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Keywords: NSP-ELISA, RT-PCR, sensitivity, specificity, real time PCR, focal screening.

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I. Introduction

Foot-and-mouth disease (FMD) is a devastating viral disease effecting cloven hoofed animals including cattle, pigs, sheep, and goats. The burden of the disease is manifested through reduced productivity and limitation of international trade in live animals and their product causing serious economic losses (Syed & Graham, 2013). It is a highly contagious, trans-boundary, acute, vesicular disease of cloven-hoofed animals including those in the wild (Alexandersen & Mowat, 2005) which act as reservoirs of the virus for transmission to the domestic animals (Anderson, Anderson, Doughty, & Drevmo, 1975). The causal agent of FMD is called foot-and-mouth disease virus (FMDV). It is a small, non-enveloped, single stranded RNA virus 8.5 kb long with a positive polarity surrounded with icosahedral capsid symmetry belonging to the genus *Aphthovirus* of the *Picornaviridae* family (Boothroyd *et al.,* 1981). It has seven serotypes A, O, C, Asia 1 and the Southern African territories (SAT) 1-3 of which all have occurred in most East African countries (Vosloo, Bastos, Sangare, Hargreaves, & Thomson, 2002) except Asia 1 (Rweyemamu, 1982). Studies have shown that the predominant FMDV serotypes in Uganda are O and SAT-2 (Balinda *et al.,* 2010). Other serotypes reported include SAT-1 and SAT-3 (Vosloo *et al.,* 2002), serotype C was last recorded in early 1971 (Vosloo *et al.,* 2002).

The disease is characterized by short lasting fever, epithelial lesions on the tongue, dental pad and inner mouth area leading to excessive salivation and drooling and lesions on the feet causing lameness

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(Margo, E Chase-Topping Handel et al., 2013). The initial virus multiplication takes place in the pharynx epithelium producing vesicles and lesions and later vesicles appear on the feet (Burrows *et al.,*1981) making the tissues in these areas preferred specimens for diagnosis (Sutmoller, 1992).

In Africa the epidemiology of FMD in Africa is not well understood (Ayebazibwe *et al.,* 2010). The widespread movement of animals, the wide host range of the virus involving wild and domestic animal reservoirs and the presence of multiple strains and sub-strains complicating the epidemiology of the disease.

In Uganda the assays commonly used assays for detection of FMD include conventional reverse transcription polymerase chain reaction (Kasambula, Belsham, Siegismund, H.R Muwanika1, & C, 2012) and antibody ELISA (Mwiine et al., 2010). A recent study by Namatovu et al., 2013 showed that the exclusively collected sample in East African countries in general and Uganda in particular is serum. So in East Africa nearly all the national referral laboratories use antibody ELISA (Namatovu et al., 2013) because it is cheap and can be used to test large volume of samples (OIE, 2009) and does not depend on virus isolation (Paixao et al., 2008) or the expensive molecular techniques such as real time RT-PCR and conventional RT-PCR (Kafeero et al., 2016). In the same study by Namatovu et al. 2013, national reference laboratories are understaffed yet most molecular methods rely on services of well trained staff. This makes antibody ELISA the major assay used in diagnosis of foot-and-mouth disease. In the study by Kafeero et al. 2016, foot-and-mouth disease virus reverse transcription loop mediated assay has been evaluated. It was found to have a comparable sensitivity as the foot-and-mouth disease virus real time RT-PCR giving hope for FMD diagnosis even in the field with high sensitivity. None the less despite its high popularity due to the high sensitivity, specificity, rapidity, cost-effectiveness, field applicability, colorimetric detections (Notomi et al. 2000, Mori et al. 2001, Nagamine & Hase, T Notomi 2002, Matovu et al. 2010, Hopkins et al. 2013, Atuhaire et al. 2014, Kafeero et al. 2016), it has not received a lot of attention.

In this study we report the diagnostic challenges of foot-and-mouth disease virus in Uganda by comparing the results from the two commonly used assays of NSP ELISA and conventional PCR in national and research laboratories in Uganda. The results from the two assays were compared with real time quantitative PCR as the gold standard (OIE 2008).

