Significance of sequence variation in the P1 and 3A genes of foot-and-mouth-disease virus isolates from southern Africa.

By

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I declare that the dissertation, which I hereby submit for the degree M.Sc (Agric) Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

[Signature]

Lwio E. Heath.

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Date.
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Abstract

Title: Significance of sequence variation in the PI and 3A genes of foot-and-mouth disease virus isolates from southern Africa.

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Foot-and-mouth disease (FMD) virus is a highly contagious pathogen responsible for one of the most economically devastating diseases in cloven-hoofed animals. The disease threatens the economies of southern African countries that are dependent on agricultural export of animal origin. The majority of outbreaks in southern Africa are caused by the 3 SAT serotypes. The geographical distribution of these serotypes is largely restricted to sub-Saharan Africa with SAT 1 and 2 accounting for more than 80% of the outbreaks in domestic livestock. Due to this, research on the third of the SAT types has been somewhat neglected. The lack of genetic information with regards to the structural-protein coding region of the SAT type 3 viruses limits the determination of the level of correlation between the genetic characteristics of prevalent isolates and strains currently being used in vaccine production. In order to broaden the understanding of the genetic characteristics of the SAT 3 serotype, the structural-protein-coding region of the FMDV KNP/10/90/3 isolate was determined and compared to representative isolates of six FMDV serotypes. Nucleotide and amino acid sequence analyses showed the genetic makeup and protein organisation of KNP/10/90/3 to be typical of FMDV. As would be expected the isolate shared a high level of sequence homology with Bec/1/65/3 and was more closely related to the other SAT serotypes than to European FMD viruses. Data indicate that intratypic variation for the PI region of the SAT type viruses is significantly higher compared to that of serotypes A, O and C.

In addition to the four structural proteins (P1 precursor), the FMD genome encodes multiple mature polypeptides and intermediate cleavage products. The mature polypeptides include the 3A protein. This 153 amino acid protein, as well as its precursor (3AB), has been shown to be involved in viral RNA replication and death of infected cells. Changes in 3A have been associated with altered host range in the hepatoviruses, rhinoviruses, and enteroviruses. Recently a direct correlation was shown to exist between a 10 amino acid deletion within the 3A protein FMDV and attenuation of the virus in cattle. Although the 3A coding region has been shown to be highly conserved among several European FMD virus isolates, nothing is known about the sequence characteristics of the 3A of the SAT type viruses. In light of this, the nucleotide sequences of the 3A non-structural-coding sequence of several African FMD virus isolates were determined and comparatively analysed. We compared this region of the genome among different SAT serotypes and with that of European, South American and Asian isolates in order to assess the extent of genetic variation within the 3A coding region of naturally occurring viruses in sub-Saharan Africa. Our results indicated that the 3A region of the SAT isolates differed markedly from that of the European isolates but were closely related within the serogroup. The percentage site conserved in all isolates examined was calculated to be 47.4% at nucleotide sequence level and 50.3% at amino acid level. The different SAT serotypes were indistinguishable within the phylogenetic arrangement.
Chapter 1: Literature Review
1.1 General introduction to the disease

Foot-and-mouth disease (FMD) virus is a highly contagious pathogen responsible for one of the most economically devastating diseases affecting cloven-hoofed animals (Bachrach, 1978). FMD threatens the economies of southern African countries, like Botswana, Namibia, Zimbabwe and South Africa that are dependant on agricultural export of animal origin. The disease is characterised by a non-lethal infection culminating in temporary oral and pedal vesicles, which can result in a significant reduction in the production of meat or dairy products (Shahan, 1962).

Due to the infectious nature of the virus, trade embargoes are enforced against regions where FMD is endemic (Bachrach, 1968; Bachrach, 1978). Countries that are free of FMDV maintain rigid quarantine and import restrictions to prevent the introduction of the disease, while control programs to eradicate FMD include the slaughter of infected or exposed animals and vaccination using chemically inactivated vaccines.

The virus is classified in the family Picornaviridae, which contains nine genera viz. enterovirus, rhinovirus, cardiovirus, hepatovirus, parechovirus, erbovirus, kobuvirus, teschovirus and aphthovirus (van Regenmortel et al., 2000). Historically FMDV has been the only member of the aphthovirus genus, but recently Equine Rhinitis A virus, formerly known as Equine Rhinovirus 1, was included within this group and FMDV appointed as the type species of the aphthoviruses.

There are seven recognised FMDV serotypes of which six occur on the African continent. The South African Territories (SAT) types 1, 2 and 3 are three serotypes unique to sub-Saharan Africa and are predominantly responsible for outbreaks of the disease in the region (Thomson, 1994). In addition of these, serotypes A, O and C are also occasionally found to be associated with outbreaks in Africa.

Under experimental conditions the viruses show little or no cross-protection against infection (Brooksby, 1982). This would account for reports that a single dose of monovalent vaccine fail to protect against challenge with heterologous virus (Cartwright et al., 1982). The level of serological intratypic variation differs for each of the seven FMDV
serotypes. A number of identifiable subtypes exist for both serotypes A and O while intratypic variation within the C and Asia-1 serotypes appear to be markedly less. Despite the high degree of variation within the SAT types, no serological distinct subtypes have been identified for these serotypes (Esterhuysen et al., 1988). Traditionally subtyping has been based on results generated by cross-neutralization assays and compliment fixation tests and shows good correlation with cross-protection in vivo (Thomas, 1986).

Molecular characterisation of field isolates have aided significantly in broadening our understanding of the epidemiology of the disease and enabled researchers to more effectively address the problems associated with the disease. A characteristic feature of FMDV epidemiology is the frequent emergence of new strains with altered antigenic properties and is due to the genetic instability of the virus (Domingo et al., 1996).

Although the European types of FMDV have been studied intensively, little is known about the genetic makeup of the SAT type viruses.

1.2 Epidemiology of the disease

1.2.1 Geographical distribution and recent history of FMDV

FMD has a global distribution occurring in South America, Europe, Asia, the Middle East, the Far East and Africa. The geographical distribution of the seven virus types is heterogeneous. Types A, O and C is widely distributed and has been responsible for outbreaks of FMD in South America, Europe, Africa, the Middle East and Far East. In contrast, the geographical distribution of the Asia 1 serotype is relatively restricted, since it is confined to the Far and Middle East and the Balkan states (Daborn, 1982; Rweyemamu et al., 1982). All three SAT types are largely restricted to sub-Saharan Africa although there have been incursion of SAT 1 and 2 into North Africa and the Middle East respectively (Pereira, 1981).

FMD is classified as a List A disease defining it as a transmissible disease of one or more animal species, that has the potential for very serious and rapid spread, irrespective of national borders, that it is of serious socio-economic or public health consequence and that it is of major importance in the international trade of animals and animal products. Countries affected by the disease is required to report all cases of FMDV to the Office
International des Epizooties (OIE) as often as necessary to comply with Articles 1.1.3.2 and 1.1.3.3 of the International Animal Health Code. The FMD status of individual countries are continually monitored by both the World Reference Laboratory (Pirbright) UK, and the OIE. A graphical representation of the current global distribution of FMDV as published by the EIO is given in Figure 1.2.1.

In Europe and North America the effects of FMD have historically been great and vast amounts of money have been spent in attempts to eradicate or control the incidence of the disease. Northern America is considered to be free of FMD while in northern Europe incidence of the disease has decreased to such an extent that the disease is now considered to be exotic to all member states of the European Economic Community (Kitching, 1998). Despite recent reports of the eradication of the disease in western Europe and the declaration of Paraguay and Argentina as “Free from FMD with vaccination” areas, little progress have been made in the global control of FMDV. The impact of these and other significant advances, have been offset by the reappearance of the FMD in regions such as Malaysia and the introduction of the disease into areas formally free of FMDV such as Taiwan. Political and socio-economic instability in the Independent States of the former Soviet Union and frequent outbreak in the countries of southern Europe constantly pose a threat of large outbreaks in Europe (Kitching, 1998).

The continual global threat posed by FMD is highlighted by recent developments in Europe. Since the cessation of vaccination in the European union in 1991, there have been outbreaks of FMD in Italy in 1993 and in Greece in 1994 and 1996. Sporadic outbreaks of FMD were also recorded in Bulgaria, Russia, Albania and the former Yugoslavia at different times over the same period. Of particular concern to Europe is the situation in Turkey. Until 1989 a buffer zone existed on the northern border of Turkey, in which susceptible animals were routinely vaccinated. The zone was moved into Anatolia and vaccination discontinued in Turkish Thrace. However, due to the endemic status of FMD in Anatolia, outbreaks soon spread into Thrace posing a renewed threat to the Balkan states north of Turkey. Due to the nomadic culture of the people of the Middle East, it has proven difficult for countries to control the disease in isolation. In addition to this, wars and a general lack of infrastructure within the region have hampered efforts to control FMD.
Foot & Mouth Disease - Status based on recorded outbreaks 1996-2000 & current OIE classification

Figure 1.2.1. The global distribution of Foot-and-mouth disease virus. Spatial Information Research Centre. University of Otago, 2000.
FMD serotypes A, O, C and Asia-1 are widespread through India and the Far East. Although FMD is endemic to most of the region, Malaysia had been free of FMD up until 1990. In 1991 an isolated outbreak occurred in quarantine station on Malaysian border with Thailand. This was however followed by annual outbreaks in the surrounding areas between 1993 and 1996. Since then sporadic outbreaks of the disease have occurred in Malaysia with the most recent involving an isolated outbreak of FMD in the southern province of Kar.

A significant event in the recent history of FMD was the report of an outbreak in Taiwan during 1997. Until then, Taiwan had been free of FMDV. Although early outbreaks were brought under control by the mass slaughter of pigs, FMD is still prevalent in the region with cases of FMD occurring as recent as February 2000. Genetic characterisation of the viruses responsible for outbreaks in the region revealed that they significantly differ from historical strains of FMD isolated in the Far East and reconfirmed the importance of genetic evolution in the epidemiology of the disease.

In contrast to the situation in Europe and Asia, the incidence of FMDV in South America has greatly reduced. This is mainly due to marked improvement of in the efficiency of FMD control programs in the region. Several countries in the region have remained free of FMD for consecutive years. The last outbreak of FMDV in Uruguay occurred in 1990 and in 1993 the country was awarded the then unique status of “free of FMD with vaccination”. In 1994 Uruguay ceased prophylactic vaccination and in 1995 declared itself free of FMD. Argentina and Paraguay have report similar successes in controlling FMD and was declared “free of FMD with vaccination” in 1997. Regional co-operation to control FMD has undoubtedly contributed to the general decrease of FMD incidence in South America (Kitching, 1998).

FMD is endemic to the most part of sub-Saharan Africa. It is however difficult to accurately determine the distribution of the disease on the subcontinent due to inconsistent reporting of the disease in several countries (Thomson, 1994). SAT type 1, 2 and 3 have been responsible for the overwhelming majority of outbreaks in cattle in sub-Saharan Africa, although sporadic outbreaks of FMD have been caused by serotypes A, O and C. Of the SAT types, the highest incidence in domestic animals is caused by SAT 2 (48%).
Outbreaks of SAT 1 make up approximately 36% of cases in cattle, while SAT 3 are only responsible for the remaining 16% of outbreaks. SAT 3 has a restricted distribution and has never been diagnosed in domestic animals outside of southern Africa (Thomson, 1994).

Extensive phylogenetic characterisation of SAT1 viruses isolated from African buffalo has revealed that these viruses evolve independently in at least three distinct geographical locations within southern Africa and can broadly be grouped in one of three topotypes (Bastos et al., 2001). Most southern African countries have a single SAT 1 topotype within their borders, which is shared by at least two neighboring countries. Similar observations of strict geographical clustering of strains have been also reported for the SAT 2 and 3 type viruses respectively (Bastos 1999, personal communication).

The implementation of improved control strategies in southern Africa has significantly decreased the incidence of the disease in domestic livestock. In South Africa, zoning and control of FMDV has lead to the restriction of FMD outbreaks to wildlife populations in the northeastern extremes of the country. However, after several years of the absence of the disease in domestic livestock two independent outbreaks occurred during the summer of 2000 in Mpumalanga and KwaZulu-Natal respectively. Eradication of the FMD from sub-Saharan Africa is unlikely due to the persistent infection of African buffalo (*Syncerus caffer*) in the region. Buffalo act as a potential source of infection of other susceptible wildlife and domestic species. Thus, the focus is on control of FMD in sub-Saharan Africa rather than the eradication.

1.2.2 Persistent infection in wildlife

African buffalo is the only species of wildlife known to be persistently infected by FMDV (Hedger, 1976). The vast majority of infections are however clinically inapparent. Infection in a single animal can be sustained for at least five years with the continuous circulations of virus in the pharyngeal region of the animal. In isolated herds, SAT 1 and 2 isolates can stably be maintained for 24 years by continual circulation within the population (Condy et al., 1985). In cattle as in buffalo, virus levels in the pharyngeal region begin to decline soon after initial infection (Burrows, 1966). Six months post infection virus levels become nominal, but virus may persist in 20% of the herd for up to
two years (Hedger, 1968). During this period, animals are not protected against superinfections (McVicar & Sutmoller, 1976; Van Bekkum et al., 1959).

Although several other wildlife species periodically become infected, there is little or no evidence that any other species of wildlife are capable of sustaining FMD infections over extended periods of time. In the Kruger National Park (KNP) in South Africa, regular epidemics of FMD occur in Impala (*Aepyceros melampus*), a species of gazelle found throughout southern Africa. Outbreaks among Impala are frequently characterised by the onset of severe symptoms. Serological surveillance however, showed that the virus is not maintained in the population during interepidemic periods. Despite the fact that researchers have shown that under experimental conditions, it is in fact possible to transmit FMDV from acutely infected Impala to cattle, very little evidence exits to support speculation that outbreaks in cattle in areas surrounding the park originate from Impala (Meeser, 1962; Thomson et al., 1984).

Infection rates in buffalo are exceptionally high (Hedger, 1972; Falconer and Child, 1975). African buffalo breed seasonally with most calves being born in midsummer. This causes the synchronised infection of juveniles resulting in the simultaneous onset of disease in more than 10% of a breeding herd (Thomson, 1994). This in turn leads to the significant increase in the environmental contamination and provides a source of infection for other species in the immediate vicinity. African buffalo occur in or adjacent to all endemic FMD areas in the subcontinent. In addition to this, high FMD seropositivity rates suggested that that buffalo act as a major source of infection of livestock (Condy et al., 1969). Recent studies have unequivocally confirmed that African buffalo is the predominant source of infection for outbreaks in domestic and wildlife species (Dawe et al., 1994; Bastos et al., 2000).

**1.2.3 Spread of infection**

Although indirect transfer of virus occasionally involves contaminated objects or material, most often spread is affected by direct contact between infected animals actively excreting virus and susceptible hosts (Sellers, 1971; Rweyemanu et al., 1982). FMD virus can routinely be detected in all physiological fluids of viraemic animals and excretion can occur as much as four days before the onset of clinical symptoms (Hyslop, 1970; Burrows
et al., 1971). With the exception of the oesophago-pharyngeal secretions, virus is not secreted in any of the major animal secretions or excretions for more than two weeks post-infection. (Graves et al., 1971).

Of all secretions and excretions in acutely infected animals, saliva contains the highest concentration of infectious units. This is the major contributing factor in the cross-contamination of the immediate environment and subsequent spread of the disease in susceptible populations. Due to the large respiratory volume of cattle, these animals are especially vulnerable to infection via the respiratory tract (Sellers, 1971). Cattle can be infected by inhaling doses of the virus as low as 25 infections units (Donaldson et al., 1987). The infectivity of aerosols is heavily dependent on the size of the droplets and is associated with larger droplets, which are most likely to be trapped in the upper respiratory tract. Strong circumstantial evidence exists that long-distance transmission of FMD is possible under favourable environmental conditions (Donaldson, 1979; Gloster et al., 1982). However, reports of airborne spread of infection from primary outbreaks are restricted to outbreaks in northern Europe and no convincing evidence for its involvement of FMD incidence in southern Africa has been advanced.

Domestic pigs are exceptionally efficient in excreting FMD virus and are often intensively farmed, resulting in high densities of diseased animals during outbreaks and large scale contamination of the surrounding areas (Donaldson, 1983). In contrast with cattle, pigs are equally susceptible to infection by the oral and respiratory routes (Terpstra, 1972). And circumstantial evidence suggests that infection of pigs usually result from the feeding of contaminated food (Strohmaier, 1987). Although sheep and goats are highly susceptible to infection and excrete virus at significant levels, little is known about the role of these animals in the epidemiology of the disease in southern Africa. Both species is however considered to be crucial in the spread of the disease in Turkey and some European countries (Pay, 1988).

Other secretions that contain appreciable quantities of virus are those of the nasal, pharyngeal and urogenital tract. The importance of urine and faeces in the transmission of FMD is not yet clear. Only low levels of virus have been found in these excretions and are not considered as a significant source of infection during outbreaks (Cottral, 1969;
Gainaru, 1986). Semen, however, may contain virus levels of up to $10^{4.7}$ MLD$_{50}$/ml even prior to the development of visible lesion (Sellers, 1983). Recovery of infectious virus from the genital tract of infected steers makes transmission via the sexual route a distinct possibility.

Although there is no known insect vectors implicated in the spread the virus, circumstantial evidences suggest that face flies (Musca spp.) may be involved in the mechanical transmission of FMDV via the conjunctiva (Sutmöller & McVicar, 1973). However, preliminary investigations failed to generate any supporting evidence of this (Thomson, 1985).

Cattle are immune to re-infection by homologous virus for one to three years on average and occasionally for up to four-and-a-half years (Bachrach, 1968; Brooksby, 1982). Circumstantial evidence suggests that immunity post-infection may be less for the SAT types (Thomson, 1994). Little is known concerning the duration of immunity in other species, but experimental evidence suggests that it may be shorter than in cattle (Bachrach, 1968).

1.3 The structure and antigenic properties of FMDV

1.3.1 Three-dimensional structure

The FMD virion is roughly spherical and contains no lipids. It is composed of 60 protein subunits consisting of the four structural proteins, VP4, VP2, VP3 and VP1. Five copies of VP1 are clustered around the five-fold axis, while VP2 and VP3 are positioned around the two- and three-fold axes of symmetry. These three proteins assemble into a protomeric subunit and twelve subunits form the icosahedral virus particle (Finch & Klug, 1959). VP4 of picornaviruses is completely buried at the base of the protomer.

