Persistent infection of African buffalo (Syncerus caffer) with SAT-type foot-and-mouth disease viruses: rate of fixation of mutations, antigenic change and interspecies transmission


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Transmission of a plaque-purified SAT-2 foot-and-mouth disease virus (FMDV) occurred erratically from artificially infected African buffaloes in captivity to susceptible buffaloes and cattle in the same enclosure; in some instances transmission occurred only after contact between persistently infected carriers and susceptible animals lasting a number of months. Because the rate at which FMDV mutations accumulated in persistently infected buffaloes was approximately linear (1.64% nucleotide substitutions per year over the region of the 1D gene sequenced), both buffaloes and cattle that became infected some months after the start of the experiment were infected with viruses that differed from the original clone. The nucleotide differences were reflected in significant antigenic change. A SAT-1 FMDV from a separate experiment inadvertently infected some of the buffalo in the SAT-2 experiment. The SAT-1 FMDV also accumulated mutations at a constant rate in individual buffaloes (1.54% nucleotide changes per year) but the resultant antigenic variation was less than for SAT-2. It is concluded that persistently infected buffaloes in the wild constantly generate variants of SAT-1 and SAT-2 which explains the wide range of genomic and antigenic variants which occur in SAT-1 and SAT-2 viruses in southern Africa.

Introduction

Foot-and-mouth disease (FMD) is an acute systemic disease which causes vesicular lesions in the mouths and on the feet of cloven-hoofed animals. Despite its generally low mortality rate, FMD is one of the most economically important animal diseases because of its depressive effect on the productivity of intensively farmed livestock, the multiplicity of immunological variants of the causative virus and its highly contagious nature. In southern Africa, the epidemiology of the disease appears to be unique because circumstantial evidence indicates that most epizootics of FMD in livestock are derived from free-living African buffalo (Syncerus caffer) (Condy, 1979; Thomson, 1995).

In both cattle and African buffalo which have recovered from the acute phase of the infection, FMD virus (FMDV) is able to persist in the oesophageo-pharyngeal (OP) region for several years (Van Bekkum et al., 1959; Sutmoller & Gaggero, 1965; Burrows, 1966; Sutmoller et al., 1968; Hedger, 1968, 1972, 1976; Anderson et al., 1979) although the replication strategy of persistent FMDV remains to be determined (Salt, 1993). The ability of persistently infected animals, often referred to as carriers, to transmit the infection to susceptible individuals they come in contact with has been a matter of contention for many years but there is growing evidence that although such transmission is inefficient it does occasionally occur (Hedger & Condy, 1985; Gainaru et al., 1986; Salt, 1993; Dawe et al., 1994a, b; Malirat et al., 1994).

Analysis of viruses rescued from cell cultures or animals acutely or persistently infected with plaque-purified FMDV strongly suggests that FMDV variants are generated de novo at high rates; these observations prompted Domingo et al. (1993) to postulate that antigenic variation occurs independently of immune selection. Even following a single replicative cycle in cell culture, significant amino acid variation resulting in altered antigenicity has been reported (Rowlands et al., 1983; Meyer et al., 1994). In persistently-infected cell cultures, there was
progressive modification of the viral genome as well as accompanying phenotypic changes in the virus (De la Torre et al., 1985, 1988); by cell passage 58 the RNA consensus coding for the 1D (VP1) protein differed in approximately 0.3% of residues from the original viral RNA. Nine viral clones from this population sequenced over the 1D region of the RNA showed heterogeneity of $5 \times 10^{-4}$ differences per nucleotide (De la Torre et al., 1985). In viruses recovered over a period of 18 months from cattle infected with clones of FMDV C_g, having either short or long genomic poly(C) tracts, the lengths of the poly(C) tracts of the recovered viruses fluctuated, charge shifts in capsid proteins VP1, VP2 and VP3 were detected and a rate of nucleotide replacement that ranged from $0.9 \times 10^{-8}$ to $7.4 \times 10^{-9}$ substitutions per nucleotide per year was measured (Costa Giomi et al., 1984; Gebauer et al., 1988). Comparable results were obtained more recently with FMDV 0_1 Campos where sequential virus isolates from individual cattle analysed by RNase T_1 two-dimensional mapping and nucleotide sequencing of the 1D-coding region showed irregular increases in the fixation of mutations as the infection progressed with up to 2.9% difference between the RNAs of the original infecting virus and those recovered over a period of approximately 21 months (Malirat et al., 1994). These data were interpreted as indicating the coexistence of heterogeneous populations of virus in individual cattle in which variants evolve independently of each other, any one of which may predominate at a given time. However, the antigenic changes resulting from these genome variations were calculated as being insufficient to circumvent the immune response induced by a potent vaccine incorporating the original virus (Malirat et al., 1994).

