Characteristics of Foot-and-Mouth Disease Viral Strains Circulating at the Wildlife/livestock Interface of the Great Limpopo Transfrontier Conservation Area

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Place where the work was carried out: Zimbabwe and South Africa.

Summary

Foot-and-mouth disease (FMD) inflicts severe economic losses within infected countries and is arguably the most important trade-restricting livestock disease in the world. In southern Africa, infected African buffaloes (*Syncerus caffer*) are the major reservoir of the South African Territories (SAT) types of the virus. With the progressive expansion of transfrontier conservation areas (TFCAs), the risk of FMD outbreaks is expected to increase due to a higher probability of buffalo/livestock contacts. To investigate the dynamics of FMD within and around the Great Limpopo TFCA (GLTFCA), 5 herds of buffaloes were sampled in June 2010 to characterize circulating viruses in South Africa and Zimbabwe. Three SAT-2 and three SAT-3 viral strains were isolated in both countries, including one that was genetically linked with a recent SAT-2 outbreak in Mozambique in 2011. In addition, two groups of unvaccinated cattle (n = 192) were serologically monitored for 1 year at the wildlife/livestock interface of Gonarezhou National Park (GNP) in Zimbabwe between April 2009 and January 2010, using the liquid-phase blocking ELISA (LPBE) and a test for antibodies directed against non-structural proteins (NSP). Neither clinical signs nor vaccination of cattle were reported during the study, yet a high proportion of the monitored cattle showed antibody responses against SAT-3 and SAT-1. Antibodies against NSP were also detected in 10% of the monitored cattle. The results of this study suggest that cattle grazing in areas adjacent to the GLTFCA can be infected by buffalo or other infected livestock and that cattle trade movements can act as efficient disseminators of FMD viruses to areas several hundred kilometres from the virus source. Current methods of surveillance of FMD at the GLTFCA interface seem insufficient to control for FMD emergence and dissemination and require urgent reassessment and regional coordination.

Introduction

The Great Limpopo Transfrontier Conservation Area (GLTFCA), covering about 10 million ha, encompasses Gonarezhou National Park (GNP) in the south-east Lowveld (SEL) of Zimbabwe, Kruger National Park (KNP) in South Africa, Limpopo National Park (LNP) in Mozambique and their surrounding communal lands and conservancies. The long-term plans for this vast area are primarily focused on the connection of large territories

devoted to wildlife and the development of wildlife-based tourism, and imply a greater freedom of movement for wildlife and tourists across international boundaries. These developments have the potential to greatly increase national revenues from tourism but also to increase interactions between wildlife, livestock and people over a much larger landscape than has been the case in recent decades. However, they present enormous challenges, related to the expansion of the ranges of wildlife-related pathogens and vectors, and the increase in the extent of the wildlife/livestock interfaces (Bengis, 2005; Siembieda et al., 2011). Indeed, the spread of disease from wildlife to domestic herds could impact on food security in the region and influence trade in livestock and animal products including beef exports. Equally, the spread of disease within the wildlife populations in the GLTFCA (Caron et al., 2013) can have serious ecological consequences among some key species in the region.

Foot-and-mouth disease (FMD) is one of the many contagious transboundary diseases that can spread rapidly within livestock populations and have a negative economic impact in a specific country or region. Effective control and prevention rely largely on the implementation of strategies such as separation of wildlife and livestock, repeated vaccination of cattle herds exposed to wildlife, control of animal movements and careful assessment of the risk of FMD virus (FMDV) introduction into disease-free areas (Jori et al., 2009). Various factors, including illegal transboundary movement of livestock and/or wildlife or contacts between wildlife and unvaccinated or inadequately vaccinated cattle herds, can result in disease outbreaks.

In southern Africa, the incidence of the disease in livestock has increased appreciably over the last decade, exceeding outbreak frequencies prior to the introduction of vaccines in the late 1970s. This re-emergence is evident even in countries that had effectively controlled the disease for many years, such as South Africa and Botswana (Baipoledi et al., 2004; Jori et al., 2009; Dion et al., 2011). Despite having what are arguably the most advanced FMD control strategies in the region, both countries have experienced multiple outbreaks since 2009. Of particular concern is that in 2011 both countries experienced outbreaks within recognized FMD-free areas, resulting in the suspension of beef exports to the European Union and substantial economic losses.

Between 1985 and 2000, Zimbabwe was one of the most successful beef exporters in the region, generating millions of US$ each year (Scoones et al., 2010). However, the socio-economic and political changes experienced since 2000, leading to widespread land reform and resettlements, overwhelmed the veterinary control programmes (Mavedzenge et al., 2008). Clinical FMD outbreaks in cattle became widespread in the country between 2001 and 2003, but declined after 2005, becoming more sporadic and associated with regions devoted to wildlife, especially African buffalo (*Syncerus caffer*) (Zimbabwe National Veterinary Services, unpublished data). Since 2001 to date, however, little information has been available on the circulation of FMD strains.

At a regional level, the reasons for the re-emergence of FMD remain unclear but are likely to be multifactorial. Factors such as poor vaccine efficacy, increasing elephant populations inflicting damage to veterinary cordon fences (Jori et al., 2009, 2011), the expansion of buffalo populations or the uncontrolled movement of infected livestock are likely to play a role in the re-emergence and dissemination of FMD in southern Africa. The establishment of transfrontier conservation areas (TFCAs) renders FMD control in this region more complex and may promote contact between wildlife and livestock.

The goal of this study was to document and characterize the transboundary dissemination of FMDV among buffalo populations from different countries within a TFCA and to monitor the changes in antibody titres of cattle exposed to FMDV-infected buffalo in areas surrounding the Zimbabwean side of the GLTFCA (GNP). We sampled buffalo herds along the Limpopo River (natural barrier between both countries) to isolate FMD viruses, to assess the genetic proximity of FMDV isolated with viruses from outbreaks in the region in recent years and to detect possible emerging variants of FMD viruses. In addition, naïve sentinel cattle in the communal grazing areas adjacent to GNP were serologically monitored in two different areas at 3 and 10 km from the GNP (with high and low risk of contact with wildlife) to identify antibodies against potentially circulating FMD viruses and to determine whether the close proximity of wildlife had any effect on FMDV antibody levels in the cattle population.

