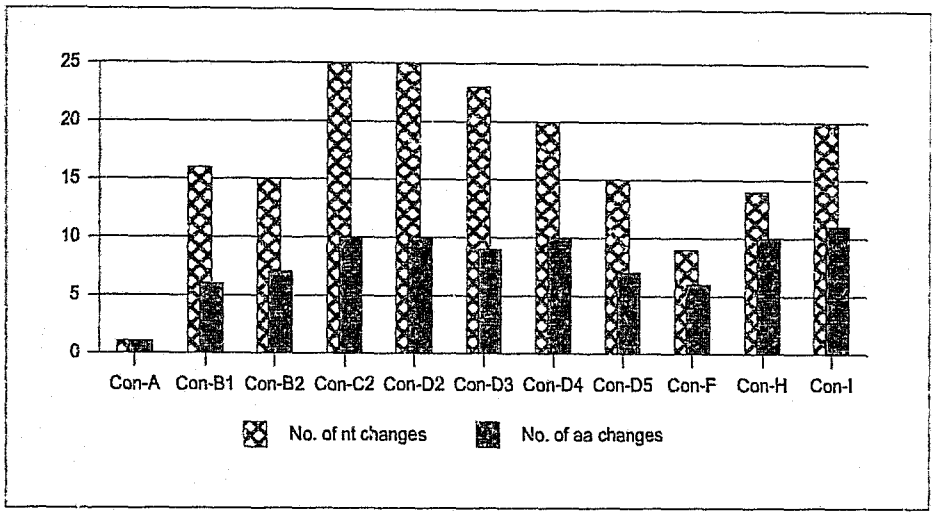

Fig. 3.9 Number of nucleotide and predicted amino acid changes for 11 consensus sequences.

Fig. 3.9 presents the number of nt and predicted aa changes for each consensus sequence representing 9 genetic groups. Groups C2 and D2 (groups 5 and 2) both carried the highest number of mutations, 25 nt changes and 10 aa changes. Fig. 3.9 illustrates a useful and simple way of preliminarily assessing a genetic drift that might have occurred in geographically related groups over time . By comparing the consensus sequences of the 2 SA wt groups, a considerable difference in the number of mutations was noted. D5 had 15 nt and 7 aa changes, whereas group I (group 7) showed 20 nt and 11 aa changes. Thus, genotype I displayed a greater number of mutations and may have evolved from the "earlier" South African viruses in subgroup D5, signifying a genetic drift. However, the aa substitution at position 85 of the N gene was characteristic for D5 viruses only. Given that this mutation was not seen in any of the recent genotype I strains and assuming that a reversion of this particular mutation did not occur, it is more plausible to consider an independent evolution for both lineages. When viruses of group I (7) and D5

that were isolated over a period of 20 were analysed, no significant change in number and type of mutations was seen over this extended period of time (Figs. 3.5 and 3.6). Therefore, it can be assumed that no siginificant genetic drift occurred in those groups. Measles virus is known to be remarkably stable over time, a fact which is illustrated by genotype A (1) viruses that have at least been circulating for 44 years and that do not show many changes over this period of time.

(B) Distance matrix analysis

The genetic distances between some consensus sequences representing the most important genetic groups currently circulating is shown in Table 3.2. The genetic distances at nt and aa levels were calculated using the DNADIST and PROTDIST programs of the Phlyip package.

Relative to Schwarz, the C2 (5) group displays the highest variability at nt level (5.5%). The variability in aa between the different genetic groups ranged from 3.7% (B1 or 6) to 6.9% for the group I (7), which included the majority of SA wt strains. As much as the SA group I (7) viruses showed the highest variability at aa level relative to Schwarz, the D5 group displayed the least changes of all groups analysed, followed by other African strains in group B1 and B2 (6) and by Wdk91/97 (Namibia) which revealed nt divergence rates of only 3.1%.

The highest variability regarding aa changes of all groups was detected between groups I (7), representing the predominant SA wt group and group C2 (5), which includes mainly European strains but also US American strains from 1994. As mentioned before, SA wt strains of group I (7) revealed a relatively high level of resemblance to group D2, D4, and D5 (2, and 3) which was confirmed by the low figures for nt and aa diversity (max. 3.8% for nt, and max. 3.6% for aa). The second SA wt group, D5 has phylogenetically been shown to group with other group D viruses and this relatedness was also reflected in the distance matrix analysis. The only 2 strains analysed here that did not group in wt lineages representing the majority of SA strains (I and D5), Wdk91/97 and SSPEs/PE97 are included in Table 3.2.

	Con-A	Con-B1	Con-B2	Con-C2	Con-D2	Con-D3	Con-D4	Con-D5	Con-I	Wdk91/97	SSPEsPE97
Con-A (1)	0	**3.7**	**4.3**	**6.0**	**6.3**	**5.5**	**6.2**	**4.3**	**6.9**	**4.9**	**6.3**
Con-B1 (6)	3.6	0	**3.0**	**4.9**	**5.1**	**5.1**	**6.4**	**4.4**	**7.1**	**5.1**	**5.1**
Con-B2 (6)	3.1	4.1	0	**6.9**	**7.1**	**6.3**	**7.1**	**5.0**	**7.7**	**5.7**	**7.1**
Con-C2 (5)	5.5	6.9	7.4	0	**7.0**	**7.5**	**8.2**	**6.2**	**8.9**	**6.9**	**7.0**
Con-D2 (2)	4.7	6.1	6.6	6.9	0	**4.2**	**2.4**	**3.0**	**3.0**	**2.4**	**0**
Con-D3 (4)	5.0	6.9	5.9	8.1	5.2	0	**4.2**	**2.4**	**4.9**	**3.0**	**4.2**
Con-D4 (3)	4.3	6.2	6.2	6.9	2.7	4.3	0	**3.0**	**3.0**	**2.4**	**2.4**
Con-D5 (?)	2.9	5.2	4.7	5.5	3.1	3.8	2.7	0	**3.6**	**1.0**	**3.0**
Con-I (7)	4.7	6.6	6.6	7.4	3.8	5.4	2.9	3.4	0	**1.8**	**3.0**
Wdk91/97 (3)	3.1	5.4	5.0	6.4	2.5	4.1	2.0	2.0	2.0	0	**2.4**
SSPEs/PE97 (2)	4.7	6.1	6.6	6.9	0	5.2	2.7	3.1	3.8	2.5	0

Table 3.2 Distance matrix of consensus sequences and 2 selected strains analysed for this study. The percent nt variation is given in the lower triangle and the percent aa divergence is given in bold letters in the upper triangle.

According to the phylogenetic trees and alignments of aa and nt, SSPEs/97PE appeared to belong to group 2 (D2) and this was clearly supported by the distance values. SSPEs/PE97 had an identical sequence to the consensus sequence that was generated for group 2 (D2). The phylogenetic tree (Fig. 3.7) shows that Wdk91/97 grouped with lineage 3 (D4) viruses although it did not branch off the same node as other members of this group. Wdk91/97 appeared to have genetic features that could be described as lying somewhat between groups 3 and 2. The distance analysis for these strains revealed identical nt values for groups D4, D5, and I (each 2.0%). The aa divergence for the Namibian virus from SA groups D5 and I is very low (1.0% and 1.8%, respectively), whereas values when compared to groups 2 and 3 (D2 and D4, respectively) were somewhat higher at 2.4% diversity (at aa level). The distance matrix analysis confirmed the phylogenetic analysis in that groups closely related to each other in a phylogenetic tree had low values for nt and aa divergence whereas groups showing little relation to each other displayed higher diversity values approaching 8.0% at nt level and 9.0 % at aa level.

3.1.6 Discussion

Molecular epidemiological data on MV in southern African countries has not been available to date. The present study aimed to establish (i) how many different genetic groups of MV are present in South Africa, (ii) what the genetic properties of these viral groups are and how they compare to strains isolated elsewhere. As discussed in 1.4, the knowledge of the molecular epidemiology of MV is necessary before indigenous and imported viruses can be distinguished, and this is important in assessing the success of local MV control and elimination programs. The WHO is moving toward setting goals for national and global control and the eventual eradication of MV and an increasing number of countries are investing considerable effort in achieving this. The estimated number of measles infections each year approaches 45 million with approximately 1 million measles related deaths. Africa, especially sub-saharan Africa still accounts for the majority of these cases. The reduction and control of measles infections in Africa will therefore be a cornerstone in achieving the goal of global MV eradication. The EPI (Extended Program on Immunization) in South Africa is currently attempting to reduce the numbers of measles infections by implementing mass vaccination campaigns and by improving routine vaccine coverage. An indicator for the continued problem of

maintaining a high vaccine coverage is reflected in the large number of measles infections seen in the country (Schoub, B.D., 1994). Over the past 10 years, an average of 15 000 notifications and 300 deaths per annum have been reported in South Africa and this is almost certainly an underestimate of actual cases (Naidoo, S. and Meyers, K., 1994). However, after the completion of 2 mass vaccination campaigns in 1996 and 1997, the above numbers of measles cases are likely to decrease considerably in the near future. The knowledge of the genetic groups of MV indigenous to South Africa will be an important means to evaluate the success of MV control programs. Neighbouring countries still have endemic levels of the virus and importations of MV across the borders will continue to occur. It is not known whether the genetic groups of MV in neighbouring countries are different to the ones currently circulating in SA. Efforts are currently being made to collect MV specimens from countries such as Zimbabwe, Namibia, Botswana, Mozambique and others to establish the molecular epidemiology of MV in the whole of southern Africa.

To determine strain variation of SA MVs, specimens were collected from 4 different provinces. Although there are still vast areas remaining in the country that could not be included in this present study as specimens were not available, the 97 samples analysed have revealed 3 distinct genetic groups that are currently in circulation. At this point, it can not be completely ruled out that more MV groups are present in SA. However, there is extensive travelling from all parts of the country into Johannesburg (Gauteng) mainly for work-related purposes, and the 82 analysed specimens collected in Gauteng are likely to reflect MV from other provinces as well. This is known for NC1/95, a specimen from Newcastle in the Natal province that was taken from a boy who contracted the disease in Natal and then travelled to Johannesburg where he became ill.

Group I

Three distinct genetic groups have been found to circulate in South Africa, 1 of which included 4 vaccine-like strains isolated between 1988 and 1996 (group A or 1). Three of these 4 isolates had identical carboxyl-terminal N sequences to the Schwarz vaccine strain. The recent strain, Jhb38/95, was isolated from a child that was hospitalised with severe measles related symptoms but further medical history for this patient was not available. The HIV status of these patients was also unknown and it can not be speculated whether the patients were immuno-suppressed. It therefore remains unclear why the vaccine strain caused such severe clinical symptoms. So far, clinical signs of infection

following vaccination have been reported to be subclinical, milder and shorter in duration than those of a natural infection (Osterhaus, A.D. et al., 1994). Patients' histories for the isolates made in 1988 and 1989 that also belonged to this group 1 (A) were unfortunately not available. Jhb25/96 displayed a sequence that differed by 2 nt from the Schwarz vaccine strain. This strain therefore appeared to be a vaccine-like wt strain rather than the vaccine strain. Substantial numbers of wt isolates of this genotype have not been made since 1971. It will be interesting to continue the analysis of MV strains to identify whether this group 1 (A) is still circulating more frequently elsewhere. Before the worldwide introduction in the 1960s of the potent measles vaccines, this genetic group was believed to be the predominant genotype in many parts of the world (Rima, B.K. et al., 1995a).

Group II

The wt genetic group II (D5) viruses have been present in the country at least from 1978 onwards. Most isolates of this group were made 12 to 20 years ago and only 2 of the 6 strains were recent (1995 and 1997). There is a possibility that this group was more frequent in the 1970s and 1980s and that it has been partly replaced by group III. Jhb4/97 carried 7 additional nt and 4 aa changes when compared to SA168/78, a strain of the same group that was collected 19 years earlier. This might indicate an evolutionary process towards the higher mutated more recent isolates.

Group III

The third and at present dominant genetic group III (I or 7) included the majority of isolates made in South Africa. A total of 87 strains (out of 97) proved to belong to this group. Twenty-nine of these strains were determined to be group III viruses by sequence analysis of the COOH-terminus of the N gene. A further 58 strains were grouped by HMA analysis (chapter 3.3). Can, an isolate made in Canada in 1989 was closely related to the recent South African MVs, sharing 10 out of 11 aa substitutions. Unfortunately, further sequences of Canadian isolates were not available, and so it is not reasonable to comment on possible importation routes of this genetic group. However, the fact that 3 SA strains from 1978 belonged to this group might demonstrate that this genotype has been longer in circulation in South Africa than in Canada.

Asymptomatic measles infections

T3/96 represented a urine sample that was taken from an acutely infected 26 year old male in Johannesburg who had a history of receiving measles vaccination at the age of 15 months and 7 years of age. The measles infection

in this case indicated primary vaccine failure. Primary vaccine failure occurs in about 5% of vaccine recipients immunized at 15 months of age (Surveillance Bulletin, 1996; Mathias, R.C. et al., 1989). To determine if this patient was indeed a case of primary vaccine failure an avidity test was performed on a serum sample by the diagnostic section of the NIV. Avidity during the first weeks following primary infection is low and increases with the maturation of IgG antibodies (Narita, M. et al., 1996). The avidity for T3/96 was low (<30%), indicating a primary infection in this patient due to primary vaccine failure. This was also confirmed by a positive IgM ELISA result in this case indicative for a primary infection (Linnemann, C.C. et al., 1973). The patient's mother (T5/96) who had a history of natural measles some 30 years ago was in close contact with the son during his illness. A urine sample obtained from the mother showed typical measles CPE after 3 passages in B95a cells. A throat swab specimen taken from the same individual, as well as urine and throat swab samples taken from the father of T3/96, remained negative in tissue culture for 6 passages. MV-specific immunofluorescence using monoclonal antibodies against the N protein performed on those samples also gave negative results. IgG and IgM ELISA testing was carried out on serum samples from both parents showed IgG positivity and avidity values of 49% for the mother and 55% for the serum sample of the father. Both parents were negative for IgM antibodies.

The fact that MV could be isolated from the urine of an asymptomatic individual who was in close contact with an acutely infected person has, to our knowledge, never been reported before. However, it has been proposed that asymptomatic individuals can develop secondary immune responses and may in fact be transiently contagious (Muller, C.P. et al., 1996; Pedersen, I.R. et al., 1989). The question remains at which frequencies these subclinical or asymptomatic cases occur as they could present an important source of viral dissemination in cases where no epidemiological link to an acute case can be found. Following this result, a study has been set up to include urine, blood, and throat swab samples from care-givers that had been in close contact with an individual acutely infected with measles. To date, 4 pairs of infected individuals and their asymptomatic care-givers have been analysed. Virus could not be isolated or detected by immunofluorescence on urine or throat swab samples from the care-givers at any stage, whereas 3 of the 4 infected children clearly were positive for measles infection.

Sequence analysis revealed that both mother and son had identical carboxyl-terminal N genes. It can not be excluded at this stage that the aforementioned MV isolation from an asymptomatic mother was in fact due to a contamination with the virus from the son. However, great care was taken to strictly separate

both samples in tissue culture and at any later stage so that a contamination seemed unlikely. It is questionable whether the viral titres in the mother would have been sufficient to infect susceptible individuals. Furthermore, no virus was isolated from the throat swab sample and MV titres in the urine must have been very low since isolation only occurred after 3 passages in tissue culture. In contrast, MV-positive CPE of samples (either urine or throat swab) from acutely infected patients generally became evident during the first passage in B95a cells (after 1 to 3 days). Further pairs of MV-infected patients and their asymptomatic care-givers will have to be analysed in order to state whether this is a common phenomenon in South Africa or elsewhere.

Analysis of stored clinical samples

Worldwide, efforts are being made to reduce the incidence of MV infections. Some countries in the developed world are moving towards the elimination of MV from within their borders, or have succeeded in interrupting transmission of indigenous virus, as has been shown for the USA (Bellini, W.J. and Rota, P.A., 1998; Rota, J.S. et al., 1996). Nevertheless, most developing countries are only now beginning to embark on MV control programmes such as mass vaccination campaigns and strategies to improve routine vaccine coverage. For countries that currently do not undertake molecular epidemiological surveillance of MV, the amplification of the carboxyl-terminus of the N gene form stored clinical samples using the PCR protocol described in 3.1.3 could be a useful tool in obtaining retrospective epidemiological data. This would allow for sequence analysis of the amplified fragments in order to establish the characteristics of MV strains that have been circulating in that country over time. The knowledge of the genetic characteristics of MV strains prior to implementation of control measures and mass vaccination campaigns, is essential for distinguishing imported from indigenous virus strains. Most techniques reported to date that allow for partial amplification of the MV genome from clinical samples such as sera and CSF's, were used only on fresh samples that had never been frozen, or the techniques proved to be rather time-consuming (Matsuzono, Y. et al., 1994). In addition, other methods amplified only conserved regions (Jin, L. et al., 1996), and resulted in PCR products of less than 200 bp in length (Nakayama, T. et al., 1995) or required further DNA purification prior to sequence analysis (Jin, L. et al., 1996; Nakayama, T. et al., 1995). The method for RNA extraction and RT-PCR of the carboxyl-terminal 450 bp of the N gene described here proved to be rapid, relatively simple, and yielded PCR products that could be used directly for sequencing analysis. Apart from the retrospective analysis of MV using stored material, this technique can further be used to obtain sequence data from recent sera in situations where

specimens for virus isolation are not available. The serum and CSF samples had been stored for 20 years at -20°C and were likely to have been frozen and thawed several times due to use of the samples for other studies. Considering the substantial degradation of RNA that occurs in samples that have been stored for prolonged periods of time and that have been frozen and thawed numerous times, positive amplification in 24% of cases was satisfying. However, the extent of RNA degradation did not permit the amplification of fragments longer than 350-400 bp in most of these samples.

Sequence comparison of different samples from one patient

The CSF and serum samples that were obtained from the same SSPE patient from the Eastern Cape revealed identical sequences for the carboxyl-terminal 456 nucleotides. Similar results were reported by Sakaguchi, M et al. (1986) who found identical sequences for a throat swab and lymphocyte sample from one patient in Japan. This was the only region of the genome that could be amplified using the method discussed above. Partial amplification of the M and H genes were negative. Previous studies have shown extensive mutations in the M gene and F gene of SSPE MV strains (Wong, T.C. et al., 1991; Cattaneo, R. et al., 1986; Cattaneo, R. et al., 1988). Cattaneo, R. et al. (1989) further showed that the carboxyl-terminus of the N gene also accumulated amino acid changes which, however, did not significantly alter the gene product. The results presented here indicate that the carboxyl-terminal N gene sequence of a SSPE case revealed a "normal" sequence that did not reflect unusual hypermutations. This sequence was shown to be closely related to US American strains that were associated with a sustained measles outbreak in this country in the late 1980s and early 1990s (Rota, J.S. et al., 1996).

In summary, this chapter presents molecular epidemiological data on South African measles viruses isolated between 1978 and 1997. The phylogenetic trees, nt and aa alignments, and distance matrix analysis showed that 2 distinct wt groups and 1 group of vaccine-like are circulating in the country. A strain from Namibia, although carrying distinct changes, grouped with the majority of SA MVs. A second MV from Namibia was shown to group with D4 (3) viruses, a group that mainly represents US American MV isolates. A CSF and serum sample taken from a SSPE patient in the Eastern Cape province were identical and were confirmed to be closely related to Chi1, the representative strain of D2 (2) viruses that were responsible for the resurgence of measles in the USA between 1989 and 1991. The analysis of consensus sequences for different genetic groups is a useful way to present genetic comparisons of newly described strains with the ones known to circulate in other parts of the world.

CHAPTER 3.2 MOLECULAR EPIDEMIOLOGY OF MEASLES VIRUS IN SOUTH AFRICA- ANALYSIS OF HEMAGGLUTININ GENES

3.2.1 Introduction

The hemagglutinin (H) gene of wt MVs displays a high variability and sequence and phylogenetic analysis of the full coding region as well as of partial sequences has been used in numerous studies to differentiate between genetic groups (Rota, J.S. et al., 1992; Outlaw, M.C. et al., 1997; Rima, B.K. et al., 1997). The H gene has been suggested to undergo more change than other regions of the genome since the encoded protein is present on the surface of the virus and is therefore likely to become exposed to increased immunological pressure (Rota, J.S. et al., 1992). The H protein is the major target for inducing neutralizing antibodies. Currently, efforts are underway to precisely determine the epitopes on the H protein that induce immune responses (Rota, P.A. et al., 1995). In this context, knowledge of sequence diversity within the H gene will contribute to understanding antigenic variations of the H proteins (summarised in 1.2.1 (B)).

In this study, full coding sequences of the H gene were determined for 21 MV strains from South Africa, selected in order to represent the 3 genetic groups circulating in the country (3.1). The analysis of H genes was particularly important to establish whether the 4 group I strains were vaccine or vaccine-like viruses (discussed in chapter 3.1). Furthermore, this analysis served to confirm the groupings obtained upon sequence analysis of the N gene. The comparison of H and N gene results for use in genptyping will be discussed. Additionally, 2 aa residues that have been suggested to govern CD46 downregulation (Lecouturier, V. et al., 1996; Bartz, R. et al., 1996) were specifically noted. A further aa residue (416) will be discussed in view of an additional glycosylation site that some wt strains contain at this position (Rota, J.S. et.al., 1992; Saito, H. et al., 1994). Partial coding sequences of H genes of several MV from the Gambia and Zambia have recently been published (Outlaw, M.C. et al., 1997). These will be compared to South African isolates and selected strains representing other genetic groups.
Group designations in this chapter will be used in order to conform to those used in chapter 3.1. The full coding sequence of H comprises 1830 nt or 610 aa. For reasons of simplicity, only a few selected strains were used for the alignment of predicted aa changes in Fig. 3.12.

3.2.2 Phylogenetic analysis of South African measles virus H genes

Hemagglutinin genes of 21 SA MV isolates were cloned (2.5) and sequenced on either ABI 373 or ALFexpress automated sequencers (2.4.3) in duplicate or in triplicate. Phylogenetic analysis was performed as described in 3.1.2 (A). Information on strains is summarised in Table 2.1.