The above findings indicate that FMDV produces a variety of phenotypes selected in response to different virus/host relationships via complex and heterogeneous distributions of genomes in accordance with the quasispecies, competitive exclusion and Red Queen's concepts (Domingo et al., 1985; Clarke et al., 1994). In buffalo populations of southern Africa, intratypic nucleotide variation of up to 29% was found in the 3' third of the 1D (VP1) gene of SAT virus types (Vosloo et al., 1995). Even within the Kruger National Park of South Africa, which is a relatively small area in the north-east of the country, intratypic variation of up to 20% was observed in SAT-2 isolates from buffalo. This finding, together with the knowledge that individual buffalo remain persistently infected for periods of at least 5 years (Condy et al., 1985), indicates that buffalo are generators of considerable variation in SAT-type FMDV.

In order to measure the rates of fixation of mutations in viruses from carrier buffalo, young animals were infected with plaque-purified virus and the genetic and antigenic variation in viruses recovered from these animals was tested over a period of 2 years. As a means of comparison, the same parameters were followed for the plaque-purified virus passaged in cell culture. Transmission of the virus between buffalo and from buffalo to cattle was also assessed by keeping infected and susceptible buffalo as well as susceptible cattle in the same pen.

**Methods**

**Animals.** The four buffaloes used in this experiment were obtained from the Kruger National Park where FMD is endemic. They were born and reared in captivity to preclude natural infection with FMDV. The procedures and pens used for the purpose have been described previously (Bengis et al., 1986). At the onset of the experiment the buffalo calves were 15–20 months old at which time maternally derived antibodies to SAT-1, 2 and 3 were shown to have declined below detectable levels.

Buffaloes 1 and 2 (Bufs 1 and 2) were infected with 1 ml of twice plaque-purified SAT-2 virus which had a titre of $10^{8.5}$ TCID$_{50}$ as determined by microplate titration using IB-RS-2 cells. The virus was inoculated in 0.2 ml quantities into five different sites on the dorsal surface of the tongue. Two uninfected (susceptible) young buffaloes (Bufs 3 and 4) were kept in close contact with the infected animals. The buffalo were examined every second day for the first two weeks and thereafter once a month for 15 months. At the time of each examination OP scappings (probang specimens) were collected for virus isolation (Hedger & Stubbins, 1971).

Two domestic cows were introduced into the pen holding the buffalo 39 days after the start of the experiment. They were obtained from a locality free of FMD and were obliged to eat the same food and drink from the same water trough as the buffaloes. The cattle were observed daily to detect obvious infection and were bled monthly for serology.

**Viruses.** A SAT-2 FMDV designated Zim 7/83 Rs_4 and which had been twice plaque-purified on IB-RS-2 cells and thereafter passaged four times on IB-RS-2 cells to obtain a high titre inoculum was used to infect Bufs 1 and 2. This virus was originally isolated from an outbreak of FMD in Zimbabwe and was received as a gift from the Botswana Vaccine Institute.

Viruses were isolated on primary bovine or pig kidney cells and stored in aliquots at $-70^\circ$C (stock virus). Designation of buffalo viruses is indicated by the prefix Buf, followed by the number of the buffalo, the day the isolate was made and the type of virus. Therefore, Buf2/2/2 indicates that this SAT-2 isolate was obtained from Buf 2 two days after the start of the experiment. The isolate Buf2/2/2E was made from epithelium collected from a lesion. The cattle isolates are indicated by the designations C1 and C2.