Materials and Methods

Study area

The study was conducted in the transboundary area of the GLTFCA, between the northern part of KNP in South Africa (Pafuri) and the southern area of GNP in Zimbabwe. The Limpopo River is the geographical border separating South Africa and Zimbabwe (Fig. 1).

Two different locations were chosen in the SEL according to their proximity to the boundary of GNP and their potential contact with wildlife. The dip tank selected in the high-risk area (HRA) lays at 3 km from the GNP boundary. The low-risk area (LRA) was located more than 10 km from the park's borders, where little or no wildlife occurs (M. de Garine-Wichatitsky, unpublished road-count data 2009–2010). In each of these areas separated by 25 km, one dip tank was chosen to monitor sentinel cattle: Pfumare

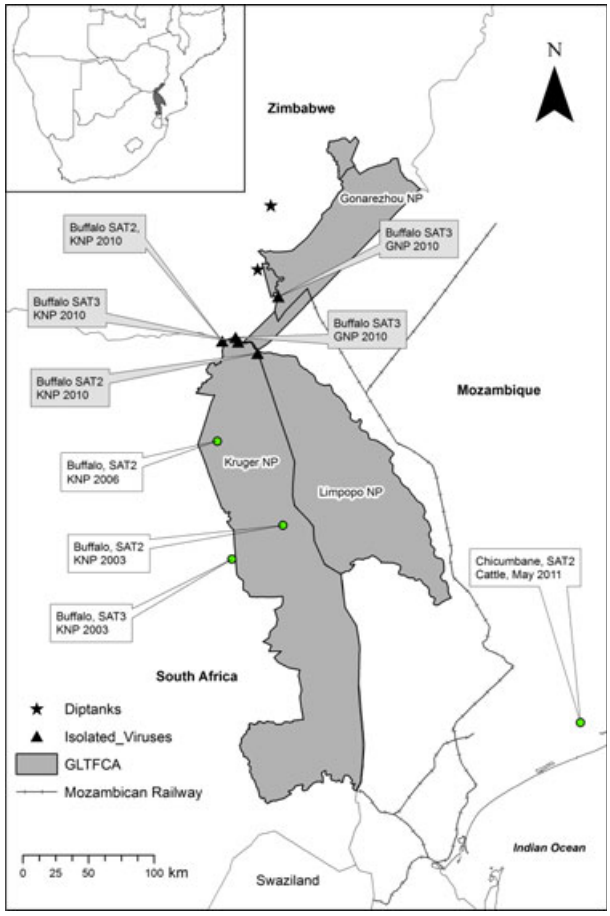

Fig. 1. Map of the study area showing the sampled dip tanks (stars), the areas where the buffalo were sampled (triangles), the FMD viruses isolated in the study (in grey frame) and the genetically linked virus isolated in Mozambique in 2011 and in South Africa in 2003 and 2006 (in white frame).

dip tank (21°25′S; 31°24′E) for the LRA and Pahlela dip tank (21°52′S; 31°19′E) for the HRA (Fig. 1).

Sampled buffalo herds

In early June 2010, a total of 40 buffalo were captured and sampled in the transboundary area of the GLTFCA between South Africa and Zimbabwe. A total of twenty-five individuals were captured in three different locations in the extreme north of the KNP, adjacent to the Limpopo River, and an additional 15 animals were captured in the south of the GNP. In each location, large buffalo herds (close to 100 individuals) were located from a helicopter, separated in smaller groups (5–10 individuals) and then darted in quick succession using a combination of 8 mg etorphine hydrochloride (M99, Novartis, Johannesburg, South Africa), 80 mg azaperone (Stresnil; Janssen Pharmaceutical Ltd., Johannesburg, South Africa) and 1500 IU hyaluronidase (Hyalase; Kyron Laboratories, Johannesburg, South Africa) in adults and 6 mg etorphine hydrochloride and 60 mg azaperone with 1500 IU hyaluronidase in subadults. All animals were positioned in sternal recumbence as soon as the ground teams moved into the capture to collect the samples. Blindfolds were placed on all buffalo, and dart wounds were treated with 200 mg cephalexine (Rilexine 200 LC; Virbac Animal Health, Centurion, South Africa). Once all sampling procedures were completed, the buffalo were injected with a combination of 50 mg naltrexone (Naltrexone; Kyron Laboratories, Johannesburg, South Africa) and 12–16 mg of diprenorphine (M50:50; Novartis, South Africa) depending on the age, to reverse the effect of the immobilizing drug.

Blood samples (30 ml) were taken from the jugular vein. Sera were obtained through centrifugation and stored at 4°C until their arrival at the veterinary laboratory in KNP. Of the 40 buffalo sampled for sera, 48% were younger than 2 years and 52% were adults.

In addition, probangs (Sutmoller and Gaggero, 1965) were inserted into the oesophageo-pharyngeal region of 25 of the 40 immobilized buffalo to collect mucosal tissue and emptied and rinsed into PBS. Only 20% were younger than 2 years, and the rest were adults. These samples were frozen and stored in liquid nitrogen directly after removal from the animals and transported to the FMD Reference Laboratory of the Transboundary Animal Disease Programme (TADP) at the Agricultural Research CouncilOnderstepoort Veterinary Institute (ARC-OVI) in South Africa.

