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Full Length Research Paper

Seroepidemiological investigation of foot-and-mouth disease virus serotypes in cattle around Lake Mburo National Park in South-Western Uganda

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Foot-and-mouth disease (FMD) outbreaks in cattle occur annually in Uganda. In this study the authors investigated antibodies against FMD virus (FMDV) in cattle in surrounding areas of Lake Mburo National Park in South-Western Uganda. Two hundred and eleven serum samples from 23 cattle herds were examined for the presence of antibodies against FMDV non-structural proteins and structural proteins using Ceditest® FMDV-NS and Ceditest® FMDV type O (Cedi Diagnostics BV, Lelystad, The Netherlands). Furthermore, serotype-specific antibodies against the seven serotypes of FMDV were determined using in-house serotype-specific Solid Phase Blocking ELISAs (SPBE). Of the sera tested, 42.7% (90/211) were positive in the ELISA for antibodies against non-structural proteins, while 75.4% (159/211) had antibodies against the structural proteins of FMDV serotype O. Titres of ≥ 1:160 of serotype-specific antibodies in SPBEs were identified in 61% (19/31), 33% (5/15), 6%7 (20/30), 37% (10/27) and 12% (4/33) of the investigated samples for serotypes O, A, SAT 1, SAT 2 and SAT 3, respectively. This study indicates that most of the FMD outbreaks in the cattle herds in the investigated area were probably caused by FMDV serotype O, A and/ or SAT-serotype(s). It also shows that the usage of non-purified, multivalent vaccines in Uganda obscures the serological diagnosis of FMDV outbreaks, and that the sampling strategy needs to be improved. Finally, it emphasizes the importance of isolation and characterization of FMD viruses responsible for outbreaks in the area.

Key words: Foot-and-mouth-disease, antibodies, cattle, Uganda.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is classified within the *Aphthovirus* genus as a member of the *Picornaviridae* family (Andrewes et al., 1978; King et al., 2000). FMDV includes seven serotypes: O, A, C, Asia 1, Southern African Territories (SAT) 1, SAT 2 and SAT 3, of which all except Asia 1 have been reported on the African continent (Vosloo et al., 2002; Rweyemamu et al., 2008a). The disease is highly contagious and causes formation of vesicles on the mouth and the coronary band of feet in all cloven-hoofed animals (Thomson, 1994). FMD is a transboundary animal disease (FAO-OIE, 2004) and is economically very important to countries which export and import animals or animal products (Rweyemamu et al.,

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2008b). The control of FMD is particularly complicated, since a high proportion of infected cattle and other species are persistently infected (Sutmoller et al., 2003). Several studies (Van Bekkum et al., 1960; Sutmoller and Gaggero, 1965; Hedger, 1968; Hedger, 1970; Burrows et al., 1971; Rossi et al., 1988) have shown that FMD virus can persist in the oro-pharyngeal region of cattle for up to 2.5 - 3.5 years post infection (Alexandersen et al., 2003) and of African Buffalo (Syncerus caffer) for up to 5 years (Condy et al., 1985).

Persistently, infected animals have constituted an obstacle in using vaccination to control FMD outbreaks since it has not been possible to differentiate if serologically positive animals were vaccinated and persistently infected animals (Sørensen et al., 1998). However, based on the fact that vaccines against FMD rarely develop antibodies against the non-structural proteins (NSP), tests for antibodies against NSP of FMDV have been developed, and several commercially available tests for this differentiation have been evaluated (Brocchi et al., 2006). Cattle that was infected or exposed to FMDV regardless of vaccination status can be identified by detection of antibodies against the NSP (Lubroth and Brown, 1995; Diego et al., 1997; Malirat et al., 1998; Sørensen et al., 1998). Moreover, the NSP test can detect antibodies against all seven serotypes of FMDV (Sørensen et al., 2005).

The area surrounding Lake Mburo National Park (LMNP) in the South-Western part of Uganda is not fenced, hence, livestock and wildlife intermingles freely across the borders of the park. For preventive and control purposes, cattle in this area are annually vaccinated against serotypes O, SAT 1 and SAT 2. Despite this effort, the surrounding district (Kiruhura district) has experienced FMD outbreaks more than twice a year with a total of eleven outbreaks registered between 2004 and 2008 (C. Rutebarika, personal communication, 2008). For this reason, it has been speculated that wildlife animals could be the cause of the periodic outbreaks of the disease in this locality.