Zim 7/83 Rs_4 was also passaged 40 times at an m.o.i. of approximately 0.5 in IB-RS-2 cells to determine the rate of change in cell culture without immune pressure.

**RNA extraction from tissue culture.** Tissue culture specimens were placed at 95°C for 2 min and RNA was extracted by a modified version of a method described by Boom et al. (1990).

**Reverse transcriptase (RT) and polymerase chain reaction (PCR) amplification.** The RT reaction was performed on approximately 2 μg of RNA using a primer described by Beck & Strohmaier (1987) (5' CCAGTGGTGACCCGGGAAG). The PCR reaction was performed on 3 μl of the cDNA mix. The primers used were 5' CCACGTATTACCTTTTGTGAC 3' (developed by W. Vosloo, which primes at position 206 of the 1D gene) and the same primer used in the cDNA reaction (Beck & Strohmaier, 1987). After an initial denaturation step at 95°C for 3 min, 30 cycles were performed at 95°C for 45 s and 72°C for 1 min.

The PCR products of approximately 500 bp were identified on a 1.5% agarose gel stained with ethidium bromide. The positive samples were excised from the gel and purified by means of the Cleanmix purification system (Talent).
Sequencing analysis. The PCR-amplified segments were sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical). Purified PCR products were heated to 95 °C for 3 min and snap cooled with liquid nitrogen. All reactions were performed at a final concentration of 10% DMSO to resolve secondary structure. The regression lines to determine the rate of change in the SAT-1 and 2 isolates were calculated using standard statistical methods.

ELISA. Antibody levels in the sera of cattle and buffalo were determined using a liquid-phase blocking ELISA (Hamblin et al., 1986).

Preparation of antisera for antigenic analysis. Two cattle were inoculated with inactivated Zim 7/83 emulsified in Freund's complete adjuvant. The cattle were bled 21 days later (primary serum) and given a booster inoculation with the same virus without adjuvant. Final bleeding took place after a further 14 days (secondary serum). Sera prepared after primary and secondary inoculation were aliquoted and stored at −20 °C.

Antigenic analysis of SAT viruses. Antigenic relationships between viruses were determined in cross neutralization tests performed in microtitre plates on IB-RS-2 cells and were calculated as r-values (Esterhuysen et al., 1988; Thomson, 1994).

Results
Development of FMD lesions and virus transmission
Of the two buffalo into which SAT-2 virus was introduced by needle inoculation (Bufs 1 and 2; Fig. 1), only Buf 2 developed lesions. These consisted of small, non-vesicular, necrotic areas at the sites of inoculation on the dorsum of the tongue and small vesicles on the upper lip and gum on the second day after inoculation. SAT-2 virus was isolated from the epithelium covering the small vesicles which had healed by day 7. Neither of the 2 ‘in-contact’ buffaloes (Bufs 3 and 4) developed any evidence of FMD during the course of the experiment. However, careful examination of the animals was only possible irregularly when they were immobilized for specimen collection (Fig. 1).

Since lesions referable to FMD were only found transiently in Buf 2, OP secretions collected using a probang cup (Hedger & Stubbins, 1971) were inoculated onto cell cultures in order to recover persistent virus. In other instances sero-conversion to SAT-2 was used to determine whether infection had occurred. The isolation of viruses from probang specimens and sero-conversions are summarized in Fig. 1 which shows that SAT-2 transmission to Buf 3 occurred soon after the start of the experiment but in the case of Buf 4 transmission only occurred 7 months later.

The two cows introduced into the pen housing the buffalo 39 days after the start of the experiment remained free of infection for approximately 10 months before FMDV infection occurred in both animals. However, the infection in the cattle was only recognized after the acute phase had passed; it was
Fig. 2 (a). For legend see opposite.
proven by isolation of SAT-2 virus from probang specimens and sero-conversion to SAT-2 in both animals first detected on day 326 (Fig. 1).