The three-dimensional structure of FMDV has been resolved by X-ray crystallography (Acharya et al., 1989). Each of the structural proteins is folded into an eight-stranded β-barrel. For VP2 and VP3 the axes of the barrel are in the plane of the capsid while the axis of VP1 is turned slightly upwards in the direction of the 5-fold axis. Residues 141 to 160 of VP1 have been identified as the major antigenic region of the virus and are able to induce
neutralising antibodies (Bittle et al., 1982; Pfaff et al., 1982). This region has been shown
to be situated within a connecting loop structure projecting out onto the surface of the
virus. With the exception of this region the virion appears to be smooth with an average
radius of between 100Å and extending to 150Å at the outer most limit. The viral genome is
complete encapsulated by the icosahedra particle and not exposed at the surface at any
point.

1.3.2 Genome structure and organisation
The FMD virus genome consists of a single-stranded RNA molecule of approximately
8700 base pairs (bp) in length. The RNA has a positive polarity and thus acts directly as
messenger-RNA. The protein-coding region of the genome is flanked on each end by a
non-translated region. In typical mRNA fashion, the viral RNA is polyadenylated at the 3'
end. The function of the polyA tail is not clear, but RNA molecules of polioviruses with
shorter polyA tracts have been shown to exhibit a lower specific infectivity (Spector &
Baltimore, 1974; Hruby & Roberts 1976; Frey & Strauss, 1978). However, in contrast to
several other picornaviruses, the specific infectivity of FMDV is not affected by the length
of the 3' polyA tract (Grubman et al., 1979). The polyA tail may further be responsible for
RNA stability by protecting the genome against enzymatic degradation within the host cell.

The 5' end of the viral genome carries a small viral protein known as the VPg. This protein
is covalently attached to the 5' terminal pU-pU sequence of the RNA through a
phosphodiester linkage involving the phenolic hydroxyl group of a tyrosine residue
(Wimmer, 1982). Three different forms of the VPg have been observed in association with
the FMDV genome (Forss & Schaller, 1982). This corresponds to the presence of a three
VPg gene tandem present within the coding region of the genome (King et al., 1980). The
VPg appears to play an important role during viral replication and possible packaging of
the virus. The 5' non-translated region can broadly be separated into three distinctive
regions. The first 400bp, known as the S fragment is capable of forming a large hairpin
structure of which the precise function is not known. The S fragment is separated from the
larger portion of the genome by a polyC tract ranging in size from 100 to 420 residues
(Harris et al., 1976; Rowlands et al., 1978). The function of the polyC tract is not entirely
known, but it has been shown to be involved in determining virulence in other picornavirus
models (Costa Giomi et al., 1984; Duke & Palmenberg, 1989) The remainder of the 5’
non-coding region consists of approximately 700bp and contain a series of three pseudoknots and the internal ribosome entry site (IBRS) (Belsham & Brangwyn, 1990). The IBRS is linked to the cellular 57kDa protein and plays a crucial role during translation (Luz & Beck, 1990; Luz & Beck, 1991).

Four functional regions can be identified within the protein-coding region of the genome (Robertson et al., 1985). The first of these is the L region starting at the 5' initiation codon and ending at the start of the structural-protein-coding region. This region encodes the leader protease, a distinctive feature of the aphthoviruses. The L region is directly followed by the P1 region encoding the precursor of the structural proteins. The P1 region is approximately 3kb in length and contains the coding sequences for all four of the structural proteins. The remaining 2/3 of the genome encodes all of the non-structural proteins and can be separated into the P2 and P3 functional regions respectively. The RNA contains an open reading frame encoding a single polyprotein. A cascade of proteolytic processing events carried out by three viral proteases result in 12 mature gene products. A graphical representation of the genome organisation and translational products are given in Figure 1.3.1.

1.3.3 Translational products
Translation of the viral RNA is initiated by the binding of ribosomes directly to the internal ribosome-binding site within the non-coding region and results in the formation of a polysome. Synthesis of a polyprotein is mediated by the use of the host cell protein synthesis machinery. Full-length (260kDa) polyprotein is ordinarily not formed, since the proteolytic processing thereof is initiated prior to the termination of translation.

Translation is initiated with equal frequency from of two in-frame AUG codons situated within the L region of the genome (Fross et al., 1984) and facilitate the formation of two alternative forms of the leader protease (Lab and Lb) sharing a common carboxyl terminus (Clarke et al., 1987; Sanger et al., 1987). During the initial stages of viral replication there is a marked decrease in translation of host mRNA. This is a consequence of the proteolytic cleavage of the 220kDa subunit of the cellular cap-binding complex (eIF-4). The cleavage is not carried out by a viral coded protease but by a latent host protease activated by the leader protease (Devaney et al., 1989).
Figure 1.3.1 A graphical representation of the genome organisation and expression of FMDV. The three C’s (ccc) at the 5’ end of the genome indicates the position of the polycytidylic acid tract. The black dot at the same end indicates the position of the internal ribosome entry site. The polyprotein is synthesised from left (N terminus) to the right (C terminus). All non-structural proteins are indicated in yellow. The four structural proteins that make up the proteinous capsid structure are represented by the blue rectangles. The three A’s (AAA) at the 3’ end of the genome indicates the position of the poly A tail associated with the genome.
During the early processing event, two auto-catalytic cleavages occur involving the leader protease (Strabel and Beck, 1986) and the 2A protein (Clarke and Sanger, 1988) respectively, resulting in the formation of the mature leader protease and the separation of the P1 fragment from the rest of the gene products. It has been shown that a 19 amino acid sequence located within the 2A protein is sufficient to mediate the cleavage of the polyprotein immediately C-terminal to 2A (Ryan et al., 1991). The 2A protein has furthermore been implicated in the shut off of host protein synthesis (Bernstein et al., 1985).

The structural proteins 1A (VP4), 1B (VP2), 1C (VP3) and 1D (VP1), are contained within the P1 precursor and associate to form the protein coat subunits (protomers). The protomers of picornaviruses have been shown to be wedge-shaped with VP1, VP2 and VP3 occupying separate domains within the structure. Alkaline removal of VP2 and VP4 does not result in the dissociation of the protomer, suggesting that VP1 and VP3 contain important protomer-bonding domains which plays a curtail role in the stabilization of the virion (Katagiri et al., 1971).

In addition to their structural role, the coat proteins are the major determinants of the antigenicity of the virus. Several findings indicate that VP1 is the major immunogenic protein of FMDV and is able to elicit neutralising antibodies in its purified form (Laporte et al., 1973; Sobrino et al., 1989). However, a relatively large dose of purified VP1 is required to induce similar levels of antibodies compared to levels induced by the intact virus, stressing the importance of conformational epitopes involving all the structural proteins (Laporte et al., 1973; Bachrach et al., 1975;).

Two conformational neutralising epitomes have been found within VP1 of type O viruses (Kitson, 1990) as well as a third epitope within the same protein of the A12 virus (Baxt et al., 1989). In addition to the before mentioned conformational epitope, several linear epitopes can also be found within the VP1 protein (Crowther et al., 1993). Experimental data showed that the major antigenic site is located in the region of amino acid residues 141 to 160. These amino acids coincide with the G-H loop loop structure, protruding from the particle surface (France et al., 1994). Trypsin mediated removal of the loop structure not only results in the reduction of its antigenecity, but also causes a dramatic loss of
infectivity. The abolishment of infectivity is associated with the loss of ability to attach to susceptible cells, due to the removal of the RGD motif (amino acids 145 to 147). The RGD motif is thought to be the cell attachment site of the virus, reacting with cellular receptors on the surface of susceptible cells (Geysen et al., 1985; Fox et al., 1989).

In addition to the epitopes located on the VP1 protein, neutralizing epitopes can also be found on VP2 and VP3 proteins (Kitson et al., 1990; Baxt et al., 1989). VP4 is rarely exposed to the service of the particle and therefore is not subjected to immunogenic surveillance. For this reason VP4 is the least antigenic of the four structural proteins. However, a distinctive feature of the VP4 structural protein, as well as the PI and VP0 precursors, is the N-terminal addition of a myristic acid to these proteins (Chow et al., 1987). A cellular enzyme rather than an enzyme of viral origin mediates the addition of the n-tetradecanoic acid. The possible role of the myristate is not clear, but it is thought to be involved in the localization of the PI precursor on the cellular membrane, assembly of the pentamers or the release of pentamers from the membrane (Rueckert, 1990).

The role of 2B and 2C is not quite clear, but initial investigations suggested that the 2B protein of rhinovirus 2 might be involved in host range determination (Yin & Lomax, 1983). Guanidine hydrochloride is known to block the initiation of RNA synthesis and is able to inhibit viral replication of several picornaviruses at millimolar concentration (Caliguiri & Tamm, 1973; Crowther & Melnick, 1961; Rightsel et al., 1961). Resistance to this drug have been mapped to the 2C region of FMDV (Saunders & King, 1982), as well as poliovirus (Anderson-Sillman et al., 1984), suggesting that the 2C protein is involved in RNA synthesis (Li & Baltimore, 1988). It has also been shown that the introduction of sitespecific mutations within the highly conserved 2C coding region, severely restricts the ability of the virus to replicate (Li & Baltimore, 1988). This result further point to the involvement of the 2C protein in RNA synthesis.

No information is available to clearly define the role of protein 3A in the picornavirus life cycle and its possible effect on the cellular metabolism. However, the 3AB polypeptide, the precursor of the genome-bound VPg protein (3B) and the 3A protein, stimulates the in vitro synthesis of poly(U) RNA, directed by the 3Dpol viral polymerase (Lama et al., 1994) and is thought to act in a similar manner in vivo. The 3AB precursor is known to associate
with cellular membranes during replication and thereby direct the replication complex to the endoplasmic reticulum (Giachetti et al., 1991; Xiang et al., 1998). The association of the precursor with cellular membranes is mediated by a hydrophobic domain present in the 3A protein (Xiang et al., 1998). Protein-membrane interaction of the 3A protein has been shown to cause a cytopathic effect due to an increase in membrane permeability, prevent surface expression of host proteins and to inhibit protein excretion from 3A expressing cells (Doedens & Kirkegaard, 1995; Doedens et al., 1997).

Despite the fact that the 3AB precursor is required for effective viral replication of poliovirus, purified 3A protein does not show RNA binding or transactivation activity. This suggests that the 3A protein acts only as an anchor or carrier of the VPg protein, which in turn acts as a primer during viral RNA synthesis (Takegami et al., 1983; Takada et al., 1986; Giachetti et al., 1991; Porter, 1993). If the 3AB precursor does in fact act as a co-factor of the viral polymerase, it would allow for the regulation of replication by means of proteolytic processing (Lama et al., 1994). The recent emergences of naturally attenuated strains of FMDV have shed new light on the possible role of the 3A protein. In 1997, a devastating outbreak of FMD in Taiwan was caused by a type O virus exhibiting atypical virulence. It produced a high incidence of disease in swine but did however not affect cattle. Genetic characterization of this and other attenuated strains of FMDV, showed an altered state of the 3A protein to be the major determinant of the atypical epidemiology (Beard and Mason, 2000). It was therefore hypothesised that the 3A protein my play a significant role in determining host range specificity and virulence. The exact mechanism by which this could be effected is however not clear.

The 3CD precursor of FMDV can be cleaved in two different ways to yield either 3C + 3D or 3C' + 3D' (Doel et al., 1978, Ruckert et al., 1979). In the case of poliovirus the alternate cleavage occur at a Tyr-Gly site and is mediated by 2A (Toyda et al., 1986). The 3C protease or its precursor 3CD, mediates the majority of the cleavages of the polyprotein with the exception of the auto-catalytic cleavages of the leader protease, the 2A protein and VP0 precursor (VP0 → VP4 + VP2) during maturation of the virion. This viral protease belongs to the family of cellular serine proteases. The catalytic triad was predicted to be His46-Asp84-Cys163 (Gorbalenya et al., 1989) and confirmed by site-directed mutagenesis (Grubman et al., 1995). In addition to the viral protein processing, the 3C
protease of FMDV cleaves the host-cell protein histone H3 and may be involved with shut-off process of host-cell transcription (Falk et al., 1990). The final protein coded for by the virus, is the 3DPol protein. This protein is cleaved from its precursor by the 3C protease and has as its main function the replication of the RNA genome. This RNA polymerase is required for copying the positive sense RNA strand to form the complementary minus stranded RNA and the consequential formation of additional the positive stranded viral RNA.

Besides the four capsid proteins, the VPg associated with the viral genome is the only protein of viral origin packaged into the mature virion. All other viral proteins are only produced during replication of the virus following the successful infection of a susceptible cell.

1.4 Variation within the FMDV genome

1.4.1 Overview of replication
Virus entry during FMD infection occurs due to receptor-mediated endocytosis. A crucial part of internalisation of the virion is the non-covalent interaction between the vitronectin host cell surface receptors (integrin \( \alpha_v\beta_3 \)) and the Arg-Gly-Asp (RGD) protein ligand (Fox et al., 1989). Internalisation are thought to occur due to the release of VP4 and the unfolding of hydrophobic regions of the VP2, 3 and 4 proteins that would result in the formation of a pore through which the RNA might be transferred to the cytoplasm (Evens & Almond, 1998).

Early on during infection the majority of viral RNA acts as mRNA, since viral replication is focused on protein synthesis. Levels of the replicative intermediate RNA increases 2 to 4 hours post-inoculation while the replicative form RNA accumulates slower and reaches lower levels during infection. RNA replication involves at least two viral coded proteins, the 3DPol and the VPg. These two proteins as, well as a 67kDa host factor protein, is tightly associated with the smooth endoplasmic reticulum and forms the 27S replicon complex. RNA synthesis is template dependent and is thought to be primed by the VPg. The 3DPol is a RNA dependent RNA polymerase and is responsible for the elongation of both positive and negative sense RNA during virus replication. Later, as RNA synthesis proceeds at a
constant rate, synthesis of positive-sense RNA predominates, and approximately 50% of these molecules are packaged into virions.

Shell assembly proceeds directly following the proteolytic cleavage of the P1 precursor. Immature protomers (5S subunits) are formed by the aggregation of VP0, VP3 and VP1. The 5S subunits assemble into pentamers followed by the assembly of 12 pentamers into either non-infectious RNA-containing provirion or naturally empty capsids. Formation of the infective 146S virions is achieved by the maturation cleavage of the VP0 precursor. Complete virus particles are ultimately released from the cell by infectious-mediated disintegration of the host cell.

It is important to note that the products of viral replication are rarely exact copies of the original virus involved in initial infection. The inherent error prone nature of viral replication is an important aspect of the biological evolution of the virus and contributes to the ability of the virus to adapt to the ever-changing cellular environment.

1.4.2 Mutations
Mutation is responsible for a significant portion of genetic variation observed after replication. Changes in the sequence of genomic nucleic acids occur at a higher frequency in single-stranded than double-stranded nucleic acids. Due to the absence of a complementary sequence, point mutations within single-stranded species become immediately effective. Mutation rates in RNA genomes are generally considerably higher than the rate observed for DNA (Steinhauer and Holland, 1987). High mutation rates observed for RNA are mainly due to the lack of an effective proofreading mechanism of RNA-dependent RNA polymerases.

Estimates of the mutation rate of FMDV were found to be in the order of $10^{-3.8}$ and $10^{-5.6}$ per base position depending on the method of investigation (Pringle, 1964; McCohen, 1981; McCohen, 1986). The rate at which mutations are fixed within the FMDV genome are heavily dependent on the time period and portion of the genome examined and were found to vary between $0.04 \times 10^2$ and $4.5 \times 10^2$ substitutions per nucleotide per year (Sobrino et al., 1986). Mutations are most frequently observed in the three major capsid proteins viz. VP2, VP3 and VP1. In fact, mutation rates for the structural-protein-coding
regions of the genome are up to six-fold higher than for any other part of the genome (Sobrino et al., 1986).

Studies on the accumulation of mutations in FMDV under field conditions revealed that in fully susceptible animals mutations only occur occasionally and that these mutations rarely persist in the progeny (McCohen, 1986). However, in partially immune populations mutations are rapidly accumulated. Nucleotide sequences of sequential isolates obtained from carrier animals revealed a similar pattern. However, specimens collected from carrier animals frequently yielded mixtures of mutated viruses (Gebauer et al., 1988).

1.4.3 Quasispecies concept
In 1971, Eigen and his collaborators developed a mathematical model to describe the dynamics of RNA replication in which a RNA genome is considered to be a population of quasispecies. The model allows for the definition of each individual mutant within the population to be defined in terms of a probability. The first experimental evidence that RNA viruses may have a quasispecies distribution was generated using phage Qβ (Batschelet et al., 1976, Domingo et al., 1976). It was shown that up to 15 percent of individual isolates differed on average at 1 to 2 positions from the phage Qβ stock virus after 50 passages (Domingo et al., 1978).

Despite the high level of heterogeneity within a virus population, high mutation rates are not always reflected in the pathology of infected individuals. Under constant conditions a single variant would dominate during infection causing a distinct pattern of pathology. However, any change in the conditions that in any way detrimentally affects the efficacy of replication of the dominant variant, would allow for the selection of a more fit quasispecies which would have the potential to replace the dominant variant (Hyslop, 1965; Domingo et al., 1980, Matau et al., 1987). The result is a highly flexible biological system, which allows the virus to evade constant immune surveillance and establish or mediate persistence through the continual selection of variant genomes. However, the effects of mutations are limited and rarely result in a large shift in the biological behaviour of the virus.
1.4.4 Recombination

Unlike mutation, recombination offers a mechanism by which large segments of genetic material can be exchanged between two viruses. Although recombination in DNA is well documented, evidence of RNA recombination is limited to only a few viral families. In 1977, Cooper reported that it was possible to construct a genetic map of the poliovirus genome using crosses between temperature sensitive mutants defective in coat protein formation and RNA synthesis. Recombination does not only occur under experimental conditions, but have also been shown to occur under natural infectious conditions. Kew and Nottay (1984) identified natural isolates of poliovirus that contained segments of all three virus serotypes. It is speculated that recombination occurs when a single cell is simultaneously infected with two or more FMD viruses (Pallansch et al., 1980).

Biological evidence suggests that recombination of picornaviruses occur due to template switching during replication (Cooper et al., 1974). The proposed mechanism by which this occurs, involves the detachment of the RNA polymerase from the original template and reattachment to an alternative template within close proximity to the replication machinery. The length of the picornavirus recombination map, 1.2% for poliovirus (Cooper, 1977) and 3% for aphthoviruses (McCohen, 1981), implies that recombination occurs with very high frequency. It is estimated that in the case of polio up to 20% of all viral genomes produced during a single cycle of replication may be recombinant. The frequency of recombination appears to be higher during synthesis of the negative strand (Kirkegaard and Baltimore, 1986). Although recombination between serotypes of FMDV do occur, crosses between different strains of the same serotype occur more readily (Tolskaya et al., 1983; McCohen et al., 1985).