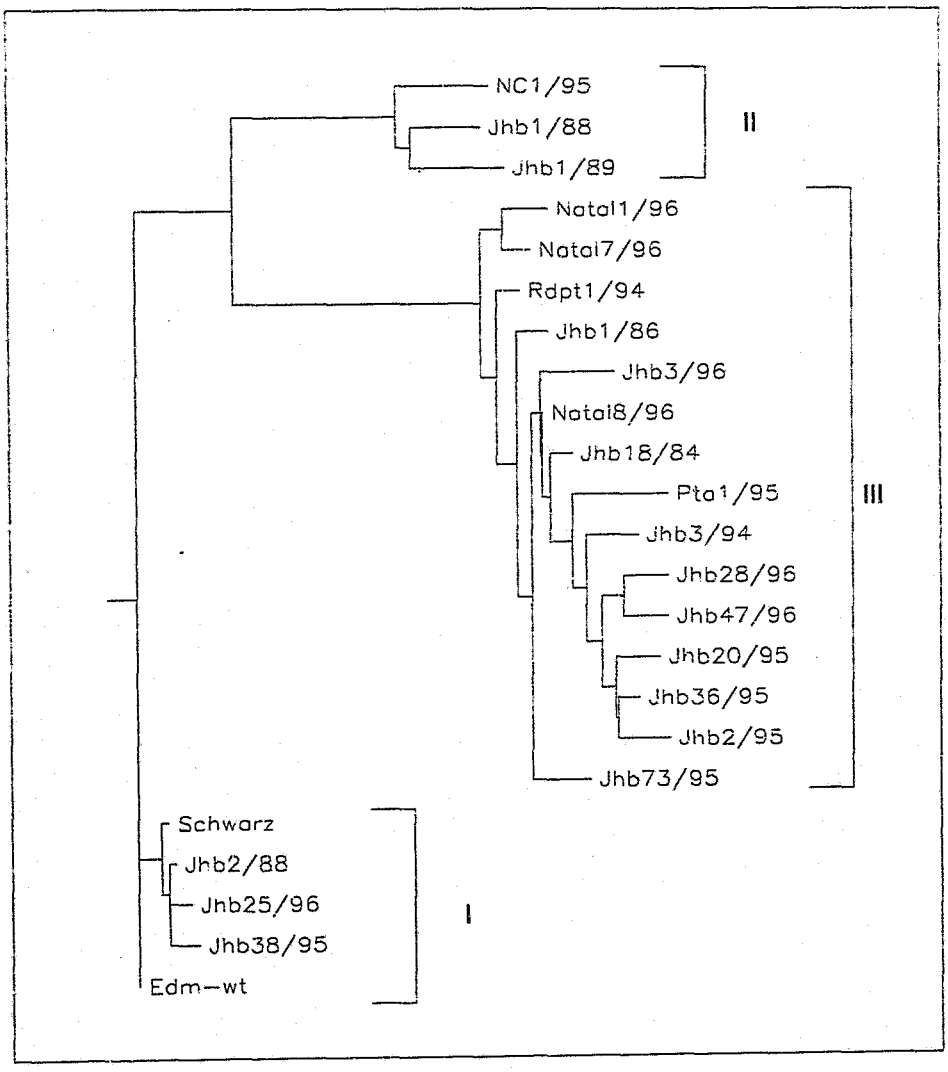

Fig. 3.10 Phylogenetic analysis of the entire coding region of the H gene for 21 South African measles virus strains. The tree is unrooted and was generated using the DNAML program (Phylip).

The phylogenetic analysis on H genes from representatives of the 3 SA genetic lineages confirmed that 2 wt groups as well as 1 group of vaccine-like viruses (Fig. 3.10) are presently circulating in SA, as was determined by partial sequencing of the N gene (3.1.2). For 3 of the 6 strains of group II (D5) H sequences were determined. Once more these viruses proved to belong to a distinct group clearly different from the majority of SA wt strains in group III. The predominant genetic group III also includes the H gene sequence of a strain that was isolated on Vero cells in 1984 (Jhb18/84), showing that this group has been circulating in SA for many years. The coding sequences of H genes from stored clinical samples (2.2.2 and 2.3.3) could not be analysed since PCR amplification of partial H fragments remained negative (data not shown). PCR amplification products of H genes for selected MV isolates were shown in Fig. 3.1. The H sequences of the 4 vaccine-like or vaccine strains (Jhb1/88, Jhb1/89, Jhb38/95, and Jhb25/96) that have been discussed before were closely related to the vaccine strain Schwarz and the Edmonston wt sequence. However, the phylogenetic tree in Fig. 3.10 indicates that the H sequences of these strains were not identical to Schwarz. The question whether these strains represent the vaccine strain or vaccine-like wt isolates will be discussed further in view of nt and aa alignments.

3.2.3 Analysis of H gene sequences from different genetic groups and comparison of phylogenetic results for H and N genes

Fig. 3.11 shows a dendrogram including several South African H sequences and strains from other parts of the world representing most of the genetic groups known to date. Although partly different strains were included in this phylogenetic analysis, the groupings for the strains analysed before (3.1.3) were very similar to the ones described here. Information on MV that were isolated in countries other than South Africa is given in Appendix B. The genetic groups and their designations have been discussed in chapter 3.1. In summary, individual strains that belonged to a certain genetic group on the basis of N gene sequences grouped with the same lineages when H genes were analysed. In addition, some recently published strains, for which corresponding N sequences were not available from the NCBI Genbank, were included in the H tree.

C2 (5) seems to be the predominant genotype in most parts of Europe. TX96 was isolated in the USA but was shown to have been imported from Italy (Rota, J.S. et al., 1998). Other isolates like AB93Ger and WTFV were circulating in Germany in 1992 and 1993, respectively. Numerous new strains from groups D and I (2,3,4, and

7) were analysed as well as additional strains from South Africa that formed part of the genetic lineage I (7).

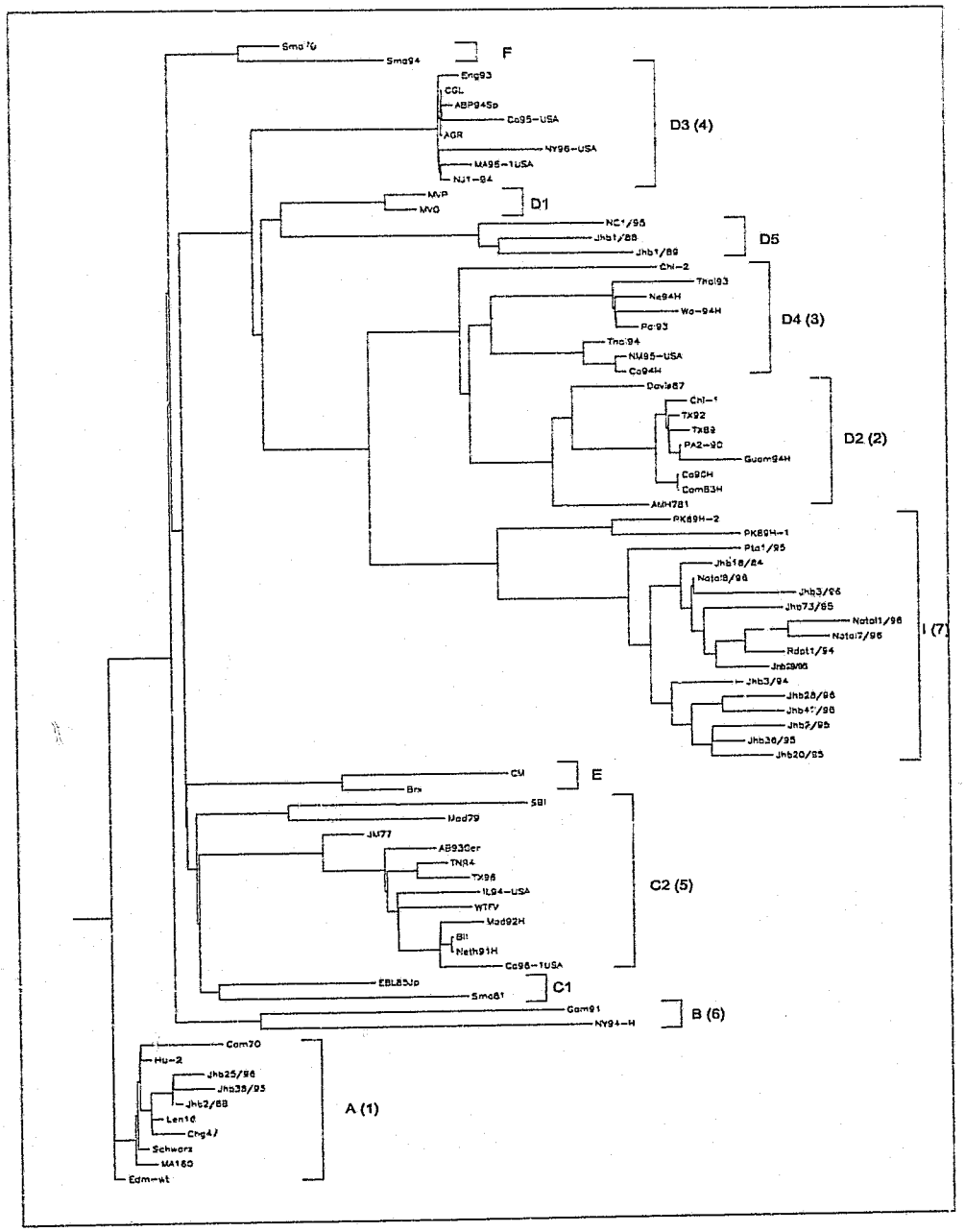

Fig. 3.11 Phylogenetic analysis of H genes.

Phylogenetic results for H and N genes compared well to each other and it appears that analysis of either gene can be used for molecular epidemiological studies. To date, there is no evidence for recombination between MV strains. Thus, simply because of the difference in length of the 2 genes, sequencing of the 456 carboxyl-terminal nt of the N gene would seem easier and more rapid.
The N gene sequence of the Canadian wt strain (Can) was shown be closely related to the predominant genetic group in South Africa (I or 7). The analysis of H genes showed that 2 wt strains from Pakistan (PK89-1, PK89-2) also grouped with the majority of SA MVs. To date, one 1989 Canadian virus (N gene) and 2 strains from Pakistan (H genes) also isolated in 1989 were very similar to South African MV strains of group I (7).

Not included in the N or H phylogenetic trees were wt isolates from China since sequences were not available at the time of writing. This group of viruses has recently been reported to be distinct and considerably different from any genetic group known to date (Bellini, W.J. and Rota, P.A., 1998).

(A) Nucleotide diversity between strains from 8 distinct genetic groups

Table 3.3 shows characteristic nt changes that seem to be useful genetic markers for differentiation of the various viral groups. An alternative method to present such data is to create consensus sequences for each genotype as was presented for the N gene sequences in chapter 3.1.

The nt substitutions at position 72 and 129 of the coding region of the H gene distinguished wt from vaccine and vaccine-like strains (A or 1). Substitutions characteristic for all group D (D2 (2); D3 (4); D4 (3); D5) and group I (7) viruses were identified at positions 525, 754, and 1476. The silent mutations at positions 154, 180, 1659, 1701, 1716, and 1818 were specific to lineage I, including SA wt strains and 2 isolates from Pakistan. A coding substitution at nt 1797 which resulted in the replacement of valine (V) by glutamate (E) was distinct for both SA wt groups (D5 and I) as well as for vaccine and vaccine-like strains. This substitution appeared to be specific to SA MV strains and has not been detected in genetic groups circulating elsewhere. Table 3.3 also demonstrates that the SA isolate Jhb38/95 (A or 1) carried 5 specific nt changes, each of which was coding for aa replacements. Although the carboxyl-terminal N sequence of this strain was identical to the Schwarz vaccine strain, the substitutions in the H gene indicate that this strain indeed was a wt isolate of group 1 (A) rather than the vaccine strain itself.

Table 3.3: Characteristic nucleotide substitutions for selected strains of 8 different genetic groups.

Nt no.	Sch (A)1	Jhb38/95 (A)1	NC1/95 (D5)	Jhb3/96 (I)7	PK89-1 (I)7	CAM83 (D2)2	Thai94 (D4)3	AGR (D3)4	IL94 (C2)5	Gam91 (B)6	aa
21	G	-	-	-	-	A	A	-	-	-	-
42	T	-	-	C	C	C	C	-	-	-	-
54	C	-	-	T	T	T	T	-	-	-	-
72	C	-	T	T	T	T	T	T	T	T	-
129	T	-	C	C	C	C	C	C	C	C	-
154	T	-	-	C	C	-	-	-	-	-	-
180	T	-	-	G	G	-	-	-	-	-	-
291	A	-	G	G	G	G	G	-	-	-	-
525	A	-	G	G	G	G	G	G	-	-	-
618	A	-	G	G	-	G	G	G	G	G	-
631	G	-	A	-	A	A	A	A	A	A	G→S
754	T	-	C	C	C	C	C	C	-	-	Y→H
885	C	-	-	T	T	T	T	-	-	-	L→F
1210	G	A	-	-	-	-	-	-	-	-	D→N
1289	G	A	-	-	-	-	-	-	-	-	G→E
1451	C	A	-	-	-	-	-	-	-	-	T→N
1476	A	-	G	G	G	G	G	G	-	-	-
1659	G	-	-	A	A	-	-	-	-	-	-
1701	A	-	-	G	G	-	-	-	-	-	-
1716	A	-	-	T	T	-	-	-	-	-	-
1769	C	T	-	-	-	-	-	-	-	-	S→F
1797	T	A	A	A	-	-	-	-	-	-	V→E
1818	C	-	-	T	T	-	-	-	-	-	-

Strains that carried most of the group-specific nt substitutions were selected to represent the relevant genetic groups in this table. This table shows a selection of nt changes only. Additional and similar nt substitutions were present in most groups but for reasons of simplicity were not listed.

(B) Genetic distances

The genetic distances between H gene sequences of SA strains corresponded well with the distances calculated for N gene sequences. South African Group A (1) viruses differed by a maximum of 0.4% (nt level) between each other. D5 viruses varied by 1.3%-1.5% when compared to each other, whereas strains from the SA lineage I (7) appeared to be more closely related with nt divergence rates ranging between 0.5% and 1.8% and averaging approximately 1%. The isolates from Pakistan that were closely related to the majority of SA wt viruses, differed by 2.4%-3.0% to other group I strains (data not shown). The nt divergence rates for the representatives of the genetic groups circulating in countries other than South Africa and Pakistan were also similar to the ones obtained in the distance matrix analysis of the N gene (Table 3.2).

3.2.4 Analysis of predicted amino acids of the H protein

(A) Alignment of amino acids for strains representing 8 genetic groups

Fig. 3.12 shows an alignment of predicted aa for a selection of MV strains representing 8 genetic groups.

Fig. 3.12

```
Sch          1   MSPQRDRINA FYKDNPHOKG SRIVINREHL MIDRPYVLLA VLFVMFLSLI   50
Jhb38/95     1   .......... .......... ....V..... .......... ..........   50
NC1/95       1   .......... .......... .......... .......... ..........   50
Jhb3/94      1   .......... .......... .......... .......... ..........   50
PK89-1H      1   .......... .......... .......... .......... ..........   50
Cam83-H      1   .......... .......... .......... .......... ..........   50
Thai94       1   .......... .......... .......... .......... ..........   50
Pal93        1   .......... .......... .......... .......... ..........   50
AGR          1   .......... .......... .......... .......... ..........   50
I194         1   .......... .......... .......... .......... ..........   50
Gam91        1   .......... .......Y.. .......... ......A... ..........   50

Sch         51   GLLAIAGIRL HRAAIYTAEI HKSLSTNLDV TNSIEHQVKD VLTPLFKIIG   100
Jhb38/95    51   .......... .......... .......... .......... ..........   100
NC1/95      51   .......... .......... .......... .......... ..........   100
Jhb3/94     51   .......... .......... .......... .......... ..........   100
PK89-1H     51   .......... Y......... .......... .......... ..........   100
Cam83-H     51   .......... .......... .......... .......... ..........   100
Thai94      51   .......... .......... .......... .......... ..........   100
```