The aim of this study was to establish the serotype-specificity of antibodies towards FMD present in the cattle population in the area surrounding LMNP.

MATERIALS AND METHODS

Study area and serum samples

In this study, a total of 211 cattle blood samples of long horned Ankole breeds aged more than four years were collected from 23 herds with a history of previous FMD outbreaks in the areas surrounding LMNP (in Kiruhura district in South-Western Uganda). Except herd A, all cattle herds were kept under the communal grazing system with animals feeding and intermingling freely in large common herds but having separate enclosed areas (kraals) where they stayed at night. Herd A was a fenced government farm where animals were grazed separated from the other herds. The area around LMNP was selected based on information of annual FMD outbreaks from the commissioner of disease control, Ministry of Agriculture, Animal Industry and Fisheries (MAAIF). The herds were selected based on recent FMD outbreaks as communicated by the field animal husbandry officer of Kiruhura district. Vaccination of cattle was last performed in 2007, at least ten months before the samples were collected.

Sample handling

The serum was extracted in the field within 12 h of sampling using a Mobilespin 12-V field centrifuge (Vulcon Technologies, Uk). Aliquots of 4.5 ml serum samples were kept on ice until being stored at -20°C.

Antibody assays

All sera were screened for antibodies against FMDV non-structural proteins (NSP) using Ceditest® FMDV-NS kit (Cedi Diagnostics BV, Lelystad, The Netherlands) and against structural proteins of FMDV serotype O (SP-O) using Ceditest® FMDV type O kit (Cedi Diagnostics BV, Lelystad, The Netherlands). Sera positive for antibodies against NSP were further investigated using an in-house Solid Phase Blocking ELISA (SPBE) system for serotype-specific antibodies against FMDV developed at Lindholm, Denmark (Have and Holm Jensen, 1983). The Ceditest® FMDV-NS kit is a blocking ELISA that detects antibodies against the non-structural 3ABC protein of FMDV of all seven serotypes and it may be used to detect infection in vaccinated animals (Sorensen et al., 2005). Standard protocol procedures were followed according to manu-facturer's instructions. Optical Density values (OD) were measured with a Multiskan Ascent spectrophotometer using dual wavelengths of 620 and 450 nm and Ascent Software, version 2.6 (Thermo Labsystems Oy, UK). The results were expressed as Percentage Inhibition (PI) as follows:

$$PI = 100 - [\text{Test serum } (OD_{450} - OD_{620}) / \text{Mean negative control } (OD_{450} - OD_{620})] \times 100$$

PI < 50% was interpreted as negative, while a PI value of ≥ 50% was positive.

Testing with Ceditest® FMDV type O kit was performed according to the manufacturer's instructions. Measurement of OD values and calculation of PI was as described above for the Ceditest® FMDV-NS kit.

Except for one sample from herd A, all sera positive in the NSP ELISA were screened at a dilution of 1:5 in SPBEs for antibodies against the seven FMDV serotypes O, A, C, Asia1, SAT 1, SAT 2 and SAT 3 of FMDV.

The method for the SPBEs is described by Balinda et al. (2009). Briefly, all seven FMDV strains as well as guinea pig and rabbit immune sera raised against the SAT-serotypes were kindly provided by the World Reference Laboratory (WRL), Pirbright, UK, while guinea pig and rabbit immune sera against serotypes O, A, C and Asia 1 were raised at Lindholm, Denmark. FMDV strains were propagated in primary or secondary bovine kidney cell cultures, or in baby hamster kidney (BHK) cell cultures, and culture harvests