King et al. (1988) showed that in addition to single cross-over events, double cross-overs were also occurring within the FMDV genome. The cross-overs were scattered throughout the genome, suggesting that it was a general rather than site-specific event (Fross et al., 1984). Nucleotide sequence data of known cross-over points within the FMDV genome suggests that although perfect homology is not a prerequisite for recombination to occur, relatively high homology (70-80%) is required. Furthermore, cross-over points are generally situated within region of high secondary structure (Wilson et al., 1988). Recombination can therefore probably occur throughout the genome.
Although there is substantial evidence that mutational and recombination events contribute substantially to the genetic heterogeneity of FMDV, the mechanisms that lead to the fixation of these mutation within successive populations are poorly understood. It is however clear that generation and the establishment of new variant populations play an important role in the evolution of the virus.

1.5 Aims of the Study
The study is composed of two distinct Sections. The first Section deals exclusively with the genetic characterisation of the structural-protein-coding region of a specific FMDV isolate originating from southern Africa. In the second Section, the relevance of the genetic variation observed for the 3A non-structural-protein-coding region of several African FMDV isolates originating from diverse geographical and host origins, is studied.

SAT 3 type viruses are rarely responsible for outbreaks of the disease in domestic livestock. This, coupled to the restricted geographical distribution of the serotype, are probable reasons why very little research on representative isolates of SAT 3 type viruses has been done. Nevertheless, SAT type 3 viruses pose a significant threat to commercial stock farming in southern Africa. This is especially pertinent in areas bordering wild life reserves where FMD is endemic. It has been shown that in the Kruger National park, South Africa, that a large portion of the resident African buffalo has significant titers of antibodies directed against SAT 3 type viruses. These animals represent a continual reservoir of virus capable of causing a severe outbreak among domestic animals in the surrounding areas.

The generation of genetic information with regards to the structural-protein coding region of the SAT type 3 viruses would greatly assist in the design of custom-made vaccines, which accurately reflects antigenic characteristics of prevalent isolates. The major objective of the study was to expand on the present knowledge concerning characteristics of the structural-protein-coding region of the SAT 3 type viruses by determining the nucleotide sequence of the entire P1 precursor of FMD virus KNP/10/90/3. This isolate was first isolated in 1990 from an oesophagus-pharyngeal sample collected during a routine survey of the endemic African buffalo population in the Kruger National Park of
South Africa and are currently being used in the formulation of vaccines to be used in southern Africa. We compared this region of the genome, consisting of the four structural proteins (VP1, VP2, VP3, and VP4), among different SAT serotypes and with that of representative isolates of serotypes A, O, and C.

The 3A non-structural protein of FMDV has been shown to be involved in viral RNA replication and death of infected cells. As previously discussed in Section 1.3.2, changes in 3A have been associated with altered host range and virulence in several picornaviruses. Although the 3A coding region has been shown to be highly conserved among European isolates, the sequence characteristics of the 3A of the SAT type viruses have not been investigated. In light of this, the nucleotide sequences of the 3A non-structural-coding sequence of several African FMD virus isolates were determined and comparatively analysed. We compared this region of the genome among different SAT serotypes and with that of European, South American and Asian isolates in order to assess the extent of genetic variation within the 3A coding region of naturally occurring viruses in sub-Saharan Africa.
Chapter 2: Genetic characterisation of the structural-protein-coding region of foot-and-mouth disease virus KNP/10/90/3
2.1 Introduction

Nucleotide sequence determination has proven to be an invaluable tool in the genetic characterisation of foot-and-mouth disease virus (Palmenberg, 1989). Phylogenetic studies based on partial genomic sequences have aided scientists in their quest to understand the development of outbreaks and more accurately predict and combat the spread of the disease (Brown, 1987).

Phylogenetic characterisation of field isolates originating from sub-Saharan Africa, based on nucleotide sequence data, has revealed that these viruses evolve independently in different geographical regions complicating the design and production of universal vaccines which effectively address the antigenic variation within the SAT types (Vosloo et al., 1995). The extension of effective FMD control to other countries in the region, as well as improved control in established areas thus requires the use of vaccines designed to address the antigenic characteristics of prevalent strains occurring in specific areas within the region (van Rensburg & Nel, 1999).

However, selection of prevalent, high producing and stable vaccine strains is a cumbersome and expensive process. To address this problem, new generation vaccines are being developed based on the ability of naked FMDV RNA to infect susceptible cells and result in the formation of intact virions (Rueckert, 1990). The first step in is approach is the construction of infectious full-length cDNA clone of a suitable vaccine strain. The antigenic characteristics of such a clone can easily be manipulated by exchanging the antigenic determinants of the virus i.e. the structural proteins with that of an alternative virus. The cDNA clone acts as a vector expressing the structural-protein cassette of an alternative virus inserted in place of the native structural-protein-coding region, resulting in the formation of a chimeric FMD virus exhibiting the antigenic characteristics of the alternative virus, while retaining the growth characteristics of the virus from which the clone was originally constructed (Sa-Carvalho et al., 1997). The chimeric viruses can then be isolated and directly applied in current vaccine production protocols.

The major advantage of the approach is the ability to construct custom designed vaccines without having to adapt newly isolated strains to cell culture for the purpose of vaccine production. This strategy has previously been shown to be successful for both type A and
O serotypes (Zibert et al., 1990; Reider et al., 1993). Due to the genetic and phenotypic diversity of FMDV, a number of crucial factors that may influence the success of such a strategy in the southern African context however remain unknown. These include the role of proteolytic variation and cleavage site differences observed in isolates form different geographical areas.

Traditionally more emphasis has been placed on the characterisation of isolates occurring predominantly in Europe and South America. Although the structural-protein-coding region of several isolates representative of serotypes A, O and C have been successfully characterised (Forss et al., 1984; Robertson et al., 1985; Singh et al., 1996), comparatively little is known of the SAT type viruses which are predominantly responsible for outbreaks in sub-Saharan Africa. To date only four reports have been published describing the genetic characteristics of the entire structural-protein-coding region of FMD viruses representative of the SAT serotypes (Knowles et al., unpublished; Newman et al., unpublished; Brown et al, 1989; van Rensburg and Nel, 1999; van Rensburg, unpublished). Of these only one deals with a representative isolate of SAT 3. Although the geographical distribution of the SAT type 3 viruses is restricted to southern Africa, it still poses a significant threat to the commercial farming in these regions. This chapter focuses on the genetic characteristics of KNP/10/90/3 and reports on the relevance of the data obtained towards elucidating the extent of genetic variation within the structural-protein-coding region of SAT 3 serotype. We furthermore briefly discuss the possible influence of genetic variation among the SAT isolates on the construction of custom-made vaccines based on the strategy described above.

2.2 Materials and methods

2.2.1 Origin of the virus used in the study
The KNP/10/90/3 virus was first isolated in 1990 from an oesophagus-pharyngeal sample collected during a routine survey of the endemic African buffalo population in the Kruger National Park of South Africa. The virus was initially isolated on pig kidney cells (primary isolation), and cultured on IB-RS-2 cells for a further 3 passages. The virus was partially

* The recipes for the preparation of all buffers and reagents referred to in the texts are summarised in appendix A.4.
purified from the third passage and aliquots stored at -70°C. In addition to this isolate, the previously published structural-protein-coding region sequences of several other isolates were included in the analysis of the data and were obtained from the Genbank nucleotide sequence database. Details of all isolates used in the study are summarised in Table 2.2.1.

Table 2.2.1. List of viruses used in comparative studies of the structural-protein-coding region of the foot-and-mouth disease virus.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Country</th>
<th>Year of Isolation</th>
<th>Species of Origin</th>
<th>Reference</th>
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<td><strong>Serotype A</strong></td>
<td></td>
<td></td>
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<tr>
<td>A12</td>
<td>United Kingdom</td>
<td>1932</td>
<td>Bovine</td>
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<td>A22</td>
<td>USSR</td>
<td>1964</td>
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<td>A10</td>
<td>Argentina</td>
<td>1961</td>
<td></td>
<td>X00429</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>1965</td>
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<td>X00871</td>
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<td>Brazil</td>
<td>1958</td>
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<td>M89781</td>
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<td>Germany</td>
<td>1962</td>
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<td>1965</td>
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<td>AF286347</td>
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</table>

* The nucleotide and amino acid sequence data for KNP/196/91/1 have been submitted to the Genbank nucleotide sequence database and have been assigned the accession number AF283429. This sequence data was generously supplied by H.G. van Rensburg (unpublished results).

* The nucleotide sequence has been submitted to the Genbank nucleotide sequence database and has been assigned the accession number AF286347.
2.2.2 Preparation of cell culture
All cell lines used in the study were subcultured as described by Freshney (1987). Briefly, the procedure involved rinsing of the cell sheet, assisted detachment of the cells from the solid surface and re-incubation of the cells after the addition of fresh maintenance medium. A single Roux flask containing an IB-RS-2 cell line monolayer was initially rinsed with warmed (37°C) versene-trypsin after which the fluid was decanted and discarded, followed by the addition of 3ml fresh solution and the incubation of the cells at room temperature for approximately 5 minutes. Versene-trypsin acts as a detergent causing a lowering of the surface tension and thereby assists in the detachment of the cells from the glass surface. Following the incubation of the cells, 5ml Modified Eagle’s maintenance medium, warmed to 37°C, was added to the flask and the cells resuspended by repeatedly dispensing the total volume of fluids using a 10ml glass syringe. Equal volumes (1ml) of the resulting cell suspension was transferred to sterile Roux flasks and 50ml, Modified Eagle’s medium, containing 5% normal bovine serum, penicillin (1000 units/ml), neomycin (75μg/ml) and streptomycin (100μg/ml) added. The cells were incubated at 37°C for approximately four days, whilst continually being rocked before being used for virus propagation.

2.2.3 Propagation of the foot-and-mouth disease virus
A single Roux flask containing a confluent cell sheet was inoculated with 1ml of the stock virus (Section 2.1.1) at a low multiplicity of infection. The inoculated cells were overlaid with 50ml warmed Eagle’s medium, containing penicillin (1000 units/ml), neomycin (75μg/ml) and streptomycin (100μg/ml), but void of normal bovine serum. The flask was incubated at 37°C for approximately 24 hours, while gently being rocked. As soon as 100% cytopathic effect (CPE) was observed, 50ml of the medium now containing the viruses release from the cells during lysis, was pored off into a second Roux flask containing a fresh uninfected monolayer of IB-RS-2 cells. This flask was incubated at 37°C for 30 minutes to allow sufficient attachment of the virus to the uninfected cells. Following incubation, all fluids were decanted and discarded. Any cell debris and unattached virus was removed by adding 10ml phosphate buffer saline (pH 7.6 @ 25°C) and gently swirling the flask for 30 seconds. The rinsate was discarded before fresh maintenance medium (Modified Eagle’s medium containing 1000 units/ml penicillin, 75μg/ml neomycin and 100μg/ml streptomycin) was added to the monolayer. The infected cells were incubated for
3 to 4 hours at 37°C, until 80% CPE could be observed. At this point the virus was harvested as described in Section 2.2.4.

2.2.4 Partial purification of the foot-and-mouth disease virus
The FMD virus was recovered using a method similar to that described by Rico-Hesse et al. (1987) for the purification of poliovirus from cell culture. Infected cells were detached from the glass and resuspended as described above (Section 2.2.2). The suspension was then transferred to a 38ml polycarbonate ultracentrifuge tube (Beckman) and successively centrifuged for 10 minute periods at 2500, 5000 and 10000 revolutions per minute respectively (Beckman SW28 rotor) to remove the cell debris. Subsequent to completion of each centrifugation step, the supernatant was transferred to a clean tube before proceeding to the next. At completion of the final centrifugation step the supernatant was again transferred to a clean tube and 2ml of a 30% sucrose solution (30% sucrose, 0.1M Tris/HCl, pH 7.6 @ 25°C) carefully placed at the bottom of the tube using a sterile 10ml glass syringe to form a distinct pellet within the aqueous phase. The tube was centrifuged at 28000 rpm for 3 hours at 4°C using a Beckman SW28 rotor. The resulting supernatant was decanted and all residual fluid drained thoroughly using a P200 micropipet. A volume of 500µl ice-cold 1 x Tris/EDTA buffer (pH 7.6 @ 25°C) was added to the tube containing the pellet and incubated at 4°C for 5 minutes. On completion of incubation, the pellet was subjected to sonication for 5 seconds at an amplitude of 20 microns (Sonifier B-12 probe) whilst on ice. Aliquots of the resuspended pellet were transferred to a 1.5ml RNase-free micro-test tube and either stored at -70°C or processed immediately.

2.2.5 RNA extraction from purified foot-and-mouth disease virus
Volumes of 5µl β-mercaptoethanol and 50µl of a 10% sodium dodecyl sulphate solution were added to the sonicated pellet. The sample was briefly vortexed after which 250µl chloroform/isoamylalcohol solution at a volume-to-volume ratio of 24:1 and 250µl 0.1M Tris/HCl-saturated phenol were added. The contents of the tube were mixed by inversion and fractionated by centrifuging the tube at maximum revolutions for 5 minutes (Hettich bench top centrifuge, equal to 16000xg). The aqueous phase was transferred to a clean 1.5ml micro-test tube, taking care not to transfer any protein precipitate present at the interface. This was followed by the addition of 500µl fresh 0.1M Tris/HCl-saturated
phenol to the transferred aqueous phase. The contents of the tube were gently mixed by inversion and centrifuged at 16000xg for 4 minutes. The aqueous phase was transferred to a clean 1.5ml micro-test tube and two volumes of diethylether added to it. The sample was centrifuged at maximum revolutions for 4 minutes to allow proper separation. The aqueous phase was transferred to a clean tube and incubated at room temperature for approximately 5 minutes to allow contaminating diethylether to evaporate. The viral RNA was concentrated by precipitation as described below in Section 2.2.6.

2.2.6 Precipitation of RNA
The RNA was precipitated from the aqueous phase by the addition of 3M sodium acetate (pH 5.2 @ 25°C) to a final concentration of 0.3M and 2.5 volumes of absolute ethanol. The sample was incubated overnight at -20°C. The precipitate was concentrated by centrifugation (16000xg for 30 minutes). The resulting pellet was washed by discarding the supernatant and adding 100μl of an 80% ethanol solution. The ethanol was discarded after brief centrifugation. The excess ethanol was removed by evaporation under vacuum and the pellet resuspended in 50μl Tris/EDTA buffer (pH 7.6 @ 25°C) containing 5U RNase inhibitor (5U/μl, Promega).

The concentration and purity of the extracted viral RNA was determined by measuring the optical density of the solution at 260nm and 280nm, respectively. Only preparations with an OD260/OD280 ratio of 1.6 to 1.9 were used for further manipulation. The RNA was aliquoted into smaller volumes of 5μl and stored as -70°C.

2.2.7 Preparation of cDNA
The oligonucleotide used to initiate the reverse transcription was previously described by Beck and Strohmaier (1987). It is designed to anneal to an area 33 nucleotides downstream of the 1D (VP1) structural-protein-coding region. This corresponds to a highly conserved portion of the FMDV genome within the 2AB region. The oligonucleotide was obtained commercially from GibcoBRL and designated WDA (5’AAGGGCCCAGGGTGGACTC3’).

The reaction was performed in a 0.5ml micro-test tubes (Roche Molecular Biochemicals). The reaction mix contained 4μl 5 x Reverse Transcriptase buffer (Promega), 0.5μl diluted
RNase inhibitor (5U/µl), 3 µl deoxyribonucleotide 5'-triphosphate mixture (2.5mM), 2 µl of the WDA oligonucleotide (10pmol/µl), 0.5 µl saturated dimethylsulfoxide solution, 0.5 µl hexanucleotides (200 pmol/µl) and 5 µl distilled H₂O. A volume of 5 µl viral RNA was added to the reaction mix. The tube was briefly centrifuged, incubated at 70°C for 3 minutes in order to destabilise any secondary structure present in the conformation of the RNA and on completion directly transferred onto ice for an additional 2 minutes. Subsequent to this, 3 µl Avian Myeloblastosis Virus Reverse Transcriptase enzyme (10U/µl, Promega) was added and the reaction mix incubated at 42°C for 1 hour. On completion of the polymerisation reaction, enzyme activity was terminated by incubating the reaction mix at 80°C for 2 minutes. The cDNA was stored at -20°C.

2.2.8 Amplification of the structural-protein-coding region
The P1 coding region of the FMDV genome was amplified by employing the polymerase chain reaction (PCR)(Erlich, 1989). The first oligonucleotide used to initiate amplification was designed to anneal to an area within the 5' non-coding region of the genome. This oligonucleotide was obtained commercially from Gibco BRL and designated NCR1 (5'-TACCAAGCGACACTCGGGATCT-3'). The WDA primer used to initiate the preparation of the cDNA was used as the second oligonucleotide in the amplification of the P1 region.

![Figure 2.2.1](image-url)  
*Figure 2.2.1 A graphical representation of the polymerase chain reaction strategy used to amplify the structural-protein-coding region of FMDV. The primers used in the reaction are represented by the red arrows (→).*
The Expand™ Long Template PCR System (Roche Molecular Biochemicals) was used to amplify the region of interest. Two separate master mixtures were prepared as prescribed by the manufacturer. The first master mixture consisted of 5µl deoxyribonucleotide 5'-triphosphate mixture (2.5mM), 1.25µl of the NCR1 oligonucleotide (10pmol/µl), 1.25µl of the WDA oligonucleotide (10pmol/µl) and 4µl distilled H₂O. The second mixture contained 2.5µl 10 x Expand™ Long Template PCR Reaction Buffer (Roche Molecular Biochemicals), 0.5µl MgCl₂ (100mM), 0.4µ Expand™ Long Template enzyme mixture and 9.85µl dH₂O. The two master mixtures were added together, mixed thoroughly and overlaid with mineral oil. A volume of 2µl cDNA was added and the reaction mixture briefly centrifuged.

The PCR was performed using an OmniGene Hybaid Thermocycler. The sample was initially denatured at 95°C for 2 minutes followed by 10 cycles consisting of denaturation at 95°C for 30 seconds followed by a 30 seconds annealing period at 60°C and ending with polymerisation for 60 seconds at 68°C. This was followed by a further 20 cycles differing from the first 10 cycles only by the stepwise extension of the initial polymerisation period of 60 seconds by 20 seconds for each new repetition of a cycle.