Sampling design for cattle monitoring

Monitoring of antibodies in sentinel cattle around the GNP was undertaken between April 2009 and March 2010. Those animals were not vaccinated during the study and were actually used as the control population for a trivalent purified FMD vaccine efficacy trial undertaken at different dip tanks. At each dip tank, approximately 100 heads of young cattle, of approximately 6 months of age and belonging to several owners, were chosen and individually identified with ear tags. Young animals were specifically chosen to mitigate the risk of residual antibody activity due to previous outbreaks or vaccinations. The longitudinally monitored were bled at T0 and thereafter at 2 weeks, and at 4, 5, 7, 8 and 10 months post-initial sampling. Blood samples were collected in plain tubes, allowed to clot at ambient temperature, stored on ice and then centrifuged in the laboratory to obtain serum.

Laboratory analysis

Sera from cattle and buffalo and probang samples from buffalo were sent to TADP at ARC-OVI to be analysed for

the presence of antibodies and virus, respectively. An initial screening of the sera was performed with a liquid-phase blocking ELISA (LPBE), and titrations were assessed for each of the 3 SAT types, as outlined in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008). The reference viruses used in the LPBE were SAR 9/81/1, Zim 7/83/2 and KNP10/90/3, respectively. These antigens were selected on the basis that they were antigenically representative or related to viruses within the topotype found in both domestic and wildlife species in the areas under investigation. For all assays, including the initial serological screening, titres ≥1.6 were considered positive. To confirm infection, samples were tested for antibodies against non-structural proteins (NSP) with the 3ABC ELISA (PrioCHEK FMDV NS; Prionics AG, Switzerland).

Virus isolation

The FMDV isolates were obtained by preparing 10% (w/v) suspensions of buffalo probang material according to standard operating procedures (SOP FMD 1, TADP). Briefly, confluent monolayer primary pig kidney cell cultures in 10 ml tubes were used to culture the virus. Probang material was ground with sterile sand and PBS, centrifuged and the supernatant used to inoculate the 10 ml tubes. The supernatant was diluted in RPMI medium, and four tubes for each dilution (neat to 10^{-3}) were inoculated. The tubes were incubated at 37°C for 1 h, and medium was discarded and replaced with fresh RPMI medium and incubated at 37°C for 24 h. The tubes were inspected microscopically for cytopathic effect (CPE). If CPE was observed, the virus was harvested by shaking the tube vigorously and centrifuging the harvest. The supernatant was then stored with sterile 2% glycerol at −70°C. If no CPE was observed after 48 h, the tubes were frozen and blind passaged on newly prepared pig kidney cells and CPE observed microscopically after 48 h. In cases where FMD virus did not multiply in primary pig kidney cells, probang material was passaged two or three times on ZZR (caprine tongue) cells (Brehm et al., 2009).

RNA isolation, cDNA synthesis, PCR amplification and sequencing

Viral RNA was extracted from infected cell lysates using the High Pure RNA Isolation Kit (Roche, Manheim, Germany) or QiaAmp RNA extraction kit (Qiagen) according to the manufacturer's instructions and used as template for cDNA synthesis as previously described (Vosloo et al., 2006). The partial 1D gene region (495 nucleotides) was amplified using the WDA (Beck and Strohmaier, 1987) and VP1-AB (Bastos, 1998) primer set. This primer set was selected on the basis of its complementarity to the most conserved areas among different serotypes in the 2A-2B region, 33 nucleotides down-stream of the carboxyterminal of 1D. Standard dye-terminator sequencing was performed using the same primer set with Bigdye version 3.1 (ABI; Life Technologies, Johannesburg, South Africa) on an ABI genetic analyser. Consensus sequences were compiled from the overlapping forward and reverse runs and analysed using BioEdit (Ibis Biosciences, Carlsbad, CA, USA), Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) and MEGA version 3.0 (Biodesign Institute, Tempe, AZ, USA).

The degree of viral sequence diversity of the isolates was assessed as follows: A nucleotide divergence of ≤5% between isolates was considered indicative of a close relationship proper of a genotype, whereas a nucleotide divergence ≤20% between isolates was considered proper of topotypes geographically and epidemiologically linked (Bastos et al., 2001; Knowles and Samuel, 2003; Vosloo et al., 2006). This degree of nucleotide divergence is shown in Table 3.

Statistical analysis

The evolutionary divergence between viral sequences was estimated using the maximum composite likelihood method in MEGA4 (Tamura et al., 2004, 2007). The number of base substitutions per site from analysis between the isolates was calculated based on pairwise analysis. Standard error estimates were obtained by a bootstrap procedure (500 replicates). All positions containing gaps and missing data were eliminated from the data set.

Serological data from cattle at different time points represented by antibody titres at the minimum dilution were entered into an Excel spreadsheet and analysed with Stata 11 (StataCorp, College Station, TX, USA). Seroprevalence for each of the three SAT types was expressed as the proportion of cattle in each group with antibody titre ≥1.6 (positive). Seroprevalence was compared between groups using Fisher's exact test.

Results

Serological and virological status of the buffalo

Thirty of the 40 buffalo sampled (75%) had high levels of antibodies (>2.2) to one or more of the 3 SAT types, the highest percentages being for SAT-2 (81.8%) and SAT-3 (81.4%) (Table 1).

Virus isolation

Six viruses (3 SAT-2 and 3 SAT-3) were isolated from the 25 probangs collected, half of them in animals older than 2 years (Table 2). The SAT-2 viruses were isolated from

Table 1. Proportion of animals with antibody titres against the different SAT types reported in the sera of the buffalo analysed with the LPB ELISA. Titres ≥1.6 are highlighted in light grey. Titres >2 are highlighted in dark grey

Titre	SAT 1	SAT2	SAT3
<1.3	8.7	4.5	4.7
1.4	–	–	2.3
1.5	–	–	2.3
1.6	–	–	2.3
1.7	–	2.3	–
1.8	6.5	2.3	–
1.9	4.3	–	4.7
2	–	–	–
2.1	4.3	9.1	2.3
2.2	2.2	–	–
>2.2	73.9	81.8	81.4
TOTAL	100	100	100

two groups captured at Crook's Corner and Banyini Pan areas in the north of the KNP (Fig. 1). One SAT-3 virus was isolated in the Lalapalm Windmill area of the KNP. Two additional SAT-3 viruses were isolated in each of the western and eastern groups captured in the South of GNP in Zimbabwe (Fig. 1 and Table 2). All the viruses, except KNP/1/10/2, were isolated on ZZR cells.