were inactivated with ethyleneimine and used as antigens. Optimal dilutions of antigens and guinea pig and rabbit sera were predetermined for each of the seven serotype-specific antibody ELISAs, and the tests were run on separate microtitre plates (Nunc-Maxisorp, Roskilde, Denmark) using a volume of 100 µl per well. The plates were incubated at room temperature and rinsed three times in ELISA-buffer (0.015 M Na_2HPO_4, 0.0025 M KH_2PO_4, 0.5 M NaCl, 0.05% Tween-20) in-between steps except for the last wash, which included five rinses. Plates were coated with serotype-specific guinea pig immune sera (O-BFS 1981, A-Iraq 1996, C-Turup 1961, Asia 1 Shamir, SAT 1 (BOT 1/68), SAT 2 (ZIM 5/8 V72) and SAT 3 (ZIM 4/81)) for one hour, followed by a one hour incubation with inactivated FMDV antigens [O-Manisa, A-Iraq 1996, C-Noville, Asia 1 Shamir, SAT 1 (BOT 1/68), SAT 2 (ZIM 5/8 V72) and SAT 3 (ZIM 4/81)]. Then test sera and control sera were added. Each plate contained duplicates of strong and weak positive control sera and four wells with negative control serum. The plates were incubated on an orbital shaker overnight at room temperature, followed by incubation with serotype-specific rabbit antisera diluted in ELISA buffer with 10% normal calf serum (NCS) for one hour, peroxidase-conjugated swine anti-rabbit IgG (Dakopatts P0217) diluted 1:1000 in ELISA buffer with 10% NCS and 1% normal guinea pig serum for 30 min and finally TMB substrate (Tetramethyl-benzidine). The reaction was stopped after suitable colour development (15 min) by adding 1 M sulphuric acid.

Measurement of OD values was as described previously for the Ceditest® FMDV-NS kit, but results were expressed as OD percentage (ODP) were as follows: ODP = [Test serum (OD_{450} - OD_{620}) / Mean negative control (OD_{450} - OD_{620})] x100. The cut off values varied between serotypes. Sera were considered positive in the screening, if the ODP was < 50% for serotypes O, SAT 1, SAT 2, SAT 3, <45% for type A and <35% for serotypes C and Asia 1 (H Balinda et al., 2009).

For each of the seven serotype-specific ELISAs, 33 - 50% of the sera with positive reactions were titrated from 1:5 to 1:640. These sera were selected so they constituted 20 - 100% of the sera with antibodies against NSP from each positive herd, except for herds H and R which could not be investigated further due to depletion of sera. The antibody titres were calculated as the reciprocal of the last positive dilution. A serum was considered positive for antibodies against a given serotype, if $\log_{10}$ (titre) was ≥160. Titers of ≤ 40 were considered negative, while titre 80 was considered inconclusive.

Statistical analysis

Descriptive statistics were used and frequency distributions calculated (Thrusfield and Bertola, 2005). Seropositivity rates were determined by dividing the number of positive serum samples by the total number of samples tested.

RESULTS

Screening for antibodies against NSP and SP-O

A total of 211 cattle sera were subjected to ELISAs for antibodies against NSP and SP-O (Table 1). Ninety of the 211 cattle samples (42.7%) were positive for antibodies against NSP of FMDV, while 159 (75.4%) were positive for antibodies against SP-O of FMDV. Most farms had more animals' positive in the SP-O ELISA than in the NSP ELISA, except for farms K, L, M, O, Q and W which had more positives in the NSP ELISA. The numbers of positive animals for the two tests were the same on farms P and T.

Screening and titration in serotype-specific antibody ELISAs

A total of 89 serum samples from 20 cattle herds were selected based on positive reactions in the NSP ELISA and screened at dilution 1:5 for serotype-specific antibodies (Table 2). The vast majority of serum samples were positive in the ELISAs for antibodies against serotypes O (92%), SAT 1 (100%), SAT 2 (74%) and SAT 3 (96%), while fewer samples were positive for Antibodies against serotype A (52%) and serotype C (49%), and only 18% of the samples were positive in the ELISA for antibodies against Asia 1. Titration of a representative sub-set of these samples in the relevant SPBE ELISAs resulted in very low titres (≤40) for all titrated reactions in the serotype C ELISA (14 of 44 positive reactions titrated) and the serotype Asia 1 ELISA (8 of 16 positive reactions titrated).

The numbers of sera with titres of 80 and above are shown in Table 3. When using a cut-off for positive titre set at titre ≥160 many sera had evidence of recent exposure of the animals to serotypes O (61%), A (33%), SAT 1 (67%), SAT 2 (37%) and SAT 3 (12%). This table also shows that the picture would not be significantly different whether a cut-off titre of 80 or 160 is used. Serotype-specific antibody titres for serotypes O, A, SAT 1, SAT 2, SAT 3 are shown in Table 4. Generally, most farms had evidence of presence of antibodies against more than one serotype with highest titres towards serotypes O and SAT 1, and to some extent serotype SAT 2. With regard to serotype SAT 3, only four sera had titres ≥ 160, and these were all in combination with higher titres against serotypes O, SAT 1 and/or SAT 2.