After completion of the thermocycling the amplified fragments were separated in a 1.0% agarose gel by standard electrophoreses techniques (Sambrook, 1987). The gel was prepared by dissolving 1.0g of Multi-Purpose Agarose (Boehringer Mannhein) in 100ml 1 x TAE buffer. After allowing sufficient time for the gel-mix to cool to approximately 60°C, 2µl Ethidium bromide (10µg/ml) was added, the gel cast and left to set at room temperature. A volume of 5µl DNA loading buffer (40% sucrose solution coloured with bromo-phenol-blue) was added to the PCR reaction mixture and 50µl of loaded onto the set gel. A commercial DNA marker (HindIII restricted phage φX17 genomic DNA) was loaded onto the gel as a reference. The gel was run at 50V for approximately 45 minutes after which the fragment was visualised by exposing the gel to short wavelength ultraviolet light using a bench top transluminator. Recovery of the appropriate amplified fragment from the agarose gel was done as described in Section 2.2.9.
2.2.9 Agarose gel purification of the P1 amplicon

The Amplified P1 fragment was recovered from the agarose gel according to the protocol set forward by the manufacturers of the Nucleospin Extract 2 in 1 DNA extraction kit (MacHerey-Nagel). The DNA fragment (~3.1kbp) was excised from the gel with the minimum excessive agarose and transferred to a clean 2ml micro-test tube. The fragment was weighed and 300μl NT1 buffer (supplied in the kit) added for each 100mg gel excised. The mixture was incubated at 50°C for approximately 10 minutes and periodically inverted to ensure proper degradation of the agarose gel. The melted sample was loaded into the NucleoSpin column containing a silica filter (supplied as one unit in the kit). The column was inserted into a clean 2ml micro-test tube and centrifuged for 60 seconds at maximum revolutions using a Hettich bench top centrifuge. The resulting filtrate was discarded and 750μl NT3 buffer (supplied in the kit) added onto the filter. The column was again inserted into a 2ml micro-test tube and centrifuged for 60 seconds at maximum revolutions. The filtrate was discarded and the NucleoSpin column placed in a 1.5ml micro-test tube where after the DNA was eluted from the filter by adding 50μl 1M Tris/HCl buffer (pH 8.8 @ 25°C) and centrifuging the column for 60 seconds at 16000×g. The filtrate containing the purified DNA was stored at -20°C.

2.2.10 Ligation of the purified P1 fragment into a suitable plasmid vector

The pGEM-T-Easy vector system (Promega) was chosen as a suitable vector for cloning of the purified P1 PCR fragment (Figure A.1.1). This system has been optimised using a 1:1 molar ratio of insert to vector DNA. The appropriate amount of PCR product included in the ligation reaction was calculated by applying the following formula:

\[
\text{Amount of insert (ng)} = \frac{\text{Amount of vector (ng) x Size of the insert (kbp) x insert:vector molar ratio}}{\text{Size of vector (kbp)}}
\]

Table 2.2.2 summarises all ligation reactions set up during this experiment. Reactions were performed in 0.5ml micro-test tubes at a final volume of 10μl and incubated at 4°C for approximately 48 hours after which it was directly used in transformation reactions.
2.2.11 Preparation of competent *Escherichia coli* cultures

Competent cells were prepared using a method described by Tang *et al.* (1994). A pure culture of the *Escherichia coli* strain JM109 was inoculated into 100ml LB liquid medium and shaken at 37°C for approximately two hours. The optical density at 600nm was monitored every 20 minutes to examine the growth of the culture. After reaching a cell density of 5 x 10^7 cells/ml (OD_600 ::::; 0.3), the cells were harvested by brief centrifugation and resuspended in 10ml of an ice cold solution consisting of 80mM calcium chloride and 50mM magnesium chloride. The resuspended cells were placed on ice for 10 minutes. This treatment was repeated twice before the cell pellet was finally resuspended in 5ml 0.1M calcium chloride. An equal volume of 50% Glycerol was added and 200ul aliquots of the cells frozen in liquid nitrogen. The aliquots were stored at -70°C.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Standard Reaction</th>
<th>Positive Control</th>
<th>Background control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x T4 Ligase Buffer</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>pGEM-T-Easy vector (50ng/µl)</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
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<td>2µl</td>
<td>Not applicable</td>
</tr>
<tr>
<td>PCR Product (50ng/µl)</td>
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<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>T4 Ligase (3U/µl)</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>dH_2O</td>
<td>6µl</td>
<td>5µl</td>
<td>7µl</td>
</tr>
</tbody>
</table>

2.2.12 Transformation of the ligated pGEM-T-Easy vector

A 200µl aliquot of competent *E. coli* JM109 cells, prepared as described in Section 2.2.11, was slowly thawed on ice for each of the respective ligation reactions (Table 2.2.2). The total volume of the ligation reaction (10µl) was added to each aliquot. Each sample was left on ice for a further 30 minutes and subsequently incubated at 37°C for 5 minutes. A volume of 800µl SOC culture medium was added to each sample after incubation, the contents of each tube gently mixed by inversion and incubated at 37°C for 30 minutes. A representative volume (100µl) of each sample was transferred to SOB-agar culture plates, containing ampicillin (100ng/ml), X-Gal solution (2%) and IPTG (10mM). The plates were incubated at 37°C for 14 hours after which all visible colonies with an estimated diameter.
of at least 0.5mm were counted. The transformation efficiency was calculated using the data obtained by the transformation of the positive control ligation reaction by applying the following formula:

\[
\text{Transformation efficiency (} \frac{\# \text{ cfu/} \mu \text{g of DNA}}{\mu \text{g of DNA}}) = \frac{\# \text{ of transformants} \times \text{Final Reaction volume (} \mu \text{l})}{\text{Quantity DNA transformed (} \mu \text{g}) \times \text{Volume plated (} \mu \text{l})}
\]

Suspected recombinant colonies, indicated by the absence of blue pigment, were transferred to fresh SOB-agar culture plates, incubated at 37°C for 18 hours and stored at 4°C.

2.2.13 Isolation of recombinant plasmids
Cultures suspected of containing recombinant plasmids were inoculated into 200ml flasks containing 50ml TB liquid medium containing 50ng/ml ampicillin. The flasks were incubated overnight at 37°C while continuously being shaken at 26 revolutions per minute. The cells were harvested from 3ml liquid medium by successively transferring 1ml of the cell suspension to a 1.5ml micro-test tube, centrifuging the tube at 8000 x g using a Hettich bench top centrifuge and subsequently discarding the supernatant. The resulting cell pellet was resuspended in 250µl SET buffer (pH 8.0 @ 25°C). To this was added 500µl of a freshly prepared solution consisting of 1% sodium dodecyl sulphate and 0.1N sodium hydroxide. The contents of the tube was mixed by inversion and incubated at 65°C. After a 30 minute incubation period, 375µl of a 3M sodium acetate solution (pH 5.2 @ 25°C) was added and the sample transferred onto ice for approximately 15 minutes. Cell debris was removed by centrifuging the samples at 16000xg for 15 minutes. The resulting supernatant was transferred to a clean 1.5ml micro-test tube and a volume of 300µl polyethylene glycol/sodium chloride solution added to it. The sample was left on ice for a further 2 hours and subsequently centrifuged at 16000xg for 15 minutes using a bench top centrifuge. The resulting supernatant was discarded, the pellet washed with 500µl 70% ethanol and resuspended in 400µl 1 x TE buffer (pH 7.6 @ 25°C). A volume of 400µl 5M ammonium acetate was added to the resuspended pellet followed by the addition of 600µl isopropanol. The sample was placed on ice for 30 minutes and the precipitated DNA concentrated by centrifugation the sample 16000xg. On completion of centrifugation the supernatant was carefully removed. Any remaining ethanol was removed by evaporation.
under vacuum. The pellet was resuspended in 50\mu l 1 x TE buffer (pH 7.6 @ 25°C) and stored at -20°C.

2.2.14 Sequence determination of the cloned structural-protein-coding region
The nucleotide sequence of the cloned fragment was determined independently for both the positive and negative strand of the DNA using oligonucleotide primers designed to generate partially overlapping DNA sequences of the entire P1 genomic region. A list of oligonucleotides used in the sequence determination, as well as a graphical representation of their respective positions on the genome are summarised included in the appendix (Table A.1.1 and Figure A1.2). These primers were designed based on nucleotide sequence data available on Genbank as well as the data generated in this study, as sequence data became available.

Two independent methods were employed to determine the nucleotide sequence of the structural-protein-coding region of the FMDV isolate in question. The first method was previously described by Zimmerman and Kaesberg (1978) and later modified by Xie et al. (1987). This method of sequence determination is based on the chain termination due to the incorporation of 2,3-dideoxy-D-ribose analogues of the deoxyribonucleoside 5'-triphosphates during template directed polymerisation of deoxynucleotides (Sanger et al. 1977). The newly synthesised DNA is labelled by the incorporation of $^{32}$P-labelled deoxyadenosine 5'-triphosphate. Nucleotide sequencing by chain termination involves the following methodology.

Purified plasmid DNA (5\mu l) was denatured by adding 5\mu l of a 0.4M sodium hydroxide solution containing EDTA at a concentration of 0.04M to it and incubating the mixture at 37°C for 30 minutes. The denatured plasmid was then precipitated by adding 1\mu l of a 3M sodium acetate solution (pH 5.2 @ 25°C) and 3 volumes of absolute ethanol to the mixture and incubating it overnight at -20°C. The precipitated DNA was concentrated by centrifuging the mixture at 16000xg for 10 minutes using a bench top centrifuge. The supernatant was removed with a P1000 micropipet and the pellet dried under vacuum to remove any excess fluids. The DNA was resuspended in 12\mu l dH$_2$O.
An annealing reaction consisting of 4μl of the resuspended plasmid DNA, 2μl 5 x Sequentase Reaction Buffer and 4μl of a oligonucleotide primer (10pmol/μl), was set up and incubated at 65°C for a period of 2 minutes before being slowly cooled to 35°C. The labelling reaction mix, containing 1μl DTT, 2μl diluted labelling solution, 0.5μl 32p α-ATP (800Ci/mmol) and 2μl Sequenase enzyme, was prepared and 5μl thereof added to the annealing reaction mixture. The contents of the micro-test tube was mixed thoroughly and incubated at room temperature for 5 minutes.

The polymerisation reactions were performed in a 96 well polyethene plate. An 2.5μl aliquot of each dideoxynucleoside-triphosphate terminator (ddNTP) was transferred to separate wells and 3.5μl of the annealing reaction mixture added to each well. The contents of the wells were mixed by repeatedly dispensing the entire volume using P10 micropipet. The mixture was incubated at 37°C for 5 minute and polymerisation reaction terminated by adding 4μl formamide dye mix to each well. The constructed DNA was denatured by incubating it at 75°C and immediately processed.

Two micro-litres of the contents in each well was loaded onto a 12% polyacrylamided gel in order A, C, G and T according to the ddNTP present the respective wells. The gel was run at 60W for approximately 60 minutes, using 0.5 x TBE buffer (pH 8.3 @ 25°C) as conductive fluid, to effectively separate the fragments constructed during the polymerisation reaction. After completion of the run, the gel was carefully submerged in an acetic acid/methanol/water solution (1:1:8 v/v) for 10 minutes. It was then transferred onto 3MM filter paper (Whatman), dried at 80°C for 45 minutes using a gel drier and placed in a development cassette in direct contact with a X-ray film. The film was exposed to the gel for approximately 2 days at room temperature and subsequently developed using Ilford Phenisol developer and fixer at the prescribed concentrations. The nucleotide sequence was read manually from the developed X-ray film and transferred onto computer in order to construct a digital record thereof. The digital version was used computer assisted analysis of the nucleotide sequence.

Alternatively, the ABI PRISM™ BigDye™ Terminator Cycle Sequencing ready reaction kit (Perkin-Elmer Applied Biosystems) was used to determine the nucleotide sequence of the cloned fragment. The kit offers a non-radio active alternative to the method discussed.
above by incorporating florescent labelled synthetic terminators. Sequencing reactions were set up as prescribed by the manufacturer and consisted of 2μl Terminator Ready Reaction Mix, between 250 to 300ng double-stranded DNA template and 20pmols of the appropriate oligonucleotide. The final volume of each reaction was adjusted to 20μl with the appropriate volume of deionised water. Thermocycling was performed on the GeneAmp 2400 PCR System (Perkin-Elmer Applied Biosystems) as specified by the manufacturer (ABI PRISM™ BigDye™ Terminat or Cycle Sequencing ready reaction kit protocol, 1997).

After completion of the thermocycling, 20μl 65% ethanol was added to the reaction mixture before incubation thereof at room temperature for a period of 1 hour. The tube was centrifuge for 30 minutes at 16000xg and the supernatant removed from it using a P20 micropipet. The resulting pellet was washed with 70% ethanol and dried under vacuum before being gently resuspended in 3μl sequencing loading buffer. The sample was submitted for processing to the Department of Genetics at the University of Pretoria. The completed sequence was supplied in both electropherogram format and digital text format. The data were verified manually and the necessary corrections made before being analysed as discussed in Section 2.2.15

2.2.15 Computer assisted analysis of the obtained nucleotide sequence
The nucleotide sequence of the entire P1 polyprotein-coding region of FMDV KNP/10/90/3 was constructed by computer assisted alignment of the partially overlapping DNA sequences generated as described in Section 2.2.14 (DAPSA ver.4.31: Harley, 1998). The reconstructed full-length nucleotide sequence was aligned to the corresponding region of several other FMDV isolates (Table 2.2.1) using the DAPSA program. The alignment was used to determine the phylogenetic relationship of these isolates. All phylogenetic analyses were performed using the GCG program (Wisconsin Package ver.10). More detailed analysis of each of the specific structural proteins coded for within the P1 genomic region was carried out using either the Antheprot (Delaeye, 2000) or DNAMAN programs (Lynnon Biosoft) depending on the task performed.
2.3 Results

2.3.1 Amplification and cloning of the structural protein-coding region

Initial attempts to amplify the P1 region of the FMD virus KNP/10/90/3 yielded five distinct DNA products ranging in size from approximately 620 to 3100bp. Subsequent attempts to optimise the reaction conditions and thereby reduce non-specific amplification were unsuccessful (results not shown). However, the size of the largest fragment (3100bp) corresponded to the size of the product expected to contain the P1 region as predicted by computer assisted analysis of several published FMD virus genomes. In order to verify whether this product indeed contained the P1 coding region, it was purified from the agarose gel, cloned into the pGEM-T-Easy vector system (Promega) by means of T/A cloning and sequenced. Competent *E. coli* JM109 cells were transformed as described in Section 2.2.12. The efficiency of the transformation was calculated to be $4 \times 10^6$ colony forming units per $\mu$g DNA transformed. Since the pGEM-T-Easy vector system contains a selection protocol based on the insertional inactivation of the *LacZ* operon, the absence of the distinctive blue colour generated by the catalytic conversion of XGal, was taken to be indicative of the successful incorporation of the desired fragment. The ratio of white to blue colonies was calculated to be 5:1.

Five white colonies were randomly selected and cultured for the purpose of plasmid extraction. The respective isolated plasmids were independently sequenced inwards from both cloning sites using the NCR1 and WDA primers. Sequences obtained for all five clones using the NCR1 oligonucleotide corresponded to the unpublished leader protease coding sequence of KNP/10/90/3 (van Rensburg, unpublished results). However, only the sequence of one clone generated using the WDA oligonucleotide, corresponded to the published VP1 genomic sequence of KNP/10/90/3 (GenBank accession number: AF0235). Results indicated that the remaining four clones contained a truncated version of the P1 region and lacked approximately 70 nucleotides at the 3' end of the VP1 coding region. The presence of the complete coding regions of both the leader protease and VP1 structural protein suggested that the entire structural-protein-coding region was contained within at least one of the clones. This clone, designate pGEM.SAT3P1, was used in determining the nucleotide sequence of structural-protein-coding region of KNP/10/90/3.
2.3.2 Nucleotide sequence of FMD virus isolate KNPI/10/90/3

The nucleotide sequence of the cloned fragment was determined independently for both the positive and negative strand of the DNA using oligonucleotide primers designed to generate partially overlapping DNA sequences of the entire PI genomic region. The reconstructed sequence translated into a single open reading frame of 2784 base pairs. The derived amino acid sequence contained recognisable sequences of the leader protease, the VP4, VP2, VP3, VP1 structural proteins and the 2A non-structural protein when compared to published sequence data of viruses from the O, A and C serotypes. The nucleotide as well as the deduced amino acid sequences of the structural-proteins-coding regions VP0, VP3 and VP1 is represented in Figures 2.3.1. a to c. The nucleotide sequence of KNP/10/90/3 was submitted to the NCBI nucleotide sequence database and was assigned the accession number AF286347.

Nucleotide frequency were calculated to be 19%, 30%, 26% and 25% for the bases T/U, C, A and G respectively (DNAMAN, Lynnon Biosoft). A striking characteristic of the coding region is the apparent bias in favour of G/C (55%) over A/T (45%). This type of bias has previously been shown to be common for at least four of the seven FMDV serotypes and are not restricted to the structural-protein-coding region but rather persist throughout the genome (Brown et al., 1989). Similar biases are not commonly observed in other members of the picornavirus family (Racaniello and Baltimore, 1981) and seem to be a distinguishing characteristic of FMDV within the taxon. The bias towards G/C is further reflected in the codon usage during translation and is particularly evident in the third position of the codons were a 1.62:1 G/C:A/T ratio is well above the overall ratio of 1.2:1.

2.3.3 Computer assisted analysis of the structural proteins.

Analyses of the nucleotide and amino acid sequence of the PI region were performed on a homologous region starting at the N-terminal amino acid of the VP4 structural protein and ending in the C-terminal amino acid of the VP1 structural protein. Sequence identity across all serotypes analysed are given in Table 2.3.1.

Nucleotide homology over the entire structural-protein-coding region when compared to representative isolates of the SAT 1 and 2 were calculated to be approximately 60% while
an expected higher homology was observed between the SAT 3 isolates (73.6 %). When the nucleotide sequence of the KNP/10/90/3 isolate was compared to the corresponding region of three European serotypes, the homology was found to be markedly lower (= 50%). This is in contrast with a previously study where the P1 region of another SAT 3 isolate, Bec/1/65/3, was compared to the same European isolates which found the sequence identity amongst these isolates to be in the region of 60% (Brown et al., 1989). It is however important to note that both studies found the amino acid homology of the isolates to be approximately 60%. The intratypic and interpypic variation calculated for the individual structural proteins VP4, VP3, VP2 and VP1 are summarised in Table 2.3.1.

Table 2.3.1 Percentage homology between KNP/10/90/3 and 6 other FMDV serotype at nucleotide and amino acid levels.