Phylogenetic analysis of the partial VP1 region of the genome revealed that all of the viruses clustered within SAT-2 and SAT-3 topotypes (Figs 2 and 3, respectively) restricted to the northern parts of South Africa, western Mozambique and southern Zimbabwe (Vosloo et al., 2002). All of the SAT-2 viruses isolated in this study grouped as a single topotype (topotype I), but in two distinct genotypes (Fig. 2). The SAT-2 buffalo isolate from Crook's Corner (KNP1/10) was closely related to the cattle virus 01/MZ/11 isolated in the coastal area of Mozambique during a recent disease outbreak investigation (Fig. 2). The pairwise nucleotide divergence between the individual isolates collected from buffalo is shown in Table 3. The genetic difference between the two isolates was <3%. Both isolates were also related to a virus isolated during buffalo survey in KNP in 2003 (KNP/9/03). The SAT-3 isolates from topotype I grouped into three distinct genotypes. Interestingly, two of those isolates from the same location (Sengwe Corridor) clustered in two different genotypes. (Fig. 3).

Serological status of the sentinel cattle

At the start of the survey (T0), 12.3% (n = 42) of the initial 228 cattle monitored were seropositive to one or more SAT serotypes (LRA, 13.2%; HRA, 11.4%; P = 0.84) (Table 4). In addition, 10.1% of cattle sampled showed antibodies against NSP (LRA, 11.4%; HRA, 8.8%; P = 0.52) (Table 4). Those 42 animals that tested positive for antibodies against the SAT serotypes or NSP at T0 were thus excluded from all subsequent observations and analyses.

As a result, none of the 186 remaining cattle included at T0 had discernible levels of antibodies to any of the SAT types and NSP at the start of the observation period.

The number of cattle sampled at each time point during the study varied between 186 and 83 (Table 5). Significant rises in seroprevalence occurred between 4 and 10 months (corresponding to the period between August and December 2009) for all three serotypes (Fig. 4a), although they were less significant for SAT-2 (SAT-1: $P < 0.001$; SAT-2: $P = 0.01$; SAT-3: $P = 0.009$). Indeed, at 4 months, antibodies to one or more of the 3 SAT types were detected in 28% of the monitored animals (31% in the LRA and 26% in the HRA; $P = 0.57$). As shown in Table 5, the proportion of seropositive animals to any SAT type increased to 73% at 5 months (78% in the LRA and 70% in the HRA; $P = 0.40$) and decreased slightly at 7 and 8 months, but remained consistently high at 10 months (56% in the LRA and 71% in the HRA; $P = 0.18$).

The change in seroprevalence over time for each of the different SAT serotypes in the LRA and HRA is shown in

Table 2. Summary of the genetically linked FMD viruses isolated or mentioned this study from buffalo or cattle in South Africa, Mozambique and Zimbabwe

Species	Sex	Age	Location	Group	Country	Serotype	Isolates	GenBank Accession Number
Cattle	NA	NA	Chokwe	NA	Mozambique	SAT 2	01/MZ/11	JQ950549
Buffalo	Female	2 years	Pafuri	Crook's Corner	South Africa	SAT 2	KNP/1/10/2	JX088744
Buffalo	Female	4 years	Pafuri	Banyini Pan	South Africa	SAT 2	KNP/2/10/2	JQ950550
Buffalo	Female	18 months	Pafuri	Banyini Pan	South Africa	SAT 2	KNP/3/10/2	JQ950548
Buffalo	Male	2 years	Pafuri	Lalapalm Windmill	South Africa	SAT 3	KNP/4/10/2	JQ950551
Buffalo	Female	7 years	Sengwe Corridor	Limpopo River	Zimbabwe	SAT 3	ZIM/2/10/3	JQ950552
Buffalo	Female	3 years	Sengwe Corridor	South GNP	Zimbabwe	SAT 3	ZIM/3/10/03	JX088745

NA, non-applicable.