Fig 3.12, continued

```
Pal93      51  .......... .......... .......... .......... ..........  100
AGR        51  .......... .......... .......... .......... ..........  100
Il94       51  .......... .......... .......... .......... ..........  100
Gam91      51  .......... .......... .......... .......... ..........  100

Sch       101  DEVGLRTPQR FTDLVKLISD KIKFLNPDRE YDFRDLTWCI NPPERIKLDY  150
Jhb38/95  101  .......... .......... .......... .......... ..........  150
NC1/95    101  .......... ......F... .......... .A........ ..........  150
Jhb3/94   101  ........H. ......F... .......... .......... ..........  150
PK89-1H   101  ......A... ......F... .......... SG........ ..........  150
Cam83-H   101  .......... ......F... .......... .......... ..........  150
Thai94    101  .......... ......F... .......... .......... ..........  150
Pal93     101  .......... ......F... .......... .......... ..........  150
AGR       101  .......... ......F... .......... .......... ..........  150
Il94      101  .......... ......F... .......... .......... ..........  150
Gam91     101  .......... ......F... .......... .......... ..........  150

Sch       151  DQYCADVAAE ELMNALVNST LLETRTTNQF LAVSKGNCSG PTTIRGQFSN  200
Jhb38/95  151  .......... .......... .......... .......... ..........  200
NC1/95    151  .......... .......... .......... .......... ..........  200
Jhb3/94   151  .......... .......... ...A.A.... .......... ..........  200
PK89-1H   151  .......... ...D...... .......... .......... ..........  200
Cam83-H   151  .......... .......... ...A.A.... .......... ..........  200
Thai94    151  .......... ........A. ...A.A.... .......... ..........  200
Pal93     151  .......... ........A. ...A.A.... .......... ..........  200
AGR       151  .......... .......... .......... .......... ..........  200
Il94      151  .......... .......... .......... .......... ..........  200
Gam91     151  .......... ....T..... .......... .......... ..........  200

Sch       201  MSLSLLDLYL GRGYNVSSIV TMTSQGMYGG TYLVEKPNLS SKRSELSQLS  250
Jhb38/95  201  .......... .......... .......... .......... ..........  250
NC1/95    201  .......... SL........ .......... .......... ..G.....M.  250
Jhb3/94   201  .......... .......... .......... .......... ..G.....P.  250
PK89-1H   201  .......... S......... .......... .......... ..G.......  250
Cam83-H   201  .......... S......... .......... ....G..... ..G.......  250
Thai94    201  .......... S......... .......... .......... ..G.......  250
Pal93     201  .......... S......... .......... .......... ..G.......  250
AGR       201  .......... S......... .......... .......... ..G...P.P.  250
Il94      201  .......... S......... .......... .......... ..G...L...  250
Gam91     201  .......... S......... .......... ..I.....P. ..G.......  250

Sch       251  MYRVFEVGVI RNPGLGAPVF HMTNYLEQPV SNDLSNCMVA LGELKLAALC  300
Jhb38/95  251  .......... .......... .......... .......... ..........  300
NC1/95    251  .H........ .......... .....F.... ...FN...L. ..........  300
Jhb3/94   251  .H........ .......... .....F.... .D.F...V.. .....F....  300
PK89-1H   251  .H........ .......... .....F.... .H.F...... .....F....  300
Cam83-H   251  .H........ .......... .....F.... ...F...... ....RF....  300
Thai94    251  .H........ .......... .....F.... .D.F...... .....F....  300
Pal93     251  .H........ .......... .....F..S. ...F...... .....F....  300
AGR       251  .H........ .......... .....F.... ...F...... ..........  300
Il94      251  .......... .......... .....F.... .......... ..........  300
Gam91     251  .......... .......... .....F.... .......... ..........  300

Sch       301  HGEDSITIPY QGSGKSVSFQ LVKLGVWKSP TDMQSWVPLS TDDPVIDRLY  350
Jhb38/95  301  .......... .......... .......... .......... ..........  350
NC1/95    301  .......... .......... .......... .......... ..........  350
Jhb3/94   301  .R..A..... .......... .......... .......... ..........  350
PK89-1H   301  .R.....V.. ....Q..... ........Q. .......... ..........  350
Cam83-H   301  .R...V.V..     ..... .......... .......... ..........  350
Thai94    301  .R........ .......I.. .......... .......... ..........  350
Pal93     301  .R........ .......... .......... .......... ..........  350
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Fig 3.12, continued

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AGR          301  ..........  ..........  ..........  ..........  ..........  350
I194         301  ..G.......  ..........  ..........  ........F.  ..........  350
Gam91        301  ..........  ..........  ..........  ..........  ..........  350

Sch          351  LSSHRGVIAD  NQAKWAVPTT  RTDDKLRMET  CFQQACKGKI  QALCENPEWA  400
Jhb38/95     351  ..........  ..........  ..........  ..........  ..........  400
NC1/95       351  ...Q.....N  ..........  ..........  ......E...  ..........  400
Jhb3/94      351  ..........  ..T.......  ..........  ......R...  ..........  400
PK89-1H      351  ..........  ..........  ..........  ..........  ..........  400
Cam83-H      351  ..........  ..........  ..........  .........N  ..........  400
Thai94       351  ..F.......  ..........  ..........  ..........  ..........  400
Pal93        351  ..........  ..........  ..........  ......Q...  ..........  400
AGR          351  ......I...  ..........  ..........  ..........  ..........  400
I194         351  ..........  ......I...  ..........  ..........  ..........  400
Gam91        351  ..........  ..........  ..........  ..........  ..........  400

Sch          401  PLKDNRIPSY  GVLSVDLSLT  VELKIKIASG  FGPLITHGSG  MDLYKSNHNN  450
Jhb38/95     401  ...N......  ..........  .........E  ..........  ..........  450
NC1/95       401  ..........  ..........  ..........  ..........  ..........  450
Jhb3/94      401  ..........  .....N....  ..........  L.........  ..........  450
PK89-1H      401  ..........  ..........  ..........  ..........  ..........  450
Cam83-H      401  ..........  .....N....  ..........  ..........  .....T....  450
Thai94       401  ..........  .....N....  ..........  ..........  ..........  450
Pal93        401  ..........  .....N....  ..........  ..........  ..........  450
AGR          401  ..........  ..........  ..........  ..........  ..........  450
I194         401  ....H.....  ..........  ..........  ..........  ..........  450
Gam91        401  ..........  ..........  ..........  ..........  ..........  450

Sch          451  VYWLTIPPMK  NLALGVINTL  EWIPRFKVSP  YLFTVPOKEA  GEDCHAPTYL  500
Jhb38/95     451  ..........  ..........  ..........  ...N......  ..........  500
NC1/95       451  ..........  ..........  ..........  N....Q....  ..........  500
Jhb3/94      451  ..........  ..........  ..V.......  N.........  .G........  500
PK89-1H      451  ..........  ..........  ..........  N.........  ..........  500
Cam83-H      451  ..........  ..........  ..........  N.........  ..........  500
Thai94       451  ..........  ..........  ..T.......  N.........  ..........  500
Pal93        451  ..........  ..........  ..........  N.........  ..........  500
AGR          451  ..........  ..........  ..........  N.........  ..........  500
I194         451  E.........  ..........  ..........  N.........  ..........  500
Gam91        451  .........R  ..........  ..........  N.........  ..........  500

Sch          501  PAEVDGDVKL  SSNLVILPQD  DLQYVLATYD  TSRVEHAVVY  YVYSPSRSFS  550
Jhb38/95     501  ..........  ..........  ..........  ..........  ..........  550
NC1/95       501  ..........  ..........  ...H......  ..........  ..........  550
Jhb3/94      501  ..........  ..........  ..........  ..........  ..........  550
PK89-1H      501  ..........  ..........  ..........  ...G......  ..........  550
Cam83-H      501  ..........  ..........  ..........  ..........  .....G....  550
Thai94       501  ..........  ..........  ..........  .F........  ..........  550
Pal93        501  ..........  ..........  ..........  ..........  ..........  550
AGR          501  ..........  ..........  ..........  ..........  ..........  550
I194         501  ..........  ..........  ..........  ..........  ..........  550
Gam91        501  ..........  ..........  ..........  ..........  ..........  550

Sch          551  YFYPFRLPIK  GVPIELQVEC  FTWDQKLWCR  HFCVLADSES  GGHITHSGMV  600
Jhb38/95     551  ..........  ..........  ..........  .........F  .........E  600
NC1/95       551  ........V.  ..........  ....L.....  ..........  .........E  600
Jhb3/94      551  ..........  ..........  ..........  ..........  .........E  600
PK89-1H      551  ..........  ..........  ..........  ..........  ..........  600
Cam83-H      551  ..........  ..........  ..........  ..........  ..........  600
Thai94       551  ..........  ..........  ..........  ..........  ..........  600
Pal93        551  ..........  ..........  ..........  ..........  ..........  600
AGR          551  ..........  ..........  ..........  ..........  ..........  600
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Fig 3.12, continued

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Il94        551  .......... .I........ .......... .......... ..........  600
Gam91       551  .......... .F........ .......... .......... ..Y.......  600

Sch         601  GMGVSCTVTR ---------- ---------- ---------- ----------  650
Jhb38/95    601  .......... .......... .......... .......... ..........  650
NC1/95      601  .......... .......... .......... .......... ..........  650
Jhb3/94     601  .......... .......... .......... .......... ..........  650
PK89-1H     601  .......... .......... .......... .......... ..........  650
Cam83-H     601  .......... .......... .......... .......... ..........  650
Thai94      601  .......... .......... .......... .......... ..........  650
Pal93       601  .......... .......... .......... .......... ..........  650
AGR         601  .......... .......... .......... .......... ..........  650
Il94        601  .......... .......... .......... .......... ..........  650
Gam91       601  .......... .......... .......... .......... ..........  650
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Fig. 3.12 Alignment of predicted amino acids from selected strains of 8 genetic groups. Position 1 corresponds to the first amino acid of the translated mRNA. The major hydrophobic region is underlined and potential glycosylation sites are indicated in italic and bold letters.

Amino acid substitutions at positions 117, 243, 276, and 481 are markers that distinguish wt from vaccine strains. A Y→H replacement at 252 and a L→W replacement at 284 were characteristic changes only seen in group D and group I virus strains.

Group-specific changes for South African D5 viruses only, were located at residue numbers 211, 285, and 289 (R→L, S→N, and V→L, respectively). Group I (7) strains shared 3 conserved changes at positions 288 (M→V), 305 (S→A), and 473 (I→V). These changes could be interpreted as unique markers useful for differentiation between imported and indigenous MV in SA. The aa substitution V→E (position 600), corresponding to the nt substitution at position 1797 represented a distinct change for SA MV strains of all 3 groups that was not seen in any other genetic group. On average the primary sequence of the H protein of these SA group I (7) strains differed by 57 nt and 22 aa from the Schwarz vaccine strain. D5 strains carried 42 and 16 conserved aa changes when compared to the vaccine strain.

(B) Characteristics of South African vaccine and vaccine-like strains

The Schwarz vaccine strain carries characteristic aa substitutions at positions 117 (F→L), 484 (N→T), and 600 (E→V) when compared to the Edmonston wt strain. Except for the first aa replacement, none of the South African vaccine-like strains of group 1 (A) showed these changes, but displayed the "original" residues that are present in Edmonston. Apart from these mutations, the 2 recent isolates in group 1, Jhb25/96 and Jhb38/95, carried further non-conserved nt and aa changes. This clearly indicates that both strains represent wt isolates of group 1 (A), differing on average by 4 nt compared to the Edmonston strain.
The 2 earlier strains, Jhb2/88, and Jhb2/89 had identical COOH-terminal sequences when compared to Schwarz and apart from the 2 mutations mentioned above, identical aa relative to the H gene of Schwarz. Therefore, these earlier isolates are more likely to represent re-isolated vaccine strains.

Nucleotides of SA group 1 (A) viruses were also aligned with vaccine strains other than Schwarz (Edmonston-derived) and the Edmonston wt strain (data not shown). CAM70 (Brazil), Len16 (Russia), and Chg47 (China) are vaccine strains that were derived from wt strains isolated in those countries. The SA group 1 (A) viruses shared 4 coding nt substitutions with all attenuated vaccine strains mentioned above. These changes at positions 137 (C→T), 631 (A→G), 1441 (A→T), and 1636 (G→A) seem to be highly conserved among group 1 (A) but were not present in the Edmonston wt strain (nt position 137 here corresponds to nt 157 in Rota, J.S. et al., 1994). However, the 2 earlier SA isolates in this group each carried 1 silent nt change, and both recent strains (Jhb38/95, and Jhb25/96) each shared 3 unique nt changes when compared to other vaccine strains. As mentioned above, the SA strains had certain characteristics with other vaccine strains in common. Nevertheless, particularly the 2 recent isolates displayed several specific nt and aa changes identifying these strains as wt isolates of genotype 1.

(C) Specific amino acid changes within the H protein

Glycosylation site at residue 416
It has been shown that residue 416 can contain an additional glycosylation site in some wt strains (Rota, J.S. et al., 1992). This aspartate (D) to asparagine (N) substitution has been detected in wt isolates from groups D2 (2) and D4 (3) as well as in the majority of SA wt viruses of group I (7). Only a single strain,

Natal1/96, did not carry this substitution (data not shown). None of the SA group A (1) and D5 viruses displayed this additional potential glycosylation site. Whether this site is utilised in vivo for additional glycosylation is not clear. The antigenic properties of SA strains related to this aa substitution will be discussed in more detail in chapter 3.4.

Amino acid changes that govern CD46 downregulation

Two amino acid substitutions in the H protein have been reported to influence the ability of certain MV strains to downregulate the cellular receptor CD46 (Lecouturier, V. et al., 1997; Bartz, R. et al., 1997). Table 3.4 summarises the nt and aa at these positions for representatives of 8 genetic groups and the theoretical effect that these mutations could have on the possible downregulation of the CD46 receptor.

Table 3.4 Identification of nucleotide and amino acid changes responsible for CD46 downregulation in 8 different genetic groups

	nt 1352 aa 451		nt 1441 aa 481		possible CD46 downregulation
Sch	T	V	T	Y	+
Edm	T	V	T	Y	+
SA-Group I (A)	T	V	T	Y	+
SA-Group II (D5)	T	V	A	N	-
SA-Group III (I, 7)	T	V	A	N	-
PK89-1 (I, 7)	T	V	A	N	-
CAM83 (D2, 2)	T	V	A	N	-
Thai94 (D4, 3)	T	V	A	N	-
Pal93 (D4, 3)	T	V	A	N	-
AGR (D3, 4)	T	V	A	N	-
IL94 (C2, 5)	A	E	A	N	-
Gam91 (B, 6)	T	V	A	N	-

The aa substitutions at position 451 (valine (V)→ glutamate (E)) and 481 (tyrosine (Y)→ asparagine (N)) have been determined for several vaccine and wt strains. It was found that valine at residue 451 and tyrosine at residue 481 led to the downregualtion of CD46, HeLa cell fusion, and hemadsorption (Lecouturier, V. et al., 1997). Furthermore, Shibahara, K. et al. (1994) showed that B95a MV isolates that were previously unable to agglutinate AGM-RBC (African Green Monkey-red blood cells) gained this ability after 20 passages in Vero cells. Two of the 3 strains investigated acquired the 481 substitution after prolonged passage in Vero cells.

The V(451) and Y(481) aa that were present in all strains of group 1 (A) have been shown to induce downregulation of CD46. Most wt isolates investigated in previous studies carried mutations at position 481 which would theoretically abrogate the ability to influence the expression of CD46 on the cell surface. South African strains of group 1 (A) showed a downregulating genotype carrying aa V (451) and Y (481) (Table 3.4). All SA wt isolates of groups D5 and I (7) shared the valine at 451 with the vaccine strains but carried the Y→N substitution at residue 481. Thus, the SA wt strains should theoretically not be able to downregulate CD46. The possible effects that the downregulation of CD46 could have in vivo, are summarised in 1.3. and will be discussed further in chapter 3.5.

The only strain described in Table 3.4 that displayed the V→E substitution at position 451 was IL94 from group C2 (5). This particular change appeared to be characteristic for C2 viruses and was not found in strains belonging to other genetic groups. The representative strains for the remaining genetic groups carried the genotypic markers at 451 and 481 identical to the SA wt isolates mentioned above.

3.2.5 Phylogenetic analysis of partial coding sequences of African measles virus strains

Outlaw, M.C. et al. (1997) have recently described partial coding sequences of the H gene (1200 nt) for several African MV strains from the Gambia and Zambia. Two Zambian strains isolated in 1992 and a selection of Gambian viruses (1994 and 1995) that have been shown to group together were compared to South African isolates. The only other African sequences described before (Gam91, NY94-H, and several 1983 isolates from Cameroon and Gabon) grouped in lineage B (6) and did not reveal significant similarities to South African MV strains (Figs.3.7 and 3.11). Fig. 3.13 shows the phylogenetic analysis for selected

African viruses and representatives of other genetic groups. Further, 1 strain that was isolated in the Coventry area in England (1993) was included in this tree. Outlaw, M.C. and Pringle, C.R. (1995) concluded that this strain represented a wt isolate closely related to genotype 1 (A) viruses. None of these isolates could be included in the phylogenetic analysis in Fig. 3.11 which was based on the full coding region of the H gene, as only 1200 nt of the coding sequence of H were available for the strains described by Outlaw, M. C. et al. (1997) and Outlaw, M.C. and Pringle, C.R. (1995).

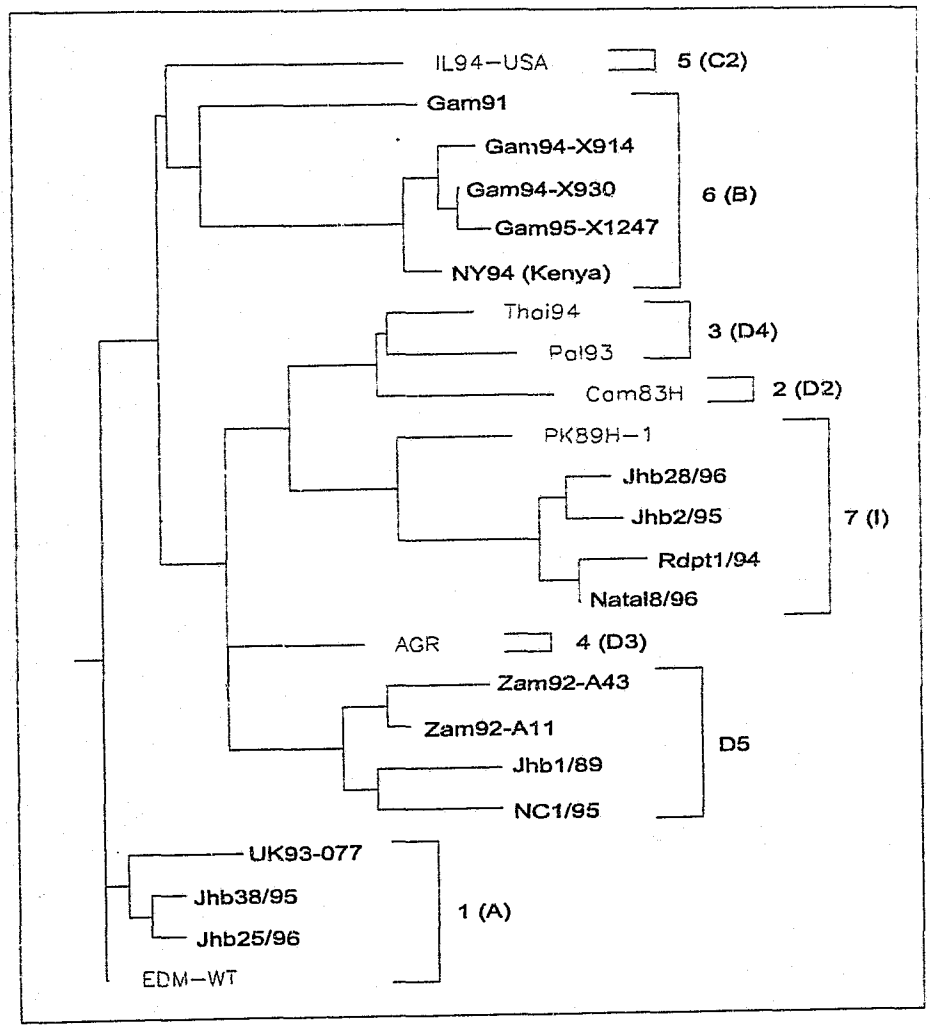

Fig. 3.13 Analysis of partial coding sequences of H for a selection of African strains and representatives of other genetic groups.

This phylogenetic tree clearly indicates that the Coventry isolates indeed grouped with vaccine and vaccine-like viruses in group 1 (A). Two Zambian strains from 1992 (Zam92-A43, Zam92-A11) clustered with South African D5 strains. This is the first time that MV similar to the SA isolates in this group were shown to circulate in other countries within Africa. In view of these results it would be interesting to obtain more sequences from southern Africa to investigate whether this a common lineage in this part of the world. Table 3.5 shows nt and aa changes of the partial coding sequence of H for 2 Zambian strains and South African D5 viruses. MV from these 2 countries shared 10 conserved nt changes of which 5 were coding for aa replacements. Four of 5 aa changes listed below were unique to D5 strains only, confirming the genetic similarity between South African D5 strains and the 2 Zambian isolates from 1992.

Table 3.5 Characteristic nucleotide and amino acid changes in MV strains from South Africa and Zambia.

Nt #	South Africa-D5 (1988-1995)	Zambia (1992)	aa
687	G→A	+	-
854	G→A	+	L→F
865	G→T	+	V→L
891	A→G	+	-
1078	G→A	+	D→N
1116	A→T	-	-
1158	-	T→C	-
1164	-	T→C	-
1215	C→T	+	-
1398	A→G	+	-
1451	C→A	-	-
1675	A→G	+	I→V
1724	A→T	+	Q→L
1755	T→G	+	-

+ nucleotide change present; - nucleotide or amino acid substitution absent

Eighteen west African MV from the Gambia (1994 and 1995) have been demonstrated to be closely related to each other (Outlaw, M.C. et al., 1997). Therefore, only 3 of these isolates were selected for the phylogenetic analysis in Fig. 3.13. The Gambian viruses grouped with other west African strains described before in lineage B (6). This group includes isolates from Cameroon, Gabon, and Gambia (1991) as well as a strain that was imported into the USA from Kenya (NY94) (Rota, J.S. et al., 1996). The Gambian isolates shown in Fig. 3.13 appeared to be more closely related to this Kenyan virus than to another strain that was isolated in this country 3 or 4 years earlier. It seems plausible to assume that more genetic groups will be found as more isolates from other African countries will become available.

3.2.6 Discussion

Groupings of South African MV strains established on the basis of H gene sequences confirmed the results based on the carboxyl-terminal N gene sequences. Two distinct genetic groups of wt MV and one group of vaccine-like strains were identified and have been discussed before.