A separate experiment using SAT-1 virus to infect impala (Aepyceros melampus) in pens near those containing the buffalo and cattle was initiated 5 months after the start of the SAT-2 investigation. By some unexplained means, SAT-1 from the impala investigation infected all the buffalo; the pattern of SAT-1 isolation from probangs and seroconversion to SAT-1 (Fig. 1) indicated that transmission to the buffalo occurred on at least two separate occasions (days 177–219: Bufs 2, 3 and 4 and day 639 for Buf 1) (Fig. 1). There was equivocal serological evidence that the cattle may also have been infected with SAT-1 virus but this type was never isolated from probangs taken from the cattle (results not shown).

Genomic changes in viruses isolated from animals and cell cultures

The nucleotide and deduced amino acid sequences of the carboxy-terminal part of 1D of SAT-2 isolates are shown in Fig. 2 (a and b). All the SAT-2 viruses isolated from cell cultures or from lesion material or probangs of both buffalo and cattle had nucleotide homologies of > 97% with the plaque-purified

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**Fig. 2.** (a) Nucleotide sequences of all the SAT-2 isolates sequenced in this study. (b) Deduced amino acid sequences of all the SAT-2 isolates included in this study. Dashes indicate sequence same as first isolate, asterisks indicate sequence not determined.
starting virus. Furthermore, in the SAT-2 viruses the rate at which mutations occurred remained constant as shown by the linear regression (Fig. 3). The correlation coefficient \( r \) was 0.88 (\( a = 0.2484 \) and \( b = 0.00441 \)). This also shows that all the SAT-2 isolates were derived from the same virus. From Fig. 3 it was calculated that the rate of change was 1.64% per year in buffalo over the region sequenced.

Ten of the 25 nucleotide changes in the SAT-2 isolates occurred at the third position of the codon and were silent whereas 15 occurred at either the first or second positions and resulted in amino acid changes, except one first codon change which was silent (Fig. 2b). Most of the nucleotide changes were transitions; two transversions occurred at positions 292–294 (ACC to AAC; Thr to Asn) and position 455 (GCA to CTA; Ala to Leu). A few changes seemed to become fixed with time. At position 253–255 GAA changed to AAA in isolates made after day 219. Similarly, at position 478–480 the cell-passaged viruses and the isolates made from Buts 1, 2 and 3, 2–15 days after initial infection had AAA, but all the isolates made after that had GAA leading to substitution of Glu for Lys (Fig. 2a and b). A similar change occurred at 634–636 where the first isolates from Buts 1 and 2 (needle inoculated with the plaque-purified virus) had AAG (Lys) while all the other isolates had GAG (Glu).

The cattle isolates had the same changes as Bu1/326/2 and Bu4/366/2 (Fig. 2a and b). For example at position 331–333 the latter had AAT where all the other isolates had AAC. This was also observed at position 586–591 where GCC TAC were replaced by GGT TAT in the cattle and two buffalo isolates. All these changes were silent. Of the 10 amino acid changes which occurred in all the data available on SAT-2, 9 were non-conservative (Fig. 2b).

The nucleotide and deduced amino acid sequences of the SAT-1 isolates are indicated in Fig. 4(a and b). The percentage changes with the original virus (SAR 9/81) ranged between 0.44% at day 0 to 2.67% at day 545. The rate of fixation of mutations is shown in Fig. 5. In this instance the \( r \)-value was 0.99 (\( a = 0.3983 \) and \( b = 0.0042 \)). Although fewer values were used for the SAT-1 regression (6) than for SAT-2 (16), the correlation was better. From Fig. 5 it was calculated that the rate of change for SAT-1 was 1.54% per year.

Thirteen silent mutations occurred of which one was at the first position of the codon (605), while 4 other changes occurred at the first position of the codon (Fig. 4a and b). Two of the changes were transversions. Some of the changes appeared to become fixed with time viz. at 478–480 ATT became ATC after 420 days in all the isolates and at 508–510 TTC became TTT after 420 days. However, at positions 574–576 CTC became CTT only in Buts 1 and 4 after 420 days but not in Bu2 (Fig. 4a). This also occurred at 595–597 where TTG became CTG only in Buts 1 and 4 after 420 days (Fig. 4a) but did not lead to an amino acid change (Fig. 4b). Only four amino acid changes occurred over the region sequenced and all were non-conservative (Fig. 4b).