<table>
<thead>
<tr>
<th></th>
<th>A111</th>
<th>O1 Kaufheuren</th>
<th>C1 Germany</th>
<th>Bot 1/68/1</th>
<th>Zim 7/83/2</th>
<th>Bec/1/65/3</th>
</tr>
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<tbody>
<tr>
<td>VP4</td>
<td>75.2 (90.5)</td>
<td>76.0 (94.1)</td>
<td>74.8 (90.4)</td>
<td>81.0 (95.2)</td>
<td>81.9 (95.2)</td>
<td>81.1 (95.2)</td>
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<tr>
<td>VP2</td>
<td>62.9 (63.1)</td>
<td>62.7 (66.3)</td>
<td>61.7 (62.3)</td>
<td>63.7 (72.3)</td>
<td>69.4 (71.4)</td>
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<tr>
<td>VP3</td>
<td>57.4 (52.2)</td>
<td>48.6 (50.0)</td>
<td>55.5 (49.3)</td>
<td>57.1 (57.5)</td>
<td>68.3 (54.4)</td>
<td>75.6 (70.7)</td>
</tr>
<tr>
<td>VP1</td>
<td>38.4 (44.7)</td>
<td>42.4 (45.4)</td>
<td>47.3 (42.1)</td>
<td>49.1 (48.8)</td>
<td>48.0 (55.9)</td>
<td>75.6 (81.7)</td>
</tr>
<tr>
<td>Total*</td>
<td>52.1 (58.3)</td>
<td>46.4 (60.5)</td>
<td>51.8 (56)</td>
<td>61.3 (67.7)</td>
<td>81.6 (63.7)</td>
<td>75.6 (82.0)</td>
</tr>
</tbody>
</table>

Nucleotide and amino acid (in brackets) homology determined using DAPSA (Harley, 1998).
*Total homology was calculated for the polypeptide region spanning from the N-terminus of VP4 structural protein and C-terminus of VP1.

The intratypic and interpypic variation calculated for the individual structural proteins VP4, VP3, VP2 and VP1, are summarised in Table 2.3.1. The highest level of homology was found within the VP4 protein. In contrast to VP4, the VP1 structural protein showed remarkably low levels of homology.

The cleavage of the P1 precursor at the VP2/VP3, VP3/VP1 and P1/P2 junction sites are carried out by the 3C viral protease in a sequence dependent manner (Baxt et al., 1989; Belsham et al., 1993). Using data generated in this study, comparative investigations of these sites revealed notable differences in at least one of the cleavage sites of the SAT isolates compared to the equivalent site of the European serotypes (Figure 2.3.4). The junction between VP0 and VP3 was marked by the substitution of the glutamate residue (E) by a glutamine residue (Q) at position 303 of the polyprotein.
2.3.4 Phylogenetic relationship of FMDV based on the PI protein

Phylogenetic analyses were carried out for a data set containing 14 orthologous taxonomic units using neighbor-joining and maximum likelihood methods. Two representative sequences of each of the 7 FMDV serotypes were included in the analysis. Similar results were obtained in both cases and only results of the neighbor-joining method are depicted in Figure 2.3.2. Isolates grouped strictly according to serotype. Two distinct lineages were obtained, the first representing the European types (Green) and the second representing the SAT types of FMD viruses (Blue). Variation for the entire PI region of the SAT 3 serotype was calculated to be 16.4% \((N=2)\) and was found to be similar to the intratypic variation present within the same region of the SAT 2 serotype (16%, \(N=3\), van Rensburg et al., 1999). It is however important to note that only two isolates were used to determine variation within the PI region of the SAT 3 serotype and that the degree of intratypic variation for this serotype may change with the addition of more representatives. Results furthermore indicated that the variation for the PI region of the SAT type viruses was higher compared to that of serotypes A (11%), O (7%) and C (9%) (van Rensburg et al., 1999). This corresponded to results of previous studies that showed intratypic variation for the VP1 protein of SAT types to be as much as two to three times higher than variation observed within the corresponding protein of serotypes A, O and C (Bastos et al., 1998).

2.5 Discussion

Nucleotide and amino acid sequence analyses showed the genetic makeup and protein organisation of KNP/10/90/3 to be typical of FMDV. As would be expected the isolate shared a high level of sequence homology with Bec/1/65/3 and was more closely related to the other SAT serotypes than to European FMD viruses.

The VP4 protein-coding region showed the highest level of homology among SAT3 isolates. This protein is concealed within the capsid and is therefore not exposed to antibody surveillance. Furthermore, it exists in close association with the viral RNA. It has been hypothesized that the autocatalytic processing of the VP0 precursor involves a serine residue situated in close proximity of the cleavage site within VP2 (Arnold et al., 1987). However, the lack of an adjacent histidine residue required for serine protease activity suggested that an alternative proton donor is involved. It has been proposed that a base
within the viral RNA might fulfil this role (Rossnam et al., 1985). A high degree of structural conservation is needed to consistently maintain this association. The applicability of the hypothesis towards the proteolytic processing of KNP/10/90/3 is however questionable, since no serine residues within VP2 can be found in close proximity to the VP4/VP2 cleavage site.

In contrast to VP4, the VP1 structural protein showed remarkably high levels of intratypic variation. The VP1 protein contains the major immunodominant region of the virion and is therefore under constant selective pressure. This contributes to the high levels of variation within this protein. As would be expected however, the homology for the VP1 protein within the SAT 3 serotype (75.6%) was noticeably higher than observed across serotypes (~40% for European serotypes and ~48% for SAT1 and 2). The VP2 and VP3 structural proteins have also been shown to contain neutralising epitopes, but to a lesser extent than VP1 and therefore displayed intermediate levels of homology.

In addition to the general composition of the P1 precursor, the biochemical characteristics of several distinct regions within the precursor are important to note. During an early post-translational processing event, a myristic acid is added to the N-terminus of the P1 precursor. This modification has been observed for viruses in all genera of the picornaviridae, as well as several other virus families (Chow et al., 1987). The covalent linkage of the n-tetradecanoic acid is determined by the presence of the consensus moiety Gly-X-X-X-Ser/Thr/Ala (where X represents any other amino acids) at the N terminus of VP4 (Chow et al., 1987). The N-terminal amino acid sequences of the VP4 protein from the KNP/10/90/3 isolate conforms to this consensus and therefore could potentially be myristylated.

Virus entry during FMD infection occurs due to receptor-mediated endocytosis. A crucial part of internalisation of the virion is the non-covalent interaction between the vitronectin host cell surface receptors and the Arg-Gly-Asp (RGD) protein ligand (Fox et al., 1989; Evens & Almond, 1998). The RDG sequence is located between the G and H β strands of VP1 and is conserved across all seven FMD serotypes. As would be expected this moiety was present within VP1 of KNP/10/90/3. In addition to containing the RGD moiety, the G-H loop also contains the major immunodominant epitope. The hypervariable region of
Bec/1/65/3 (amino acid 142 to 165) corresponding to the immunodominant epitope of O1 Kaufbeuren (amino acids 141 to 160) is characterised by the presence of five additional amino acids (Brown et al., 1989). The exact same insertions were found within the VP1 protein of KNP/10/90/3. Similar differences within this region were observed for both serotypes A and C involving 7 and 9 amino acids respectively when compared to KNP/10/90/3. The implications of the high homology of the additional amino acids observed within the G-H loop of both SAT 3 isolates however remain unexplored.

As mentioned earlier in the chapter, efforts are underway to circumvent problems associated with the selection of suitable strains of FMDV for vaccine production by constructing chimeric viruses (refer to paragraph 2.1.1). However, a number of crucial factors that may influence the success of such a strategy in the southern African context should be considered before the implementation of such a strategy. The most significant factor is the role of variation of the cleavage sites within the P1 precursor among different viruses.

Despite the high degree of variation found within the VP1 structural protein of different FMD virus isolates, the VP1/2A cleavage site was the least variable of all the junction sites within the P1 precursor. The two amino acids directly at the cleavage site (positions 739 and 740) were conserved across all isolates included in the analysis. The VP3/VP1 junction site (positions 523 and 524) of all the SAT types corresponded with that of serotypes A and C. In all SAT type viruses included in the study, the junction between VP0 and VP3 was marked by the substitution of the glutamate residue (E) by a glutamine residue (Q) at position 303 of the polyprotein. Structurally and spatially these two amino acids are similar, but differ significantly in the net charge of their respective side chains. Because of the carboxylate present in the side-chain of glutamic acid, this amino acid is negatively charged at neutral pH. However, the side-chain carboxyl group frequently bind NH₂ to form the side-chain amide group, yielding the analogue amino acid, glutamine (Campbell, 1995). In contrast with glutamate, glutamine has a polar side-chain that is electrically neutral at pH 7.

It is important to note that regions flanking the reactive sites could possibly play a role in the recognition of the cleavage sites by the protease and that variation within these regions
could further compromise the effective processing of the P1 precursor. Comparative analyses of the flanking regions indicated that these regions seem to be conserved within serotypes, but show a greater degree of variation across serotypes. The precise effect of the variation within the P1 precursor remain unknown, but in our opinion do not pose a potential problem in the construction of chimeras SAT type viruses using the cDNA backbone of the European isolates.
VP0 Structural protein precursor

Open Reading Frame: 1-909
Total amino acid number: 303, MW=33021Da

Figure 2.3.1.a The nucleotide and deduced amino acid sequence of the VP0 precursor of FMD virus isolate KNP/10/90/3. (U residues present in the viral RNA are shown as T.) The VP4/VP2 cleavage site is indicated in red bold italics. The molecular weight of the precursor was predicted by computer modeling and not determined empirically.
VP3 Structural-protein-coding region

Open Reading Frame: 910-1632
Total amino acid number: 221, \text{MW}= 24422

910  GGCATCGTCCCCGGTCCACGACGCTACGCGGGTTTCCAGAACACA
304  GIVPVVACHDGYYGFQNT
961  GACCCCAAGCACGGGGACCTATCTACGCGGTACGCGGTGCGCTGCCGAGCAGGACT
321  DPKTADPIYGLVSNNPPRTAF
1021 CCCGCCGTTGCAACACCTGTGGGACCTGCGCTGCAACGCTGCGCTGCGCA
341  PGRFTNLGRRCSPVPNFLWT
1081 TCAGCGGAGCGTGTTAACAGAACGACAGAGTGGAGGTCAGACACACACACACACAC
361  STGHRTRLPSTTVAARYSHT
1141 TTGACTTGCGATATTGGGCAAGAAGGTTAATTAGAGCACTTACGCGAGGCTGACG
381  LTWHLGTRSFKNTYVAGLAQ
1201 TAACTCGGGAATGCGACCACTTGAGTCACACATGCAACAGGCGTCCGCGCA
401  YYAQSGLNLMYTMPTQ
1261 TCAAAGCAGGTTCTAAGTGGTGCATCTCAACACGCGTCCGCGCA
421  SKARFMVAYIPPSTDLFRHS
1321 CGAGCGACGAGCACACTGCTCCACTCGGAGTGGGACACGCGACTGAACTCAGCG
441  RGSSTLLHSEWDTGLNSKFT
1381 TCGACGGGCAACCAGATGCGGAGAGCAGCTTGGGTACATATGAGGACGCTGAAA
461  FTGQPTWRADFAYYCDEPE
1441 CAGCCGCTCCAGCAAGAAGTGCTGCAAGATATCAATCAGAACATGAGGCCCTGTAT
481  QASAQGGVTVTVYQITDTHDPD
1501 TCAGCGTGCTTTGCTCGTGTCGCTACGCCGCCTGGGTGCTGCTCCAATCAAC
501  SAVLVSVSAGADLEFRLPIN
1561 CCTGGCGACAA
521  PVAQ

Figure 2.3.1.b The nucleotide and deduced amino acid sequence of the VP3 structural-protein-coding region of FMD virus isolate KNP/10/90/3. (U residues present in the viral RNA are shown as T.) The molecular weight of the precursor was predicted by computer modeling and not determined empirically.
VP1 Structural-protein-coding region

Open Reading Frame: 1572-2217
Total amino acid number: 215, MW=23681

Figure 2.3.1.c The nucleotide and deduced amino acid sequence of the VP1 structural-protein-coding region of FMD virus isolate KNPIIOI9013. (U residues present in the viral RNA are shown as T.) The molecular weight of the precursor was predicted by computer modeling and not determined empirically.
Figure 2.3.2 A Neighbor-joining tree depicting the structural-protein gene relationships between fourteen FMD virus isolates representative of the seven serotypes. A trial number of 1000 was applied. The analysis was performed using the deduced amino acid sequences of the structural-protein-coding region of the genome. Isolates originating from Europe, Asia and South America are indicated in green, while isolates originating from southern Africa are indicated in blue. Equine Rhinitis virus A was used as an outgroup.
<table>
<thead>
<tr>
<th></th>
<th>VP0/VP3 Junction</th>
<th>VP3/VP1 Junction</th>
<th>VP1/2A Junction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A12</td>
<td>YVHVAGELPSK</td>
<td>ELRLPIDSRS</td>
<td>KQKIIAPG</td>
</tr>
<tr>
<td>A32</td>
<td>H.</td>
<td>QT</td>
<td>KQ</td>
</tr>
<tr>
<td>O1 Kaufbeuren</td>
<td>N.</td>
<td>V.A.A.E</td>
<td>V.V.KQ</td>
</tr>
<tr>
<td>O1 Campos</td>
<td>N.</td>
<td>V.A.A.E</td>
<td>V.V.KQ</td>
</tr>
<tr>
<td>C1 Germany</td>
<td>N.Y.</td>
<td>V.A.Q.T</td>
<td>PL.A.KQ</td>
</tr>
<tr>
<td>C3 Resende</td>
<td>N.</td>
<td>V.A.L.T</td>
<td>RL.A.KQ.S</td>
</tr>
<tr>
<td>India/68/73</td>
<td>H.</td>
<td>F.V.A.R.TT</td>
<td>E.E.KQ.V</td>
</tr>
<tr>
<td>Lebanon/83</td>
<td>EG.V.A.</td>
<td>F.V.A.Q.TA</td>
<td>E.E.KQ.V</td>
</tr>
<tr>
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<td>F.M.S.SR.TQ</td>
<td>TTLVKA.KQ.S</td>
</tr>
<tr>
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<td>N.Y.K.A.</td>
<td>F.M.S.SR.TQ</td>
<td>RTA.TK.V.KQ.C</td>
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<tr>
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<td>N.Y.G.</td>
<td>F.F.VR.TQ</td>
<td>FDAP.GVA.KQ</td>
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<tr>
<td>Zim 7/83/2</td>
<td>N.F.K.A.</td>
<td>F.F.V.VR.TQ</td>
<td>FDSP.GVE.KQ.C</td>
</tr>
<tr>
<td>Beo 1/65/3</td>
<td>N.Y.R.</td>
<td>N.AT.TQ</td>
<td>TPLVK.D.KQ.MC</td>
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<td>KNP 10/90/3</td>
<td>N.Y.K.T.</td>
<td>F.N.VA.TQ</td>
<td>T.LV.D.KQ.C</td>
</tr>
</tbody>
</table>

|                |                  |                  |                 |
|                | 294              | 313              | 514             |
|                | 534              | 730              | 752             |
|                | 313              | 514              | 534             |

Figure 2.4.1. A multiple alignment of the deduced amino acid sequence of the proteolytic cleavage sites VP0/VP3, VP3/VP1 and VP1/2A of twelve FMDV isolates representative of the seven FMDV serotypes. Isolates originating from Europe, Asia and South America are indicated in green, while isolates originating from southern Africa are indicated in blue. The precise catalytic sites are indicated in bold. Residues within the cleavage sites represented in red italics indicate amino acid differences compared to the deduced sequence of the A12 isolate.
Chapter 3:
Analysis of the 3A non-structural-protein-coding region of foot-and-mouth disease virus isolates from Africa
3.1 Introduction

The mature 3A protein of FMDV has been shown to be highly conserved among European isolates (Knowles et al., in press). This 153 amino acid protein is known to be involved in viral replication and has been shown to mediate the relocation of the RNA replication machinery to the cellular membrane (Xiang et al., 1998). The 3AB polypeptide, the precursor of the 3A and 3B viral proteins, stimulates the in vitro synthesis of poly(U) RNA, directed by the 3Dpol viral polymerase (Lama et al., 1994). FMDV is unique among the picornaviruses in that it encodes three copies of genome-bound 3B (VPg) protein, which serves as a primer for genome replication (Rueckert, 1996). The association of the 3A protein with the cellular membrane is known to prevent the surface expression and excretion of cellular proteins thereby contributing to the death of infected cells (Doedens et al., 1997). Changes in 3A have been associated with altered host range in the hepatoviruses (Lemon et al., 1991; Morace et al., 1993; Graff et al., 1994), rhinoviruses (Heinz & Vance, 1996), and enteroviruses (Lama et al., 1994).

Historical accounts of FMD outbreaks commonly showed a marked species restriction of FMD virus isolates involved. For example, Brooksby (1950) cited reports of epidemics in Germany during the 1920’s and Britain during the 1930’s in which pigs were predominantly affected. Cattle were almost completely excluded in these outbreaks and it was experimentally shown that the virus isolates were readily transmitted from pig-to-pig, but not from pig-to-cattle. More recently, during 1997, four million pigs were slaughtered in order to contain an outbreak of FMD in Taiwan. The outbreak caused by serotype O viruses (01 Taiwan), demonstrated an extensive porcineophilic phenotype while being completely absent in the bovine population. Results of several in vitro and in vivo studies showed that the viruses isolated during the outbreak were unable to infect bovine thyroid cells or to cause typical disease symptoms following needle infection of cattle (Dunn et al., 1997).

Genetic characterization of the causative isolate revealed distinct differences in the 3A sequence when compared to that of representative European and South American isolates. The most significant of these is a 10 amino acid deletion corresponding to codons 93 through 102 of the European and South American isolates (Beard and Mason, 2000). These
authors were able to demonstrate a direct correlation between the presence of this deletion within the 3A protein and attenuation of the FMDV in cattle. Similar deletions were previously found within the 3A genomic region of two viruses (O1C-O/E and C3R-O/E) attenuated through repeated passages in chicken embryos. These attenuated viruses exhibited similar porcinephilic phenotypes to that observed for the O1 Taiwan isolate (Giraudo et al., 1990). In all three cases the deletion was tolerated without affecting the pathogenicity of the virus in swine, while severely restricting growth in other systems. These results strongly suggest the involvement of the 3A protein in virulence and possibly host range specificity (Beard and Mason, 2000). A subsequent study on FMD viruses from the Southeast Asia region (Knowles et al., 2001) indicated the presence of this deletion in the oldest isolates from the region. In contrast to previous results (Beard and Mason, 2000), these viruses did not display similar attenuation on bovine keratinocytes, suggesting that this deletion (residues 93 –102) in the 3A region alone cannot be attributed to the observed growth restriction (Knowles et al., 2001). Additional mutations in the 3A genome observed for O1 Taiwan might however be responsible for the species specificity.