Though most antibody titres against serotype A were negligible, five of seven titrated sera from farm A had titres ≥ 160. One of these five sera (F1-16) had a higher titre towards serotype A than towards other serotypes; another (F1-1) had the same titre as towards serotypes O and SAT 1 (Table 4). Hence, there was some evidence of exposure to serotype A in herd A, either by infection or vaccination.

DISCUSSION

In this study, antibodies against NSP were detected in

Table 1. Number of animals with antibodies towards FMDV NSP and SP-O in cattle herds surrounding LMNP in Kiruhura district.

District	Herd	No of sera collected (n)	Positive sera on NSP (%)	Positive sera on SP (%)
Kiruhura	A	73	36 (49.3)	70 (95.9)
	B	8	5 (62.5)	7 (87.5)
	C	5	2 (40.0)	5 (100.0)
	D	5	2 (40.0)	5 (100.0)
	E	6	0	2 (33.3)
	F	12	1 (8.3)	8 (66.7)
	G	15	3 (20.0)	14 (93.3)
	H	4	1 (25.0)	2 (50.0)
	I	5	2 (40.0)	5 (100.0)
	J	11	2 (18.2)	9 (81.8)
	K	10	5 (50.0)	4 (40.0)
	L	5	5 (100.0)	1 (20.0)
	M	6	6 (100.0)	2 (33.3)
	N	5	0	4 (80.0)
	O	5	5 (100.0)	4 (80.0)
	P	6	3 (50.0)	3 (50.0)
	Q	4	4 (100.0)	2 (50.0)
	R	2	1 (50.0)	2 (100.0)
	S	5	0	1 (20.0)
	T	5	2 (40.0)	2 (40.0)
	U	5	1 (20.0)	3 (60.0)
	V	6	2 (33.3)	3 (50.0)
	W	3	2 (66.7)	1 (33.3)
Total		211	90(42.7)	159(75.4)

42.7% of the tested cattle sera (90/211), while antibodies against SP-O were detected in 75.4% (159/211). Similar studies using NSP test for FMD sero-diagnosis in Southwestern Ethiopia (Gelaye et al., 2009) showed that seropositivity in cattle was 12.08% (n = 276), while in Western Uganda, 14% (n = 143) and 22% (n = 55) of goats and sheep, respectively, were found positive in this assay(Balinda et al., 2009).

In this work, 15 herds had a higher proportion of the samples positive in the SP-O ELISA than in the NSP ELISA, which would indicate vaccination and in some animals in combination with infection. However, the vaccines used in Uganda are non-purified trivalent vaccines and the animals in the study were adult and were likely to have been vaccinated several times during their life, and this vaccination history in itself is likely to elicit antibodies towards NSP (Sutmoller et al., 2003). On the other hand, six herds (K, L, M, O, Q and W) had more samples positive in the NSP ELISA than in the SP-O ELISA, indicating infection, though presumably not with serotype O. This is in line with the findings in Ugandan non-vaccinated African buffalos (*Syncerus caffer*), where serological evidence of exposure to SAT-serotypes was not recognized by the SP-O ELISA (Ayebazibwe et al., unpublished data). The latest recorded outbreak of FMD in the area took place in April-June 2007 and the vaccination was completed during the following two months, while the sampling was carried out in April 2008, a year after the outbreak. Thus, it is likely that the antibodies demonstrated in the investigated sera were a mix of vaccine-elicited and outbreak-elicited antibodies.

Moreover, though the non-structural 3ABC protein (NSP) ELISA test kit has aided sero-diagnosis of FMDV in many countries by detecting antibodies towards all seven serotypes of FMDV (Sørensen et al., 1998; Bronsvoort et al., 2006), its ability to differentiate between vaccinated and infected animals is hampered when applied in areas like Uganda, where non-purified vaccines are used. To circumvent this, Sutmoller et al. (2003) recommended sampling of young animals with a maximum of one vaccination to define whether vaccinating farms have been infected. In the current work, investigation of serotype-specificity of antibodies gave a diverse picture, but with highest titres towards serotypes

Table 2. Screening at a dilution of 1:5 of sera from cattle herds surrounding LMNP in Kiruhura district for serotype-specific antibodies towards FMDV.