II. Methods and Materials

a) Study sites

The study was carried out between July 2014 to July 2015 on samples collected from Bungokho county Mbale district and Kamonkoli County in Budaka district during the foot-and-mouth disease 2014/2015 outbreak in our country.

b) Study design

A cross-sectional study was carried out following reports of foot-and-mouth disease outbreaks in Mbale district, Bungokho County and in Budaka district, Kamonkoli County as described in our previous study (Kafeero et al., 2016). Purposive sampling was done based on animals having clinical symptoms like oral lesions, history of infection but having healing lesions and any other asymptomatic cattle in the same farm/kraal or grazing with the symptomatic cattle as reported by the Sub-count Veterinary Officer and or the farmers. The inclusion criteria were cattle with clinical symptoms and the asymptomatic ones in the same farm while exclusion criteria were cattle in farms without any clinical signs or history of clinical signs. All farmers in the villages where sampling was done keep few cattle on average 3-4 animals per house hold and on zero grazing basis, transmission of the virus was assumed to be low between kraals/farms.

c) Sample size determination

The desired confidence interval for sensitivity estimates was 95% (width of 0.05). The specificity of NSP ELISA in previous studies by Diego, Brocchi, Mackay, & De Simone, 1997 was in the range 99%. This was consistent with the studies by Minga et al., 2015 which gave a diagnostic specificity of 99.4% and a diagnostic sensitivity of 64.00%. Sample size at the required absolute precision level for sensitivity was calculated by applying Buderer's formula (Buderer, 1996).For sample size calculation, an estimate of specificity of 95% and a precision of 5% within the 95% confidence level was considered. In addition, a prevalence of 50% as recommended in outbreak cases was used (Buderer, 1996). From this a total of 176 cattle were used from which 176 sera were obtained for NSP ELISA test. 176 tissues/ swabs were obtained for nucleic acid tests of real time RT-PCR as the gold standard (Office International des Epizooties (OIE), 2008) and gel based PCR. The sensitivity, specificity, likelihood ratios and disease prevalence values of the two assays relative to the real time PCR as the OIE recommended gold standard(Office International des Epizooties (OIE), 2008) were established.

d) Sample collection

Samples were collected from Mbale and Budaka Districts of Eastern Uganda during the 2014-2015 foot-and-mouth disease outbreak in Uganda as previously described in our study (Kafeero et al., 2016). Briefly, samples were collected from cattle with clinical signs, those which had healing lesions in the mouth, dental pad or on the feet and the asymptomatic animals in same kraals/ from the same farmer. Three types of samples were collected from animals; epithelial tissues

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(ETs), oral swabs (OSs) and blood. The ETs were obtained from animals with vesicles in the mouth, feet or teats. The OSs were obtained from animals with no clinical signs but sharing the same kraal with those having clinical signs. Blood was obtained from all the study animals from which serum (S) sample was also obtained. Exclusion criterion involved cattle from kraals with no any animal having clinical signs. These were taken as the non-cases.

After the identification of the animal as a case, it was restrained and blood was collected from either the caudal vein or the jugular vein into red top vacutainers by a trained technician using disposable vacutainer needles and given a field identification number. Blood was left to stand at the ambient temperature for serum to separate out and the red blood cells to sediment to the bottom of the tube and later separated in the evening of each day and aliquoted into crayon vials then kept on ice. Epithelial tissues and swabs were collected in the crayon vials containing virus transport medium PBS/Glycerol, given a field identification number and kept in liquid nitrogen. The date of sample collection, district, county, sub-county, parish, GPS number, type of sample collected as well as the presence of clinical signs were all recorded in the field book. All samples were transported to the virology laboratory, College of Veterinary Medicine Animal Resources and Bio security, Makerere University. The tissues/ swabs were kept at -80°C while the serum was kept at -20°C pending further use.

A total of 176 cattle were used in this study. From all animals (n=176), blood to be used for obtaining serum (100%) was obtained. From 30 animals (n=30) epithelial tissues (17.05%) were obtained. From 148 animals (n=146) oral swabs (82.95%) were obtained (Table 1). Serum was used for serological test using the NSP ELISA while swabs and epithelial tissues were used for molecular assays of real-time PCR and conventional PCR.

Table 1 : Total number of samples and sample type collected.

Sample type	Number of Sample (%)
Serum	176 (50%)
Epithelial Tissues	30 (8.5%)
Oral Swabs	146 (41.5%)
Total	352 (100%)

All the epithelial tissue, ET (n=30) and oral swabs from the dental pads, OS (n=146) were used for molecular diagnosis while all the sera samples (n=176) were used for serological tests using the NSP ELISA.