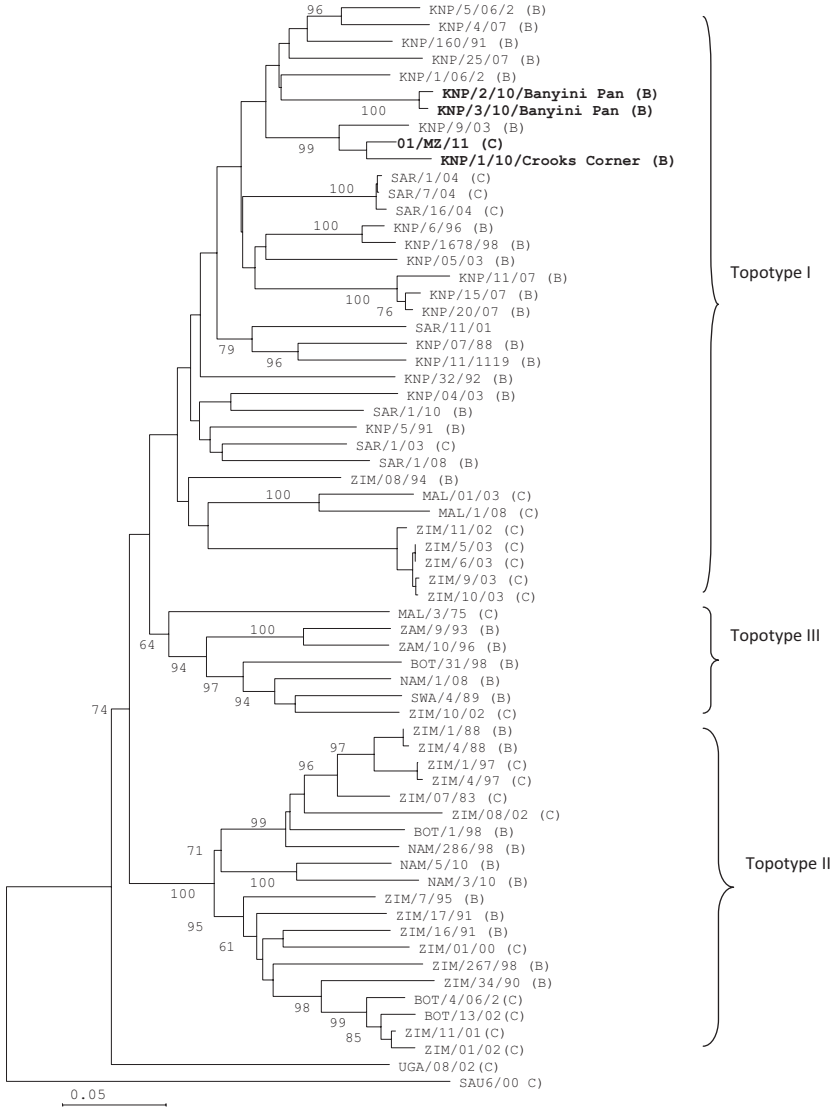

Fig. 2. Neighbour-joining tree depicting **partial VP1-gene (495 nucleotides) relationship of** all SAT 2 foot-and-mouth disease strains characterized from buffalo in the GLTFCA **and the strain isolated in cattle in Mozambique** (indicated in bold) **and other contemporary and reference viruses. A distance of 5% is depicted by the scale.** All strains cluster within a single genotype within the topotype I. B indicates buffalo, and C indicates cattle.

Fig. 4a. Those changes over time followed similar patterns along the study for the three SAT types although at different proportions of seropositivity, indicating possible cross-reactions. The proportion of animals showing antibody titres ≥1.6 against SAT-3 started increasing after 2 weeks, representing 17% of the animals at 4 months and reaching a peak of 68% at 5 months (76% in the LRA and 62% in the HRA; $P = 0.16$). After 7 months, the proportion of animals showing SAT-3 antibody titres decreased in the LRA but remained high in the HRA, apart from an unexpected dip in seroprevalence for all SAT types at 7 months. At 10 months, the overall SAT-3 seroprevalence was 33% (16% in the LRA and 51% in the HRA; $P = 0.001$). There was a large increase in the proportion of animals positive to SAT-1 at 5 months (38% of animals); after decreasing slightly, it increased sharply between 8 and 10 months (45% of animals). The proportion of animals with antibodies against SAT-2 followed a similar pattern, although it was lower than for SAT-1 and SAT-3 types (Fig. 4a).

The proportion of animals with antibodies to NSP represented 4.8%, 7.0% and 16.3% of all cattle at 5, 7 and 10 months, respectively (Fig. 4b). These proportions were similar in the LRA and HRA, except at 10 months, when it

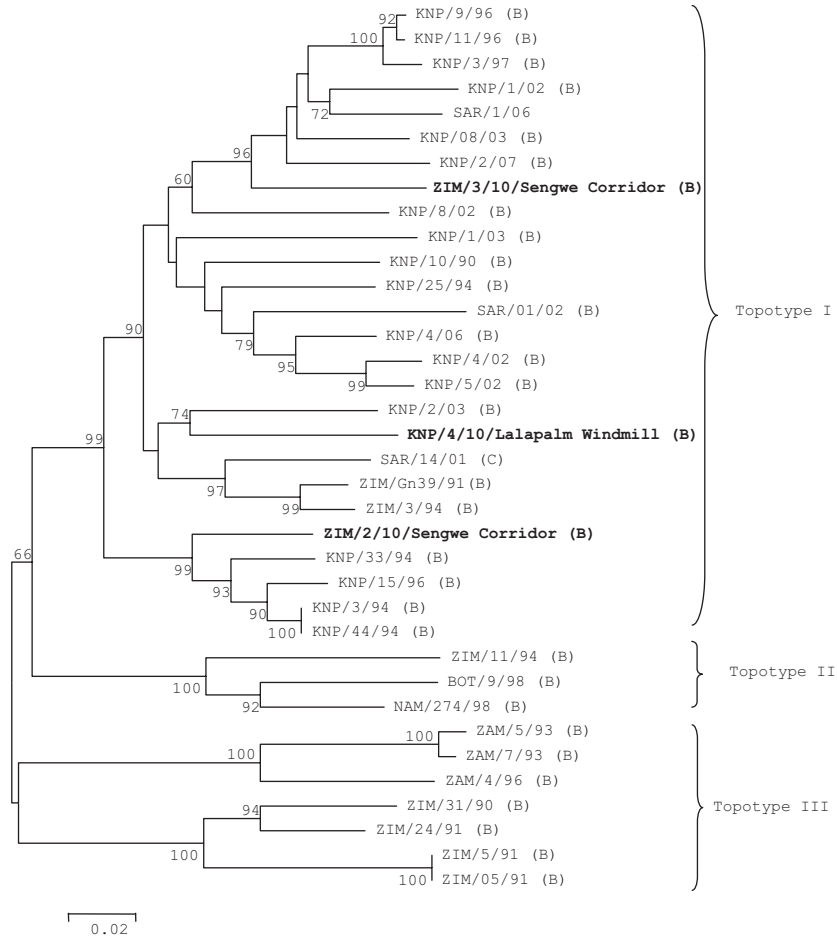

Fig. 3. Neighbour-joining tree depicting **partial VP1-gene (495 nucleotides) relationship of all SAT 3 FMDV isolates characterized from buffalo in the GLTFCA (indicated in bold) and other contemporary and reference viruses. A distance of 2% is depicted by the scale**. Each strain clusters within a separate genotype within the topotype I of SAT 3 viruses. B: buffalo and C: cattle.