The occurrence of a vast diversity of wildlife and domestic livestock across the African continent presents researchers with a unique opportunity to study the epidemiology of FMDV. It is generally accepted that African buffalo (Syncerus caffer) occurring across sub-Saharan Africa are persistently infected with FMDV without exhibiting any of the classical symptoms of the disease (Thomson, 1994). However, occasional outbreaks of FMD in impala (Aepycerus melampus), an abundant species of antelope found in southern Africa, are characterised by the development of clinical symptoms ranging in severity. In contrast with the persistent infection of buffalo, there is no clear evidence that the virus is maintained within these antelope through interepidemic periods (Thomson, 1994). It is generally accepted, based on nucleotide sequence data, that buffalo are the source of infection of other susceptible species (Bastos et al., 2000).

Although the 3A coding region of several European isolates have previously been described, the sequence characteristics of the 3A of the SAT type viruses have not been investigated. In light of this, the nucleotide sequences of the 3A non-structural-coding sequence of several African FMD viruses isolated form different host species were
determined and comparatively analysed. We compared this region of the genome among different SAT serotypes and with that of European, South American and Asian isolates.

3.2 Materials and Methods

3.2.1 Origin of the viruses
FMDV isolates representative of all seven serotypes, originating from diverse geographical and host origins were selected for this study. Isolates indicated with asterisks were used to generate additional nucleotide sequence data for this study. Where possible, primary isolations of the viruses were used in direct RNA extraction. In all cases where primary isolations were not available, low passage cell culture samples were used. The complete passage history of each virus is summarised in Table 3.2.1.

3.2.2 RNA extraction from foot-and-mouth disease virus
RNA was extracted from cell culture specimens by a modified guanidium-based nucleic acid extraction method (Boom et al., 1990). A tissue culture sample, previously stored at -70°C, was slowly thawed on ice and 100μl transferred to an RNase-free 1.5ml micro-test tube. A volume of 1ml L6 lysis buffer was added to the sample together with 40μl particulate silica. The sample was briefly vortexed and incubated at room temperature for 5 minutes after which it was thoroughly mixed by inversion and centrifuged at maximum revolutions for 15 seconds using a Hettich bench top centrifuge. The supernatant was discarded and the resulting pellet resuspended in 900μl L2 buffer before again being concentrated by centrifugation. The supernatant was again discarded and the pellet washed by resuspension in 900μl 70% ethanol. The sample was centrifuged at 16000xg, the supernatant discarded and 900μl acetone added to the pellet. The silica was concentrated by centrifugation, the bulk of the supernatant removed using a micropipet and residual acetone evaporated by incubating the tube at 56°C for 20 minutes. The RNA was eluted from the silica by adding 30μl prewarmed (56°C) 1x TE buffer, containing 5U of RNase Inhibitor (Roche Molecular Biochemicals) to the pellet and incubating at 56°C for a further 2 minutes. The aqueous phase containing the RNA was carefully transferred to a clean micro-test tube and stored at -70°C.

* The recipes for the preparation of all buffers and reagents referred to in the texts are summarised in appendix A.4.
Table 3.1. (Continued) List of FMD viruses used in comparative studies of the 3A non-structural-protein-coding region.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Country</th>
<th>Year of Isolation</th>
<th>Species of Origin</th>
<th>Passage History</th>
<th>Reference</th>
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<td>AF335014</td>
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<td></td>
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<td>Girauda et al. 1990</td>
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</table>

* Indicates the viruses of which the 3A region was successfully sequenced.
N/A Not Available
**Table 3.1.1. List of FMD viruses used in comparative studies of the 3A non-structural-protein-coding region.**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Country</th>
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<th>Species of Origin</th>
<th>Passage History</th>
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<td>Argentina</td>
<td>1961</td>
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<td>1932</td>
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<td>KEN/1/91/O*</td>
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* Indicates the viruses of which the 3A region was successfully sequenced.
N/A Not Available
3.2.3 Preparation of cDNA
The oligonucleotide used to initiate the reverse transcription was designed from the partial nucleotide sequence of the SAT 2 isolate, ZIM/7/83 (H. G. van Rensburg, unpublished data). It was designed to anneal to an area approximately 100 nucleotides downstream of the 3A non-structural-protein-coding region (Figure. 3.2.1). The primer was obtained commercially from Gibco BRL and designated P445 (5'-ACCATCTTTTGCAAGTC-3'). The cDNA synthesis reaction was performed as described in Section 2.2.7 with the only alteration being the substitution of the WDA primer with the P445 oligonucleotide. After completion of the reaction, the cDNA was stored at −20°C.

3.2.4 Amplification of the 3A non-structural-protein-coding region
The 3A non-structural-protein-coding region of the FMDV genome was amplified by PCR using cDNA prepared as described above. The first oligonucleotide used during the amplification was designed to anneal to an area within the 2C non-structural-protein-coding region of the genome (Figure. 3.2.1). This primer was obtained commercially from Gibco BRL and designated P444 (5'-GGCCGTTGAAATGAAGAGA-3'). The P445 oligonucleotide was used as the second primer in the amplification.

![Diagram of RNA structure](image)

Figure 3.2.1 A graphical representation of the polymerase chain reaction strategy used to amplify the 3A non-structural-protein-coding region of FMDV. The primers used in the reaction are represented by the red arrows (→).

* The author would like to thank Dr. Peter Mason (PIADC, USA) for kindly supplying the oligonucleotides.
Reaction conditions were optimised using a method described by Cobb et al. (1985). This involved setting up a series of reactions in which the effect of a chosen number of variables on the outcome of the reaction was tested. Three variables, namely deoxyribonucleotide concentration, primer concentration and magnesium chloride concentration were chosen, limiting the number of reactions needed to test all permutations of these variables to seven. A summary of the reactions set up in order to optimise the reaction conditions is presented in Table 3.2.2. The PCR was performed using an OmniGene Hybaid machine. The sample was initially denatured at 94°C for 2 minutes followed by 30 repetitions of a cycle starting off with denaturation at 95°C for 30 seconds followed by a 30 seconds annealing period at 53°C and ending with polymerisation for 90 seconds at 72°C.

After completion of the thermocycling the amplified fragment was separated from the unincorporated primers on a 1.5% agarose gel by standard electrophoreses techniques (Sambrook, 1987). The gel was prepared by dissolving 1.5g of Multi-Purpose Agarose (Roche Molecular Biochemicals) in 100ml 1 x TAE buffer. After allowing sufficient time for the gel-mix to cool to approximately 60°C, 2µl Ethidium bromide (10µg/ml) was added, the gel cast and left to set at room temperature. A 10% volume of DNA loading buffer (40% sucrose solution coloured with bromo-phenol-blue) was added to the PCR reaction mix and 50µl of the mixture loaded onto the gel. A commercial DNA marker (HindIII restricted phage øX174 genomic DNA) was loaded as a reference. The DNA was electrophoresed at 100V for approximately 30 minutes after which the fragment was visualised by exposing the gel to short wavelength ultra-violet light using a bench top transilluminator. Recovery of the amplified fragment (0.8kbp) from the agarose gel was done as previously described in Section 2.2.9. The purified DNA was stored at -20°C until needed.

3.2.5 Sequence determination of the amplified 3A non-structural-protein-coding region
The ABI PRISM™ BigDye™ Terminator Cycle Sequencing ready reaction kit (Perkin-Elmer Applied Biosystems) was used to determine the nucleotide sequence of the amplified fragments. The reaction was performed as described in Section 2.2.14. Sequencing reaction mix was set up as prescribed by the manufacturer and consisted of 2µl Terminator Ready Reaction Mix, between 90 to 150ng double-stranded DNA template and
20pmols of the appropriate oligonucleotide. The final volume of each reaction was adjusted to 20μl with the appropriate volume of deionised water.

To ensure the validity of the data, both the negative and positive sense strands of the amplified fragment were independently sequenced using the P444 and P445 primers respectively. The data obtained was verified by manual inspection of the electropherograms and analysed as discussed in Section 3.2.6.

### Table 3.2.2. Composition of reactions used to optimise the polymerase chain reaction conditions used during the amplification of 3A non-structural-protein-coding region.

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<th>*dNTP’s</th>
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</tr>
</tbody>
</table>

* Indicates variables tested

### 3.2.6 Computer assisted analysis of the nucleotide sequences

In addition to isolates sequenced in this study, the 3A non-structural-protein-coding region sequence of several isolates obtained from GenBank and included in the analysis (National Centre for Biotechnology Information). All nucleotide sequences determined in this study have been submitted to GenBank under the accession numbers indicated in Table 3.2.1.

Nucleotide sequences, as well as predicted amino acid sequences, were aligned using the DAPSA program (Harley, 1998). These multiple sequence alignments were subsequently used to determine the phylogenetic relationships of the isolates. Phylogenetic analyses for the full data set (N=21) were carried out using DNAMAN version 4.13 (Lynnon Biosoft). Bootstrap supported trees were constructed using a random seed generator of 111 and 1000
bootstrap trials. The 3A coding region of equine rhinitis A virus (GenBank Acc. No. X96870) was selected as outgroup. Secondary structural protein predictions were carried out using the Garnier, Gibrat and DPM algorithms contained in Antheprot version 4.9 (Delaeg, 1999), while DNAMAN version 4.13 (Lynnon Biosoft) was used to determine the hydrophobic regions within the predicted proteins.

3.3 Results

3.3.1 Amplification of the 3A non-structural-protein-coding region
Initial attempts to amplify the 3A non-structural-protein-coding region of the A10 FMD virus yielded four distinct DNA products ranging in size from approximately 420 to 860bp (results not shown). The size of the largest fragment (~860bp) corresponded to the size of the product expected to contain the 3A coding region as predicted by computer assisted analysis of the FMDV genome. In order to verify whether this product indeed contained the 3A coding region, it was purified from the agarose gel and sequenced. The results indicated that the fragment did contain the 3A coding region.

After verifying that the 3A non-structural-protein-coding region of the A10 FMDV was being amplified, amplification of the corresponding region of several other isolates was attempted. In addition to the non-specific amplification, the concentrations of the products varied significantly depending on the isolate used. It was therefore necessary to optimise the PCR reaction conditions in order to minimise amplification of non-target regions and to maximise the yield of the specific target region. KNP/10/90/3 was chosen as a representative isolate and subsequently used in all experiments designed to optimise the reaction conditions.

PCR conditions were optimised by employing a strategy described by Cobb et al. (1985). Results of the optimisation experiments indicated the optimal reaction mixture to consist of 5µl 10x Biotools DNA polymerase reaction buffer, 3µl dNTP’s (2.5mM), 3µl of the P445 oligonucleotide (10pmol/µl), 3µl of the P445 oligonucleotide (10pmol/µl), 2µl polymerase enzyme [(1U/µl) Biotools SA] and 30µl dH2O. Amplification of the target region using the optimal conditions yielded a single fragment of approximately 860bp at a concentration 4
times higher than that observed during initial experiments. Similar results were obtained for several other isolates tested under these conditions.

3.3.2 Genetic heterogeneity of the 3A non-structural protein

To determine the genetic heterogeneity of the 3A non-structural-protein-coding region of African isolates of FMDV, representatives of the six serotypes occurring on the continent were selected and compared to isolates originating from Europe, South America and Asia (Table 3.3.1). Comparative analysis of these sequences demonstrated that none of the 11 isolates of which the nucleotide sequences were determined in this study, contained any insertions (Figure 3.3.1). However, a single amino acid deletion was observed in all the SAT type 3A proteins, located 7 amino acids from the carboxyl terminus of the protein when compared to the corresponding region of types A, O, C and Asia-1.

The nucleotide sequence identity calculated for all isolates amounted to 47.4%, while the amino acid identity was calculated as 50.3%. Nucleotide sequence identity within the 3A coding region of the SAT types (group A, Figure 3.3.2) was calculated to be 73.6%, which was markedly higher than the 63% calculated for group B, containing types A, O, C and Asia-1 (Figure 3.3.2). The amino acid sequence was found to be highly conserved within the N-terminus region of the protein.

In Figure 3.3.1, only secondary structural motifs that could be predicted with the most certainty are indicated. It is evident that these motifs were conserved among all the different FMDV serotypes in the N-terminus region of the 3A protein. Limited amino acid substitutions could be found for the SAT types in \( \alpha \)-helix 1 and 2 (Figure 3.3.1a). Secondary structure predictions in the C-terminus region of the protein were more complicated and only three regions could be indicated with confidence (Figure 3.3.1b). It is worthy to note that the presence of \( \alpha \)-helix 6 in the two isolates from Southeast Asia, O1 Taiwan and O1 Chupei, is not well supported (results not shown). The most variable regions of the 3A protein for all the isolates were found to be located between residues 113 and 151 (Figure 3.3.1b).

A distinct and conserved hydrophobic domain is situated between residues 61 and 77 (Figure 3.3.1) of the FMDV 3A protein (Beard & Mason, 2000) and is common to all
picornaviruses (Xiang et al., 1998). The hydrophobic domain was found to be highly conserved in all isolates belonging to types A, O, C and Asia-1 (Figure 3.3.1b). Although the identity of residues 65 to 67 as well as 73, differed for the SAT type viruses, the hydrophobicity of the region remained conserved. These differences involved the change of CLT (residues 65-67) in types A, O, C and Asia-1 to VVV in the SAT types, with the exception of SAR/9/81/1 (VVG) and KEN/3/57/2 (CLA). Residue 73 changed from V found in A, O, C, Asia-1 and KEN/3/57 to I in the SAT type isolates. According to the prediction made using the DNAMAN program, the hydrophobicity of all the 3A proteins remained constant. Therefore, the variation in amino acid sequence should not affect the functionality of the domain.

3.3.3 Phylogenetic relationship of FMDV based on the 3A protein
Genetic relationships of the FMDV viruses were determined by phylogenetic analysis of the 3A gene sequence data. Consistent and comparable results were obtained with the use of parsimony, maximum likelihood and neighbor-joining methods when analysing the nucleotide sequence data (results not shown). A neighbor-joining tree based on the alignment of 3A nucleotide sequence data is shown in Figure 3.3.2.

All SAT type viruses, with the exception of KEN/3/57/2, were found in a single distinct phylogenetic cluster (group A, Figure 3.3.2) separate from the other types and supported by a bootstrap value of 100. In contrast, isolates of serotypes A and O which originated from Africa, as well as the SAT 2 isolate KEN/3/57/2, formed part of a second phylogenetic cluster in which types A, O, C from Europe and South America and Asia-1 were also contained (group B, Figure 3.3.2). This cluster was similarly supported by a significant bootstrap value of 98. However, sub-grouping within these clusters was limited. The only well-supported sub-groupings in cluster B involved the grouping of the Southeast Asian isolates (O1Taiwan and O1Chupei) with a high bootstrap value 100. The other significant groupings were that of O1Campos together with O1C-E/O, the virus that was derived by passaging O1Campos through eggs and O1Kaufbeuren and C3Resende and its egg-derived strain C3R-E/O.

Phylogenetic analysis using the predicted amino acid sequences of the isolates yielded similar results as discussed above (results not shown). Isolates were again found in two major phylogenetic clusters consistent with the pattern generated using the nucleotide
sequence data. Although the phylogenetic arrangement of isolates belonging to subgroup B were somewhat different from that represented in Figure 3.3.2, bootstrap values were generally very low and did not statistically support the pattern generated using the amino acid sequence data.

### 3.4 Discussion

Previous results indicated that the 3A protein might play a role in host range specificity (Beard and Mason, 2000), and in this study, 3A genes of FMDV isolated from different species in Africa were investigated for obvious amino acid differences. The 3A proteins of the SAT type viruses were conserved in length, although a single amino acid deletion corresponding to residue 145 of types A, O, C and Asia-1 was observed for all the SAT type isolates investigated. As this deletion was found in virus isolates obtained from impala, cattle and buffalo, it is not thought to play a role in host range specificity. Knowles et al. (in press) also found that for the East-Asian isolates mutations other than deletions seem to play a role in host range specificity. The possible effect of this deletion on the functional role of the 3A protein was not investigated in this study.

Furthermore, the hydrophobic domain within the FMDV 3A protein (Beard & Mason, 2000), thought to mediate the association of the 3A protein with cellular membranes (Xiang et al., 1998), was found to be conserved in all isolates investigated. Despite the amino acid changes observed in the SAT isolates within the domain when compared to the O, A, C and Asia-1 isolates, the predicted hydrophobicity was consistent. Therefore, the variation in amino acid sequence should not affect the functionality of the domain. The amino acid sequences of the hydrophobic domain of African isolates belonging to type A and O were identical to that of the European isolates.

Phylogenetic analysis based on the nucleotide and amino acid sequence data of the 3A coding region confirmed that the SAT type viruses differ significantly from types A, O, C and Asia-1 isolates and group within a single distinct phylogenetic group. This is in contrast with phylogenetic studies based on the VP1 (containing the major antigenic determinant) coding sequences where viruses group strictly according to serotype, as would be expected (Bastos, 1998). FMDV, being a single-stranded RNA virus has a high
rate of mutation as the virus has no proof-reading ability during replication (Holland et al., 1982). It is generally accepted that more variation can be tolerated in the structural proteins than in the functional, non-structural proteins, although the rate of mutations across the whole genome would be similar. Selection would be on the functional level (Sobrino et al., 1983; McCahon 1986; Sobrino et al., 1986). Therefore, although the VP1 gene sequences vary by up to 34-40% between SAT serotypes (Bastos, 1998), variation of only 26.4% was found between the limited number of 3A gene sequences investigated in this study.

Notably a single SAT isolate, KEN/3/57/2, grouped consistently within the European lineage using both nucleotide and amino acid sequence data of 3A. KEN/3/57/2 groups with the SAT 2 serotype when VP1 gene sequences are compared, but groups with types A, O and C isolates when comparing 3A non-structural-proteins sequence data, suggesting that this isolate may have been the product of a recombination event. This is further supported by the multiple alignment of the amino acid sequence of 3A, which showed that the hydrophobic domain of KEN/3/57/2 more closely resembled that of the European serotypes than other SAT isolates. More isolates from all over Africa need to be investigated to determine whether the 3A genes of SAT isolates from certain regions on the continent are more similar than from other regions.

