Differential persistence of foot-and-mouth disease virus in African buffalo is related to virus virulence

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Abstract

Foot-and-mouth disease virus (FMDV) circulates as multiple serotypes and strains in many endemic regions. In particular the three Southern African Territories (SAT) serotypes are maintained effectively in their wildlife reservoir, the African buffalo, and individuals may harbour multiple SAT-serotypes for extended periods in the pharyngeal region. However the exact site and mechanism for persistence remain unclear. FMD in buffaloes offers a unique opportunity to study FMDV-persistence, as transmission from carrier ruminants has only convincingly been demonstrated for this species. Following co-infection of naïve African buffaloes with three SAT-serotypes isolated from field buffaloes; palatine tonsil swabs were the sample of choice for recovering infectious FMDV up to 400 days post infection (dpi). Post-mortem examination identified infectious virus for up to 185 dpi and viral genome up to 400 dpi in lymphoid tissue of the head and neck, mainly focussed in germinal centres. Interestingly viral persistence *in vivo* was not homogenous and the SAT-1 isolate persisted for longer than SAT-2 and SAT-3. Co-infection and passage of these SAT isolates in goat and buffalo cell lines demonstrated a direct correlation between persistence and cell killing capacity. These data suggest FMDV persistence occurs in the germinal centres of lymphoid tissue but the duration of persistence is related to virus replication and cell killing capacity.

Importance

Foot-and-mouth disease virus (FMDV) causes a highly contagious acute vesicular disease in domestic livestock and wildlife species. African buffalo (*Syncerus caffer*) are the primary carrier host of FMDV in African savannah ecosystems, where the disease is endemic. We have shown the virus persists for up to 400 days in buffaloes and there is competition between viruses during mixed infections. There was similar competition in cell culture; virus that killed quickly persisted more efficiently in passaged cell cultures. These results may provide a mechanism for the dominance of particular viruses in an ecosystem.

Introduction

The picornavirus, foot-and-mouth disease (FMD) virus (FMDV), continues to be a major burden for livestock owners in endemic countries (1). Controlling transboundary diseases such as FMD at source is therefore a shared interest between endemic and free countries (2). FMD in sub-Saharan Africa is unique, not only are the Southern African Territories (SAT) serotypes almost exclusively endemic, but they are also maintained in wildlife (3). Therefore, FMD control in livestock is dependent in part on an understanding of the pathogenesis and transmission from African buffaloes. SAT-serotypes differ from each other with respect to their incidence. SAT-2 is the most widely distributed and is also the serotype most often associated with outbreaks in livestock and wildlife, followed by SAT-1 and then SAT-3 (4-6). However, SAT-1 is the serotype most frequently isolated from buffaloes (personal communication Nick Knowles). The SAT-serotypes are known to exist as mixed infections, with all three isolated on occasions from buffalo oesophageal-pharyngeal fluid (OPF), similarly there are field examples of mixed infections of type O and Asia-1 viruses in cattle (7, 8). It is unclear why certain FMDV serotypes exist in discrete geographies, while other serotypes, for example type O, have a more global distribution (9). The geography of isolated host populations certainly plays a role, however it is unclear whether viral competition or interference plays a role in viral distribution.

In the Kruger National Park (KNP) the SAT-serotypes are highly prevalent and over 98% of buffaloes develop antibodies to all three-serotypes by age two (10, 11). After the acute-period of infection, FMDV can be recovered from OPF, with prevalence varying from 40-60%, although virus recovery from OPF is intermittent and unreliable (7, 11, 12). This ‘carrier state’ is considered unique to ruminants and is defined as the period after 28 days post infection (dpi) in which infectious FMDV may be detected in OPF (13). The carrier state has been reported for up to five years in buffaloes, but the proportion of carriers under field

and experimental conditions decline after three years of age (11, 14). The carrier state is controversial, and to date there are no unequivocal reports of transmission from persistently infected domestic livestock. In contrast, transmission from carrier buffaloes to cattle has been demonstrated (15, 16). Nevertheless, the conditions for transmission from carrier buffaloes are not well understood and are difficult to replicate. In fact, most attempts to effect transmission from carrier buffaloes have failed, even under immunosuppression or viral co-infection (11, 12, 14, 17-19). In addition, the mechanism of persistence remains unclear and the sites for persistence in buffaloes have not been identified. In carrier cattle, virus replication has been detected within pharyngeal epithelium and it has been proposed that FMDV persists through a sustained low level replication (20, 21). Virus particles have also been detected in lymphoid tissue germinal centres (GCs) in ruminants (22, 23). Although virus replication has not been demonstrated, GCs have been proposed as a virus reservoir with the potential for immune-complexed virus to infect lymphoid cells that migrate through GCs to peripheral epithelium.

Here we describe the dynamics of three SAT-serotypes during co-infection in buffaloes; infectious FMDV and RNA were readily detected after acute infection, mainly in palatine tonsil swabs. However, detection occurred significantly less frequently over time. These data provide support for field observations that FMDV is cleared from buffaloes. Importantly, there were significant variation in the quantity of viral RNA detected for the different serotypes. Using the FMD-challenge viruses, we explored the correlation between killing capacity and viral dynamics in cell cultures of mixed infections. Here, we demonstrated differential survival of SAT-isolates during multiple passages in cell culture.

Materials and Methods

Viruses

The SAT-viruses were isolated from buffaloes in the KNP in primary pig kidney (PK) cells (1 passage) and propagated in IB-RS-2 cells (RS cells, 4 or 5 passages) to prepare challenge viruses KNP/196/91/1 PK1 RS5, KNP/19/89/2 PK1 RS4 and KNP/1/08/3 PK1 RS4 (4, 24).