District	Herd	NSP sera screened positive	Number of positive sera per serotype						
			O	A	C	Asia1	SAT1	SAT2	SAT3
Kiruhura	A	35	35	33	29	11	35	34	35
	B	5	5	2	1	0	5	3	4
	C	2	2	1	1	0	2	2	1
	D	2	2	0	0	0	2	1	2
	F	1	1	0	1	0	1	1	1
	G	3	3	1	1	2	3	3	3
	H	1	1	0	0	0	1	1	1
	I	2	2	1	1	0	2	1	2
	J	2	2	1	2	0	2	1	2
	K	5	3	1	0	0	5	1	4
	L	5	3	1	1	0	5	1	4
	M	6	5	0	2	0	6	5	6
	O	5	5	1	2	1	5	5	5
	P	3	2	2	1	0	3	2	3
	Q	4	4	1	1	1	4	0	4
	R	1	1	0	0	1	1	1	1
	T	2	2	0	1	0	2	1	2
	U	1	1	1	0	0	1	1	1
	V	2	2	0	0	0	2	2	2
	W	2	1	0	0	0	2	0	2
	Total	89	82	46	44	16	89	66	85

Table 3. Number of sera with high titres of antibodies towards FMDV serotypes.

Titre*	Number of sera per FMDV serotype						
	O	A	C	Asia 1	SAT 1	SAT 2	SAT 3
80	5/31	0/15	0/14	0/8	7/30	8/27	6/31
≥ 160	19/31	5/15	0/14	0/8	20/30	10/27	4/31

*Expressed on log10 and cut-off ≥160.

O, SAT 1 and SAT 2. Except for an incursion of serotype A in 2002 (WRLFMD, 2009), this is in agreement with the diagnosed FMDV serotypes in Uganda since 1978 (WRLFMD, 2009), as well as with the serotypes included in vaccines used after 2002 (O, SAT 1 and SAT 2) (C. Rutebarika, personal communication, 2008). Antibody titres against serotype SAT 3 were lower than O, SAT 1 and SAT 2 titres in the same sera, and antibody titres against serotypes C and Asia 1 were negligible as were most of the antibody titres against serotype A. Hence, these low titre reactions were most likely cross reactions of antibodies against other serotypes.

In contrast, five of seven serum samples from farm A had high titres of antibodies against serotype A, and two of these were higher than or equal to titres towards serotypes O, SAT 1 and SAT 2, thus providing some evidence of exposure to FMDV serotype A in herd A. As serotype A vaccines have not been used in Uganda since 2002, it is not likely that these antibodies were remains from vaccination, and therefore relatively recent infection with serotype A could be suspected. The relatively high level of cross-reactivity recorded in this study is somewhat surprising, as the Solid Phase Competition ELISA has been shown to have a higher specificity than both the

Table 4. Serotype-specific antibody titres for serotype O, A, SAT 1, SAT 2, SAT 3 in 18 herds around LMNP.

Herd	No. of Sera samples tested	No. of NSP positive	Animal ID	Specific FMD serotypes				
				O	A	SAT1	SAT2	SAT3
A	73	36	F1-16	40	320	160	40	80
			F1-35	160	nd	nd	80	nd
			F1-1	640	640	640	160	40
			F1-19	>640	40	160	320	80
			F1-39	>640	160	320	320	160
			F1-21	>640	160	>640	320	160
			F1-63	nd	160	nd	nd	nd
B	8	5	F2-5	20	20	40	10	20
			F2-1	80	nd	80	20	10
			F2-3	160	*	320	40	10
C	5	2	F3-1	80	*	80	160	10
			F3-5	160	5	320	40	*
D	5	2	F4-2	40	*	nd	*	nd
			F4-87	160	*	320	320	40
F	12	1	F6-100	320	*	320	160	20
G	15	3	F7-117	160	*	320	80	80
			F7-110	320	5	>640	320	160
I	5	2	F9-128	160	10	>640	80	40
J	11	2	F10-134	320	nd	nd	*	nd
			F10-137	nd	*	320	nd	80
K	10	5	F11-143	20	*	320	10	20
			F11-146	40	*	nd	*	40
L	5	5	F12-153	>640	5	nd	80	40
			F12-155	nd	*	80	*	20
M	6	6	F13-157	nd	*	40	40	40
			F13-156	nd	*	>640	80	10
O	5	5	F15-167	320	*	320	80	80
			F15-170	>640	40	>640	20	320
P	6	3	F16-56	80	5	80	80	20
Q	4	4	F17-58	40	*	80	*	20
			F17-60	160	20	320	*	80
T	5	2	F20-66	80	*	320	40	10

Table 4. Contd.