Measles virus genetic groups in South Africa:
The 2 recent SA vaccine-like strains were demonstrated to be wt isolates of group 1 (A) which includes vaccine and vaccine-like sequences from different parts of the world. Jhb25/96 and Jhb38/95 carried 4 and 5 different coding nt changes relative to the Schwarz vaccine strain that has been used in South Africa for many years. Jhb2/88 and Jhb2/89 however, displayed only 1 nt change. Patients' histories for these isolates were not available but there is a possibility that these early strains (isolated on Vero cells) were in fact derived from the Schwarz vaccine strain. This single nt change may have been acquired during replication of the virus in the patients. At this point in time, it is not possible to establish beyond doubt whether these viruses represent vaccine strains or wt isolates of group 1. In this context, vaccine-like wt strains have recently been isolated in England (Outlaw, M.C. and Pringle, C.R., 1995). One strain from the Coventry area revealed 1 coding nt change only when compared to the MMR vaccine. Another 4 viruses from this area were also shown to be vaccine-related although a premature termination signal at residue number 583 in these strains shortened the H protein by 35 aa. Genotype 1 is believed to have been the predominant genetic group in the world before the widespread introduction of

measles vaccines. The last wt MV of group 1 (A) was isolated in 1970 (Rima, B.K. et al., 1997). Almost 30 years later, 9 viruses of this genotype were found to circulate in South Africa and England. There is a strong possibility that more group 1 viruses will be identified as more countries participate in routine molecular surveillance of circulating MV strains.

The ratio of expressed over silent mutations within the 2 South African wt groups D5 and I, was 0.35 and 0.38, respectively. Both ratios are indicative of random drift rather than a direct genetic drift due to immunological pressure. Evolutionary rates for South African MV were extremely low. The earliest H sequence within group I was derived from an isolate made in 1984 in Johannesburg. Comparison of Jhb18/84 to recent SA wt strains revealed only 1 additional nt change in recent isolates and this equalled a mutation rate of 5.5 x 10^{-5} per annum. The immunological pressure due to high vaccine coverage has been proposed to possibly drive the evolution of MV (Rima, B.K. et al., 1997), as has been demonstrated for Influenza A virus which is known to accumulate mutations under increased immunological pressure (Domingo, E. and Holland, J.J., 1994). The evolutionary pressure caused by elevated vaccine coverage was certainly relatively low in African countries as well as in South Africa. The coverage rates for South Africa over the past 10 years averaged 76% for the first dose and 63% for individuals who have received a second dose of vaccine (Epidemiological Comments, 1995). Thus, it is reasonable to assume that MV isolated in Africa are likely to display an even higher degree of genetic stability than viruses from developed countries. This has been reported before for African strains (NY94-H from Kenya and Cameroon strains from 1983) that were isolated in 1983 and 1994. The 11 year time span between isolations did not show a significant evolution and nt differences only attained 1.5% over time (Rota, J.S. et al., 1996).

Comparison of South African MV strains with those isolated in other parts of Africa:

Another recent study by Outlaw, M.C. et al. (1997) analysed wt strains that were isolated in the Gambia during 1994 and 1995 as well as 2 isolates from Zambia (1992). The region analysed (1200 nt) covered the majority of the open reading frame (ORF) for the external domain of the H protein and was therefore likely to represent the majority of significant neutralizing epitopes. Eighteen Gambian isolates have been shown to group together. Phylogenetic analysis was performed on a selection of Gambian strains, the 2 Zambian strains in addition to a selection of South African MV (Fig. 3.13). The Zambian isolates were related to

group D5 of South African strains. This is interesting since viruses of this group have only been found to circulate in these 2 southern African countries to date. D5 is certainly not the predominant genetic group in South Africa at present, but it could be the major lineage in other southern African countries from which individual strains could have been imported into South Africa. Efforts will be made to obtain more samples from Zambia and other neighbouring countries in order to establish whether this group is circulating in higher numbers elsewhere. Previously described African strains from the Gambia (Gam91), Kenya (NY94-H), Cameroon and Gabon (Rota, J.S. et al., 1996; Giraudon, P. et al., 1988) were shown to be related to each other and all belonged to one genetic group (B or 6). The recently published 13 sequences from the Gambia (1994 and 1995) (Outlaw, M.C. et al., 1997) proved to cluster together and were closely related to group B viruses. Although geographically distant, 1994/95 Gambian MV were more closely related to a Kenyan isolate from 1994 (NY94) and not to the Gambian strain (Gam91) that was isolated in 1991.

Comparison of phylogenetic groupings on the basis of H and N sequences:
The results for the genetic groupings presented here were based on the entire coding region of H and compared well with the phylogenetic groupings obtained using partial coding sequences of N. No major changes were apparent when analysing the 2 different genes. However, Rima, B.K. et al. (1997) have noted that the central nodes in such phylogenetic trees are not well supported in bootstrap analysis and that changes can occur depending on the data set of sequences included in the analysis. In this particular study (Rima, B.K. et al., 1997), the only significant changes that were detected when comparing N and H genes occurred with SSPE strains. The majority of wt strains from cases of natural measles remained unchanged. To date, recombination between different MV strains has not been shown to exist and this seems plausible as genetic groups appear to be restricted to certain geographical areas and the time period for necessary co-infection with 2 viruses is very short in natural measles infections. Thus, it would be reasonable to state that either gene, H or N, could be used for establishing phylogenetic groups of MV.

Genetic groups of measles virus:
The question of how many genotypes currently exist in the world can only be answered when sequences from more countries, especially from Africa and Asia, become available, and as soon as clear guidelines as to how to define MV genetic groups have been agreed upon. Depending on the criteria used for definition of MV genotypes, generally 8 distinct genetic groups have been

described to date, most of which disclose a clear geographic restriction. The majority of South African wt strains grouped in lineage I (7) similar to strains from Pakistan and 1 Canadian isolate. This group revealed several common changes with genotype D viruses, which appears to be the predominant genetic group currently circulating in many parts of the world. Therefore, group I could also be interpreted as a subgroup within genotype D. The D5 group representing 6 South African strains as well as 2 Zambian isolates were shown to be even more closely related to genotype D measles strains, and thus could certainly be considered as a subgroup within this major genetic group. The nucleotide and amino acid alignments as well as the analysis of genetic distances supported the genetic clusters established by phylogenetic analysis, as related groups of viruses shared certain silent or coding nt substitutions and exhibited low genetic distance values. This was equally true for analysis of H and N genes.

Additional glycosylation site in the H protein

The D→N aa substitution in some wt strains has been shown to create a new potential N-linked glycosylation site (Rota, J.S. et al., 1994). The alignment of predicted aa in Fig. 3.11 demonstrated that viruses in groups D2 (2), D4 (3) and the South African strains of group I displayed this change, whereas the isolates from Pakistan, closely related to I, did not carry this substitution. Whether this 6th glycosylation site is used in vivo and what the relevance of this additional sugar could be is not clear at this point in time. However, other morbilliviruses such as canine distemper virus and rinderpest virus also contain an additional glycosylation site at or near the one described for MV (Rota, J.S. et al., 1994), providing evidence that this phenomenon might indeed have in vivo relevance.

Amino acids responsible for CD46 downregulation

Two amino acid changes at positions 451 and 481 have been proposed to play a major role in the ability of MV strains to downregulate CD46 (Lecouturier, V. et al., 1997; Bartz, R. et al., 1997). Both groups used site-directed mutagenesis to introduce valine (V) at position 451 and tyrosine (Y) at position 481 into previously non-downregulating measles strains. Apart from other mutations that might be of minor importance for a downregulating phenotype, these 2 aa substitutions were able to clearly induce downregulation of CD46. Apart from this phenomenon, Lecouturier, V. et al. (1997) further investigated the ability of 2 MV strains to fuse HeLa cells as well as hemadsorption patterns, properties that were also influenced by the 2 mutations mentioned above. Table 3.4 showed the residues that were present at positions 451 and 481 in a selection of MV strains. The downregulating genotype is characterised by aa V451 and Y481. The

vaccine strain Schwarz and the Edmonston wt strain as well as viruses related to group 1 (including 4 South African strains) carried these phenotypic markers (V and Y). Except for IL94 (group C2 or 5), all other wt isolates proved to have the valine residue at 451 but displayed a glutamate (N) at position 481. This change has been demonstrated to abrogate fusion of HeLa cells as well as the ability to downregulate CD46 (Lecouturier, V. et al., 1997). However, Bartz, R. et al. (1997) have identified several wt strains that contained the N residue at 481 instead of tyrosine (Y). All of these wt strains were initially isolated on Raji or HeLa cells. The relevance of these aa substitutions and the ability to downregulate CD46 of some MV strains will be discussed further in chapter 3.5.

CHAPTER 3.3 GENETIC SCREENING OF MEASLES VIRUS STRAINS

3.3.1 Introduction

Measles virus continues to circulate endemically in most parts of Africa as well as in South Africa. Although the incidence of the disease is believed to have been markedly reduced by 2 mass vaccination campaigns in 1996 and 1997, an average of 15 000 notified cases per annum over the past 10 years (1986 to 1996) have been reported (Epidemiological Comments, 1994-1997).

The standard and most commonly used method for determining strain diversity in MV has been sequence analysis of the carboxyl-terminus of the nucleocapsid gene (Rota, P.A. et al., 1994; Rima, B.K. et al., 1995a). Nevertheless, the abundance of circulating wt MV in South Africa and the need to obtain molecular epidemiological data on multiple specimens in a short period of time demanded a more rapid, less complex, but still reliable technique to identify strains for further characterisation by sequence analysis. This study was aimed to adapt and evaluate techniques, other than sequencing, allowing for rapid and reproducible pre-screening of MV isolates. Efforts were concentrated on the Restriction Length Fragment Polymorphism (RFLP) technique and the Heteroduplex Mobility Assay (HMA).

RFLP analysis relies on the identification of nt changes that destroy a known restriction enzyme recognition site or create novel recognition sites that can then be cleaved by selected enzymes. PCR-amplified fragments are digested with a single or multiple restriction enzyme(s) and the resulting fragments are separated on agarose gels. This approach has been successfully used by Mori, T. et al. (1994) to differentiate between wt and vaccine strains. The technique is easy to perform and results are generated rapidly. However, it generally depends on the detection of single mutations only that could alter restriction enzyme recognition. The loss of a previously present site or the creation of a new restriction site can subsequently be detected by the differences in resulting cleavage fragments on an agarose gel.

The HMA technique was initially applied to detect deletions and insertions in human genes (Prior, T.W. et al., 1993; Soto, D. and Sukumar, S., 1992; Ruano, G. and Kidd, K.K., 1992) and more recently in time for analysis of genetic relationships among HIV isolates (Delwart, E.L. et al., 1993; Strunnikova, N. et al., 1995). The

technique utilises polymorphic differences in fragment mobility relative to a fragment of known sequence. To obtain heteroduplex formation, PCR amplified fragments from known and unknown isolates are mixed together, denatured and then re-annealed. The resulting heteroduplexes show differences in migration due to the effects of primary sequence changes forming mismatches which cause bulges in the ds DNA fragment. The location, type (transition or transversion), the actual number of mutations and the spacing between mismatches influence the mobility and migration pattern of heteroduplexes in a non-denaturing gel electrophoresis (Lilley, D.M.J., 1995; Hsieh, C.H. and Griffith, J.D., 1989). Thus, the HMA allows the genetic screening of unknown DNA fragments in a sequence dependent manner (White, M.B. et al., 1992).

The HMA proved to be the most useful technique for screening and assigning individual MV isolates to genetic groups. A total of 97 isolates were genotyped, 47 of which were confirmed to belong to the predicted group by sequence analysis of either the carboxyl-terminus of the N gene or the entire coding region of the H gene. The methodology for both RFLP and HMA analysis are described in section 2.6.

3.3.2 RFLP analysis of South African measles virus strains

RFLP analysis has been used previously to genetically characterise MV on the basis of mutations in the H genes of viruses belonging to different genetic groups (Mori, T., 1994; Saito, H., et al., 1995). In this study, the carboxyl-terminus of the N gene was used to characterise South African MV isolates. However, a number of variable regions of the genome could be used for RFLP. By generating restriction enzyme maps of the relevant genomic region, restriction patterns unique to a certain genetic group could easily be identified.
Table 3.5 shows the cleavage patterns for the 3 genetic groups of MV currently circulating in SA. Consensus sequences were created for these 3 lineages and used to generate restriction enzyme maps. Con-A includes group 1 viruses that are related to vaccine strains, Con-D5 included 6 wt strains which are distinct from the majority of SA strains which belong to Con-I. To confirm the results regarding consensus sequences, original sequences of several isolates were used to generate restriction maps that were compared to the ones obtained for consensus sequences. Suitable enzymes for cleavage of the original sequences were mostly identical to the ones shown here.

Table 3.5 summarises the group-specific fragment sizes that were obtained upon cleavage of the carboxyl-terminus of the N gene (456 nt) with the listed enzymes. In addition to the enzymes mentioned below, further enzymes were identified resulting in specific fragments that would distinguish 2 or all 3 of the South African genetic groups. For reasons of simplicity only some of the suitable enzymes are presented.

Table 3.5 Fragment sizes and cleavage sites within the carboxyl-terminal 456 nt of the N gene on consensus sequences of 3 South African genetic groups for selected restriction enzymes.

Enzyme	Con-A (I)	Con-D5 (II)	Con-I (III)	differentiates between:
Bgl II	- (456)	441 (15)	441 (15)	vaccine versus wt
Dde I	- 83 (83) 364 (281) 405 (41) (41)	- 83 (83) 364 (281) 405 (41) (41)	41 (41) 83 (42) 364 (281) 405 (41) (41)	wt group I versus vaccine and group D5
Hae III	242 (242) - (214)	- (456) -	322 (322) 375 (53) (81)	all groups
MspI	239 (239) 284 (41) (172)	239 (239) - (217)	239 (239) - (217)	vaccine versus wt

con: consensus sequence
sizes of resulting fragments are given in brackets behind the location of the cleavage site

Further restriction enzymes that could distinguish between wt and vaccine-like viruses of group 1 were AflI and Cfr13I. However, some of the fragments created by

these enzymes differed only by several nt between distinct genetic groups so that it was difficult to visualize the difference in size on a 3% agarose gel. Bgl II, as shown in Table 3.5, cleaved the carboxyl-terminal 456 nt of the N gene at position 441. This is one example of an enzyme not suitable for routine RFLP analysis since the differences in size of 15 bp between different groups were difficult to detect on an agarose gel. Further enzymes for differentiation between group I and group D5 strains were: AvrII, BbvI, Cfr13I, NspI, StyI, and Frm4HI.

HaeIII, AsuI, BbvI, and PalI were the only enzymes that could distinguish between all 3 South African genetic groups. Three of the above mentioned enzymes have been used to differentiate between wt and vaccine viruses (data not shown) but HaeIII proved to work best because of the sizes of fragments generated after cleavage.

Fig. 3.14 illustrates the HaeIII cleavage fragments for selected strains representing the 3 genetic groups currently present in South Africa. The vaccine strains Schwarz and 2 SA wt strains of this group 1 (A) were cleaved into 2 fragments of 214 and 242 bp. In group D5 viruses, the HaeIII restriction site was abolished by a C→T mutation at position 244. Group I viruses too, did not contain this recognition site at 242 but due to conserved mutations at positions 322 (A→G) and 375 (A→G), 2 new HaeIII restriction sites were created. This particular enzyme proved to be highly suitable for clear discrimination between 3 viral groups.

Fig. 3.15 shows cleavage results for MspI digestions. MspI cleavage differentiated between wt and vaccine-related strains of group 1. These viruses were cleaved into 3 fragments of 45, 172, and 239 bp in length. Wild-type strains, irrespective whether they belonged to groups I or D5 were cleaved into 2 fragments of 217 and 239 bp. This difference in size of the various fragments could be visualised on a 3% agarose gel.

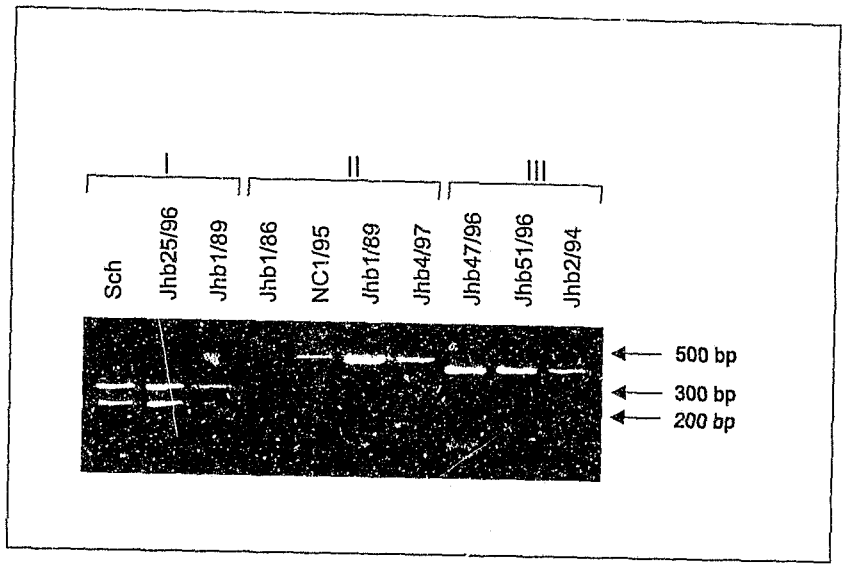

Fig. 3.14 RFLP analysis of selected strains of 3 distinct genetic groups. N-carboxyl-terminal fragments were cleaved with HaeIII and separated on a 3% agarose gel.

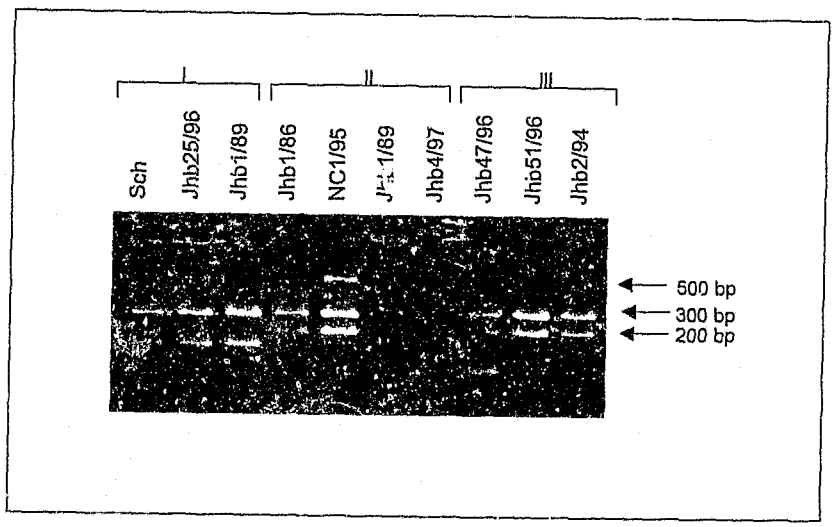

Fig. 3.15 Cleavage of carboxyl-terminal 456 nt of the N gene of representatives from 3 South African genetic groups, digested with *MspI*.

3.3.3 HMA screening of measles virus

HMA analysis has previously been used to assign HIV, HCV, and Polio viruses to different groups or clades (Novitsky, V. et al., 1996; Louwagie, J. et al., 1994; Buanaguro, L. et al., 1995; Delwart, E.L. et al., 1994; Chezzi, C. and Schoub, B.D., 1997). In this study, HMA analysis has been applied to the genetic characterisation of MV isolates belonging to different lineages. The methodology described previously by Delwart, E. et al. (1994) had to be modified in order to sensitise the method for detection of heteroduplex fragments of MV in gel electrophoresis. To find the optimal conditions for maximum separation of hetero- and homoduplex fragments, several methodologies were tested including different annealing protocols, different gel matrices and varying electrophoresis conditions. Fig. 3.16 shows diagrammatically the formation of homo- and heteroduplex bands.

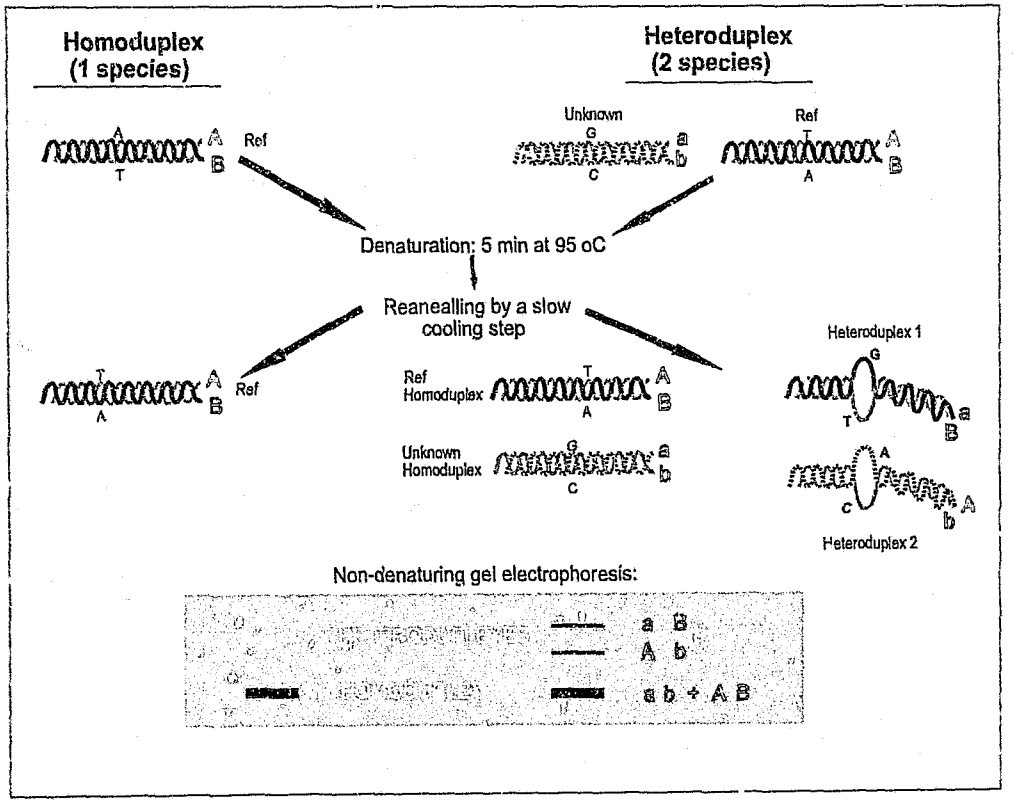

Fig. 3.16 Diagram showing the principle of heteroduplex and homoduplex formation during HMA analysis.

The conditions described in section 2.6.1 were found to be optimal for pre-screening of MVs. The MDE gel matrix proved to be superior to polyacrylamide gels (data not shown), providing a higher resolution needed to detect heteroduplexes formed between South African MV strains which varied at most by 4.9% in their nucleotide sequences and the the Schwarz vaccine strain. Best annealing results were obtained without the addition of annealing buffer and by a very slow cooling step in a pre-heated water bath.

(A) HMA analysis of South African measles virus strains

Fig. 3.17 shows the heteroduplex formation of South African MV. PCR-amplified fragments of the carboxyl-terminal 589 nt were first heat-denatured and then annealed to the corresponding fragment of the Schwarz vaccine strain by a slow cooling step. Homoduplex bands stained more intensely since they contain double the amount of DNA compared to the heteroduplex bands (Fig. 3.16).