In vivo experiments

Challenge-virus pathogenicity was confirmed in two Nguni cattle, each animal received 1×10^4 $TCID_{50}$ of each serotype intradermolingually in three distinct sites, one site per serotype.

Nine female and seven male African buffaloes from FMDV free herds were donated by Ezemvelo KZN Wildlife (29) and confirmed free of FMDV SAT-antibodies by the OIE Regional Reference Laboratory. Buffaloes were then transferred to Skukuza, KNP and housed in four experimental pens of four buffalo each.. Buffaloes were also co-infected, with 6×10^5 $TCID_{50}$ of each serotype by intradermolingual challenge. Buffaloes were immobilised with etorphine hydrochloride and xylazine for challenge, veterinary examination and sample collection (blood, OPF and palatine tonsil swabs). OPF was diluted in 3 ml probang buffer and snap frozen (30). Buffaloes were culled in groups of four at 35, 95, 185 and 400 dpi. Tissues were stored in RNAlater (Life Technologies) or snap frozen in 50% glycerol/PBS solution or O.C.T. (Tissue-Tek). Tissues collected previously from three buffaloes, screened as negative for FMDV RNA by universal qRT-PCR, served as controls.

Four FMDV-antibody free Drakensberger heifers were maintained in direct contact with buffaloes from 35 dpi, sharing hay-racks and water troughs in two pens. From 35 to 95 dpi, two heifers were placed in each pen containing six buffalo each. From 96 to 185 dpi, each pen contained two heifers and four buffalo. From 186 to 400 dpi, the four heifers and

four remaining buffalo were maintained in a single pen. Blood and OPF were collected from cattle at least monthly, coinciding with veterinary examinations. All experimental procedures with animals were approved by the ARC-Onderstepoort Veterinary Institute (ARC-OVI) Animal Ethics Committee (project number KNP-BC-02) according to national animal welfare standards and performed with the permission of the Department of Agriculture, Forestry and Fisheries (DAFF), Section 20 permit number 12/11/1/1a).

Palatine tonsil swabs

The tonsil sinuses were swabbed individually using nylon brushes (Cytotak Transwab) (31). Laryngoscopes used to depress the tongue were disinfected in citric acid and rinsed in PBS. Nylon brushes were transferred to 0.5 ml probang buffer and snap frozen. At processing, cryotubes containing the brushes were vortexed and centrifuged for 5 minutes at 3000 g. The brushes were then removed and the pellet re-suspended in the remaining sample before qRT-PCR or virus isolation (VI).

Physiological stress

On 156 and 163 dpi, four of the buffaloes in one pen (in direct contact with two cattle) were treated with 4mg Synacthen Depot (Novartis, approximately 1.3 I.U. adrenocorticotropic hormone per kg). The four buffaloes in the second pen, in contact with two cattle, served as controls. EDTA blood was collected pre-treatment and 45 minutes post-treatment to determine cortisol levels (Coat-A-Count Cortisol radioimmunoassay, Siemens Medical Solutions Diagnostics, (32)).

Laser-capture microdissection, RNA extraction and qRT-PCR

Laser-capture microdissection (LMD) was performed as described previously (22). Multiple GC, epithelium or crypt epithelium samples were measured and then dissected

(LMD7000 system, Leica). RNA was extracted from 1 mm^2 of dissected tissue sample using RNeasy Micro Kits (Qiagen) and processed by qRT-PCR.

RNA was extracted from OPF and tonsil swabs using Trizol and from plasma and VI positive cell culture supernatant using MagNA Pure LC Total Nucleic Acid Isolation Kits (Roche). RNA was extracted from RNAlater preserved tissues using MagNA Pure LC RNA isolation Kits III. cDNA was synthesised with random hexamers and TaqMan RT reagents (Applied Biosystems).

The PAN-SAT PCR targeted 1D using the WDA and VP1-AB primer set (33, 34). Challenge virus 1D/2A regions were targeted to generate RNA standards for serotype specific qRT-PCR, using the primers (Table 1) as described previously (35). The serotype specific 1D primers/probe combinations did not cross-react with 1×10^{12} disparate RNA copies/µl and were able to detect one RNA copy/µl. The pan-reactive 3D qRT-PCR assay was used for validation (36). TaqMan universal PCR master mix (Applied Biosystems) was used for qPCR reactions on the MX3005P QPCR systems (Stratagene). Copies/µl were calculated using the standard curve method and MxPro software, samples with no fluorescence above threshold after 50 cycles were considered negative (35). 28S was used as a housekeeping gene for LMD, detected using 28S primers/probe and RNA standards for buffalo 28S adapted from methods described previously (22). For RNAlater tissues, input RNA was normalized to 50ng/µl (NanoDrop 8000, Thermo Scientific) and FMDV copies expressed per µg total RNA.

Virus isolation

Virus was isolated from OPF and tonsil swabs in RS cells and from tissue samples in ZZ-R 127 cells as described previously (28, 37). Samples with no cell death after 3 passages were considered negative. Culture supernatants were analysed by serotype specific qRT-PCR.

Assays for FMDV-specific antibodies

Antibody responses were assessed by liquid-phase blocking ELISA (LPBE (38)) and virus neutralisation test (VNT) assay (13).

Interferon assay

An Mx-CAT reporter assay was used to determine the levels of biologically active type-1 interferon (IFN) in serum samples, as described previously (44).

Leucocyte count

Total EDTA-whole blood leucocyte counts were analysed on a Coulter T-890 (Beckman).