This study is the first to investigate the genetic characteristics of the 3A non-structural-protein-coding region of SAT type FMD virus isolates. The results of this exploratory study will prove useful in identifying significant variations in the 3A coding region of prevalent isolates in sub-Saharan Africa.
Figure 3.3.1.b Multiple alignment of the predicted amino acid sequence (codons 81-153) of 3A non-structural protein of several FMDV isolates. Secondary structural motifs as predicted with the Garnier, Gibrat and DPM algorithms, are indicated with $\alpha$ and $\beta$. Deletions are shown as $\text{-}$.

Amino acids identity: 100%, $>$75%, $>$50%, $<$50%.
Figure 3.3.1.a. Multiple alignment of the predicted amino acid sequence (codons 1-80) of 3A non-structural protein of several FMDV isolates. Secondary structural motifs as predicted with the Garnier, Gibrat and DPM algorithms, are indicated with $\alpha$ and $\beta$. [The hydrophobic domain characteristic of all picornaviruses is highlighted in yellow. The green shaded boxes within the highlighted area (□) indicate specific points of amino acid substitutions. Amino acids identity: $100\% \geq >75\% >50\% <50\%$.]
Figure 3.3.2. A neighbour-joining tree based on the nucleotide sequence of the 3A non-structural-protein coding region, depicting the relationship of FMDV isolated originating from diverse geographical and host origin. Equine Rhinitis virus A (ERA Y) was selected as appropriate outgroup. A trial number of 1000 was applied. The different FMDV subgroups are indicated by the brackets. Bovine isolates are indicated by a superscripted §, Porcine isolates by a superscripted #, African Buffalo isolates by a superscripted *, and Impala isolates by a superscripted @.
**A.1 Experimental strategies and Oligonucleotide sequences**

**Figure A.1.1.** A graphical representation of the cloning strategy used to construct pGEM.SAT3P1. The amplified fragment (dark blue) was ligated into a linearised pGEM-T-Easy vector by employing a T/A cloning strategy. Insertion of the foreign fragment leads to the inactivation of the Lac Z gene (ampr). This inactivation, in conjunction with resistance to ampicillin conferred by the Amp' gene ( ), is used for the selection of possible recombinant clones.

**Figure A.1.2.** A graphical representation of the relative positions of the oligonucleotides used in the nucleotide sequence determination of the P1 precursor. The structural-protein-coding region is represented in blue. The position and orientation the oligonucleotides are represented by the red arrows.
Chapter 4: General Discussion
4.1 Significance of the sequence variation in the P1 region of FMDV

Due to the economical impact of the disease, FMD is of particular importance to countries dependent on animal production and related agriculture. Trade embargoes are instituted not only to regions where FMD is endemic, but also where outbreaks occur in non-endemic areas. Countries that are free of FMDV maintain strict import restrictions to prevent the introduction of the disease. Continued global surveillance of the disease is therefore crucial.

A case in point is the recent outbreak of an exotic strain of FMD in KwaZulu-Natal, a province of South Africa free of FMDV since 1958. The outbreak occurred after infected swill taken from an Asian ship was fed to pigs at a local farm and lead to the introduction of a new serotype into South Africa. The causative isolate was shown to be a type O virus originating from the Far East. Within hours of the first reported case the surrounding area was quarantined and a general restriction placed on the movement of any cloven hoofed animals to and from the affected area. In order to contain the outbreak all susceptible animals on the affected farm, as well as the surrounding farms, were destroyed and a major vaccination program launched in the region. The international community responded by banning the import of all meat and meat products from the whole KwaZulu-Natal province until the outbreak cleared. Several countries banned imports from the whole of South Africa until several months after the outbreak was brought under control. Farmers in the region suffered significant losses in profits during this period.

The involvement of wildlife in virus maintenance and disease transmission in sub-Saharan Africa makes the eradication of disease in the region highly unlikely. For this reason more emphasis is placed on the control of the disease in Africa, rather than outright eradication. Control of FMD in southern Africa is predominantly achieved through the implementation of vaccination programs in areas where FMDV is endemic (Hunter, 1998). Although vaccination proved to be an effective strategy to control the disease in sub-Saharan Africa, current vaccines frequently fail to adequately address the extent of antigenic variation within the SAT types of FMDV (Domingo et al., 1990). One effective approach to address this problem could be to develop recombinant vaccines. Such a strategy is discussed in detail in Section 2.4, but briefly involves the development of recombinant FMD viruses by
substituting the antigenic determinants of a full-length infectious clone. Under ideal conditions this approach would be modular, allowing the rapid development of effective recombinant vaccines to be used in specific geographical locations. During the course of this study, an investigation into the approach of infectious recombinant virus vaccines was started in a parallel PhD study within the program (H.G. van Rensburg).

The major consideration in developing recombinant viruses is the effect of genetic variation within the genome on the post-translational processing events. Of specific consequence is the ability of the 3C protein to effectively process the cleavage sites within the P1 precursor. We have shown that the amino acids situated at the catalytic centre of two of the three cleavage sites within the P1 precursor, are conserved across six serotypes (With H.G. van Rensburg, PhD thesis; This study provided the SAT 3 sequence data towards the analysis). An amino acid substitution found at the remaining cleavage site in all of the SAT type viruses, was of such a nature as to suggest that it would not affect functionality of the site (Section 2.4). This conclusion is supported by reports showing that the 3C protease is able to cleave the P1 protein of heterologous European serotypes (Clarke & Sanger, 1988) and suggests that the specificity of the viral protease resides in structural features at the cleavage sites rather than the precise identity of the amino acids involved (Brown et al., 1989).

An alternative approach to the substitution of the entire P1 coding region would be to exchange only parts of this region (van Rensburg & Nel, 1999). The VP1 gene contains the major immunodominant sites and has been shown to be antigenic in its purified form (Laporte et al., 1973; Sobrino et al., 1989). The advantage of this alternative approach would be the exclusion of foreign proteolytic sites limiting the effect of genetic incompatibility. However, protection after vaccination with different regions of the capsid protein VP1, produced as recombinant proteins (Giavedoni et al., 1991) or reproduced in synthetic peptides (Bittle et al., 1982), has been lower than that achieved with classical vaccines composed of inactivated virus particles (Corchero & Villaverde, 1996). This is probably due to the improper exposure of the neutralising epitopes (Blanco et al., 2000). The disruption of conformational neutralising epitopes due to partial substitution of the P1 polyprotein may limit the efficiency of such an approach in a similar manner.
4.2 Significance of sequence variation in the 3A non-structural-protein-coding region of FMDV.

Phylogenetic reconstruction based on the nucleotide and amino acid sequence data of the 3A protein showed that the SAT type viruses differ significantly from the European serotypes. This is consistent with results of phylogenetic studies based on the VP1 structural-protein-coding sequences of FMDV (Bastos, 1998). The level of genetic variation within the 3A coding region of this subgroup was found to be markedly lower than the level of variation within the same region of the European serotype. This may be due to the fact that geographical distribution of the SAT types is restricted to sub-Saharan Africa whilst the European isolates are more globally distributed.

Although the nucleotide sequences of the 3A protein of SAT type viruses differ from that of the European serotypes, the proteins appear to be similar in structure. This would be expected, since the 3A protein plays a crucial role during infection and virus replication and is thus under significant pressure to remain functionally conserved (Beard and Mason, 2000). This is especially evident when one compares functional domains within the protein. The hydrophobicity of the domain responsible for mediating the association of the 3A protein with cellular membranes (Xiang et al., 1998), were found to be conserved between the two subgroups despite the fact that the SAT type viruses contained several amino acid substitutions within this domain when compared to that of the European serotypes.

Historically, the VP1 gene has been used to determine the phylogenetic relationship of FMDV virus isolates. Characterisation of naturally occurring variants by phylogenetic analyses of the VP1 gene facilitates the rapid determination of the serotype and geographical origin of the isolates (Bastos et al., 2000). It has further revealed that the SAT type viruses evolve independently in different geographical areas (Vosloo et al., 1995). In contrast to the phylogenetic studies based on the VP1 gene, no distinction could be made between SAT isolates belonging to different serotypes or originating from different geographical localities, based on the 3A sequence data. This can again be contributed to the fact that mutations occur more readily within the structural protein-coding regions of the genome and that the level of genetic changes fixed within the 3A coding region is restricted by the functionality of the resulting protein. The high level of homology between
the 3A proteins of diverse isolates and reduced number of informative sites thus limits the depth of the phylogenetic information attainable from these sequences.

One of the major questions of this study was whether the 3A protein of the SAT types is conserved or variable in length and features. Previous reports have shown that the 3A protein plays a role in determining the host range specificity of FMD virus isolates circulating in the Far East (Beard and Mason, 2000, Núñez et al., 2001), but nothing had been known about the involvement of the 3A protein in determining the host range of the SAT viruses. We have shown that the 3A proteins of the SAT type viruses are conserved in length and structure. We found no evidence to suggest that 3A variation may be implicated in the host range specificity of the SAT type viruses. Although isolates were carefully selected to represent diverse viruses, the limited number of isolates in this study leaves room for more expanded research in this regard.
References


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</tr>
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<tr>
<td>SEQ12</td>
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Table A.1.1 (Continued) Summary of oligonucleotides used in the sequence determination of the P1 fragment of FMDV KNP/10/90/3.

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<td>SAT3LH (20 mer)</td>
<td>5'-CCCTCACCTGCCTGTTTGT-3'</td>
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<td>5'-GTTAAACGACGCGACAGT-3'</td>
<td>Nucleotide sequencing</td>
</tr>
<tr>
<td>REV (17 mer)</td>
<td>5'-GTTTCCAGTCACGAC-3'</td>
<td>Nucleotide sequencing</td>
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A.3 **Abbreviations**

- **µg** Micro-gram
- **µl** Micro-litre
- **32P** Radio-active Phosphorus
- **A** Adenine
- **ATP** Adenosine 5'-triphosphate
- **C** Cytosine
- **Cdna** Complementary Deoxyribonucleic Acid.
- **Cfu** Colony forming units
- **Ci** Curie
- **CPE** Cytopathic effect
- **dATP** Deoxyadenosine triphosphate
- **dCTP** Deoxycytidine triphosphate
- **ddNTP** Dideoxy-ribonucleotide 5'-triphosphate
- **dGTP** Deoxyguanosine triphosphate
- **dH2O** Distilled Water
- **DMSO** Dimethylsulfoxide
- **DTT** Ditioltrytol
- **dTTP** Deoxyribosylthymine triphosphate
- **EDTA** Ethelene diaminetetraacetate
- **FMD** Foot-and-mouth disease.
- **FMDV** Foot-and-mouth disease virus.
- **G** Guanine
- **HCl** Hydrochloric Acid
- **IPTG** Isopropyl-β-D-thiogalactoside
- **Kbp** Kilo base pairs
- **M** Molar
- **mg** Milligrams
- **MgCl2** Magnesium chloride
- **ml** Millilitre.
- **mm** Millimetre
- **mM** Millimolar
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information.</td>
</tr>
<tr>
<td>ng</td>
<td>Nanno-grams</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius.</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density (the applicable wavelength is indicated in subscript)</td>
</tr>
<tr>
<td>P1</td>
<td>First polyprotein precursor coding region of foot-and mouth disease virus consisting of the structural protein coding region and the 2A non-structural-protein-coding region of the genome.</td>
</tr>
<tr>
<td>Par</td>
<td>Section</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pmol</td>
<td>Pico-moles</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid.</td>
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<tr>
<td>RPM</td>
<td>Revolutions per Minute</td>
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<tr>
<td>SAT</td>
<td>Southern African Territories.</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris/Acetic acid/EDTA buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume to volume ratio</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4chloro-3indol-β-galactoside</td>
</tr>
</tbody>
</table>
A.4 Buffers and Reagents

*Versene-Trypsin*
- 2M NaCl
- 0.1M KCl
- 0.1M NaHCO₃
- 0.1M Glucose
- 0.01M EDTA
- 5g/L Trypsin
- 0.035% Neomycin
- 0.015% Penicillin
- 0.032% Streptomycin

*Phosphate Buffer Saline (PBS)*

**Solution A:**
- 0.1M NaCl
- 0.03mM KCl
- 0.1mM Na₂HPO₄·2H₂O
- 0.01MmKH₂PO₄

**Solution B:**
- 0.01mM CaCl₂
- 0.01mM MgCl₂·6H₂O

*Autoclave separately and add 800ml Solution A to 200ml Solution B.*

*1M Tris/HCl Buffer (pH 8.0 @ 25°C)*

- 121.1g Tris
- 800ml dH₂O

*Adjust pH to 8.0 using HCl*

*Adjust final volume to 1L using dH₂O*
0.1M Tris/HCl Buffer (pH 7.6 @ 25°C)
100ml 1M Tris/HCl buffer (pH 8.0 @ 25°C)
800ml DH2O
Adjust pH to 7.6 using HCl

10% Sodium Dodecyl Sulphate solution
100g SDS
900ml dH2O
Adjust pH to 7.2 using HCl

0.1M Tris/HCl saturated Phenol
Melt the crystalline phenol at 68°C. Add 8-hydroxyquinoline to a final concentration of 0.1%. Extract consecutively with 1.0M Tris/HCl (pH 8.0), 0.1M Tris/HCl (pH 8.0) and β-mercaptoethanol until the pH of the aqueous phase is 7.6.

5 x Reverse Transcriptase Buffer
250mM Tris/HCl (pH 8.3 @ 25°C)
250mMKCl
50mM MgCl2
2.5mM Spermidine
50mM DTT

Deoxynucleotide-triphosphate mixture (2.5 mM)
2.5mM ATP
2.5mM TTP
2.5mM GTP
2.5mM CTP

10 x T4 Ligase Buffer
300mM Tris/HCl (pH 7.8 @ 25°C)
100mM MgCl2
100mM DTT
10mM ATP
**10 x Expand™ Long Template PCR Reaction Buffer**

- 20mM Tris/HCl (pH 7.5 @ 25°C)
- 100mM KCl
- 1.0mM DTT
- 0.1mM EDTA
- 0.5% Tween 20 (v/v)
- 0.5% Nonidet P40 (v/v)
- 50% Glycerol (v/v)

**1 x TAE Buffer**

- 242g Tris
- 5.7g Glacial acetic acid
- 100ml 0.5M EDTA (pH 8.0 @ 25°C)
- 900ml dH₂O

_Dilute 1:50 in dH₂O_

**LB Liquid medium**

- 10g Tryptone
- 5g Yeast extract
- 10g NaCl
- 1L dH₂O

**SOC Liquid medium**

- 20g Tryptone
- 5g Yeast extract
- 0.5g NaCl
- 10ml 250mM KCl (pH 7.0 @ 25°C)
- 965ml dH₂O

_Add after sterilisation:

- 20ml 1M Glucose (Filter sterilised)
- 5ml 2M MgCl₂ (Filter sterilised)
**SOB-agar culture plates**

20g  Tryptone  
5g  Yeast extract  
0.5g  NaCl  
10ml  250mM KCl (pH 7.0 @ 25°C)  
15g  Agar  
985ml  dH2O  

*Add after sterilisation:*

5ml  2M MgCl2 (Filter sterilised)

**2% X-Gal solution**

0.1g  X-Gal  
5ml  N,N-dimethylformamide

**100mM IPTG**

0.119g  IPTG  
5ml  sterile dH2O

**1% Sodium Dodecyl Sulphate / 0.1M Sodium Hydroxide Solution**

1ml  10% Sodium Dodecyl Sulphate  
200μl  10M NaOH  
8.8ml  dH2O

**SET Buffer**

50mM  Tris/HCl (pH 8.0 @ 25°C)  
50mM  EDTA  
20%  Sucrose

**Poly-ethylene Glycol / Sodium Chloride Solution**

27g  Poly-ethylene Glycol 6000  
66ml  5M NaCl  
34ml  dH2O
**Formamide dye mix**

- 90% deionised Formamide (v/v)
- 10mM Tris/HCl (pH 7.4)
- 5mM EDTA
- 0.1% Xylene cyanol (w/v)
- 0.1% Bromophenol blue (w/v)

**1 x TBE Buffer**

- 0.1M Tris/HCl (pH 8.0)
- 0.1M Boric acid
- 0.2mM EDTA

_Dilute 1:10 using dH₂O_

**Biotools DNA polymerase reaction buffer.**

- 75mM Tris/HCl (pH 9.0)
- 2mM MgCl₂
- 50mM KCl
- 20mM (NH₄)₂SO₄
List of conferences and symposiums at which the work was presented.

1999  International Union of Microbiological Societies Sydney, Australia

**XIth International congress of Virology**
*Toward the construction of a recombinant vaccine against Foot and Mouth Disease.*

1999  Conference of Workers in Animal Diseases Chicago, USA

**80th Annual meeting of the Conference of workers in animal diseases**
Poster Presentation: H.G. van Rensburg, L.E. Heath, L.H. Nel & P.Mason
*Aspects in the development of recombinant vaccines against FMDV.*

1999  Agricultural Research Council Pretoria, RSA

**Biennial report of the Exotic Disease Division, Onderstepoort Veterinary institute.**
*Development of recombinant vaccines against foot-and-mouth disease.*

2000  South African Society for Microbiology Grahamstown, RSA

**XIth Biennial congress of the Soth African society for microbiology**
*Genetic characterisation of the structural-protein-coding region of several Southern African Type foot-and-mouth disease virus.*

2000  South African Society for Microbiology Grahamstown, RSA

**XIth Biennial congress of the Soth African society for microbiology**
*The Possible role of the foot-and-mouth disease virus 3A non-structural-protein-coding region in determining host range specificity and virulence.*
Publication to be submitted to the Onderstepoort Journal of Veterinary Research
Analyses of the 3A non-structural-protein coding region of foot-and-mouth disease virus isolates from southern and eastern Africa

L.E. HEATH¹, H.G. VAN RENSBURG¹, W. VOSLOO² AND L.H. NEL¹.

ABSTRACT
L.E. HEATH¹, H.G. VAN RENSBURG¹, W. VOSLOO² AND L.H. NEL¹. Analyses of the 3A non-structural-protein coding region of foot-and-mouth disease virus isolates from southern and eastern Africa.¹

The 3A non-structural protein of foot and mouth disease viruses is a highly conserved protein of 153 amino acids. Recently correlation between the presence of a 10 nucleotide deletion as well as other mutations within the 3A non-structural-protein coding region and attenuation of FMDV in cattle was demonstrated (Beard & Mason, 2000). Although the 3A coding region of several type A, O and C isolates have previously been described, nucleotide sequence data of the 3A coding region of the South African Types (SAT) 1, 2 and 3 viruses, is limited. Therefore, the 3A non-structural-coding region of different SAT serotypes were determined, analysed and compared to that of European, South American and Asian isolates. Our results indicated that the 3A region of the SAT isolates investigated differed markedly from that of types A, O, C and Asia-1, but were closely related within the group.