The isolates analysed by HMA broadly fell into 3 different banding patterns, 2 of which indicated distinct heteroduplex formation and 1 pattern which showed the formation of a homoduplex band only when compared to the Schwarz-homoduplex control. The 3 patterns were designated group I (1 or A), II (D5) and III (I or 7). Lanes 2, 3, and 4 in Fig. 3.17 illustrate the formation of homoduplexes of the 3 strains Jhb2/88, Jhb2/89, and Jhb38/95, respectively when mixed with the Schwarz vaccine strain, indicating a high sequence homology which was confirmed by sequence analysis of the H and partial N genes. Group II was represented by 4 wt isolates Jhb1/86, Jhb1/88, Jhb1/89, and NC1/95, which have been discussed previously in section 3.1. The 2 remaining strains belonging to this genetic group (Jhb4/97 and SA168/78) also revealed the characteristic group II migration patterns (data not shown). The heteroduplex fragments of group II viruses and the Schwarz reference fragment migrated as a single band which was retarded relative to the homoduplex control. Group III heteroduplexes migrated as typical double bands.

HMA between isolates within the same genetic group yielded homoduplexes only (lanes 15, 16 and 17). The degree of nt divergence between the group II pair Jhb1/86 and NC1/95 (lane 16) and the group III pair Jhb29/95 and Jhb73/95 (lane 17) was 1.6% and 2.9%, respectively while the group I pair Jhb2/88 and Jhb2/89 were almost identical to each other. Thus HMA appeared to be only able to detect divergence greater than 3.0 % when analysing SA MV wt strains. Predicted amino acids were aligned for SA viruses as was discussed in chapter 3.1.

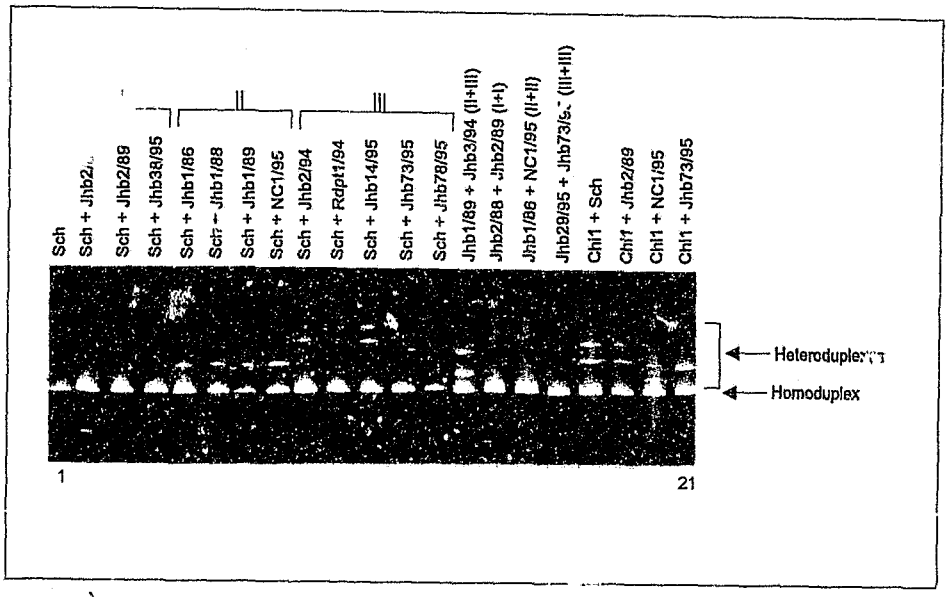

Fig. 3.17 HMA analysis of South African measles virus strains

This analysis indicated that group II MVs carried 3 nt substitutions at positions 18, 253, and 321 (G→A silent, G→A coding, and C→A silent, respectively), which were characteristic for this group only. The recent lineage of South African wt showed 3 characteristic coding nt substitutions at positions 138 (C→A), 238 (A→G), and 419 (C→T), which were distinct changes for this genetic group I (7). These results suggest that the changes characteristic for either group might be responsible for the formation of specific bulges and kinks in each genotype which subsequently determine the mobilities of heteroduplex fragments. Although the individual isolates within a genotype differed by scattered mutations throughout the analysed region of the genome, it appears that only the above mentioned substitutions alter the composition of mismatches enough to be detected by HMA analysis.

To determine the effect that a different reference strain would have on migration patterns of the analysed isolates and to investigate whether the use of a reference strain other than the Schwarz vaccine strain would allow discrimination between viruses within 1 of the 2 wt lineages, heteroduplexes were formed between South African MVs and 2 wild-types from the USA, Chi1 and JK (provided by Dr P.A. Rota,

CDC). A selection of the group I, II and III viruses were therefore annealed to either Chi1 or JK. Mobility of heteroduplexes formed with either of the 2 American strains was clearly different compared to heteroduplex fragments with the Schwarz vaccine strain, but confirmed in all cases the established groupings of the examined isolates (data not shown). Lanes 20 and 21 (Fig. 3.17) show the migration patterns of 2 South African wt MV (group II and group III) with the American wt strain Chi1 (genetic group D2 or 2). Thus, using Chi1 or JK as a reference fragment, the same groupings of viruses were obtained, but differentiation between strains belonging to 1 genotype was still not detectable, supporting once more the observation that the composition of substitutions within 2 fragments of relatively high similarity rather than the absolute number of changes, influences the mobility of formed heteroduplexes.

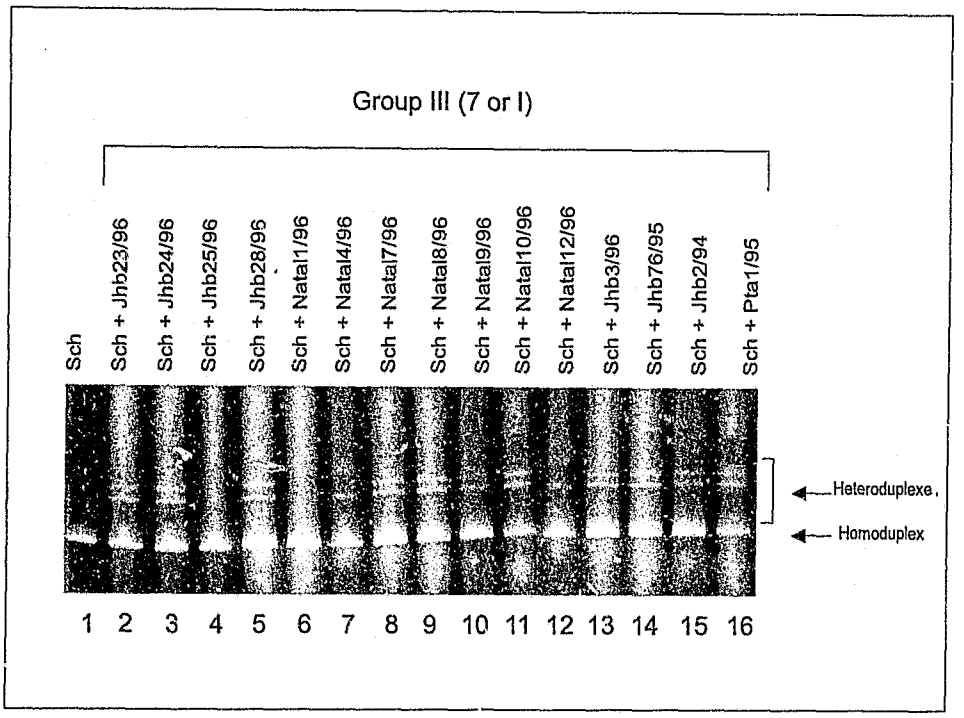

Fig. 3.18 HMA analysis of recent measles virus strains from South Africa.

Fig. 3.18 presents further HMA results for MV isolates made in 1996. Most strains tested in this analysis displayed the double heteroduplex band that was shown to be characteristic for South African group III (7) viruses. However, 3 strains from Natal (Natal 1/96, 4/96, and 12/96) showed a wider spacing between the 2 heteroduplex bands. These strains were therefore sequenced to determine whether unusual nt changes could be identified that may cause this different migration pattern. According to sequence and phylogenetic analysis, these 3 Natal strains clearly belonged to group III (I or 7) viruses (Fig. 3.4). However, they carried an additional C→T mutation at position 420 of the COOH-terminus of the N gene, adjacent to another C→T replacement at position 419 which was present in all other strains of this genetic group. Since no other significantly different changes were found, this double mutation at position 419 and 420 must have caused the shift between the heteroduplex bands.

Clinical samples of which the carboxyl-terminus of N could be amplified in 1 fragment (as discussed in section 3.1.3) were also analysed by HMA. Two further sera that were collected in 1978 (SA414/78 and SA572/78) revealed a banding pattern identical to group III strains and can therefore be grouped with lineage I (7) (data not shown).

A total of 97 samples were analysed by HMA. Four isolates were shown to belong to group 1, 6 strains formed group D5, and the remaining 85 samples proved to be part of the major genetic lineage in South Africa (I or 7). Two clinical samples from the Eastern Cape and Windhoek, Namibia were shown to be related to lineages other than the 3 circulating in South Africa. Table 3.6 summarises genotyping results on all specimens that were analysed for this study.

Table 3.6 Summary of genetic groupings of measles virus strains analysed by HMA and/or sequencing

Origin	year of isol.	total no.	Group1 (A) I	Group D5 II	Group 7 (I) III	other
?, South Africa	1978	4	-	1	3	-
Johannesburg	1984	1	-	-	1	-
	1986	1	-	1	-	-
	1988	2	1	1	-	-
	1989	2	1	1	-	-
	1994	3	-	-	3	-
	1995	51	1	-	50	-
	1996	16	1	-	15	-
	1997	2	-	1	1	-
Natal	1995	1	-	1	-	-
	1996	7	-	-	7	-
Mpumalanga	1995	3	-	-	3	-
Durban	1997	1	-	-	1	-
Eastern Cape	1997	1	-	-	-	1
Namibia	1997	2	-	-	1	1
Total		**97**	**4**	**6**	**85**	**2**

(B) HMA analysis of measles viruses from 8 different genetic groups

The HMA has been shown to be a successful method to reliably distinguish between MV strains from South Africa that belonged to distinct genetic groups. This study was undertaken to determine whether the HMA could also discriminate between MV isolated from 9 different countries that were known to belong to 8 distinct lineages.

The practical work for this study was carried out at the Centers for Disease Control and Prevention (CDC) in Atlanta during a study visit in 1997. The reagents and MV strains that were isolated in countries other than South Africa were kindly provided by Dr P.A. Rota and J. Rota. Information on these isolates is summarised in Table 3.7.

Table 3.7: Measles strains from 8 genetic groups used for HMA analysis

No.	Name	Country of isolation	Country of origin	Date of isolation	Genotype
1	Moraten	USA	USA	1968	1
2	Arg1199[1]				1
3	Jhb25/96[2]	South Africa	South Africa	1996	1
4	CA1990[3]	USA		1990	2
5	TX-1 1989[3]	USA		1989	2
6	WA:3/14/96[1]	USA	Japan	1996	3A
7	MA:1/20/96[1]	USA	unknown	1996	3A
8	NM:1/31/95[1]	USA	unknown	1995	3B
9	CO1994[3]			1994	3B
10	NY:3/31/96[1]	USA	Austria	1996	4
11	AK:2/16/96[1]	USA	unknown	1996	4
12	MN:12/6/95[1]	USA	unknown	1995	5
13	NY:4/4/96[1]	USA	Germany	1996	5
14	Gambia1086	Gambia	Gambia	1991	6
15	Y22	Cameroon	Cameroon	1983	6
16	PA:4/24/97[4]			1997	7
17	WA:6/12/95[1]	USA	Pakistan	1995	7
18	932/China[5]	China	China		8
19	936/China[5]	China	China		8
20	Jhb33/96	South Africa	South Africa	1996	7
21	Jhb2/94[2]	South Africa	South Africa	1994	7

Ref [1] Rota, J.S., personal communication, [2] Kreis, S. et al., 1997, [3] Rota, J.S.et al., 1997, [4] Rota, P.A. et al.,199 [5] Xu, W-B., personal communication.

The HMA results for selected MV strains from Table 3.7 representing 8 genotypes are shown in Fig. 3.19. Distinct migration patterns for the hetroduplexes became evident for the different genotypes and are discussed below. HMA results corresponded in all cases to sequencing results and thus confirmed the reliability of this assay for rapid genotyping of MV isolates.

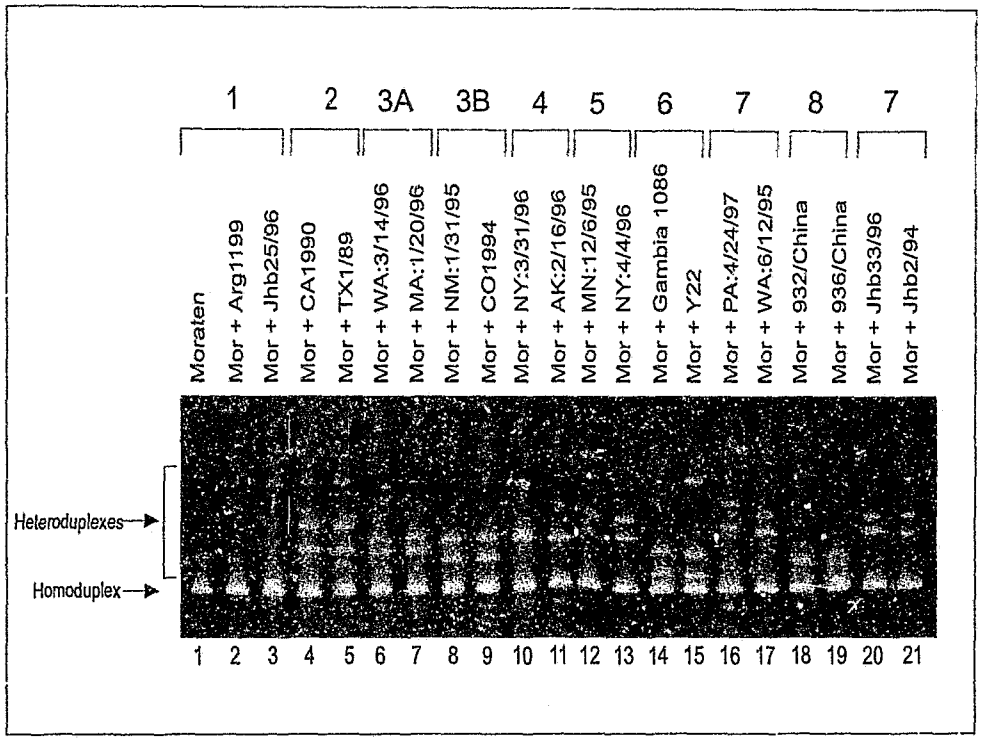

Fig. 3.19 HMA analysis of viruses representing 8 different genotypes.

All PCR-amplified fragments of the COOH-terminus of the N gene were annealed to Moraten, a vaccine strain that has been used in the USA for many years. Lane 1 (Fig. 3.19) represents the homoduplex control of the reference strain (Moraten) only.

Lanes 2 and 3 show the migration patterns for 2 vaccine-like strains. When mixed with the vaccine strain Moraten, the 2 strains Jhb25/96 and Arg1199 (Table 3.7) only revealed homoduplex formation, indicating their close relationship to group 1 (A) viruses, which was confirmed by sequence analysis. The remaining lanes in Fig. 3.19 show the differences in heteroduplex formation and subsequent migration for 2 selected wt isolates from each genotype (groups 1 to 8). The 2 isolates representing genotype 2 (CA 1990 and TX-1989) had identical heteroduplex formation apart from a non-specific band in TX-1989 which migrated below the

heteroduplexes (lane 5) . This band most likely represents an unspecific PCR amplification product that did not interfere with the formation of the group-specific heteroduplexes. Non-specific bands can easily be identified by comparing specific heteroduplex bands to the bands present in the homoduplex control for a certain strain.

Lanes 6 to 9 show that the HMA was able to distinguish subgroups (3A and 3B) within a genetic lineage. Although the top band of the heteroduplexes for all 4 viruses from this genotype (3) migrated equally, the lower band ran faster in subgroup 3B resulting in a different spacing between the 2 bands for both subgroups.

AK:2/16/96 migrated with a single heteroduplex band, whereas NY:3/31/96 showed a typical double band with the 2 bands migrating closely together (lanes 10, 11). The nucleotide (nt) alignment for these viruses illustrated that these 2 strains were identical except for 1 nt change in NY: 3/31/96 at position 169 (A→G) which probably caused the formation of the double heteroduplex bands (data not shown). Migration of both isolates was identical apart from this tight second heteroduplex band.

Lanes 14 and 15 show HMA results for African strains from the Gambia and Cameroon that both revealed a distinct mobility pattern of heteroduplexes. Lanes 16 and 17 represent members of group 7 including WA:6/12/95 from Pakistan. The relationship of isolates from Pakistan to the South African wt viruses of this group has been discussed before (chapter 3.2). The HMA migration of this isolate is identical to 2 SA strains in lanes 20 and 21 illustrating their genetic relatedness. Group 8 is represented by 2 Chinese viruses (lanes 18, 19) that are very distinct and do not seem to resemble to any other genetic group (Bellini, W.J. and Rota, P.A., 1998). 936/China had multiple non-specific bands which made it difficult to detect the specific heteroduplex band that was seen in 932/China.

In summary, HMA proved to be a suitable technique to reliably determine genetic groupings of multiple samples within a short period of time. In order to be able to assign new isolates accurately to a certain genetic group, representatives of these lineages would have to be analysed in parallel to serve as reference standards for each genetic lineage.

3.3.4 Discussion

The goal to control and eventually eradicate MV has been pursued by the WHO for a number of years. Serological as well as molecular epidemiological surveillance plays a critical role in any attempt to achieve this goal (as discussed in chapter 3.1). The knowledge of MV genetic groups indigenous to a country will enable us to establish the geographic origin of viruses associated with residual cases of measles as successful vaccination programs reduce the incidence of the virus. The standard method for determination of MV strains variations has been sequence analysis of the carboxyl-terminus of the N gene or the H gene. Particularly in Africa, sequencing facilities are not commonly available to clinical laboratories. Therefore, there was a need to develop simple, cost-effective, rapid and reliable techniques to genetically characterise MV. Using such techniques, researchers or medical personnel in poor resource settings would be able to rapidly screen a great number of cases and select samples that need to be sent for further characterisation by sequencing analysis.

In this study the RFLP and HMA techniques were applied and evaluated to predict genetic groupings of MV isolates or clinical samples. Analysis was focussed on the PCR amplified carboxyl-terminal 589 bp of the N gene. It has been shown that this region is the most variable of the MV genome and therefore suitable for strain characterisation (Griffin, D.E. and Bellini, W.J., 1996). Seventy-eight samples, collected between 1978 and 1997 in South Africa and Namibia were included in this study. Sequence and phylogenetic analysis performed on 47 of the 78 viruses confirmed the HMA and RFLP results in all cases.

Overview of screening techniques other than RFLP and HMA:
Apart from the techniques presented here other methods have been described for genetic screening or detection of point mutations in heterogenous samples. Single strand conformation polymorphism (SSCP) or dideoxy fingerprinting (ddF) could have been used alternatively to the HMA. SSCP allows for the detection of single base substitutions by denaturation of ds DNA and immediate electrophoresis on a non-denaturing gel (Spinardi, L. et al., 1991; Orita, M. et al., 1989); similar to RFLP analysis. The advantage of SSCP over RFLP is that it can detect single base substitutions at a variety of positions in DNA fragments. However, the SSCP technique involves the use of radioactive material and therefore restricts its application to specialised laboratories only. The ddF screening method is a hybrid between SSCP and Sanger dideoxy sequencing. It is very efficient but relatively complex and also uses radioactive isotopes (Blaszyk, H. et al., 1994). RNAse protection or mismatch assay is one of the early screening methods and has been

used to assess heterogeneity in RNA viruses. RNA is cleaved by Rnase A at a mismatch site in a RNA probe hybridised to mutant target DNA. This method only detects 70% of mutations and is demanding as well as it involves considerable amounts of radioisotopes (Myers, R.M. et al., 1985; Aranda, M.A. et al., 1995). One of the few non-radioactive screening techniques is the low-stringency single specific primer PCR (LSSP). This special PCR application takes advantage of of limited number of base differences in PCR fragments. By reamplification of the DNA at a very low annealing temperature (30-40 °C) in the presence of excessive amounts of a single primer and Taq polymerase, specific gene signatures are generated. The mode of action lies in the fact that extension of partial hybrids between primer and template takes place by the thermoresistent enzyme before actual dissociation of the illegitimate combination between the 2 DNA molecules. Thus, a novel DNA template is created in a specific, sequence dependent manner (van Belkum, A., 1995; Pena, S.D.J. et al., 1994). As part of this study, LSSP was also evaluated for use in MV genotyping (data not shown). Screening results obtained with LSSP were dissatisfying, resulting in too many bands on the gel as well as smear of non-specific DNA making it difficult to distinguish and read the results. Group-specific differences in the banding pattern for the MV strains analysed could not clearly be detected. Further screening techniques have been reviewed by Cotton, R.G.H. (1992).

RFLP analysis:

RFLP analysis of selected MV strains was performed on the carboxyl-terminal 589 bp of the N gene. Previous studies have focussed on partial sequences of the H gene and were able to distinguish wt from vaccine strains and different types of hemagglutination types (Mori, T., 1994; Saito, H. et al., 1995). The RFLP method is easy to perform and, except for PCR facilities, does not require sophisticated laboratory equipment. Purified PCR fragments were digested with selected restriction enzymes. Consensus sequences were created for the different genetic groups and a restriction map was generated on these sequences by the WDNAsis program. By comparison of the restriction maps for the 3 groups, enzymes that were suitable for differentiation of lineages could easily be specified. Several restriction enzymes have been identified for this purpose and were listed under 3 3.2. HaeIII appeared to be most adequate in distinguishing between the 3 genetic groups of MV currently circulating in South Africa, resulting in restriction fragments of a size that could readily be detected on standard agarose gels (2-3%). The technique is simple, affordable and produces results rapidly. However, RFLP analysis can only take single, previously known nt substitutions into consideration. If a certain mutation has a heterogenous distribution between individual strains belonging to 1 genetic group, RFLP can give conflicting and misleading results. Therefore, the selected enzymes for RFLP analysis should ideally target highly conserved changes within genetic

groups. However, if only such conserved substitutions are object of the analysis, it may be difficult to find adequate restriction enzymes that could cleave at the relevant site. Thus, it seems that, even though RFLP is a feasible method, the HMA is the more versatile and reliable technique for genetic characterisation of MV.

HMA analysis:
Several protocols for HMA have been described for detection of point mutations and single deletions or insertions in various human genes (Prior, T.W. et al., 1993; Soto, D. and Sukumar, S., 1992; Wang, Y-H. et al., 1992). Furthermore, the HMA has been used successfully for discrimination between HIV subtypes and quasispecies (Delwart, E.L. et al., 1993 and 1994) as well as for differentiation of Polio vaccine-related and wt viruses (Chezzi, C. and Schoub, B.D., 1997). This simple but efficient technique relies on sequence differences between an unknown and a reference sequence that result in mismatched base pairs. The mismatches form bulges and kinks in the RNA or DNA molecules that are causing an electrophoretic retardation (Lilley, D.M.J., 1995; White, M.B. et al., 1992). Important criteria for heteroduplex formation are both the number and the position of mutations within the analysed region. Gaps formed by unpaired nt have a greater effect on heteroduplex mobility than simply mismatched nt (Hsieh, C-H. and Griffith, J.D., 1989). Base pairs neighbouring a mismatch can affect the degree of kinking caused by the unpaired pair and thus also impact on the resulting mobility (Wang, U-H. and Griffith, J.D., 1991). Furthermore, it has been shown that purine-bulged bases produce greater electrophoretic retardation than pyrimidine bases. The simplest explanation for this phenomenon is that the bulge-induced kinks are relatively static, with the kinking angle being greater for the purine bases due to their greater size (Wang, U-H. and Griffith, J.D., 1991). Finally, Bhattacharyya, A. and Lilley, D.M.J. (1989) have demonstrated that the type of substitution (transversion/transition), its context and its position in a DNA fragment affect mobilities of heteroduplexes. Mutations at the termini of a fragment generate a greater retardation than those located in the centre. Thus, the HMA allows for screening of heterogenous fragments in a sequence dependent manner and- in contrast to RFLP analysis- can reflect the type and number of all mutations in the electrophoretic migration patterns of the heteroduplexes.

HMA analysis of South African measles strains:
HMA was performed on 78 samples, 47 of which were confirmed by sequence analysis. The strains analysed showed 2 distinct heteroduplex migration patterns. Group I (1 or A) isolates did not form heteroduplexes but migrated as homoduplex bands only, when annealed to Schwarz, indicating the close similarity of these viruses to vaccine strains. Wild-type MV from South Africa fell into 2 HMA groups.