Cell killing assay and buffalo kidney cell line

The capacity of FMDV to kill ZZ-R127 and buffalo kidney (BK) cells was determined by adapting a previously described method (45). The primary BK cell line was established from a Hluhluwe–iMfolozi Park buffalo. The killing assay was performed in 96-well plates seeded with 10^4 cells. Eight hours later, the cells were infected with serial dilutions of challenge virus previously plaque-titrated in the specific cell line (46). All three serotypes were evaluated on the same plate and non-infected rows were included as controls. Cells were stained with methylene blue at different times post-infection. Complete killing was confirmed by microscopy and results of triplicate experiments expressed as the mean number of plaque forming units (PFUs) for killing as a function of time.

In vitro co-infection assays

T25 flasks containing ZZ-R 127 or BK cells were co-infected with all three challenge viruses, each at MOI=2. Co-infection with SAT-1 at MOI=1 and SAT-2 and SAT-3 at MOI=2 or SAT-1 at MOI=0.5 and SAT-2 and SAT-3 at MOI=2 were run in parallel. Single infections at MOI=2, and non-infected flasks served as controls. Complete cell death was observed in 24 hours in all cases and 5 µl of supernatant was passaged into a fresh flask,

cultures were maintained up to passage ten. Supernatants from each passage were analysed by serotype specific qRT-PCR. Results of triplicate experiments were expressed as the number of serotype specific FMDV copies/ml cell culture supernatant.

Statistical analysis

The probability of detecting infectious FMDV or viral RNA in OPF or tonsil swabs was analysed using a generalised linear mixed model with binomial errors and a logit link function. The probability of detecting FMDV in tissues and FMDV RNA in LMD samples was analysed using generalised linear models with binomial errors and a logit link function. Killing assays and co-infection in cell culture were analysed using linear models, with the data for each cell type/virus analysed independently. For VNT titres a linear mixed model was used. All analyses were implemented in R version 3.1.0 (48) using the packages nlme (49), MASS (50) and multcomp (51).

Results

Acute response to co-infection with multiple FMD viruses. FMDV-challenged Nguni cattle developed extensive lesions on the tongue, buccal cavity and feet. By contrast, lesions in buffaloes did not extend beyond the trauma caused by needle inoculation despite receiving 60-fold more virus. Buffaloes were hypersalivating and nasal discharges were observed 1 dpi.

Despite the absence of vesicles, all buffaloes had detectable viral RNA for all three serotypes by qRT-PCR (data not shown). SAT-1, SAT-2 and SAT-3 RNA could be detected over at least 1-7, 3-5 and 1-3 days respectively. FMDV challenge stimulated an IFN response, though peak serum IFN titres in buffaloes were highly variable (peak titres from 3 to 97 I.U/ml). Circulating IFN (>1 I.U/ml) lasted at least five days in the majority of buffaloes and up to six days in challenged cattle (n=2, peak titres of 21.6 and 26.6 I.U/ml).

Similar to reports for cattle (52) FMDV-challenged buffaloes did not develop leucopenia during acute infection (data not shown).

All sixteen buffaloes seroconverted to all serotypes with VNT titres ≥ 45 from 6 to 11 dpi. High titres were maintained until study termination, except buffalo 60, whose titre to SAT-3 was less than 45 after 8 dpi, SAT-3 titres were not significantly different between day 35 and later time-points for all other animals. Surprisingly after 35 dpi, SAT-1 and SAT-2 titres continued to increase ($P<0.05$, data not shown).

The persistence of FMDV occurs in the germinal centres of lymphoid tissue in buffaloes. Viral RNA was detected from 29 to 35 dpi in OPF or palatine tonsil swabs in all buffaloes by pan-SAT diagnostic PCR. In order to compare persistence between serotypes, samples from 8 buffaloes between 35 to 185 dpi and from 4 buffaloes up to 400 dpi were analysed by serotype specific qRT-PCR (FIGURE 1) and VI (FIGURE 2). The proportion of FMDV-RNA positive samples decreased over time and at a rate that differed significantly amongst serotypes; samples at later time-points were more likely to be positive for SAT-1 ($P<0.001$, FIGURE 1). No SAT-2 RNA was detectable after 172 dpi and no SAT-3 after 214 dpi; by contrast, SAT-1 RNA was still detectable at 400 dpi (FIGURE 1). Significantly more SAT-1 RNA copies/ml were detected in swabs compared to OPF at 35 dpi ($P<0.001$) and 80 dpi ($P=0.001$) data not shown, but there were no significant differences in copy numbers between sample types at later time-points.

Significantly more swabs were positive for infectious virus compared to OPF ($P<0.001$, FIGURE 2a &b) and similar to RNA detection, the proportion of positive samples declined significantly over time ($P<0.001$, FIGURE 2a &b). Supernatant from VI positive cultures of tissue samples were screened by serotype specific qRT-PCR, significantly more SAT-1 was isolated compared to SAT-2 and SAT-3 ($P<0.001$, FIGURE 2c).