Table 3. The pairwise nucleotide divergence between the 6 isolates collected from buffalo on the Zimbabwean and South African sides of the GLTFCA (Standard error estimates are shown in square brackets)

Serotype	SAT 2			
Isolate	KNP /1/10/2	KNP/2/10/2	KNP/3/10/2	01/MZ/11
KNP /1/10/2	0	[0.007]	[0.028]	[0.008]
KNP/2/10/2	0.169	0	[0.031]	[0.033]
KNP/3/10/2	0.153	0.016	0	[0.030]
01/MZ/11	0.027	0.185	0.168	0

Serotype	SAT 3		
Isolate	KNP/4/10/3	ZIM/2/10/3	ZIM/3/10/3
KNP/4/10/3	0	[0.023]	[0.024]
ZIM/2/10/3	0.173	0	[0.025]
ZIM/3/10/3	0.207	0.212	0

Table 4. Seroprevalence of antibodies to SAT 1, 2 and 3 foot-and-mouth disease viruses and non-structural proteins among cattle in high-risk (wildlife contact) and low-risk (no wildlife contact) areas at the beginning of the survey in April 2009 (T0)

Area	Number tested	Seroprevalence (%)				Number seronegative
		SAT-1	SAT-2	SAT-3	NSP	
Low risk	114	12.3	6.1	9.6	11.4	89
High risk	114	10.5	3.5	8.8	8.8	97
Total	228	11.4	4.8	9.2	10.1	186

was somewhat higher in the HRA (24%) than the LRA (9%), although this difference was not statistically significant ($P = 0.08$). No clinical signs were observed or reported despite seven repeated observations of the monitored cattle by animal health technicians during the study. No vaccinations were ever reported during the 12 months

Table 5. Percentage (number tested) of initially seronegative cattle testing seropositive for antibodies to one or more SAT foot-and-mouth disease virus serotypes at different sampling points between April 2009 and March 2010 in high- and low-risk areas

	Time (months)						
Area	0	0.5	4	5	7	8	10
Low risk	0.0% (89)	2.8% (71)	31% (61)	78% (41)	79% (47)	32% (22)	56% (45)
High risk	0.0% (97)	1.1% (95)	26% (80)	70% (84)	12% (81)	54% (61)	71% (41)
Total	0.0% (186)	1.8% (166)	28% (141)	73% (125)	37% (128)	48% (83)	63% (86)

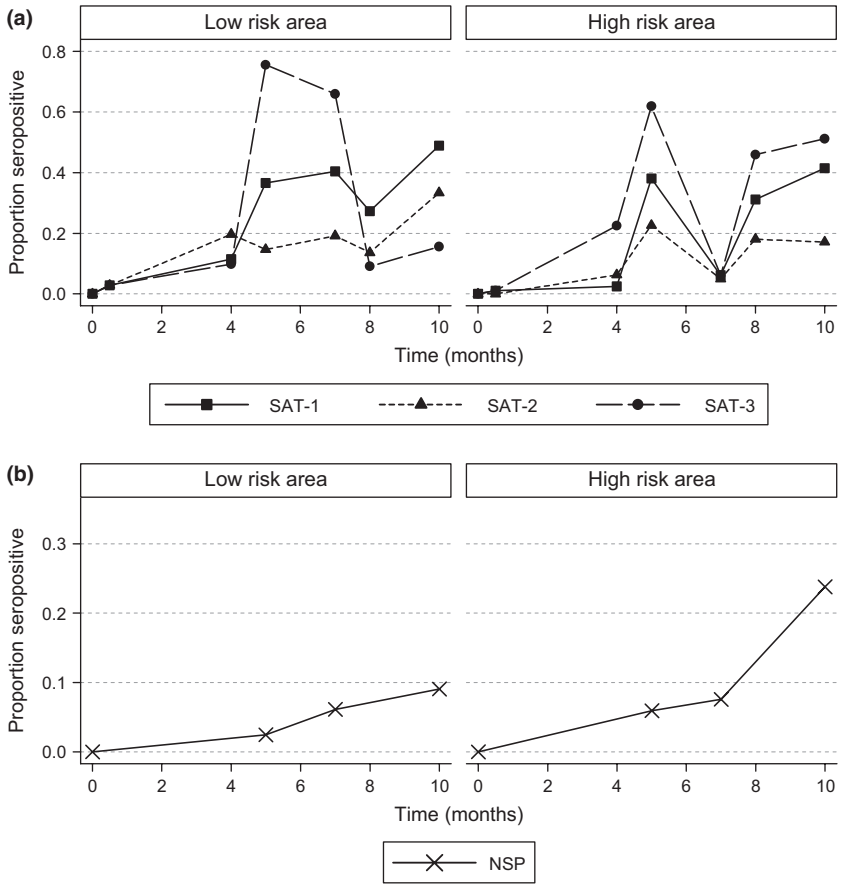

Fig. 4. (a) Proportion of sentinel cattle with antibodies against the three SAT serotypes (titre ≥1.6) and (b) proportion of antibodies against non-structural proteins (NSP) in the low- and high-risk areas between April 2009 and March 2010.

period that cattle were monitored, except for the administration of a trivalent FMD vaccine implemented during the efficacy trial, which took place in animals at different dip tanks from the ones presented here.