D5 (group II) viruses showed a single heteroduplex band, whereas group III (7) migrated with the more typical double band. Although some scattered mutations were detected when comparing individual strains within a genetic group, they generally displayed identical heteroduplex mobilities, demonstrating that these changes did not influence the mobility of heteroduplex fragments. The formation of distinct migration patterns is rather affected by the majority of specific mutations that are common to all members of a certain lineage. However, the nt replacement C→T at position 420 in 3 isolates from Natal caused a shift of the upper heteroduplex band, as was shown in Fig. 3.18. This mutation was adjacent to another C→T replacement that was present in all of South African group I (7) viruses. The double mutation in the Natal strains could cause a considerable increase in the size of the bulge at this position and this could certainly affect the retardation of the heteroduplexes. However, the lower band of the 2 heteroduplexes remained unchanged and only the upper band was slightly shifted resulting in a wider spacing. This proves that even single point mutations can be detected by HMA if they affect the composition and size of bulges or kinks. Apart from the Natal isolates, no difference in mobility of heteroduplex bands could be detected between individual isolates of the 2 South African wt groups and thus a standard curve could not be constructed in order to calculate genetic divergence by measuring different heteroduplex mobilities, as it has been described previously for HIV (Delwart, E.L. et al., 1994; Strunnikova, N. et al., 1995). The HMA, applied to MV, could clearly distinguish between viruses grouping in different lineages but failed to discriminate between viruses of one genetic group. Although the sequences variation within group II (D5) and III (I or 7) could reach up to 2.5%, this did not cause a detectable shift in mobility, when wt fragments were annealed to the Schwarz N gene fragment. The reasons for this are several. Firstly, absolute sequence variation between measles viruses is relatively low, compared to other RNA-viruses or HIV (Delwart, E.L. et al., 1995) and averages at most 8.0% in the carboxyl-terminus of the N gene. Geographically restricted MVs from 1 country or region, which group together in a distinct lineage, vary to an even lesser extent, with an average of 1-2% divergence in their sequences and thus separation of heteroduplex fragments cannot be as effective as in HIV, that can differ up to 35% (Buonaguro, L. et al., 1995). Secondly, deletions and insertions in the N gene are extremely rare and were not detected in any of the South African isolates. These types of point mutations create gaps, leading to the formation of more kinks, which subsequently affect migration patterns far more than simple substitutions. HMA analysis was also performed on the entire coding region of the N gene (data not shown) which did not provide sufficient resolution of heteroduplex fragments. This was probably due to the fact that parts of the N gene, especially the amino-terminus are very conserved and do not display a sufficient number of mutations- relative to the total size of the fragment (1683 nt)-

to allow for strain differentiation.

HMA analysis of MV strains representing 8 genetic groups:
The feasibility of HMA analysis for differentiation of MV belonging to 8 different genetic groups was investigated. Results indicated that the HMA is a highly suitable technique for reproducible and rapid differentiation between all viruses tested. Even viruses grouping in different subgroups within a given genotype could be distinguished in most cases (group 3). Thus, the HMA allows for effective screening and rapid assignment of unknown isolates to the various genotypes. Furthermore, pre-screening of multiple specimens from 1 country or from an outbreak using the HMA can identify similar strains and out-grouping isolates and thus can help reduce the amount of sequencing analysis needed for further investigations.

CHAPTER 3.4 ANTIGENIC PROPERTIES OF SOUTH AFRICAN MEASLES VIRUS HEMAGGLUTININ PROTEINS

3.4.1 Introduction

The MV H protein is a type II transmembrane glycoprotein and its antigenic properties have been shown to vary between strains from different genetic groups (Hu, A. et al., 1994; Tamin, A. et al., 1994). Variations affecting efficiency and salt-dependency of hemagglutination, loss of binding to specific mAb, the molecular weight of H proteins, and the ability to downregulate CD46 have all been described (Saito, H. et al., 1992; Shirodaria, P.V. et al., 1976; Sakata, H. et al., 1993; Rota, J.S. et al., 1992; Hummel, K.B. and Bellini, W.J., 1995; Giraudon, P. and Wild, T.F., 1985; Bartz, R. et al., 1997). However, a study on wt strains from Africa isolated between 1983 and 1984 failed to show any antigenic variation of the H protein compared to the vaccine strains (Giraudon, P. et al., 1988). Specific mAb against the MV H protein do not exhibit any cross-reactivity with other members of Paramyxo- or Morbilliviruses (Sheshberadaran, H. and Norrby, E., 1986) reflecting the specificity of the various viruses for different hosts and thus for different receptors. Anti-H mAb have been used in epitope mapping studies to locate the functional domains in H and some of these studies have demonstrated that conserved as well as variable epitopes exist in wt MV strains (Obeid, O.E. et al., 1993; Tamin, A. et al., 1994; Hu, A. et al., 1993). The construction of chimeras has been employed by others to investigate and characterise the stability of cross-reactive epitopes in H (Hummel, K.B. and Bellini, W.J., 1995; Bankamp, B., personal communication). To date, the question remains whether these antigenic differences together with the potential loss of neutralising epitopes (escape mutants?) might, in the future, render the current vaccines ineffective.

MV H proteins from various wt and vaccine strains have previously been analysed with regard to their molecular weight and the differences in antigenic epitopes (Tamin, A. et al., 1994; Bankamp, B., personal communication; Hummel, K.B. and Bellini, W.J., 1995; Rota, J.S. et al., 1992). In this study, the electrophoretic mobility of the H proteins of 10 South African MV strains was investigated by Western Blot (WB) (2.7.2) and RIPA analysis (2.7.1). Furthermore, H proteins from 6 South African isolates were immunoprecipitated with 6 different mAb to determine whether antigenic epitopes in H differed among SA MV strains.

3.4.2 Western Blot analysis of MV H proteins

Six South African MV strains were analysed by WB (method discussed in section 2.7.2) and results are shown in Fig. 3.20. Total protein extracts were obtained from MV-infected B95a cells. Electrophoresed and blotted H proteins were incubated with the mAb B2 (kindly provided by Dr P.A. Rota, CDC). This antibody immunoprecipitates a protein of ~80 kDa and reacts with all MV strains tested so far. B2 is a positive control antibody that was expected to recognise all H proteins tested. Fig.3.20 shows that all isolates representing the 3 SA genetic groups were recognised by mAb B2 with the expected molecular weight of the H proteins of 80 kDa.

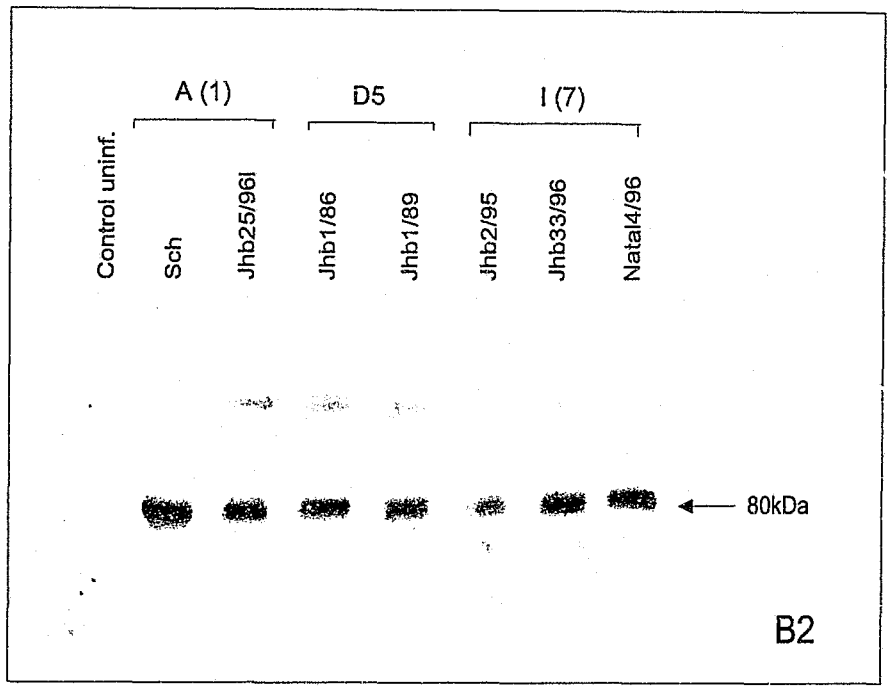

Fig. 3.20 Western Blot analysis of H proteins from 6 South African MV strains and the Schwarz vaccine strain with the mAb B2. Uninfected B95a cells served as a control in lane 1.

Another 6 wt isolates of group III (I or 7) were also analysed by WB (data not shown) and revealed identical recognition and migration patterns as the ones shown above. WB analysis certainly represents a feasible method to determine the presence of antigenic epitopes as well as the molecular weight of certain proteins. However, the method that has generally been applied to analyse MV specific epitopes as well as the sizes of different H proteins, is the RIP assay.

3.4.3 Analysis of South African H proteins by RIPA

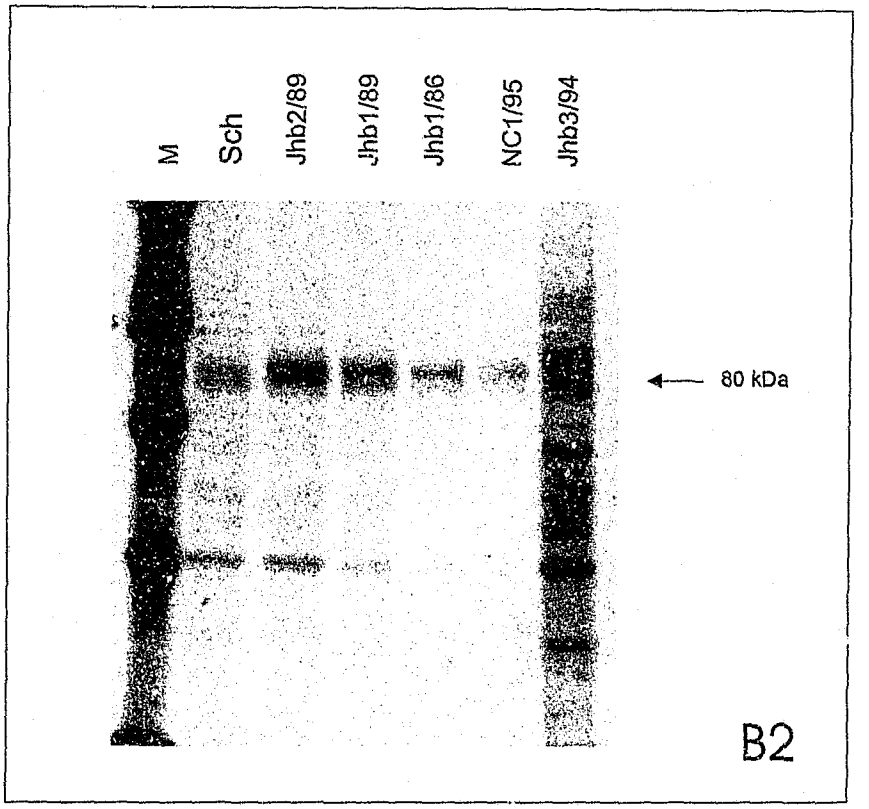

Fig. 3.21 Immunoprecipitation of MV H proteins with the mAb B2.

RIPA analysis involves the precipitation of radio-labelled proteins in solution by specific antibodies. Precipitated immuno-complexes are then separated by PAGE followed by the visualisation of specific complexes by autoradiography. Here, this method has been applied to visualise MV specific H proteins that were precipitated with a panel of mAb. Fig. 3.21 and 3.22 show the RIPA results obtained with the selected mAb, B2 and CV4 antibody on 6 SA MV strains.

All H proteins tested and shown in Fig. 3.21 appeared to have identical molecular masses, except for Jhb3/94 . Although there was considerable non-specific binding in this sample, the upper band in lane 7 is likely to be the H specific band, which migrates slightly slower compared to other wt strains as well as the Schwarz vaccine strains. A slightly increased molecular weight has also been shown to exist for several Japanese wt strains when compared to the Edmonston vaccine strain which generally has an apparent size of ~73 kDa (Saito, H. et al., 1992) as well as for 2 US American wt isolates (Rota, J.S. et al., 1992). However, the results shown here indicate that the Schwarz vaccine strain had an identical molecular weight when compared to the majority of South African wt strains. There is a possibility that the lower molecular weight (~73 kDa) of the Edmonston strain relative to other wt or vaccine strains strains (~80 kDa) is a particular property of Edmonston only. To establish whether the increase in weight could be explained by the use of an additional glycosylation site, the H proteins of several SA MV strains, including Jhb3/94 were treated with Endoglycosidase F/N-glycosiadse F prior to electrophoresis (data not shown) as was described previously (Rota, J.S. et al., 1992; Hu, A. et al., 1993). This enzyme mixture hydrolyses several types of N-glycans. Due to the removal of the N-linked sugars, the molecular weights of all strains tested were shifted by approximately 4 to 5 kDa. However, Jhb3/94 continued to migrate slower compared to other South African strains. Very similar results for Endo H or Endo F treatment have been reported (Rota, J.S. et al., 1992; Hu, A. et al., 1993). This indicates that the differential mobility of Jhb3/94 in gel electrophoresis was probably not caused by additional glycosylation.

Fig. 3.22 shows results obtained with the mAb CV4 which were similar to those obtained with B2 except for NC1/95 which was not recognised by CV4 (lane 6). The incubation with mAbs during RIPA analysis were performed on the same lysates for all strains shown in Figs. 3.21 and 3.22. Thus, the failure of CV4 to bind the H protein of NC1/95 was unlikely to be due to insufficient amounts of protein. Another 7 wt strains were tested in RIPA with CV4 and NC1/95 remained the only isolate that could not be precipitated by CV4 (data not shown). Again, Jhb3/94 in lane 7 appeared to have a slightly heavier H protein compared to other SA MVs. Except for

Jhb3/94, all other South African H proteins revealed the expected molecular mass of ~80 kDa. The genetic sequences (nt and aa) of the H gene of Jhb3/94 did not display any unusual changes that could account for this difference in molecular weight.

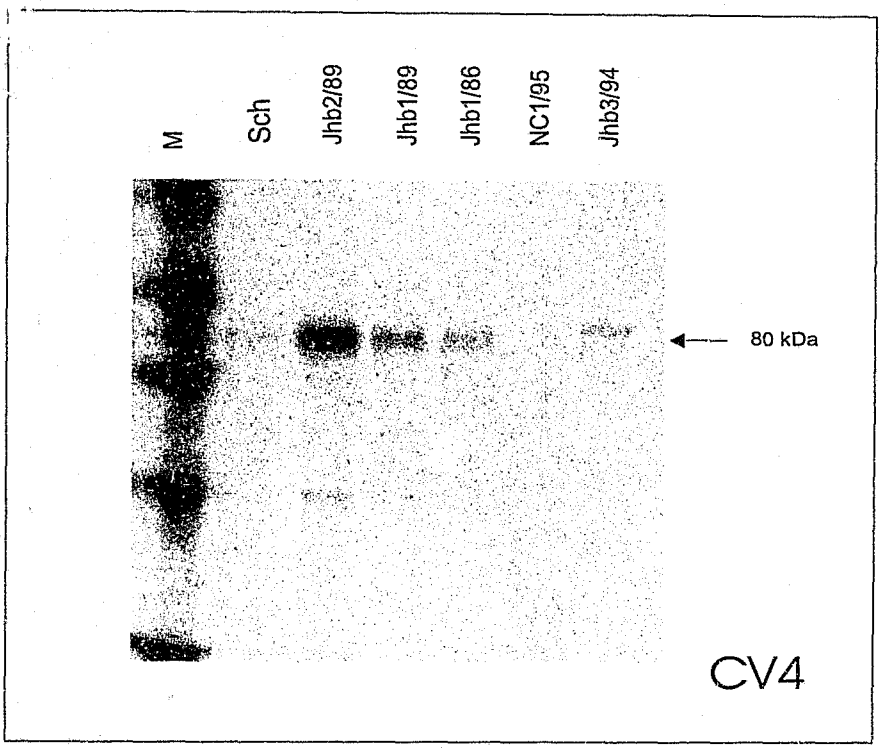

Fig. 3.22 RIPA analysis of H proteins of SA wt strains with the mAb CV4.

Fig. 3.23 (below) illustrates the precipitation results for 2 representative strains of South African MV with 6 different antibodies. The H proteins of Jhb1/86 were precipitated by antibodies 366, B2, V17, and CV4, but not by CV2, and CV5. Jhb1/86 shows typical results that were obtained for all other SA isolates analysed (Table 3.8). NC1/95 showed a similar profile except for the absence of the CV4 epitope. The lack of CV4 binding has also been detected in 2 recent wt strains from

China (Xu, W., CDC, personal communication). H gene nt sequences and predicted aa for NC1/95 and the 2 Chinese strains were therefore aligned (data not shown). No similarity or unusual mutations were found to be shared between these isolates and thus the CV4 epitope could not be mapped to an exact site within the H protein.

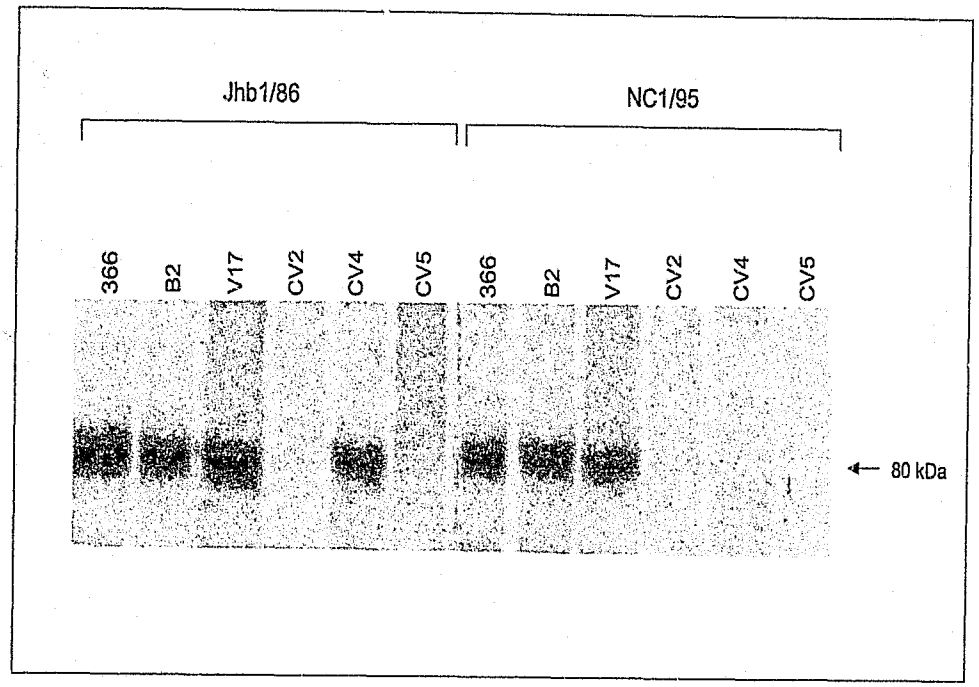

Fig. 3.23 RIPA results for 2 South African wt strains precipitated with 6 different antibodies.

Table 3.8 summarises the reactivity results for several South African MV strains with a panel of mAb.

Table 3.8 Reactivity of MV strains with a selection of mAb

MV strain (Group)	366	B2	V17	CV2	CV4	CV5	apparent mol. weight (KDa)
Sch (1or A)	+	+	+	-	+	-	~80
Jhb25/96 (1 or A)	+	+	+	-	+	-	~80
Jhb1/86 (D5)	+	+	+	-	+	-	~80
Jhb1/89 (D5)	+	+	+	-	+	-	~80
NC1/95 (D5)	+	+	+	-	-	-	~80
Jhb3/94 (7 or I)	+	+	+	-	+	-	~85
Jhb2/95 (7 or I)	+	+	+	-	+	-	~80
Jhb33/96 (7 or I)	+	+	+	-	+	-	~80
Natal1/96 (7 or I)	+	+	+	-	+	-	~80
Natal4/96 (7 or I)	+	+	+	-	+	-	~80

The mAb V17 has previously been mapped to react with aa 211-291 (Hummel, K.B. and Bellini, W.J., 1995).

Tamin, A. et al. (1994), and Bankamp, B. (personal communication) have examined the reactivity of several MV representing 4 distinct genetic lineages with the above mentioned panel of monoclonal antibodies. It was demonstrated that B2 and 366 reacted with H proteins from all lineages tested and as shown here also reacted with all South African strains analysed. The antibodies B2 and 366 therefore appear to target an epitope that is highly conserved between viruses from different genetic groups. CV4 recognised strains representing genetic groups 1 (A), 5 (C2), 3 (D4), 4 (D3), 6 (B), US American MV isolates made between 1977 and 1989, as well as the South African wt strains of group 7 (I). However, the only isolate that could not be precipitated by CV4 was the D5 strain NC1/95. The antibody V17 was interesting since it bound to all SA MV strains but only precipitated vaccine strains of group 1 (A) in other studies (Tamin, A. et al., 1994; Bankamp, B., personal communication). CV2 and CV5 precipitated all SA strains tested but only recognised few of the viruses that were analysed in the studies by Tamin, A. et al. (1994) and Bankamp, B. (personal communication).

3.4.4 Discussion

The H protein is the major MV antigen that induces neutralising antibodies. Genetic changes that have occurred in the H gene over time are well documented (Rima, B.K. *et al.*, 1997; Rota, J.S. *et al.*, 1992; Outlaw, M.C. *et al.*, 1997). Currently, many studies are focussing on the antigenic properties of the H protein to establish whether the genetic drift in the gene is reflected in changing antigenicity of the corresponding protein (Hu, A. et al., 1993 and 1994; Tamin, A. et al., 1994; Giraudon, P. and Wild, T.F., 1985). This study examined electrophoretic mobilities of H proteins from South African MV strains as well as their antigenic properties using a panel of mAbs. Studies are in progress to relate the changes observed to biological or phenotypic characteristics, such as neutralisation abilities and the ability of different H proteins to agglutinate red blood cells.

Increased molecular weight due to additional glycosylation?

One wt isolate from group 7 (I), Jhb3/94 showed a slower mobility in PAGE due to a slight increase in molecular weight. It has been shown previously that MV H proteins display variable molecular sizes in PAGE (Shibahara, K. et al., 1994; Rota, J.S. et al., 1992; Saito, H. et al., 1992; Tamin, A. et al., 1994). It was demonstrated that the Edmonston vaccine strain had a slightly lower molecular mass than several wt isolates from the USA. South African wt strains, with the exception of Jhb3/94, appeared to have identical apparent sizes relative to the Schwarz vaccine strain. South African MV isolates will be compared to the Edmonston strain in the near future to establish whether the relative lower mass is a pecularity of the Edmonston wt strain only.

Differential use of the potential glycosylation sites in the MV H protein could have been responsible for this phenomenon. However, Endo F treatment indicated that additional glycosylation in this isolate did not affect the mobility of H. Furthermore, no unusual nt or aa substitutions were detected in this strain when compared to other SA MVs. The reasons for this increased weight are not known at present. Heterogeneity of H protein glycosylation has been observed (Rota, J.S. et al., 1992; Saito, H. et al., 1992) and is believed to be determined by the primary aa sequence of H and is probably not dependent on host cell factors (Hu, A. et al., 1994). RIPA or WB analysis are useful approaches to select viruses that show differences in their apparent molecular sizes or interesting mAb binding characteristics for further studies. The advantage of the WB over RIPA lies in the fact that no radioactivity is involved making it less hazardous and it has been used before to study antigenic properties of MV H proteins (Saito, H. et al., 1992).

Specific epitopes on the H protein:

Several antibody binding sites and neutralisation epitopes on the MV H protein have been mapped. There appear to be 3 or 4 partially overlapping binding groups that include several specific mAb antigenic sites (Sheshberadaran, H et al.,1983; Giraudon, P. and Wild, T.F., 1985; Hu, A. et al., 1994; Tamin, A. et al., 1994). The mapping of point mutations in neutralisation escape mutants as well as the identification of helper T cell epitopes have added useful information to the knowledge of distinct antigenic sites of the H protein (Obeid, O.E., et al., 1993; Hu, A. et al., 1993). In this preliminary study, MV H proteins from South Africa have been analysed with 6 different mAb to determine whether differential binding characteristics could be related to the previously known aa changes. A study by Bankamp, B. (personal communication) showed that monoclonal antibodies CV2 and CV5 reacted with only 2 and 3, of the 9 isolates analysed respectively. CV2 binding was also analysed by Tamin, A. et al. (1994), who showed that US American wt isolates from 1989 were recognised by this antibody, but not isolates that were obtained in 1977 or 1983. The CV2 and CV5 antibodies did not bind to any of the SA H proteins, irrespective of the year of isolation. Interestingly, NC1/95 was not recognised by CV4, a mAb that otherwise reacted with all MV strains tested so far (except for the 2 Chinese strains mentioned above). The binding site for CV4 has not yet been mapped, but the loss of this antigenic site in NC1/95 must be due to an aa replacement between residue 132 and 524. Alignment of predicted aa revealed that NC1/95 carried 5 unique aa substitutions in this region, none of which was detected in other group D5 viruses, including the 2 Zambian isolates (as discussed in chapter 3.2). Four of these 5 mutations were located between residues 354 and 524.Therefore the binding site for CV4 is likely to be located between the aforementioned residues. This has been confirmed by binding studies on chimeras between different MV strains, that placed the CV4 binding site within the carboxyl-terminal 56% of the H protein. Bankamp, B. (personal communication) and Tamin, A. et al. (1994) also showed that V17 only recognised H proteins from vaccine viruses of group 1 (A). Here, V17 reacted with all South African MV strains representing 3 distinct genetic groups. It is unlikely that the aa substitution at position 600 (V→E) detected in SA strains of all 3 groups but in none of the other genetic groups (see chapter 3.2) confers V17 binding since this change was not present in the Edmonston wt and Moraten vaccine strain both of which could be precipitated with V17 (Bankamp, B, personal communication). Apart from this change, no specific aa substitution could be found that was common to all SA wt viruses and the group 1 (A) strains. It is therefore more plausible to assume that the V17 antigenic site was newly created in South African MV strains by multiple mutations in the epitope region.

Neutralising epitopes:
The target sequence for N-linked glycosylation has been shown to have a N-X-S or T motif but not all of these motifs are used in vitro (Hu, A. et al., 1994). Jhb1/89 and Natal1/96 shared 2 remarkable characteristics. Both strains carried a serine (S) to proline (P) substitution at aa 189 and this change has been shown to abrogate glycosylation of the 2nd potential site in H that originally displays the glycosylation motif N-C-S. Strains with this mutation have been demonstrated to represent neutralisation escape mutants and proved to have a slower mobility in a PAGE than strains without this substitution (Hu, A. et al., 1994). However, altered mobilities were not detected for the 2 SA strains that exhibited this change, indicating that this is unlikely to be sole criteria for different mobilities. An aa change at position 189 may also result in the possible loss of a linear B cell epitope that has been mapped to residues 188-199 (Mäkelä, M.J. et al., 1989). However, it is not known whether the carbohydrates at the 2nd glycosylation site are directly involved in the formation of this antigenic site (Hu, A. et al., 1993). Furthermore, the 2 isolates Natal1/96 and Jhb1/89 did not display the 416 mutation. Strains that carry this mutation at residue 416 carry a potential additional glycosylation site and have been shown to bind erythrocytes less efficiently than non-mutated isolates (Saito, H. et al., 1992). The electrophoretic mobilities of H proteins from these isolates were identical to the ones shown in the results section above. Whether these strains have altered neutralisation abilities or altered abilities to bind to erythrocytes will be examined in the near future.