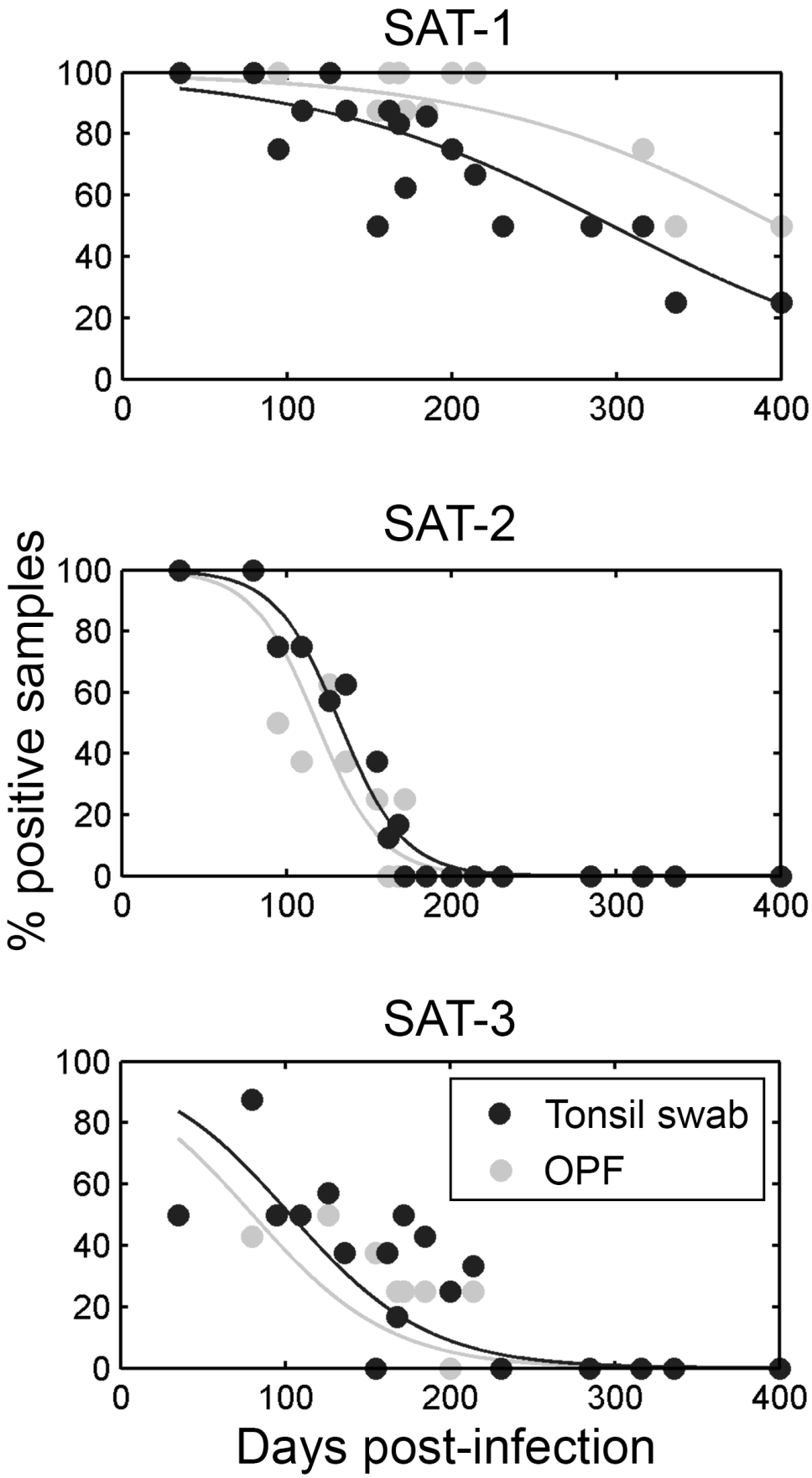

Fig. 1: Summary plots of qRT-PCR data from buffalo palatine tonsil swabs (black and OPF (grey). The lines represent the estimated probability of a positive result, the proportion of positive samples decreased over time at a rate that differed significantly between serotypes ($P<0.001$).