Passage of Zim 7/83 RS4 in cell culture resulted in one detectable nucleotide change which occurred at passage 10 (Fig. 2a). At this position (259–261) ZimRS10 had GCG whereas all the other isolates (including the buffalo isolates) had GTG. This was verified by repeating the amplification and sequencing reactions.
Antigenic variation

To determine the extent of antigenic variation during persistent infection in buffalo, the viruses isolated from Buf 1 at various intervals over a period of 366 days were tested in cross neutralization tests using primary and secondary sera from cattle immunized with Zim 7/83. Also included were the viruses isolated from cattle (C1/359/2 and C2/399/2) and the plaque-purified stock of Zim 7/83 passaged 4 and 41 times in IB-RS-2 cells without immune pressure.

The r-values calculated for viruses isolated from the buffalo are shown in Fig. 6(a). A progressive decrease in r-values was observed over a period of 177 days, after which they stabilized at a value of between 0.2 and 0.4. All the isolates examined gave slightly higher r-values with secondary antisera than with primary. Fig. 6(b) shows r-values calculated for the viruses isolated from the cattle in contact with the persistently infected buffalo, as well as those for Zim 7/83 determined after serial passage in IB-RS-2 cells. While the two cattle isolates tested had r-values > 0.4 and < 0.2 respectively, 41 passages of the same virus in IB-RS-2 cells resulted in no appreciable antigenic change (Fig. 6b).

The situation with three SAT-1 isolates from buffalo obtained either 28 or 420 days after the first isolation of SAT-1 virus from Buf 2 (day 219, Fig. 1) was different. All three viruses had r-values ≥ 0.8 (results not shown).

Discussion

In common with the genomes of other RNA viruses, FMDV has a high mutation rate (Holland et al., 1982; McCahon, 1986) which is thought to be constant along the length of the genome (Villaverde et al., 1991). Genetic heterogeneity among field isolates recovered during FMD outbreaks is well established (King et al., 1981) and it has been shown in vitro that even cloned populations of virus consist of a complex distribution of variant genomes (Villaverde et al., 1991). Immune pressure, although it may be involved to some extent, is not a prerequisite for antigenic change which can result from single critical amino acid replacements. Conversely, antigenic
change is not an inevitable consequence of viral persistence (Domingo et al., 1993). The implication is that individual animals have the potential to rapidly generate immunologically altered or tissue-tropic variants. Thus, although antigenic variants recovered from carriers of FMDV have been documented (Burrows, 1966), the converse, viz., antigenic stability of persistent viruses, has also been reported (Hedger, 1970; Malirat et al., 1994).

Examples of both these situations were obtained in this study. Antigenic analysis of 9 SAT-2 isolates made from Buf 1 between days 4 and 366 of the experiment showed that antigenic variation (ie. lowered r-values) occurred rapidly and were measurable within 4 days (Fig. 6a), although no nucleotide changes were detected in that isolate over the region sequenced (Fig. 2a). After 177 days r-values < 0.3 were obtained in Buf 1 and in one of the two cattle isolates tested (Fig. 6b) the r-value was < 0.2 after 399 days. It is possible that a vaccine prepared from the original (Zim7/83 RS4) would not protect against infection with the mutated viruses. Buf1/177/2 had 2 amino acid changes in the 140–160 region as well as 2 changes in the 200–213 region. These amino acid changes in the main antigenic regions could explain the amount of antigenic variation measured. The possibility exists that alterations outside the main antigenic regions can lead to
conformational changes, which can also influence the antigenicity of the isolates. Conversely, the SAT-1 viruses isolated from buffalo differed little antigenically from the parent virus (results not shown). The sequence analysis of these isolates indicated no changes within the main antigenic regions, but a few upstream of the 140–160 region. These changes had very little influence on the antigenicity of the isolates. Therefore, although the overall rate of change measured for SAT-1 and SAT-2 were similar (1.54% and 1.64% respectively), it seems that selection against changes in the main antigenic regions of the SAT-1 isolates occur.