The study of MV antigenic epitopes on the H protein has identified several regions that were recognised by specific mAb (Mäkelä,M.J. et al., 1989; Tamin, A. et al., 1994; Hu, A. et al., 1993). Whether these epitopes relate to functional domains is not yet known and the role that these antigenic variations may play in the general epidemiology of the virus still needs to be established. Variations concerning several antigenic properties, such as hemagglutination efficiency, the presence of T and B cell epitopes, glycosylation characteristics and others have been described above. The biological relevance for most of these variations that is observed between MV strains from different genetic groups has not been established yet. However, it can not be excluded at this point in time, that the accumulation of aa changes in key immunogenic epitopes may give rise to escape mutants that might render the current vaccines ineffective. Therefore, continued attempts to identify such epitopes and the knowledge of their variability between different MV isolates might prove essential in future.

CHAPTER 3.5 DIFFERENTIAL DOWNREGULATION OF THE CELLULAR RECEPTOR CD46 BY MEASLES VIRUS VACCINE AND WILD-TYPE STRAINS

3.5.1 Introduction

CD46, or MCP (membrane cofactor protein) is a member of the RCA (regulators of complement activation) protein family and has been established to act as a receptor for MV (Dörig, R., E. et al., 1993; Naniche, D. et al., 1993). CD46 functions to recruit and bind activated C3b and C4b complement proteins, thus permitting their proteolytic cleavage by a plasma serine protease (Liszewski, M.K. et al., 1991), and thereby inhibiting complement-mediated lysis of the cell. Certain MV strains have recently been shown to induce the downregulation of CD46 (Lecouturier, V. et al., 1997; Schneider-Schaulies, J. et al., 1995; Schnorr, J.-J., et al., 1995). This phenomenon could have profound consequences in vivo, possibly leading to an increased susceptibility of cells to complement-mediated lysis and thus inhibiting the spread of the virus (Schnorr, J-J. et al., 1995). On the other hand, a MV strain that does not downregulate CD46 may have a selective advantage within the host by gaining time to complete a full replication cycle thus increasing its infectivity (Schneider-Schaulies, J. et al., 1995). This has also been observed for HIV which, by incorporation of either CD46, CD55, or CD59 can be protected from complement-mediated destruction (Saiffudin, M. et al., 1997). Several MV wt strains, especially those that were isolated on B95a cells, appear to be unable to modulate CD46, whereas all vaccine strains tested so far caused profound downregulation of the receptor on Vero cells (Hirano, A. et. al., 1996; Naniche, D. et al., 1993b; Schneider-Schaulies, J. et al., 1995; Yanagi, Y. et al., 1994). So far, 9 vaccine and vaccine-like viruses of genetic group 1 (A) have been shown to more or less effectively downregulate CD46 from the surface of infected cells. Several wt strains representing 5 genetic groups have also been analysed in view of their potential to modulate CD46. To date, it seems that only Vero grown MV or viruses isolated in other monkey-derived cell lines appear to have the potential to downregulate CD46 (Schneider-Schaulies, J. et al., 1995a and 1995b; Bartz, R. et al., 1996). In this context, a recent study by Tanaka, K. et al. (1998) showed that the Edmonston strain did not downregulate CD46 on B95a cells. This is evidence that the cell type used for isolation, as well as prolonged adaption of viruses to non-human cells does influence a CD46 downregulating phenotype, as opposed to the origin of the

specimen (throat swab verus PBMC) or the strain of virus (Buckland, R. and Wild, T.F., 1997; Schneider-Schaulies, J. et al., 1995 and 1995a; Tanaka, K. et al., 1998). In view of these results, several authors have recently suggested that a molecule other than CD46 must act as a receptor or co-receptor for MV wt strains on marmoset B cells (Tanaka, K. et al., 1998; Hsu, E.C. et al., 1998; Buckland, R. and Wild, T.F., 1997).
Lecouturier, V. et al. (1997) and Bartz, R. et. al. (1997) have recently shown that 2 aa substitutions within the H protein at positions 451 and 481 can induce a downregulating phenotype in strains that have previously not been able to modulate CD46 (as was discussed in chapter 3.2). However, it has not been clearly established whether these 2 changes, if present in a certain wt, always relate to the ability to downregulate CD46, irrespective of the cell type that is used for isolation.

The ability to modulate CD46 was tested in several South African MV strains, the majority of which were isolated from urine or throat swab samples using the B95a cell line (Table 2.1). Vaccine strains that grew in Vero cells were also analysed and results compared to those obtained for the same strains on B95a cells. A total of 9 South African MV strains isolated on B95a cells, were adapted to Vero cells in order to determine whether the nature of the cell type influences the CD46 downregulating phenotype. So far, only 1 wt strain has been successfully adapted to Vero cells. Four MV isolates were still being processed at the time of writing and 5 isolates were considered negative after 14 passages in Vero cells as no CPE was evident and samples remained negative by measles specific IFA. To further establish whether CD46 functions as the sole receptor for wt and vaccine strains on Vero or B95a cells, an infection inhibition assay was performed and results are shown in section 3.5.4. Studies are still in progress analysing different cell culture systems (apart from Vero and B95a) in order to determine whether MV has the potential to modulate CD46 in primary cells, such as monocytes and dendritic cells.

3.5.2 CD46 downregulation by MV isolated on lymphoblastoid cells

For this study, several South African wt MV strains as well as 3 vaccine strains were grown in B95a cells. A plaque reduction assay (PRA) was used to determine the titre of viruses (2.9). B95a cells proved to be relatively fragile under the agarose overlay that is used in this assay. Cells survived for no longer than 2-3 days under the overlay and for some viruses this was too short a period of time for detectable plaques to develop. Different media and agarose overlay compositions have been

tested and the medium as described in 2.9 proved to be most suitable for use with B95a cells. However, titres could vary by a factor of up to 30 between identically repeated experiments using the same virus. Therefore, standard serial dilutions of the virus were also used for infection of monolayers and subsequent FACS analysis, which was generally performed in triplicate for each virus tested. Potential downregulation of CD46 was investigated under different experimental conditions. As mentioned above, varying concentrations of infecting virus were used. Furthermore, results for different incubation periods of viruses were obtained. The amount of H protein that was expressed on virus-infected cells was determined in order to confirm active replication of the virus in the 2 cell lines used. For this purpose, cells were incubated with the anti-H mAb B2, (described in chapter 3.4), and then incubated with the appropriate FITC-labelled secondary antibody. Specific fluorescence was subsequently measured by flow cytometry. For all experiments shown below, non-specific fluorescence was determined by staining a fraction of the cells with IgG1 control antibody, as outlined in section 2.8. The mean fluorescence of IgG1 was subtracted from values obtained for CD46 fluorescence staining. These adjusted values for CD46 expressed on MV-infected cells were then compared to the uninfected control cells stained in the same way.

Representative results for a selection of SA wt MV strains are shown below. None of the viruses that were initially isolated on B95a cells showed any tendency to downregulate the CD46 receptor. Fig. 3.24 shows results obtained for the B95a grown vaccine strains Schwarz and Edmonston. The Moraten vaccine strain also did not show the ability to downregulate CD46 on B95a cells (data not shown).

Figs. 3.25, 3.26 and 3.27 illustrate that neither South African vaccine-like viruses of group 1 (A) nor wt viruses of groups D5 and I (7) could downregulate CD46 from the surface of B95a cells, irrespective of the infecting viral titre or varying incubation periods used .

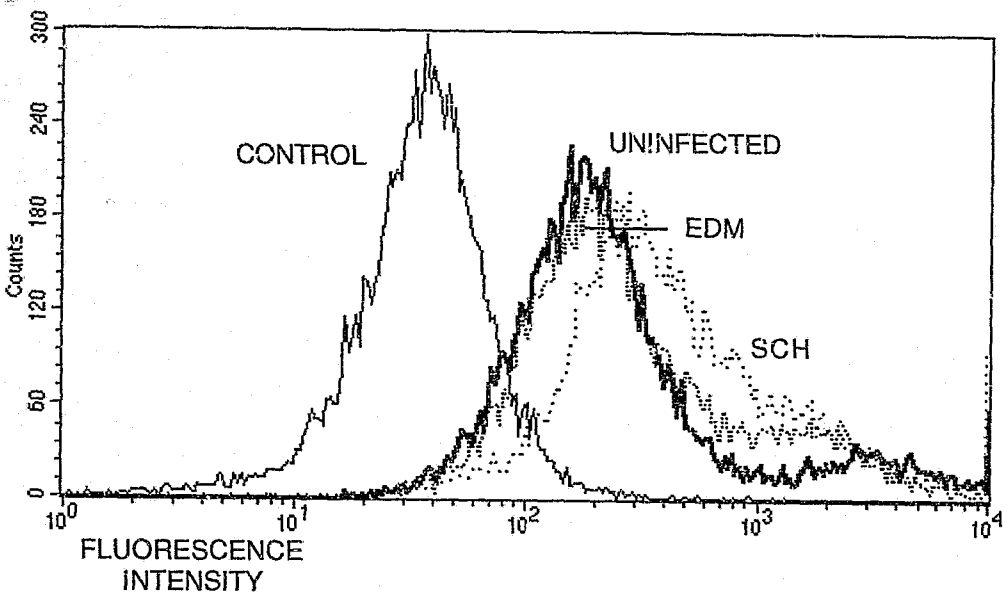

Fig. 3.24 Fluorescence histogram showing CD46 expression on uninfected B95a cells and cells infected with the Edmonston and the Schwarz vaccine strains.

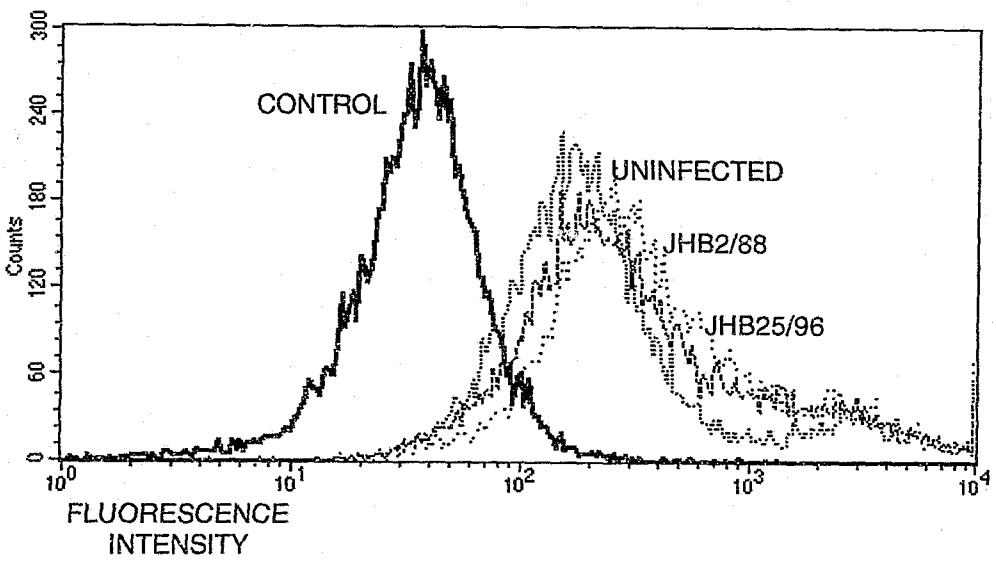

Fig. 3.25 CD46 expression on uninfected B95a cells and cells infected with either Jhb25/96 or Jhb2/88 of group 1 (A).

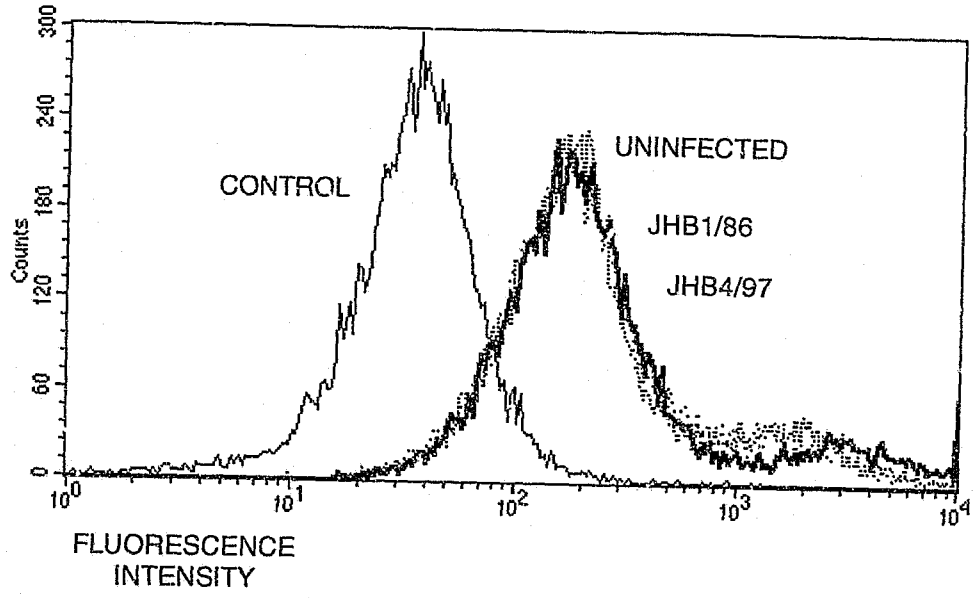

Fig. 3.26 CD46 expression on uninfected B95a cells and cells infected with either Jhb4/97 or Jhb1/86 of group D5.

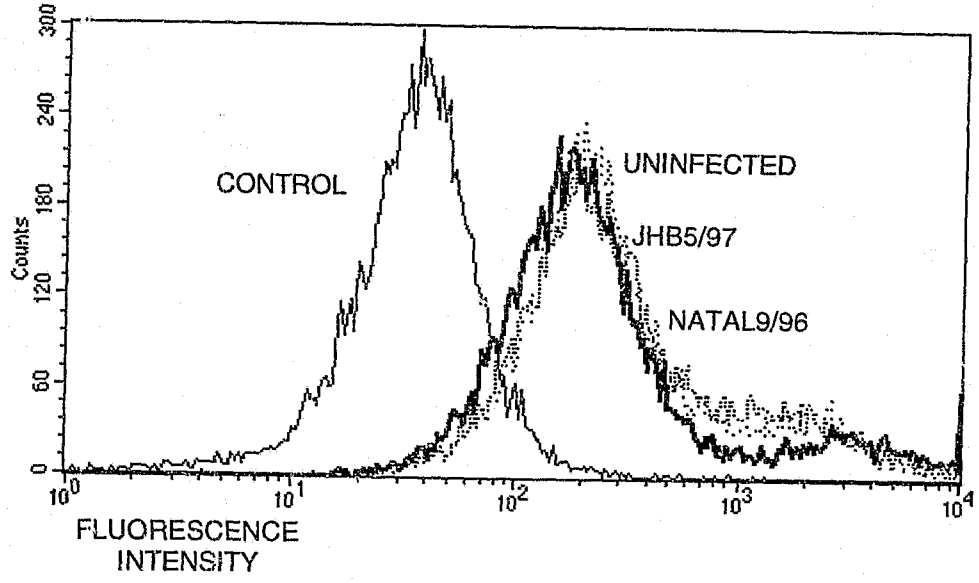

Fig. 3.27 CD46 expression on uninfected B95a cells and cells infected with representative strains of group I (7), Natal9/96 and Jhb5/97.

As shown in the representative figures above, none of the viruses analysed showed any potential to downregulate CD46 from the surface of the marmoset B cells, B95a, under any conditions. Furthermore, the Edmonston wt strain nor the vaccine strains Schwarz and Moratein were able to modulate expression of CD46 on the surface of B95a cells. It was also investigated if higher concentrations of infecting virus could induce a downregulation. Four serial 10-fold dilutions were therefore made on several viral stocks and used to infect B95a monolayers as well as different multiplicities of infection (moi) ranging from 0.01 to 3 were used. Even an input of virus at a moi of 3 that was incubated for up to 48 h pi resulting in a CPE of 90-100% did not modulate CD46 (data not shown). Thus, a downregulating phenotype is likely to be an intrinsic property of the virus and is unlikely to be altered by different experimental conditions.

The expression of H protein on MV-infected Vero or B95a cells was measured to confirm whether viruses were actively replicating in cells expressing CD46 and this was further used as a means to standardise the amount of replicating virus. The expression of H was slightly lower on Vero cells when compared to infected B95a cells. However, the amount of H protein staining was very similar when equal concentrations of different virus strains were used to infect either B95a or Vero cells (data not shown).

3.5.3 CD46 downregulation by MV isolated on monkey kidney cells

Several South African MV strains, initially isolated on B95a cells, were adapted to grow in Vero cells. Of the 9 strains selected (representing the 3 SA genetic groups) only 1 vaccine-like virus, Jhb25/96, showed CPE at passage number 6 so far. At the time of writing, 4 strains were still in tissue culture at passage number 14 without having developed CPE at any stage. Jhb1/86, a group D5 virus, was isolated on Vero cells in 1986. Some of the initial virus stock was still available and could therefore be grown in Vero cells. Unfortunately no further stocks of MV originally isolated on Vero cells were available (see Table 2.1). Therefore, only Jhb25/96, Jhb1/86 and the Schwarz and Edmonston vaccine strains could be tested for their ability to downregulate CD46 on Vero cells. Fig. 3.28 shows results for the 2 vaccine strains. Neither Jhb25/96 nor Jhb1/86 showed any effect on the expression of CD46 on Vero cells when stained with anti-CD46-FITC mAb, clone 122-2.

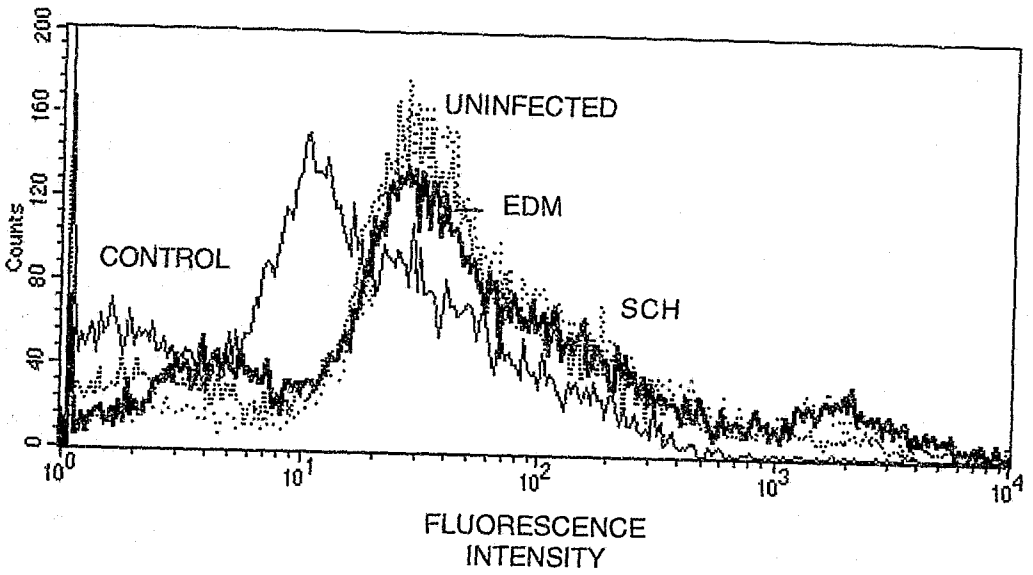

Fig. 3.28 Fluorescence histogram showing CD46 expression on uninfected Vero cells and cells infected with the Edmonston and Schwarz vaccine strains.

Although not clearly visible in Fig. 3.28, there was indeed a downwards shift in CD46 expression on vaccine virus infected Vero cells. The mean fluorescence intensity (mfi) was reduced by 18 and 21% when infecting Vero cells with Schwarz or Edmonston, respectively when compared to the uninfected control. In other similar experiments, Edm downregulated CD46 by up to 27%; the 18% downregulation by the Schwarz vaccine strain shown here was the maximum that was detected (data not shown). However, percentages for CD46 downregulation of below or around 20% could probably be considered irrelevant in vivo as the vast majority of complement protein appears to remain on the cell surface.

In summary, the results here indicate that vaccine strains have the ability to modulate CD46 to some extent from the surface of Vero cells only. None of the vaccine and vaccine-like strains tested here had the capability to modulate CD46 on

the lymphotropic B cell line, B95a. Furthermore, none of the South African wt strains, irrespective of the genetic group they belong to, showed any effect on the expression of CD46 cells on either Vero or B95a cells.

Table 3.9 summarises results on the potential modulation of CD46 on Vero and B95a cells for all MV strains tested to date.

Table 3.9 Summary of CD46 downregulation results for all strains tested.

MV	Vero CD46 downreg. (%)		B95a CD46 downreg. (%)
Sch	-	(+/- 9)	-
Edm	14	(+ 13)	-
Jhb1/86	-	(+ 12)	-
Mor	Nd		-
Jhb2/89	Nd		-
Jhb38/95	Nd		-
Jhb25/96	-		-
Jhb2/94	Nd		-
Rdpt1/94	Nd		-
Jhb4/97	Nd		-
Jhb5/97	Nd		-
Natal1/96	Nd		-
Natal4/96	Nd		-
Natal9/96	Nd		-

Nd: not done
Numbers in brackets indicate the maximum extent of CD46 downregulation which was obtained in other experiments performed under identical conditions.

Jhb1/86, as shown in Table 3.9 and Fig. 3.26, generally did not modulate CD46 on Vero cells. However, in a single experiment a downregulation of 12% was detected on Vero cells when infected with Jhb1/86. This result could not be reproduced in 3 similar experiments. This shows that experimental repeats of FACSscan results are important to establish what the true effect on CD46 modulation of a certain virus is.

3.5.4 Infection inhibition of B95a cells

To determine whether infection of B95a cells could be inhibited by blocking the CD46 receptor with an anti-CD46 mAb (clone J4/48), cells were pre-incubated with the mAb and then infected with various MV strains as described in 2.10. Schneider-Schaulies, J. et al. (1995 and 1995a) have recently shown that infection of HeLa or the human lymphotropic BJAB cell lines with a variety of MV wt and vaccine strains, could be inhibited by pre-incubation of these cells with an anti-CD46 mAb. However, results shown here indicate that infection with MV could not be inhibited using the marmoset lymphotropic B cell line B95a. The mAb used here (clone J4/48) does not recognise the monkey homolog of CD46 that is present on Vero cells (Schneider-Schaulies, J. et al., 1995). This was confirmed by infection inhibition assays on Vero cells using several SA wt MV strains and the vaccine strains Schwarz and Edmonston. Infection with none of the isolates tested could be inhibited using clone J4/48 (data not shown). Studies are currently in progress to investigate the inhibition of infection of Vero cells using anti-CD46 mAb that can recognise and bind the CD46 homolog present on monkey cells.
In this study different strains of wt and vaccine-like MV were incubated in parallel with and without anti-CD46 mAb. CPE appeared in most strains at the same time and with the same magnitude when comparing pre-treated and untreated samples (data not shown). As an indicator for MV replication in pre-treated cells, the expression of H was measured and compared to untreated cells.

Fig. 3.29 shows the expression of H on B95a cells infected with Jhb3/94. According to these data, the replication of this wt strain seemed to be unaffected by the prior saturation of the CD46 receptor with mAb, indicating that an alternative receptor is present on B95a cells that can support MV entry and infection of these cells. The expression of H protein on the surface of untreated cells and cells pre-treated with anti-CD46 mAb was further measured for another 5 SA wt strains as well as for the Schwarz and Edmonston strains. Replication of any of these viruses in B95a cells was not impaired by blocking of CD46 prior to infection. The CPE observed in both antibody pre-treated and untreated MV-infected cells were identical, irrespective of whether vaccine or wt MV were used. These results have been reproduced at least twice for all isolates tested, indicating that MV, or at least the strains analysed here, can use an alternative receptor on marmoset B cells if CD46 is not available. Cells were pre-incubated with 10 µg of anti-CD46 mAb per well in a standard 6-well plate and this amount should theoretically be sufficient to bind and block all CD46 receptor sites in 3×10^5 target cells. The question remains as to what the alternative mode and molecule for entry of MV is in specific cell types.

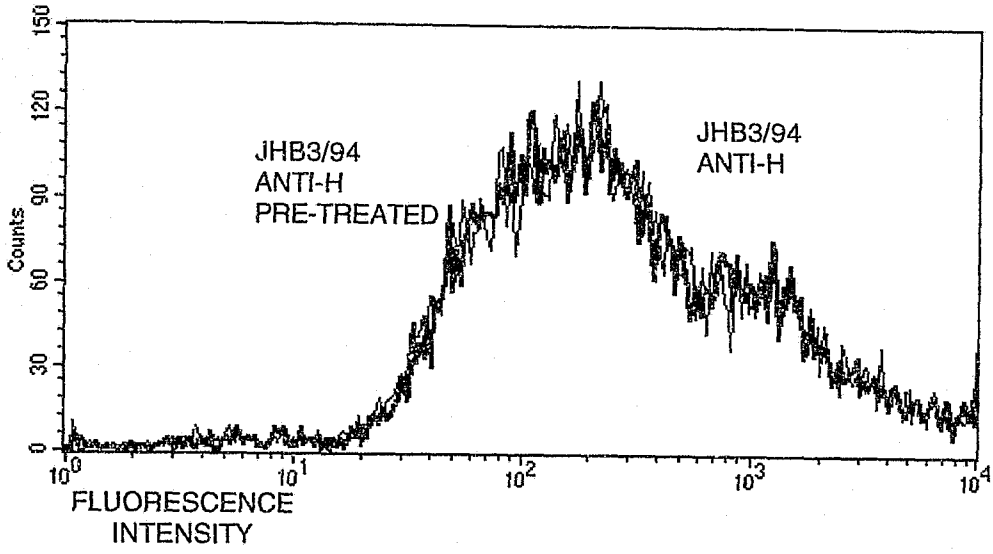

Fig. 3.29 Expression of H protein on untreated B95a cells and cells pre-treated with anti-CD46 mAb, infected with Jhb3/94 of group I (7). H-specific fluorescence of non-infected B95a control cells was not detected.

3.5.5 Discussion

CD46 has been firmly established to act as a receptor for MV. The ability to downregulate CD46 has been demonstrated for several vaccine and wt MV strains and is caused by the direct interaction of MV H protein with CD46 (Lecouturier, V. et al., 1997; Schneider-Schaulies, J., 1995 and 1995a; Naniche, D. et al., 1993). Downregulation of CD46 from the cell surface may render the infected cell more susceptible to complement mediated lysis, as was discussed in sections 1.3 and 3.1.5. Naniche, D. et al. (1993) have shown that the receptor is downregulated by rapid internalisation of CD46 from the cell surface due to interaction of the cell with the MV H protein. In most studies described to date, Vero, HeLa, Jurkat T, or BJAB cells have been used to demonstrate this phenomenon. Only recently, studies have begun to focus on B95a cells, the cell line most commonly used for MV isolation at present. Evidence has accumulated that CD46 may not be the receptor or not the sole receptor for MV on marmoset and human B cells (Tanaka, K. et al., 1998; Hsu, E.C. et al., 1998; Buckland, R. and Wild, T.F., 1997, Murakami, Y. et al., 1998).

This study was undertaken to determine whether South African MV strains representing the 3 genetic groups currently circulating in the country have the potential to modulate CD46 expression on the surface of B95a or Vero cells. In addition, the downregulating ability of 3 vaccine strains was tested. All assays were preformed under various experimental conditions to establish whether the concentration of infecting virus or the length of incubation would have any effect on the subsequent measurement of CD46 expression. An infection inhibition assay was used to investigate whether propagation of MV wt and vaccine strains could be abrogated by saturating the receptor CD46 with a mAb prior to infection.

Modulation of CD46 by SA wt MV and MV vaccine strains:
The capacity to downregulate CD46 has been demonstrated for several MV wt strains (Bartz, R. et al., 1997; Lecouturier, V. et al., 1997; Schneider-Schaulies, et al., 1995 and 1995a; Hirano, A. et al., 1996). None of these studies investigated potential CD46 downregulation by MV on B95a cells. At the time of writing however, 2 studies were published that, for the first time, analysed the influence of MV strains on the expression of CD46 on B95a cells (Tanaka, K. et al., 1998; Hsu, E.C. et al., 1998). Both studies showed that MV wt and vaccine strains had little or no effect on CD46 expression on B95a cells. In concordance with these findings, it was established that none of the SA MV strains analysed here showed any tendency to downregulate CD46 from the surface of transformed marmoset B cells. Furthermore, different experimental conditions, such as higher concentrations of infecting virus or prolonged incubation periods did not appear to induce CD46 downregulation or influence the expression of CD46 at any stage.