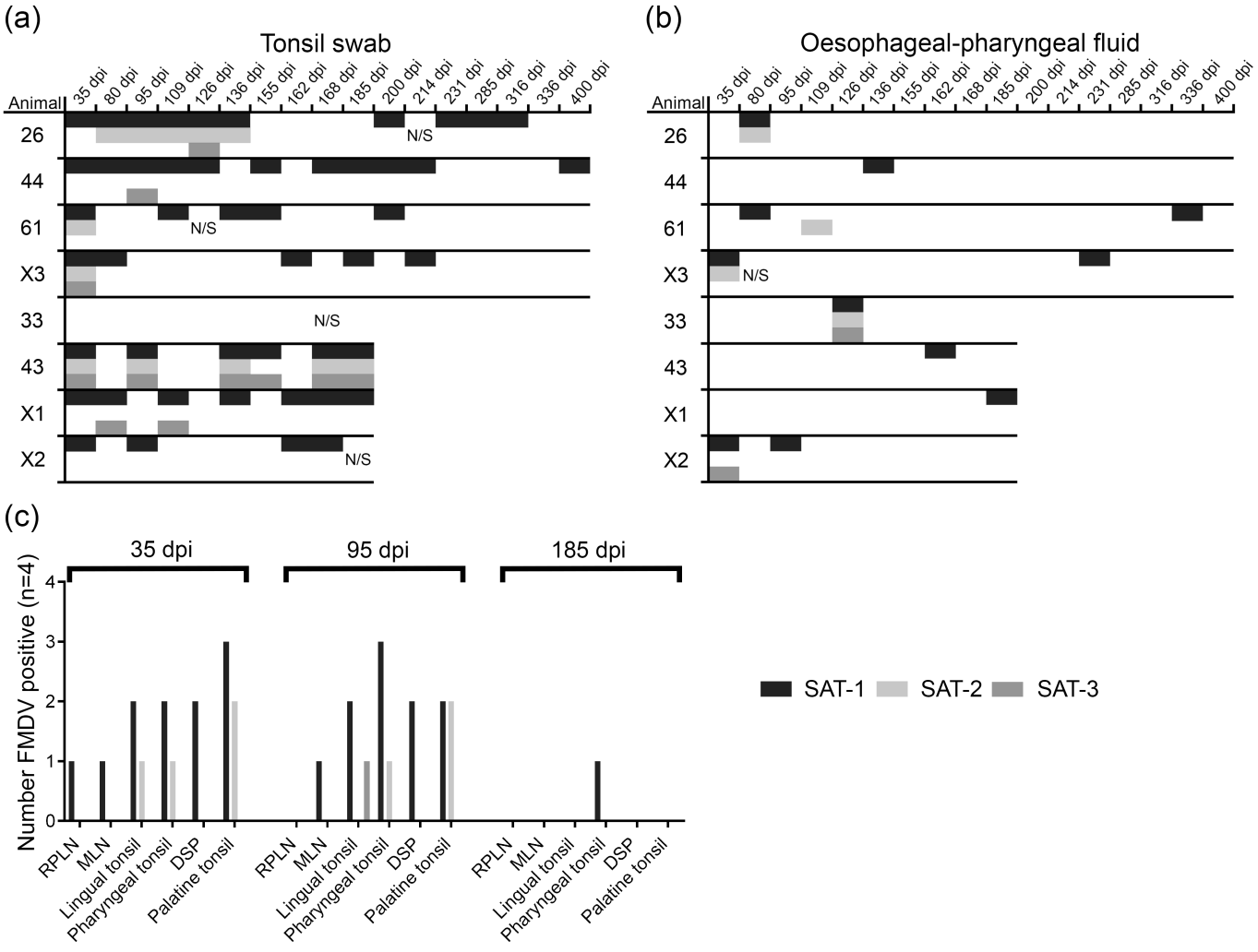

Fig. 2: VI from buffalo tonsil swabs (a) and OPF (b). Significantly more tonsil swabs were positive by VI compared to OPF ($P<0.001$). Significantly more SAT-1 was isolated compared to SAT-2 or SAT-3 ($P<0.001$). (c) VI from Buffalo tissues; RPLN=retropharyngeal lymph node, MLN=mandibular lymph node, DSP=dorsal soft palate. Only 1 sample was positive at 185 dpi, no tissues were positive at 400 dpi. Significantly more SAT-1 was isolated compared to SAT-2 and SAT-3 ($P<0.001$).

Specifically, buffalo retropharyngeal lymph node, mandibular lymph node, lingual tonsil, pharyngeal tonsil, dorsal soft palate and palatine tonsil tissue samples were screened for infectious virus and VI-positive cultures were assayed by serotype-specific qRT-PCR (FIGURE 3a). Virus was readily isolated from tissue samples at 35 and 95 dpi. However, only one tissue sample was VI positive at 185 dpi (pharyngeal tonsil, SAT-1) and no tissues were VI positive at 400 dpi. The frequency of SAT-1 detection was higher compared to SAT-2 and SAT-3 ($P<0.001$) and there were no differences between tissue types. In order to compare viral RNA persistence, whole tissue and laser micro-dissected GC or epithelium were analysed by serotype-specific qRT-PCR (FIGURE 3). SAT-1 and SAT-2 RNA were readily detected in oropharyngeal tissue samples at 35 and 95 dpi, but only two samples were positive for SAT-3 RNA. SAT-2 RNA was not readily detected at 185 and 400 dpi, whereas SAT-1 RNA was detected but was essentially limited at 400 dpi to palatine, pharyngeal tonsil and dorsal soft palate (DSP) samples. These tissues were therefore targeted for laser-capture micro-dissection (LMD) at 35, 95, 185 and 400 dpi (FIGURE 3). There was no difference between tissue types in the probability of detecting viral RNA, however detection of all serotypes by LMD decreased over time ($P<0.001$) suggesting that virus, irrespective of the serotype is cleared from tissues. The detection of different serotypes by LMD was compared and more samples were positive for SAT-1 RNA than for SAT-2 or SAT-3 ($P<0.05$). FMDV detection in GC and epithelium was compared; significantly more GCs were positive for SAT-1 compared to epithelium ($P<0.03$). Similarly, more GCs were positive for SAT-2 compared to epithelium ($P<0.007$), there was no difference for SAT-3.

Transmission of FMDV during the persistent infection stage in buffaloes. Despite direct contact for 365 days, four Drakensberger heifers did not seroconvert (VNT and liquid-phase blocking ELISA) even though virus was readily isolated from buffaloes throughout this period. At 156 and 163 dpi, four buffaloes (X1, X2, X3 and 26) in contact with two heifers,