The extent of genetic variation in SAT-2 recovered from individual animals varied from 0–2.2% in Buf 1 over a period of 366 days, to 0.7% in Buf 3 over 8 days. Buf 4 became infected only after 247 days and the isolate made at that time differed by 0.7% from the original, while the last SAT-2 isolate made at day 366 from Buf 4 differed at 2.08% of nucleotides from the original plaque-purified virus. Selection of certain genetic and antigenic subpopulations could have occurred during virus isolation on cell culture (Bolwell et al., 1989; Meyer et al., 1994), but since the first isolate (Buf2/2/2) is 100% homologous over the region sequenced, the fixed changes over time were possibly an indication of selection in buffalo and not cell culture. In cattle persistently infected with FMDV \( \text{VP1 C/58} \) for a period of approximately 630 days, the extent of variation was 0.3–1.4% in the VP1 region (Malirat et al., 1994). Thus the rate at which fixation of mutations occurred in the buffalo with SAT-2 virus was approximately the same as in cattle infected with type O virus. In an experiment similar to that described here (Dawe et al., 1994a), SAT-2 virus which was shown to be transmitted between buffalo and cattle after a contact period of 5 months, 2% nucleotide variation in the 1D region was found between the virus used to infect the buffalo and the virus recovered from the domestic cows. In this study the amount of nucleotide variation in the two cows, C1 and C2, was 2.1% after 326 days and 399 days, respectively (Fig. 2a).

The rate of change in the cell-passaged virus was comparable with that observed in other studies. Only at passage 10 did one change occur, but did not become fixed with further passage (Fig. 2a). Recently, Malirat et al. (1994) reported no genomic changes in FMDV \( \text{VP1 C/58} \) after 100 in vitro passages at high or low multiplicity of infection in the absence of immunological pressure, while Sobrino et al. (1983) reported 14–57 nucleotide differences after 28–30 passages in cell culture.

Circumstantial evidence dictates that buffalo are the usual source of FMDVs that result in outbreaks of the disease in cattle in southern Africa (Condy, 1979; Thomson, 1995), although maintenance of SAT-2 virus within the cattle population of Zimbabwe appears to have occurred between 1983 and 1989 (Vosloo et al., 1992). In particular, the role of carrier buffalo as the initiators of epizootics in cattle has been the subject of controversy and investigation (Thomson et al., 1992). Weight of evidence suggests increasingly that although transmission of FMDV from buffalo to cattle does occur it is a rare event (Thomson, 1995; Dawe et al., 1994a, b). This is reinforced by the findings of this study in that transmission of SAT-2 from the two inoculated buffalo to the two susceptible buffalo in the same pen was erratic; it occurred quickly in one animal but the other required a further 7 months to become infected (Fig. 1). Transmission of SAT-2 virus to the cows in the same pen as the buffalo only occurred about 9.5 months after their introduction to the pen (Fig. 1). It is interesting that both on this occasion as well as that reported by Dawe et al. (1994a), the cattle involved were cows and male buffalo were present in the pens on both occasions. Most previous unsuccessful experiments designed to test transmission between buffalo and cattle have involved steers (Gainaru et al., 1986; G. R. Thomson & R. G. Bengis, unpublished data).

The degree of genetic and antigenic change observed over the period of this experiment indicates that a significant amount of viral diversity can occur in a single animal (Buf 1). It also seems that the rate of change increases upon transmission to other animals as could be seen in both Bufs 3 and 4 after SAT-2 transmission (Fig. 2a). Theoretically, the extent of variation will be limited by phenotypic requirements and therefore the development of variation cannot be expected to increase indefinitely. However, since buffalo are reservoirs for FMDVs and can remain carriers for long periods of time, the rate of change observed in this study may explain the extent of variation observed in general in buffalo in the Kruger National Park (up to 29% differences within serotypes; Vosloo et al., 1995). Recombinational events too may increase genetic variation (McCaugh, 1986). These findings indicate that African buffalo are not only the usual source of SAT-type infections for domestic livestock in southern Africa but also continuously generate antigenic variants, which complicates control of the disease through immunization.

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References


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