Hsu, E.C. et al. (1998) showed that even vaccine strains did not efficiently downregulate CD46 from B95a cells. The 3 vaccine strains, Moraten, Schwarz, and Edmonston that were tested here did not modify the expression of CD46 on the surface of B95a cells. However, it is noteworthy that a study by Schnorr, J-J. et al. (1995) stated that the Edmonston strain could indeed downregulate CD46 from BJAB cells, another EBV-transformed B cell line, and from freshly isolated PBMCs (peripheral blood mononuclear cells). Lymphotropic wt strains that were initially isolated on BJAB or B95a cells appear to lack the ability to downregulate CD46 on B95a or BJAB cells (Schneider-Schaulies, J. et al., 1995 and 1995a; Tanaka, K. et al., 1998). Thus, it seems plausible to state that lymphotropic MV wt strains do not interact with CD46 on those cell lines. In this context, it has been proposed that wt MV strains may use a receptor other than CD46 on marmoset B cells (Buckland, R. and Wild, T.F., 1997; Tanaka, K. et al., 1998; Hsu, E.C. et al., 1998) and this seems likely in view of the results obtained here.

The vaccine strains Schwarz and Edmonston as well as 2 South African wt strains were analysed for their ability to downregulate CD46 on the African green monkey cell line, Vero. Vero together with the similar cell line CV-1 have been used extensively for MV isolation before the introduction of the more sensitive B95a cells. Jhb1/86 is a wt strain of group D5 that had initially been isolated on Vero cells and original stocks were still available for this experiment. Jhb25/96 had to be adapted to grow in Vero cells over 6 passages. None of the other SA wt strains could be adapted to grow in Vero cells to date. It has been reported before, that the adaption of B95a-isolated MV to monkey-derived cell lines is difficult and requires prolonged passage in tissue culture (Shibahara, K. et al., 1994). The 2 SA wt strains did not reveal any potential to modulate CD46 on the surface of Vero cells, although Jhb25/96 (group 1(A)) carried the 481Y residue. This provides further evidence that this particular residue does not always confer a downregulating phenotype to MV. In this context, Shibahara, K. et al. (1994) reported that MV strains isolated from B95a cells that previously did not hemadsorb AGM-RBC, gained the capacity to do so after 20 passages in Vero cells. It was further shown that 1 of the strains analysed had acquired the 481Y residue instead of the initial 481N residue. Lecouturier, V. et al. (1997) speculated that during multiple passages in Vero or CV-1 cells, a selection for MV with affinity for CD46 occurs that is mainly driven by the aa replacement at position 481. However, in another study the ability to downregulate CD46 could not be induced by adaptation of lymphotropic isolates to Vero cells (30 passages) (Shibahhara, K. et al., 1994).

It has been reported that MV vaccine strains efficiently downregulate CD46 from the surface of Vero cells by up to 80% (Schneider-Schaulies, J. et al., 1995 and 1995a). The 2 vaccine strains analysed here, Edmonston and Schwarz showed little ability to downregulate CD46 from Vero cell surfaces with a maximum of 27% reduction in CD46 expression in some experiments. On average downregulation did not exceed 20% under the conditions used here and the question remains whether this would be of any significance in vivo since most of the CD46 molecules remain on the cell surface. The clone of anti-CD46 mAb (122-2) that was used here clearly recognised the monkey homolog of CD46, but staining may be less efficient compared to signals obtained with other clones that specifically bind to the monkey-CD46 only. Potentially, the passage history and passage number of a cell line used for CD46 studies may be of importance. Future studies will include analysis of different passage numbers and histories for the B95a and Vero cell lines as well as different clones of anti-CD46 mAb will be used to measure modulation of CD46. The potential downregulation of CD46 from primary cells, such as dendritic cells and monocytes as well as cell lines other than Vero and B95a will also be investigated.

The 2 aa at positions 451 and 481 have been suuggested to be responsible for CD46 downregulation, HeLa cell fusion and AGM-RBC adsorption (Lecouturier, V. et al., 1997; Bartz, R. et al., 1997, and as discussed in chapter 3.2). The SA wt MV strains tested here for their ability to downregulate CD46, carried an asparagine (N) at position 481 which would theoretically render them unable to downregulate CD46. In contrast, SA MV strains of group 1 (A) displayed the tyrosine residue at this position allowing for possible modulation of the receptor. However, none of the SA group 1 (A) isolates revealed the ability to downregulate CD46 from the cell surface, indicating that residue 481 can confer a downregulating phenotype but does not necessarily do this for all MV strains. Thus, factors other than the aa replacements at positions 451 and 481 are likely to play a role in the ability of certain MV strains to downregulate CD46 from PBMCs, monkey kidney cells (Vero, CV-1) and certain other cell lines such as HeLa and Jurkat T.

Infection inhibition of B95a cells:
If CD46 is the sole receptor for binding of MV to B95a and/or Vero cells, it should theoretically be possible to inhibit infection by saturation of the receptor with an anti-CD46 antibody prior to infection. Schneider-Schaulies, J. et al. (1995a) demonstrated that infection with 17 different wt and vaccine strains could be inhibited on HeLa and BJAB cells by blocking the binding sites with a mAb against CD46. Results shown here, however, indicate that infection with MV could not be abrogated by saturating the receptor on B95a cells. This suggests that the strains tested used an alternative receptor, other than CD46, on marmoset B cells to bind to the cell. A very similar finding has recently been reported by Hsu, E.C. et al. (1998) and Tanaka, K. et al. (1998), showing that infection of B95a cells with either MV wt or vaccine strains could not be inhibited by making CD46 unavailable for virus binding. There is a possibility that this phenomenon occurs exclusively in transformed marmoset B cells. Cell lines such as HeLa, BJAB, Vero and Jurkat tested previously for infection inhibition could express CD46 as the sole receptor but may not have the alternative receptor that is present on B95a cells (Krantic, S. et al., 1995). Furthermore, different clones of anti-CD46 mAb are available and have been used in various studies. These clones may recognise different SCR of the CD46 molecule or CD46 molecules that are exclusive to a certain species or cell line (Murakami, Y. et al., 1998). For example, the clone J4/48 that was used here, does not recognise and bind to the monkey homolog of CD46. This was confirmed by using this mAb in infection inhibition assays on Vero cells. Vaccine strains are known to use the CD46 on Vero cells and entry should by abrogated by blocking the binding site of the receptor (Schneider-Schaulies, J. et al., 1995; Buckland, R. and Wild, T.F., 1997). However, infection with vaccine strains could not be inhibited by

the J4/48 mAb, indicating that this antibody was not able to bind to the monkey homolog of CD46 present on Vero cells (data not shown). These findings provide evidence that B95a cells express the receptor(s) for both wt and vaccine strains whereas monkey cells carry the functional receptor for vaccine strains and only very few wt isolates (Tanaka, K. et al., 1998). Further evidence for the existence of a receptor other than CD46 that can be used by MV strains is that vaccine strains have been passaged in chick embryo fibroblasts that do not express a CD46 homolog (Buckland, R. and Wild, T.F., 1997). It has also been proposed by the same authors that wt MV strains gradually adapt to CD46 when grown in Vero cells because these cells lack the wt receptor. In this context, it has been suggested that CD46 downregulation in general could represent an attenuation marker for MV vaccine strains (Buckland, R. and Wild, T.F., 1997). There is a possibility that by using B95a cells for virus isolation we may have selected for MV strains with altered H proteins that can bind the marmoset CD46 but no longer recognise the monkey homolog on Vero cells (Tanaka, K. et al., 1998).

MV do not appear to interact with CD46 on B95a cells since downregulation does not occur and infection with wt strains cannot be inhibited by blocking CD46 with mAb. Interestingly, the 2 cell lines used most commonly for rapid isolation of MV, B95a and BJAB, both represent EBV-transformed continuous human B lymphocytes (Murakami, Y. et al., 1998). It will be important to establish whether EBV contributes or up-regulates factors that confer binding of MV wt isolates to these cells.

In summary, lymphotropic MV wt isolates that have exclusively been passaged in B95a or BJAB cells, do not seem to interact with CD46 and are likely to utilise a receptor other than CD46 for entry into and fusion with the host cell. Thus, the differential downregulation of CD46 that is seen when analysing wt and vaccine strains in different cell lines appears to depend on the host cell rather than the virus strain. It will be important to identify what the receptor or co-receptor for MV on B cells is, and whether this receptor can be used alternatively by MV strains if CD46 becomes unavailable. Furthermore, there is a need to clearly establish whether vaccine and MV wt strains differ in their receptor usage and whether this could be attributed to experimental conditions or intrinsic factors.

4. CONCLUSIONS

Measles virus is a highly contagious disease and it is considered to be one of the most efficient pathogens known. It is estimated that approximately 40 million cases of measles and close to 1 million related deaths occur annually worldwide. Developing countries account for the vast majority of these cases. In Africa, measles continues to be a major health burden for infants, children and, as vaccine coverage improves, for young adults. However, southern African nations are currently moving toward a better control of the disease and the eventual elimination of measles. Mass vaccination campaigns have very recently been implemented in several southern African countries to reduce the overall incidence of measles and efforts will continue to focus on the improvement of routine vaccine coverage. A cornerstone in any attempt to control the disease is, apart from serological surveillance, the molecular epidemiological analysis of MV isolates. The knowledge of the genetic properties of viruses circulating in a given region or country is necessary to assign MV to genetic groups and to discriminate between indigenous viral strains and those circulating elsewhere. Molecular epidemiological studies therefore represent an important means to assess the success of MV control and elimination programs and the identification of transmission pathways of the virus.

To date, there is no consensus about the definition of MV genotypes or subgroups and how to designate them. Several investigators have used different nomenclatures for MV genetic groups. The 2 most commonly used systems for MV nomenclature (letters, A-I versus numbers, 1-7) have both been applied here to describe MV genetic groups. Currently efforts are underway to reach an agreement for the definition and nomenclature of MV genetic groups and this will certainly contribute to improving the analysis of molecular epidemiological data in future.

This was the first study to investigate molecular epidemiological as well as several antigenic characteristics of MV circulating in a southern African country. To date, little is known about the genetic properties of MV that are indigenous to other African countries. Information on a limited number of strains from West African countries, such as Gabon, Cameroon, the Gambia and Zambia has recently become available. However, with the majority of measles cases occurring in Africa, future studies will have to include the analysis of MV from many more regions within the continent, in order to determine how many genetic groups exist in Africa and whether those groups are geographically restricted to certain areas or countries. In order to accumulate information on the molecular epidemiology of the virus, efforts need to

concentrate on the analysis of MV strains from as many as possible locations within southern Africa and the rest of the continent. This information will play a critical role in attempts to eliminate MV from Africa and other parts the world. In this study, MV from South Africa were analysed at the level of gene sequences, antigenic properties and interaction with a putative cell surface receptor. Conclusions to each chapter are summarised below.

Chapter 3.1 Analysis of MV N genes:

The carboxyl-terminal 456 nt of the N gene for 97 MV strains collected between 1978 and 1997 in South Africa and Namibia have been analysed. Three distinct genetic groups were identified. The first group was represented by 4 isolates made between 1988 and 1996, which closely resembled vaccine and vaccine-like strains of genotype A (1). A second small group included 6 SA strains that shared several nt and aa substitutions with group D viruses (groups 2,3, and 4) and were therefore considered to form the subgroup D5 within this major genotype. The vast majority of SA MV strains were closely related to each other and formed group I (7). According to N gene sequences, a Canadian isolate from 1989 proved to be the only strain that clearly belonged to the same group as the majority of SA wt viruses (group I or 7).

Retrospective analysis of stored clinical samples of serum and CSF proved useful for the generation of molecular epidemiological data on viruses that circulated up to 20 years ago. For this purpose, RNA extraction and PCR amplification methods were modified in order to yield sufficient product for subsequent sequence analysis. Considering the amount of degradation that can occur in samples that have been frozen for a long time and that have been thawed several times, the amplification rate achieved here was satisfactory. This approach is particularly useful when only serum samples (for serological testing) can be obtained from interesting locations. This was the case for 2 sera from Windhoek (Namibia), one of which closely resembled D4 (3) strains rather than any other southern African isolates (lineages A, D5, I) that have been studied so far. Analysis of serum and CSF samples from a SSPE case in SA showed that this virus was almost identical to Chi1 (group D2 or 2), a strain that was isolated in the USA in 1989. One of the sera from 1978 was related to group D5 viruses, whereas 3 other viruses from 1978 serum samples grouped with the lineage I (7) strains. Thus, analysis of stored clinical samples revealed that at various times viruses from other parts of the world, although probably in very small numbers, circulated in southern Africa.

Chapter 3.2 Analysis of MV H genes:

The groupings of MV that were established on the basis of carboxyl-terminal N gene sequences corresponded well to those obtained by H gene analysis. No significant changes in groupings occurred and it was therefore concluded that either genomic region (N or H) could be used for MV strain characterisation. Partial H gene sequences of several strains from other African countries were available for a phylogenetic comparison. Recent isolates (1994/95) from the Gambia were shown to belong to group B (6) which includes other African strains from Kenya, Cameroon, and Gabon. Interestingly, 2 Zambian isolates from 1992 were closely related to SA group D5 viruses and this was the first time that isolates of this genetic group have been made in a country other than South Africa. It will be important to establish whether this group is more dominant in other parts of the continent. On the basis of partial H gene sequences, it was further demonstrated that isolates from the Coventry area (England) belonged to genotype A (1), indicating that wt strains of this group are indeed still in circulation not only in South Africa.

Chapter 3.3 Genetic screening of MV strains:

In cases were a large number of viruses need to be analysed in a short period of time, such as in outbreak situations, screening techniques other than elaborate sequence analysis are required. The HMA has been shown to be a reliable technique for rapid assignment of unknown isolates to 8 different genetic groups and thus could become a useful technique in settings where sequencing or other sophisticated laboratory facilities are not available. Other methods, such as RFLP and LSSP have also been evaluated for their potential to discriminate between MV belonging to different genetic groups. LSSP proved to deliver unsatisfactory results, whereas RFLP analysis performed on SA viruses was able to distinguish between strains from 3 distinct genetic groups. However, RFLP only takes a limited number of previously selected changes into account. In contrast, the HMA allows for differentiation of viruses according to all mutations that occur within the analysed fragments and thus would appear to be a better approach for MV strains discrimination. The HMA is easy to perform, cost-effective and is now used routinely to reliably differentiate between distinct MV in South Africa. The ability of the HMA to discriminate viruses from 8 genetic groups and 9 different countries indicates its utility for global screening of MV isolates.

Chapter 3.4 Antigenic analysis of the MV H protein:

Hemagglutinin proteins from 10 SA MV strains representing 3 genetic groups were analysed to compare their molecular weights and possible differences in antigenic epitopes. The majority of SA wt strains had an identical molecular mass to Schwarz

(~ 80 kDa) while 1 isolate of group I (7), Jhb3/94, had a greater apparent molecular mass (~ 85 kDa). Using pre-treatment with Endo F/N it was shown that the increased weight was unlikely to be due to additional glycosylation. To determine whether differences in antigenic epitopes exist among SA MV strains, reactivity of 9 SA strains with a panel of 6 anti-H mAb was investigated by RIPA. Only one of the 6 epitopes (CV4) was found to be variable. CV4 precipitated H proteins from the Schwarz vaccine strain and all SA wt strains, except for NC1/95. Alignment of nt and aa for this strain with 2 Chinese wt isolates that also did not react with CV4 failed to show similar mutations. Thus, the lack of this antigenic site in NC1/95 could not yet be attributed to unique nt/aa changes within the H gene. In summary, despite the differences in nt and aa sequences in the N and H genes, the antigenic properties examined here of SA wt strains and the Schwarz vaccine strain were for the most part identical. The presence of only minor antigenic differences between various MV wt isolates and the vaccine strains most likely account for the undoubted success of measles vaccines which protect against viruses from all genetic groups.

Chapter 3.5 Differential downregulation of CD46 by MV vaccine and wt strains:

Recently, evidence has accumulated that CD46 may not be the receptor for MV on the EBV-transformed marmoset B cell line, B95a. An infection inhibition assay using B95a cells was performed to investigate whether SA wt strains and vaccine strains can indeed utilise other molecules for entry into the host cell. Blocking of CD46 with a mAb prior to infection did not abrogate the ability of vaccine and SA wt strains to infect these cells, indicating the presence of a receptor other than CD46 on B95a cells. However, this alternative receptor or co-receptor has not yet been identified and it remains to be established whether EBV plays a role in providing or up-regulating factors that could subsequently serve as a receptor for MV. Interactions of MV H proteins with CD46 on Vero and B95a cells were analysed on a FACS cell sorter. Neither SA wt strains nor the 3 vaccine strains Moraten, Schwarz, and Edmonston showed any potential to modulate or downregulate CD46 from the surface of B95a cells and this observation was not altered under different experimental conditions. Furthermore, 2 SA wt strains did not reveal the ability to downregulate CD46 from Vero cells unlike 2 vaccine strains, Edmonston and Schwarz, which caused a downregulation of CD46 by approximately 20% (from Vero cells). These results suggest that under certain experimental conditions, CD46 on Vero cells can indeed be modulated by MV vaccine strains which have been shown to use this receptor on monkey kidney cells. It appears that usage of CD46 as a receptor can vary between MV vaccine and wt strains but may also depend on the origin of cells used for virus isolation and propagation.

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