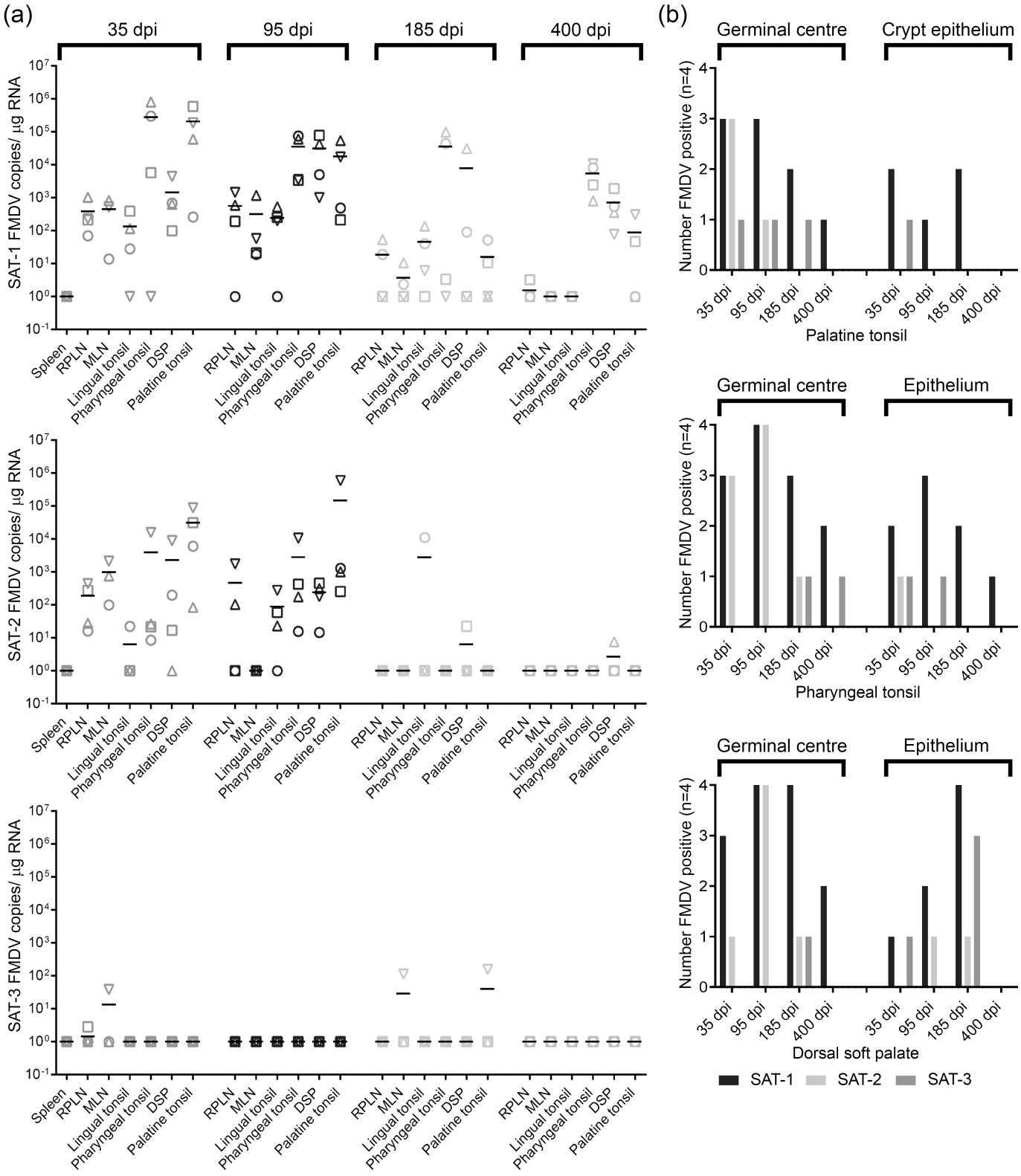

Fig. 3: FMDV RNA persistence in buffalo tissues. (a) Tissues were analysed by serotype specific qRT-PCR (n= 4, lines represent the mean). One MLN was not collected on 35 and 400 dpi. Spleen samples were excluded after 35 dpi. SAT-3 RNA was not readily detected and SAT-2 RNA was infrequently detected after 95 dpi. SAT-1 RNA was detectable up to 400 dpi but essentially limited to palatine tonsil, pharyngeal tonsil and DSP. (b) Laser microdissected GC and epithelium from the palatine tonsil, pharyngeal tonsil or DSP were analysed by serotype specific qRT-PCR (four buffaloes at each time-point, one pharyngeal tonsil was not collected at 400 dpi). There was no difference between tissue types in the probability of detecting viral RNA, however detection decreased over time for all three serotypes ($P<0.001$). Samples were more likely to be positive for SAT-1 RNA than for SAT-2 or SAT-3 ($P<0.05$). There were significantly more SAT-1 positive GC than epithelium samples ($P<0.03$), furthermore there were more SAT-2 positive GC than epithelium samples ($P<0.007$).