U	5	1	F21-73	160	5	320	160	20
V	6	2	F22-76	40	*	nd	80	nd
			F22-78	160	*	80	160	20
W	3	2	F23-81	*	*	40	*	10
			F23-82	80	*	80	*	20

*: negative at screening
nd: not done. Positive at screening, but not titrated, most often due to depletion of the sample.

liquid phase blocking ELISA and virus neutralization test (Mackay et al., 2001). This may be as a result of using a screening dilution 1:5. This will be changed in future work in endemic areas where frequent vaccination is carried out. It is possible that the WRL's (Pirbright) solid phase competition ELISA may have worked better in the present situation, since it uses a more specific guinea pig antibody for detection in contrast to the Lindholm assay in which a slightly less specific rabbit antibody is used to ensure that no reactors are missed.

The demonstration of high antibody titres against FMDV in cattle indicates previous exposure to FMDV particularly serotypes O, SAT 1 and SAT 2, and to a lesser degree serotypes A and SAT 3. Despite the vaccination history of the sampled animals, very high titres of antibodies to serotypes O and SAT 1 in a large proportion of the animals may indicate recent incursions of FMDV in some of the cattle herds, in particular the ones with high proportion of animals positive in the NSP ELISA. This is in agreement with Vosloo et al. (2002), who reviewed reports of FMD outbreaks in Uganda and other countries in sub-Saharan Africa up to 2001, and found numerous reports of outbreaks of serotypes O,

SAT 1 and SAT 2 in Uganda and its neighboring countries. Other studies (Balinda et al., 2009; Mwiine et al., 2010) have provided evidence of FMD outbreaks in Ugandan cattle in 2006 being caused by serotype O and SAT-serotypes. Balinda et al. (2009) also showed that non-vaccinated sheep and goats can be used as indicator species for serotyping circulating FMDV in a situation with a very diverse antibody response as a consequence of frequent incursions of FMDV and/or usage of non-purified vaccines.

The area around LMNP was also chosen in attempt to establish the relation between outbreaks in livestock and wildlife animals since both populations freely cross boundaries of designated areas for the national parks and interact through feeding and watering (Kalema-Zikusoka et al., 2005). Notable among the commonly observed ungulates from the national park are the African buffaloes (*Syncerus caffer*), which may play a significant role in the transmission of FMDV (Vosloo et al., 2002) due to their ability to become long-term FMDV carriers (Condy et al., 1985). In Queen Elizabeth National Park, buffaloes with antibodies against serotypes O, SAT 1, SAT 2 and SAT 3 were detected in 2005 (Kalema-Zikusoka et al., 2005), while buffalos sampled in LMNP in 2007-2008 had serological evidence of exposure to serotypes SAT 1 and SAT 2 (Ayebazibwe et al., 2010). Hence, provided part of the measured antibodies against SAT 1 and SAT 2 were elicited by infection rather than vaccination, there is an overlap between the serological evidence of FMDV serotypes in buffalo and cattle in this area. However, the mechanism of virus spread in the area can not be concluded based on the presented data.

In conclusion, there was some evidence that FMDV serotypes O and SAT 1 may have been present in cattle herds at the time of sampling. However, firm conclusions were not possible due to the annual usage of non-purified vaccines against FMDV. Therefore, there is a need to establish the actual FMDV infections in the livestock population based on serological studies of young unvaccinated cattle and other species (goats and sheep) in the area. For confirmation of FMD viruses circulating in the area, epithelial samples from clinically sick animals and probangs samples from presumably persistently infected cattle should be obtained for virus isolation and characterization. In addition, FMDV infections in buffalos should be investigated and the findings from these two species should be compared to clarify the interplay between wildlife and livestock in the epidemiology of FMDV in this area.

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