Discussion

Despite the expansion of transfrontier conservation areas in southern Africa, the increasing extent of the interface between wildlife and livestock (Bengis, 2005) and the awareness raised by some authors regarding the possible introduction of new virus topotypes through the development of the GLTFCA (Vosloo et al., 2005), studies monitoring the circulation of pathogens across the borders of different countries encompassing the TFCA are limited. This aspect has been partially addressed for the circulation of bovine tuberculosis and other diseases from South Africa to Zimbabwe (de Garine-Wichatitsky et al., 2010). In the case of FMD, serological monitoring of cattle at the interface of protected areas has rarely been undertaken until

recently (Caron et al., 2013; Miguel et al., 2013) despite the importance of FMD as a transboundary infectious disease and despite the fact that protected areas in southern and eastern Africa host large concentrations of buffaloes and represent high-risk areas for the spread of FMD from buffalo to cattle (Jori et al., 2009, 2011; Dion et al., 2011; Brahmbhatt et al., 2012).

The observation of antibodies in a high percentage of the African buffalo sampled in this study (Table 1) was expected and is consistent with previous literature reporting that more than 80% of African buffalo develop antibodies to FMD viruses within 18 months of birth in endemic areas of eastern (Bronsvoort et al., 2008; Ayebazibwe et al., 2010) and southern Africa (Thomson et al., 1992; Vosloo et al., 2002; Vosloo and Thomson, 2004; Miguel et al., 2013).

The similarity between one of the SAT-2 viruses isolated from a buffalo in Crooks Corner area in 2010 (KNP1/10/2) and a SAT-2 isolate recovered from cattle outbreaks in the Gaza Province of Mozambique during May 2011 (01/MZ/11) strongly suggests that the dissemination of genetically related viruses between susceptible species (often, but not exclusively, between buffalo and cattle) occurs within the GLTFCA and associated interfaces (Fig. 2). This SAT-2 outbreak in Mozambique was attributed to the movement of livestock by railway from the GLTFCA border between Zimbabwe and Mozambique into the southern coast of that country (http://promedmail.chip.org/pipermail/promed-eafr/2011-June/000723.html). Both isolates were also genetically related to a virus isolated from buffalo in the KNP (KNP/9/03), during a survey conducted in 2003 (Fig. 2). These observations suggest that FMDV can circulate within the buffalo populations of different countries of the GLTFCA through unrestricted animal movements and that it can travel large distances of several hundred kilometres beyond the GLTFCA boundaries if infected cattle remain undetected. This is more likely to happen if livestock are infected with strains that induce mild clinical signs or if cattle have been insufficiently immunized (Kitching, 2002). Strains inducing mild clinical signs have been previously described in Zimbabwe (Kennedy et al., 1984) and Botswana (Falconer, 1972). Recently, a longitudinal study performed in cattle from three different protected areas of Zimbabwe –including the one in our study – provided detailed evidence of an association between seroconversion to FMD and contacts between cattle and infected buffalo (Miguel et al., 2013).

In the cattle monitored in our study, only young animals of 6 months of age were chosen to exclude the possibility of them having residual antibody titres from previous exposure to virus or vaccination. To confirm absence of residual antibody activity, animals were tested at T0 to exclude potential seropositives (Table 4). Vaccination in the area had not been reported in the preceding years before those calves were chosen; thus, the presence of antibodies against SAT types and the NSP in those calves could be suggestive of a possible recent infection with circulating FMDV among the adult cattle in the current study area and subsequent transmission of antibodies to calves through the colostrum or to the natural infection of some of the calves prior to T0.

The changes in seropositivity to the three different SAT types followed a similar pattern over the time of the monitoring period although at different proportions, suggesting cross-serological reactions for SAT1 and SAT 3 (and to a lesser extent those of SAT2) in the blocking ELISA tests as described by other authors (Bronsvoort et al., 2008; Ayebazibwe et al., 2010). The proportion of cattle with antibodies against SAT-2 reached a maximum of 23% (month 5 in the HRA) for most of the study period; these levels did not suggest viral circulation. However, at month 10, SAT-2 antibodies were found in 33% of the animals in the LRA (Fig. 4a), indicating probable exposure to SAT-2 FMDV in this area towards the end of the monitoring period. In addition, the significant increase in SAT-1 and SAT-3 antibodies (Fig. 4a) suggests two other possible introductions of FMDV in the monitored cattle population: the first one in the period between 4 and 8 months (August–November 2009, during the hot dry season) and the second in the period between 8 and 10 months (November 2009 to January 2010, during the rainy season). In the first sampling points (up to month 4), the proportion of seropositive animals was significantly higher for SAT-3 in the HRA ($P < 0.001$) and slightly higher for SAT-2 in the LRA ($P = 0.20$). However, after month 4, the proportion of animals with antibodies showed a sharp increase for both SAT-1 and SAT-3 serotypes, generally being higher for SAT-3, but becoming higher for SAT-1 in the LRA in months 8 ($P = 0.35$) and 10 ($P = 0.003$). These differences suggest multiple introductions and circulation of different SAT types in the monitored cattle populations at different sampling points, either from wildlife (more likely but not exclusively in the HRA) or from other infected domestic species in both risk areas.

The observed LPBE results contrast with the low proportion of animals with NSP antibodies, which increased to 24% of the monitored population of cattle towards the end of the study (from month 7 to 10) in the HRA (Fig. 4b). This suggests that the risk of viral circulation towards the end of the study was higher in the HRA, but that the monitored cattle populations were repeatedly exposed to circulating FMD viruses in both areas.