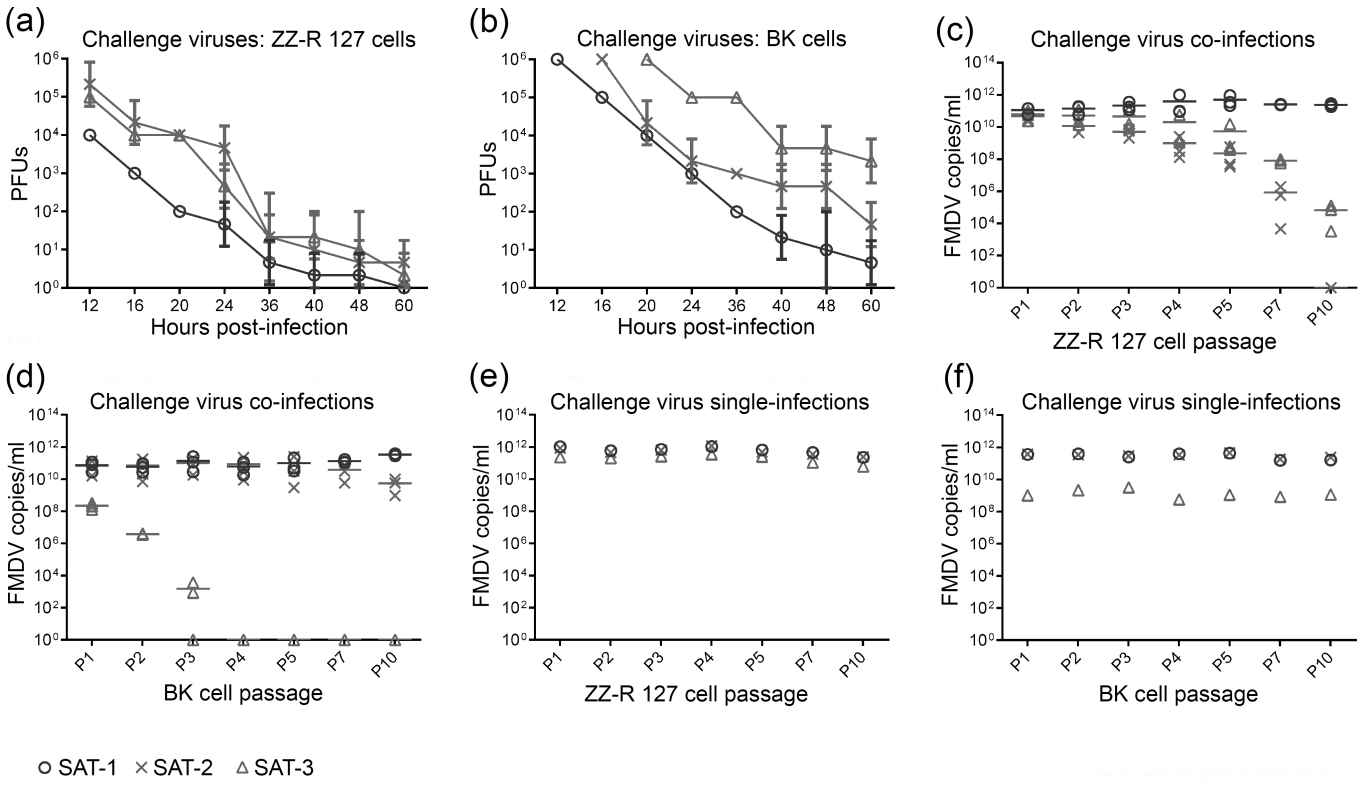

Fig. 4: Virulence and fitness in vitro. (a-b) Killing assays; each value represents the mean PFUs with standard deviation from triplicate assays for the time needed to kill 104 cells. (a) Challenge virus killing assays on ZZ-R 127 cells; SAT-1 killed more rapidly than SAT-2 and SAT-3 ($P<0.001$). (b) Challenge virus killing assays on BK cells; SAT-1 and SAT-2 killed more rapidly than SAT-3 ($P<0.001$). (c-d) Viral fitness was assessed during co-infection assays; results expressed as the number of RNA copies/ml from triplicate experiments, lines represent the mean. (c) ZZ-R 127 cells were co-infected with challenge viruses; SAT-1 (MOI=0.5) outcompeted SAT-2 and SAT-3 infected at MOI=2. (d) BK cells co-infected with challenge viruses; SAT-1 (MOI=0.5) and SAT-2 (MOI=2) outcompeted SAT-3 (MOI=2). (e-f) FMDV copies/ml cell culture supernatant harvested from (e) ZZ-R 127 or (f) BK cells after single infection with challenge virus at MOI=2.

were treated with an adrenocorticotrophic hormone analogue in order to replicate a stress response (plasma cortisol: pre-treatment 13.7-49.8 and 38.6-73.1 nmol/L, post-treatment 189.6-243.7 and 181.3-270.4 nmol/L). Treatment did not result in FMDV transmission.

The correlation between killing capacity and viral dynamics of the three challenge viruses in cultured cells. Since SAT-1 dominated co-infection *in vivo*, we investigated if viral factors play a role in persistence *in vitro.* For this purpose, co-infection and killing assays were undertaken in ZZ-R 127 cells which express $\alpha v\beta 6$, $\alpha v\beta 3$ and $\beta 1$ integrins and in buffalo kidney (BK) cells which express $\alpha v\beta 3$ and $\beta 1$ integrin (data not shown). Persistence during co-infection was broadly reflected by virulence, as assessed by cell killing capacity (FIGURE 4). SAT-1 challenge virus possessed the greatest capacity to kill ZZ-R 127 cells ($P<0.001$) and outcompeted SAT-2 and SAT-3 during co-infection (FIGURE 4c). Following co-infection with SAT-2 and SAT-3, each at a multiplicity of infection (MOI)=2, and SAT-1 at either MOI=2 or MOI=1, competition influenced viral dynamics to such an extent that SAT-2 RNA was undetectable by qRT-PCR from passage seven and SAT-3 by passage ten (data not shown). A similar correlation between viral fitness during co-infection and BK-cell killing capacity was observed (FIGURE 4d). Although SAT-3 was able to infect BK cells, RNA copy numbers were low compared to ZZ-R 127-cell infection and compared to the copy numbers achieved by SAT-1 and SAT-2. The decreased viral fitness of SAT3 on BK cells was reflected by viral RNA extinction by passage three following co-infection with SAT-2 at MOI=2 and SAT-1 at MOI=2, 1 or 0.5 (data not shown).

Discussion

Herein we demonstrated that African buffaloes are resistant to clinical FMD, however buffaloes were viraemic for up to seven days and infection induced IFN and rapid neutralising antibody responses. The incidence of clinical FMD in African buffaloes is low and it is accepted that unlike cattle, most naturally-infected buffaloes do not develop obvious

clinical signs (10, 17). There are unequivocal cases of extensive vesicles and debilitating FMD in buffalo (54, 55). The reasons for these discrepancies in disease severity are unclear and the occurrence of vesicles may depend on the interplay between virus, host and environmental factors. Considering the association between clinical signs of disease and infectiousness (37, 56) and our failed attempts to effect transmission from carrier buffaloes, understanding why FMD is occasionally severe in buffalo and whether this results in transmission is an important area of investigation.

Despite mild disease, FMDV was isolated from fifteen of the sixteen buffaloes after 34 dpi. Palatine tonsil swabs were the sample of choice for recovering infectious virus. However, FMDV isolation and RNA detection was irregular in both tonsil swabs and OPF, with samples intermittently positive for the different serotypes. There is a need for improved methods to assess the spectrum of FMDV circulating in livestock and wildlife for vaccine selection, especially when considering the challenging sampling logistics. Due to the relative ease of isolating virus from tonsil swabs compared to traditional sampling techniques, we have demonstrated that improvements are possible; however further refinement is required to overcome intermittent recovery. Over 50% of ruminants and approximately 60% of African buffaloes exposed to FMDV reportedly become carriers (7, 12, 57, 58). Our data suggest these figures may be substantially higher using refined sampling techniques and delayed viral clearance may be normal.