Key Words: Picornavirus, Foot-and-mouth disease virus, 3A non-structural protein, SAT types.

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INTRODUCTION

Foot-and-mouth disease (FMD), a highly contagious viral disease affecting cloven-hoofed animals, is characterised by a predominantly non-lethal infection culminating in temporary oral and pedal vesicles. Infections commonly result in a significant reduction in the production of meat or dairy products. Affected producers suffer substantial losses as a result of severe marketing restrictions and strict quarantine measures set in place to control the disease (Shahan 1962). Foot-and mouth disease virus (FMDV) is a positive sense RNA virus within the *Aphthovirus* genus of the family *Picornaviridae*. There are seven recognised FMDV serotypes viz. A, O, C, Asia-1 and South African Territories (SAT) types 1, 2 and 3. The SAT types are three serotypes unique to sub-Saharan Africa and are predominantly responsible for outbreaks of the disease in the region (Thomson 1994), although incidence of SAT 1 and SAT 2 has been reported in the Middle East. In addition to these, serotypes A, O and C are also occasionally found to be associated with outbreaks in Africa with Asia-1 being the only exotic FMDV type to the continent. Due to the genetic instability of the FMD virus, new viruses with altered antigenic and phenotypic properties frequently emerge (Rueckert 1990) making control of the disease by vaccination complex.

The FMDV genome contains an open reading frame encoding a single polypeptide. A cascade of proteolytic processing events carried out by three viral proteases result in 12 mature gene products. In addition to four structural proteins, the genome encodes several non-structural proteins that are involved in different stages of the viral replication cycle. One of these is the 153 amino acid 3A protein that is known to be involved in viral replication and has been shown to mediate the relocation of the viral RNA replication machinery to the cellular membrane (Xiang, Cuconati, Hope, Kirkegaard & Wimmer 1998). Furthermore, the association of the 3A protein with the cellular membrane is known to prevent the surface expression and excretion of cellular proteins, thereby contributing to the death of infected cells (Doedens, Giddings & Kirkegaard 1997). The 3AB polypeptide, the precursor of the 3A and 3B viral proteins, stimulates the *in vitro* synthesis of poly(U) viral RNA, directed by the RNA-dependant RNA polymerase (Lama, Sanz & Carrasco 1998). Changes in 3A have also been associated with altered host range specificity in
the hepatoviruses (Lemon, Murphy, Shields, Ping, Feinstone, Cromeans & Jansen 1991; Morace, Oisani, Beneduce, Divizia & Pana 1993; Graff, Normann, Feinstone & Flehming 1994), rhinoviruses (Heinz & Vance, 1996) and enteroviruses (Lama et al. 1998).

Historical accounts of FMD outbreaks have indicated that the FMDV isolates involved in some outbreaks demonstrated a marked species restriction. For example, Brooksby (1950) cited reports of epidemics in Germany and Britain during the 1920’s and 1930’s respectively, in which pigs were predominantly affected. Cattle were almost completely unaffected in these outbreaks and it was experimentally shown that the virus isolates were readily transmitted from pig-to-pig, but not from pig-to-cattle. More recently, during 1997, four million pigs were slaughtered in order to contain an outbreak of FMD in Taiwan. The outbreak, caused by serotype O virus (O1 Taiwan), demonstrated an extensive porcinophilic phenotype with no infection observed in the bovine population. Results of several *in vitro* and *in vivo* studies showed that the viruses isolated during the outbreak were unable to infect bovine thyroid cells or to cause typical disease symptoms following needle infection of cattle (Dunn & Donaldson 1997).

Genetic characterisation of the 1997 O1 Taiwan outbreak virus revealed distinct differences in the 3A sequence when compared to that of representative European and South American isolates. The most significant of these was a 10 amino acid deletion corresponding to residues 93 to 102 of the other isolates (Beard & Mason 2000). These authors were able to demonstrate a direct correlation between the presence of this deletion within the 3A protein and attenuation of FMDV in cattle. Similar deletions were previously found within the 3A genomic region of two viruses (O1C-O/E and C3R-O/E) attenuated through repeated passage in chicken embryos (Giraudo, Beck, Strebel, Augè de Mello, La Torre, Scodeller & Bergmann 1990). These attenuated viruses exhibited similar porcinophilic phenotypes to that observed for the O1 Taiwan isolate. A subsequent study on FMD viruses from the Southeast Asia region (Knowles, Davies, Henry, O'Donnel, Pacheco & Mason 2001), found a similar deletion in the earliest isolates from the region. In contrast to previous results (Beard & Mason 2000), these viruses did not display similar attenuation on bovine keratinocytes, suggesting that the deletion (residues 93–102) in
the 3A region alone can not be attributed to the observed growth restriction (Knowles et al. 2001). Additional mutations in the 3A genome observed for O1Taiwan might however be responsible for the species specificity.

The occurrence of a vast diversity of FMDV susceptible wildlife and domestic livestock across the African continent presents researchers with a unique opportunity to study the epidemiology of FMDV. It is generally accepted that most African buffalo (*Syncerus caffer*) across sub-Saharan Africa are persistently infected with FMDV without exhibiting any of the classical symptoms of the disease (Condy, Hedger, Hamblin & Barnett 1985). However, occasional outbreaks of FMD in impala (*Aepycerus melampus*), an abundant species of antelope found in southern Africa, are characterised by the development of clinical symptoms ranging in severity. In contrast with the persistent infection in buffalo, there is no clear evidence that the virus is maintained within these antelope through interepidemic periods (Thomson 1994). It is generally accepted, based on circumstantial evidence and more recently on nucleotide sequence data, that buffalo are the source of infection in impala (Bastos, Boshoff, Keet, Bengis & Thomson 2000).

Although the 3A-coding region of several European and Asian isolates has previously been described, the sequence characteristics of the 3A of the SAT type viruses have not been investigated. Therefore, the nucleotide sequences of the 3A non-structural-protein sequence of several African FMD virus isolates from different species were determined and comparatively analysed. We compared this region of the genome of different SAT serotypes with that of types A, O, C and Asia-1 isolates. Our results indicated that the 3A region of the SAT isolates differed markedly from that of types A, O, C and Asia-1, although closely related within the SAT types.

**METHODS**

**Origin of the viruses**

FMDV isolates, representative of all seven serotypes originating from diverse geographical and host origins, were selected (Table 1). Isolates indicated with asterisks were used to generate additional nucleotide sequence data for this study. Where possible, primary pig kidney cell
isolations of the viruses were used for direct RNA extraction. In all cases where primary isolations were not available, low passage cell culture samples were used. The complete passage history of each virus used is summarised in Table 1.

RNA extraction and cDNA synthesis

RNA was extracted from cell culture specimens by a modified guanidium-based nucleic acid extraction method (Boom, Sol, Salimans, Jansen, Wertheim-van Dillen & van der Noorda 1990). The viral RNA was reverse-transcribed using 10 U of AMV-RT (Promega). In addition to random hexanucleotides, an antisense oligonucleotide (P445 = 5'-ACCATCTTTTGCAAGTC-3') targeting a region within the 3B region was used to initiate cDNA construction. Oligonucleotides used in this study were designed from the partial nucleotide sequence of the SAT 2 isolate, ZIM/7/83 (H.G. van Rensburg, unpublished data).

PCR amplification

Amplification of the FMD viral genome was performed using primers targeting an 860bp region, which includes the entire 3A-coding region. The primers were designed to anneal to conserved areas within the 2C and 3B coding regions of the FMDV genome respectively. The reaction conditions were optimised using a method described by Cobb & Clarkson (1994). Reactions were performed in a 50µl volume in the presence of 0.15mM dNTP’s, 30pmol sense oligonucleotide (P444 = 5'-GGCCGTTGAAATGAAGA-3'), 30pmol antisense oligonucleotide (P445), 1 x Biotools DNA polymerase reaction buffer and 2 U of thermostable DNA polymerase (Biotools). After an initial denaturation step at 94°C for 2 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing 53°C for 30 seconds and extension at 72°C for 90 seconds were performed.

PCR product purification and sequencing
The size of amplified fragments were estimated against a DNA molecular weight marker (HindIII restricted X174 DNA, Promega) on a 1.5% agarose gel. Bands of the expected size (860bp) were excised from the gel and purified by means of the Nucleospin Extract 2 in 1 DNA extraction kit (MacHerey-Nagel). The purified products were sequenced using the ABI PRISM™ BigDye™ Terminator Cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems). To ensure the validity of the data, both the sense and antisense strands of the amplified fragments were independently sequenced using the P444 and P445 oligonucleotides.

Computer-assisted analyses of the nucleotide sequences
In addition to isolates sequenced in this study, the published 3A nucleotide sequences of several isolates were obtained from GenBank and included in the analysis (Table 1). All nucleotide sequences determined in this study have been submitted to GenBank under the accession numbers indicated in Table 1. Nucleotide sequences, as well as predicted amino acid sequences, were aligned using the DAPSA program (Harley 1998). The multiple nucleotide sequence alignments were subsequently used to determine the phylogenetic relationships of the isolates. Phylogenetic analyses for the full data set (N=21) were carried out using DNAMAN version 4.13 (Lynnon Biosoft, Copyright©1994-1999). Bootstrap supported trees were constructed using a random seed generator of 111 and 1000 bootstrap trials. The 3A coding region of equine rhinitis A virus (GenBank Acc. No. X96870) was selected as outgroup. Secondary structural protein predictions were carried out using the Garnier, Gibrat and DPM algorithms contained in Antheprot version 4.9 (Delaege 1999), while DNAMAN version 4.13 was used to determine the hydrophobic regions within the predicted proteins.

RESULTS
Genetic heterogeneity of the 3A non-structural protein
To determine the genetic heterogeneity of the 3A non-structural-protein-coding region of African isolates of FMDV, representatives of the six serotypes occurring on the continent were selected and compared to isolates originating from Europe, South America and Asia (Table 1). Comparative analysis of these sequences demonstrated that none of the 11 isolates of which the
nucleotide sequences were determined in this study, contained any insertions (Fig. 1). However, a single amino acid deletion was observed in all the SAT type 3A proteins, located 7 amino acids from the carboxyl terminus of the protein when compared to the corresponding region of types A, O, C and Asia-1.

The nucleotide sequence identity calculated for all isolates amounted to 47.4%, while the amino acid identity was calculated as 50.3%. Nucleotide sequence identity within the 3A coding region of the SAT types (group A, Fig. 2) was calculated to be 73.6%, which was markedly higher than the 63% calculated for group B, containing types A, O, C and Asia-1 (Fig. 2).

The amino acid sequence was found to be highly conserved within the N-terminus region of the protein. In Fig. 1, only secondary structural motifs that could be predicted with the most certainty are indicated. These motifs were conserved among all the different FMDV serotypes in the N-terminus region of the 3A protein. Limited amino acid substitutions could be found for the SAT types in α-helices 1 and 2 (Fig. 1a). Secondary structure predictions in the C-terminus region of the protein were more complicated and only three regions could be indicated with confidence (Fig. 1b). It is worthy to note that the presence of α-helix 6 in the two isolates from Southeast Asia, O1 Taiwan and O1 Chupei, is not well supported (results not shown). The most variable regions of the 3A protein for all the isolates were found to be located between residues 113 and 151 (Fig. 1b).

A distinct and conserved hydrophobic domain is situated between residues 61 and 77 (Fig. 1b) of the FMDV 3A protein (Beard & Mason 2000) and is common to all picornaviruses (Xiang et al. 1998). The hydrophobic domain was found to be highly conserved in all isolates belonging to types A, O, C and Asia-1 (Fig. 1b). Although the identity of residues 65 to 67 as well as residue 73, differed for the SAT type viruses, the hydrophobicity of the region remained conserved. These differences involved the change of CLT (residues 65-67) in types A, O, C and Asia-1 to VVV in the SAT types, with the exception of SAR/9/81/1 (VVG) and KEN/3/57/2 (CLA). Residue 73 changed from V found in A, O, C, Asia-1 and KEN/3/57 to I in the SAT type isolates.
According to the prediction made using the DNAMAN program, the hydrophobicity of all the 3A proteins remained constant. Therefore, it may be assumed that the variation in amino acid sequence would not necessarily affect the functionality of the domain.

**Phylogenetic relationships of FMDV based on the 3A protein**

Genetic relationships of the FMD viruses were determined by phylogenetic analysis of the 3A gene sequence data. Consistent and comparable results were obtained with the use of parsimony, maximum likelihood and neighbor-joining methods when analysing the nucleotide sequence data (results not shown). A neighbor-joining tree based on the alignment of 3A nucleotide sequence data is shown in Fig. 2.

All SAT type viruses, with the exception of KEN/3/57/2, were found in a single distinct phylogenetic cluster (group A, Fig. 2) separate from the other types and supported by a bootstrap value of 100%. In contrast, isolates of serotypes A and O which originated from Africa, as well as the SAT 2 isolate KEN/3/57/2, formed part of a second phylogenetic cluster in which types A, O, C from Europe and South America and type Asia-1 were contained (group B, Fig. 2). This cluster was similarly supported by a significant bootstrap value of 98%. However, sub-grouping within these clusters was limited. The only well-supported sub-groupings in cluster B involved the grouping of the Southeast Asian isolates (O1Taiwan and O1Chupei) with a high bootstrap value 100%. The other significant groupings were that of O1Campos together with O1C-E/O, the virus that was derived by passaging O1Campos through eggs and O1Kaufbeuren and C3Resende and its egg-derived strain C3R-E/O.
DISCUSSION

The 3A protein of the FMD virus has recently been shown to play an important role in host range specificity and virulence (Beard & Mason 2000; Knowles et al. 2001). Information regarding this protein of the SAT-type viruses is limited and prompted an investigation into the comparative characteristics of the protein among different SAT-type viruses, originating from different host species in southern and eastern Africa. The 3A proteins of the SAT type viruses were found to be conserved in length, although a single amino acid deletion corresponding to residue 145 of types A, O, C and Asia-1 was observed for all the SAT type isolates investigated. As this deletion was found in virus isolates obtained from impala, cattle and buffalo, it is not thought to play a role in host range specificity. Results obtained by Knowles and co-workers (2001) indicated that amino acid mutations surrounding the described deletion in some East Asian isolates might play a role in the observed species restriction. The functional implication of the deletion in the SAT-type 3A protein was however not investigated in this study.

The hydrophobic domain within the FMDV 3A protein (Beard & Mason 2000), thought to mediate the association of the 3A protein with cellular membranes (Xiang et al. 1998), was found to be conserved in all isolates investigated. Despite the amino acid changes observed in the SAT isolates within the domain when compared to the O, A, C and Asia-1 isolates, the predicted hydrophobicity was consistent. Therefore, the variation in amino acid sequence should not affect the functionality of the domain. The amino acid sequences of the hydrophobic domain of African isolates belonging to types A and O were identical to that of the European isolates.

Phylogenetic analysis based on the nucleotide sequence data of the 3A coding region confirmed that the SAT type viruses differ significantly from types A, O, C and Asia-1 isolates and group within a single distinct phylogenetic group. This is in contrast with phylogenetic studies based on the VP1 (containing the major antigenic determinant) coding sequences where viruses group strictly according to serotype, as would be expected (Vosloo, Kirkbride, Bengis, Keet & Thomson 1995; Bastos 1998). FMDV, being a single-stranded RNA virus with no proof-reading ability during replication (Holland, Spindler, Horodyski, Grabau, Nichol & van de Pol 1982) has a high
rate of mutation. It is generally accepted that more variation can be tolerated in the structural proteins than in the functional, non-structural proteins, although the rate of mutations across the whole genome would be similar. Selection would be on the functional level (Sobrino, Davila, Ortín & Domingo 1983; McCahon 1986; Sobrino, Palma, Beck, Davila, De la Torre, Negro, Villaneuva, Ortín & Domingo 1986). Therefore, although the VP1 gene sequences vary by up to 34-40% between SAT serotypes (Bastos 1998), variation of only 26.4% was found between the limited number of 3A gene sequences investigated in this study.

Notably a single SAT isolate KEN/3/57/2, grouped consistently within the European lineage. KEN/3/57/2 groups with the SAT 2 serotype when VP1 gene sequences are compared (Bastos 1998), but groups with types A, O and C isolates when comparing 3A non-structural-proteins sequence data, suggesting that this isolate may have been the product of a recombination event. This supposition is further supported by the multiple alignment of the amino acid sequence of 3A, which showed that the hydrophobic domain of KEN/3/57/2 did not conform to the SAT-specific pattern but rather to that of the European serotypes. However, more isolates from all over Africa need to be investigated to determine whether the 3A genes of SAT isolates from certain regions on the continent are more similar than from other regions. This study is the first to investigate the genetic characteristics of the 3A non-structural-protein-coding region of SAT type FMD virus isolates. No evidence was found to suggest that 3A variation might be implicated in the host range specificity or virulence of the SAT type viruses.

ACKNOWLEDGEMENTS

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REFERENCES


Fig 1.a.

Multiple alignment of the predicted amino acid sequence (residues 1-80) of 3A non-structural protein of several FMDV isolates. Secondary structural motifs as predicted with the Garnier, Gibrat and DPM algorithms are indicated with α and β. The hydrophobic domain characteristic of all picornaviruses is shaded in grey. The bold residues within the highlighted area indicate specific amino acid substitutions.

Fig. 1.b

Multiple alignment of the predicted amino acid sequence (residues 81-153) of 3A non-structural protein of several FMDV isolates. Secondary structural motifs as predicted with the Garnier, Gibrat and DPM algorithms are indicated with α and β. Deletions are highlighted by the shaded boxes.

Fig. 2.

A neighbour-joining tree based on the nucleotide sequence of the 3A non-structural-protein coding region, depicting the relationship of FMDV isolates originating from diverse geographical and host origins. Equine Rhinitis virus A (ERAV) was selected as appropriate outgroup. A bootstrap trial number of 1000 was applied, and significant branches of >75% are indicated. The different FMDV subgroups are indicated in brackets. Bovine isolates are indicated by §, porcine isolates by #, African buffalo isolates * and impala isolates by ©.
Table 1. List of FMD viruses used in comparative studies of the 3A non-structural-protein-coding region.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Country</th>
<th>Year of Isolation</th>
<th>Species</th>
<th>Passage History</th>
<th>Reference</th>
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<td>1932</td>
<td>Bovine</td>
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<td>BTY₁ IB-RS₂</td>
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<td>1976</td>
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* Indicates the viruses of which the nucleotide sequence of the 3A coding region was determined in this study.

N/A Not Available