Miguel et al. (2013) also observed that groups of monitored cattle located in the HRA of the GNP 1 year after our study and exposed to contacts with buffalo showed seroconversion to SAT types and NSP antibodies despite not

receiving any recent FMD vaccine application. Similar to our study, those NSP seroprevalences were also lower and shorter than expected for naturally infected populations. In fact, seroconversion to NSP and its detection depends on many factors such as virulence of the infective virus, immunocompetence of the animals, contact rates between animals and the type of test used. Therefore, interpretation of NSP results can be controversial (Brocchi et al., 2006; Sammin et al., 2007). The analysis of the serological profiles of the cattle populations monitored in the GNP interface could also suggest the possibility of accidental vaccination. This hypothesis is technically possible as these animals were the control group for a concurrent trivalent FMD vaccination trial. Nevertheless, there are several reasons why accidental vaccination was unlikely to be the cause of our observations. Firstly, the animals vaccinated in the trial were ear tagged and located at different dip tanks at least 10 km away from the ones monitored in our study, which make accidental vaccination of a large proportion of our monitored animals unlikely. Secondly, the only vaccine used in the area was a trivalent vaccine, which in the case of accidental vaccination would have resulted in significantly increased antibody titres against all 3 SAT types, as observed in the groups of animals vaccinated with the trivalent vaccine (see Figure S1). In the control groups, the proportion of animals with antibodies against different serotypes increased at different points in time (between 4 and 8 months and again between 8 and 10 months).

Thirdly, some improperly purified vaccines contain residual levels of NSP (Fukai et al., 2013) that could have been responsible for the levels of NSP and SAT antibodies observed in our monitored animals, should they have been accidentally vaccinated. However, the antibody patterns in our monitored animals do not temporally and spatially match with the time points of vaccine application in the field vaccination trial (Figure S1). Indeed, an accidental vaccination during the field trial could be responsible of a rise of NSP at M2 and M7, but not at M10 as it is shown in Fig. 4.

Finally, recently published information (Miguel et al., 2013) provided good evidence of an association between seroconversion to FMD antibodies in cattle and intensity of contacts between those cattle herds and infected buffalo in the HRA of the GNP in the absence of recent vaccination. Those contacts and associated seroconversions were more frequently observed during the hot dry season (from August to November) and rainy season (from December to March) and coincide exactly with the times where the highest proportion of animals with FMD antibodies were observed in our study. Therefore, these published observations support our findings and the hypothesis that seroconversion in our study was most likely caused by natural FMD infections, although the influence of a certain level of accidental vaccination cannot be completely ruled out.

No clinical signs were reported in the study area or in any of the neighbouring countries during the trial period, suggesting that the clinical expression of those viruses circulating in cattle was mild and the disease remained undetected. Clinical observations were not necessarily systematic, and we are not certain that all the animals were examined at all time points. However, the monitored herds were repeatedly exposed to different observers (cattle owners, technician and researchers) on at least seven different occasions during the 12 months of the study, and we assume that a clinical FMD outbreak would have been detected if FMD symptoms had been apparent. The possibility of circulation of FMDV strains characterized by mild or subclinical infection is likely to be more common than previously suspected in Zimbabwe (Miguel et al., 2013) and requires further investigation in Southern Africa. Intensive FMD surveillance assisting in the control of the disease in many areas of southern Africa is based on physical inspection at dip tanks by lay persons, technically trained personnel and/or veterinarians. Under these circumstances, the circulation of such strains could result in the undetected spread of FMD viruses out of infected zones or buffer zones, with serious implications for the control and dissemination of the disease (Jori et al., 2009).

Among all SAT types, SAT-3 viruses are reported to have had the lowest incidence of outbreaks in livestock and are also the least frequently recovered from African buffalo in South Africa (Bastos et al., 2001, 2003a,b). The recent isolation of SAT-3 viruses from buffalo in the north of KNP and the south of GNP, together with the observation of SAT-3 antibodies in the sentinel cattle at the wildlife/livestock interface in Zimbabwe and the high proportion of antibodies against SAT-3 in our sampled population of buffalo, indicates an unusual frequency of reported observations of SAT-3-related events in our study area. This observation is also supported by the fact that among the 25 clinical FMD outbreaks recorded in impala in the KNP between 1967 and 2008, the only one caused by a SAT-3 virus was isolated in 2008 at Pafuri in the extreme northern part of KNP, close to our sampling site (Vosloo et al., 2009).

Transboundary spread of FMD in southern Africa is clearly evident in the light of the events and findings described in this study although the method of spread may vary depending on the epidemiological context, such as for instance the immunological status of the susceptible animals. Observations of groups of buffalo repeatedly crossing the Limpopo River, the only physical barrier between Zimbabwe and South Africa in our study area, suggest that buffalo herd home ranges overlap at the international border, facilitating the transboundary dissemination of individuals and their pathogens. Particularly during the dry season,

when water levels in the Limpopo River fall considerably (Miguel et al., 2013), animals can easily move across the borders between South Africa, Mozambique and Zimbabwe. The viral isolates that have been recovered in the border areas of the GLTFCA countries, coupled with the serological profiles of the monitored cattle, indicate several circulating viruses, involving multiple serotypes. It should, however, be noted that the cross-border movement of cattle and small ruminants in areas adjacent to the GLTFCA, and the occurrence of strains with mild clinical expression, poses a serious additional threat of the dissemination of viruses beyond borders of the TFCAs and between different countries.

Conclusions

Our study provides evidence of the circulation of different SAT-type FMD viruses among cattle and buffalo populations living close to the boundaries of two countries encompassing the GLTFCA, and of the ability of FMDV transmitted from buffalo to cattle to travel long distances and initiate outbreaks in areas that are several hundred kilometres beyond the GLTFCA interface. It confirms that the transboundary circulation of such viruses across several countries of the GLTFCA and their spillover to cattle can seriously complicate the control of FMD outside infected zones at national and international levels (Bengis, 2005; Vosloo et al., 2005). In addition, it highlights the importance of infected livestock at the wildlife/livestock interface as potential disseminators of FMDV from TFCAs to other distant areas, which deserves further investigation. The control of FMD from a regional perspective requires international cooperation to combine efforts and create synergies between neighbouring countries facing similar challenges. In that sense, the development of TFCAs should also provide common cooperation platforms to control and monitor FMD dynamics in those high-risk areas to improve a coordinated regional control of the disease.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Proportion of antibody titers against the different SAT strains and NSP (only tested at T3) observed in the study.