FMDV was readily isolated from buffalo lymphoid and oropharyngeal tissues at 35 and 95 dpi, with 23 of the 48 tissues positive. We incorrectly anticipated that high neutralising antibody titres at these time-points would hinder recovery (59). Similarly, FMDV has successfully been isolated from lymphoid tissues from carrier sheep and cattle (60, 61). Our results, and previous data for competent carrier-species, are contrary to thorough VI-data reported for pigs; no virus could be isolated from 1140 tissue samples

harvested from 28-100 dpi despite viral RNA and capsid protein persisting in lymphoid tissue GCs (62). Viral RNA and protein localisation after the resolution of viraemia has also been described in cattle; similar to the swine data, FMDV RNA and capsid protein was restricted to GCs in OPF negative animals and no signal was detected in epithelium (22). FMDV non-structural proteins were not detectable in lymphoid or oropharyngeal tissue in pigs and non-carrier cattle (22, 62). In contrast, during FMDV persistence in cattle, cells containing non-structural proteins have been detected in pharyngeal tissue within epithelium and subjacent lymphoid tissue (21).

FMDV RNA persisted in buffalo lymphoid and oropharyngeal tissues, with significantly more GC samples positive for SAT-1 and SAT-2 compared to epithelium. Interestingly, FMDV RNA detection decreased over time for all three serotypes in LMD samples, consistent with clearance of virus and RNA from tonsil swabs, OPF and tissue over time. The VI data from tissues was most striking, with only a single tissue, a pharyngeal tonsil, positive for infectious virus at 185 dpi and no samples positive at 400 dpi. Therefore, there is a progressive reduction in virus titres over time, consistent with a delayed clearance despite the induction of high titres of neutralising antibodies soon after initial infection and antibody titres increasing significantly after 35 dpi, probably due to constant antigenic boost. Similarly, Stenfeldt *et al*. (2014) reported a decline in FMDV RNA and antigen in porcine tissue over time with clearance reported by 100 dpi and in addition, viral RNA was cleared from OPF and tonsil swabs. The retention of virus on follicular dendritic cells (FDCs) may play a key role in the delayed clearance *in vivo*. Our data, in addition to reports in cattle, sheep and pigs may represent a juncture in understanding FMDV persistence (22, 23, 62). Clearly, trapping of viral protein and RNA within lymphoid tissue follicles is a common sequel to infection, even in species in which the carrier-state, such as pigs, has not been described. In addition, it is unclear how virus in lymph tissue remains infectious despite the

presence of neutralising antibody. Recently a pathway has been described whereby FDCs protect antigen by recycling complement receptor-2 bound complement-C3d-coated immune complexes in non-degradative endosomal compartments for months (63, 64). This pathway enables FDCs to protect and present antigen in its native form. Furthermore, investigation in the context of FMDV pathogenesis, specifically the structure of FMDV-antibody/complement immune-complexes (IC) in different species and the ability of immune cells from different species to support productive replication following uptake of FMDV-IC (65) warrants further investigation.

Data from buffalo SAT co-infections demonstrated that viral factors play a role in persistence; SAT-1 dominated chronic FMD in buffaloes. SAT-1 dominance *in vivo* was broadly reflected by *in vitro* killing (virulence) and co-infection assays (fitness) in goat and buffalo cell lines. Competition between serotypes was significant, and under certain conditions SAT-2 and SAT-3 challenge viruses were undetectable at later passages by qRT-PCR. Serial cell passage of mixed type O and Asia 1 has been reported before, with virus ratios up to passage ten calculated using antigen ELISA (66). Virus extinction was not reported, and the two serotypes were shown to cycle equally through time. Interestingly, mixed infection with type O and Asia 1 has been described in cattle, with both viruses co-existing for an extended period of time (8). Therefore co-infection with different FMDV-serotypes may have distinct consequences.

When reviewing the literature, clarity on terminology is required to draw conclusions between studies. The capacity of viruses to kill cells *in vitro* may be termed virulence. However, virulence *in vivo* may or may not be related to cell killing capacity *in vitro*. Using buffalo derived viruses we demonstrated a correlation *in vitro* between viral fitness and cell killing capacity. Based on these data, one could propose that during acute infection, tissue reservoirs are established before infection is controlled by the host's immune response, most

probably in GCs. Also, the presence of live virus in tissue samples after acute infection suggests there is on-going virus replication. If there is a limited population of cells available *in vivo* to sustain virus replication then viruses that replicate and kill these cells quickly will persist more readily. This could explain the results of the buffalo co-infection studies herein and why SAT-1 clearance was delayed compared to SAT-2 and SAT-3.

The relationship between FMDV fitness and cell killing (described as virulence) has been explored previously *in vitro*, with conflicting results. Increased virulence and fitness have been reported for FMDV following passage in BHK-21 cells (45) and a correlation between virulence and fitness has been demonstrated *in vivo* for a number of other virus-host systems (67, 68). Herrera *et al.* (2007) describe the behaviour of a FMDV clone with a history of serial plaque transfer in BHK-21 cells which also deviated from the correlation between viral fitness and virulence. During passage the clone attained a very low fitness value relative to parental virus yet its virulence for BHK-21 cells was significantly higher than the reference virus. Although buffaloes were challenged with the SAT-serotypes at distinct sites, it is possible that viral competition or interference played a role in persistence. FMDV has been used as a model system *in vitro* to study competition-colonization dynamics for RNA viruses (70-72). In cell-cultures, co-infected with colonizers (highly-virulent FMDV clones) and competitors (less-virulent clones), the colonizers were outcompeted by the competitors which led to attenuation of the viral population. Our *in vivo* studies of FMDV in a natural host clearly demonstrated differential survival of viruses after mixed infection, a similar pattern of virus dominance was observed *in vitro* and the viruses that killed cells most quickly out competed other viruses.

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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