e) The RNA extraction

Total RNA was extracted from 140 µl original epithelial tissue/ swab suspension using Qiagen RNA extraction kit following the manufactures instructions as described in our previous study (Kafeero et al., 2016). Briefly, 140 µl of original epithelial tissue/ swab suspension was added to 560µl Buffer AVL- carrier RNA in the micro centrifuge tube, vortexed for 15 sec to mix and then incubated at room temperature (25°C) for 10 minutes. The tube was briefly centrifuged to remove drops from the inside of the lid, then 560µl of ethanol (96%) was added to the sample and mixed by pulse-vortexing for 15 seconds followed by brief centrifuging to remove drops from the inside lid. Then 630µl of the solution were applied to the QiAmp Mini column in a 2ml collection tube and centrifuged at 6000xg (8000rpm) for 1minute and the filtrate discarded. This procedure was performed twice. Then 500µl of Buffer AW1 was added and centrifuged again at 6000x (8000 rpm) for 1 minute. The filtrate was discarded and the column was placed in a fresh 2ml collection tube. Then 500µl of buffer AW2 were added to the column then centrifuged at 20,000 X g (14,000 rpm) for 3 min and the filtrate was discarded. Then 65 µl of Buffer AVE was added to the column, equilibrated at room temperature for 1 minute then centrifuged at 6000 X g (8000 rpm) for 1 min. The RNA samples were stored at -80°C until required for RT-LAMP and conventional RT-PCR.

f) The cDNA synthesis

This was synthesized using the Invitrogen superscript First-Strand cDNA synthesis kit following the manufacturer's instructions as described in our previous study (Kafeero et al., 2016). Briefly 2µl of 10X RNA primer mix, 0.8µl of 25X dNTPs, 2 µl of 10X RT buffer, 1µl of RNase inhibitor, 3.2µl of RNase free water and 1 µl of Supperscript III Reverse Transcriptase to a 0.5 ml microcentrifuge tube to a total volume of 10 µl. The mixture was vortexed briefly to mix then placed on ice. Then 10µl of RNA sample were dispensed to the reaction tube to make up the total reaction volume of 20µl. The mixture was incubated in a thermal cycler at 42°C for 2 hours followed by termination of the reaction at 80°C for 15minutes. The mixture was chilled at 4°C for 30 minutes then transferred to ice and 1 µl of RNase H added followed by incubation at 37°C for 20minutes to degrade the RNA template leaving only a single stranded DNA product. The cDNA was stored at -80°C until required for PCR and LAMP (Kafeero et al., 2016).

g) Real time RT-PCR reaction

In this study, the primers and probe previously described by Callahan *et.al* (2002) that detect the 3D RNA polymerase encoding gene were used as described in our earlier study (Kafeero et al., 2016). Forward Primer: 5'-ACTGGGTTTTACAAA CCT GTGA-3' Reverse Primer: 5'-GCG AGT CCT GCCACGGA-3' 3D

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Probe: (5'-FAM-TCC TTT GCA CGC CGT GGG AC-TAMRA-3'). This probe labeled with 6- (FAM) at the 5' end and the quencher tetramethylrhodamine (TAMRA) at the 3'end in Real-time RT-PCR reaction detects the $3D^{pol}$ gene sequence in all the FMDV serotypes.

The rRT-PCR reaction was based on one-step procedure combined with reverse transcription and Real-time assay. Therefore Real-time assay was carried out by Superscript III/Platinum Taq one-step rRT-PCR kit (Invitrogen). The composition of the 25 µl reaction/ Master Mix for the One-Step rRT-PCR included the following: 12.5 µl 2x- reaction buffer, 2.0 µl (10 pmol/µl) of each of the forward and reverse primer, 1.5 µl (1.5 µl) of the probe, 5.0 µl extracted RNA, 0.5 µl Superscript 111 RT/Platinum Taq mix, 1.5 µl of molecular grade H_2O. The amplification was done at the following temperature cycle: Reverse transcription (one cycle), 48 °C for 30 minutes, the initial denaturing (one cycle), 95 °C for 10 minutes; then 40 cycles consisting of 95°C for 15 seconds and 60°C for 1 minute and 72°C for 30 seconds. Negative and positive controls were included in each run. PCR amplification was carried out in the thermal cycler Rotor- Gene Q (Qiagen, German)

h) The PCR reaction

The PCR was carried out as previously described by (Moniwa, Clavijo, Li, Collignon, 2007) using primers designed to target the 3D polymerase encoding gene; forward primer: 5′ CACTTCCACATGGA TTATGGAACTG-3′ and the reverse primer: 5′ -ACATCT GAGGGATTATGCGTCAC-3′ ; Gene bank accession number JF749843 that amplified the 260 bp fragment of the highly conserved RNA polymerase (3D) gene of FMDV. Briefly, the 25 µl reaction mixture composed of 12.5 µl 2X TaqMan Universal Master Mix, 1 µl of each of the forward primers and reverse primers , 5.5 µl of PCR grade water and 5 µl of cDNA template. Negative control (nuclease free water) and positive control (field isolate) were included in each run. The reactions were carried out in an HBA Cycler machine (Mj Research Inc. USA). The following conditions: 95°C for 10 min for Taq man polymerase activation, 95°C for 15 sec for denaturation, 58°C for 30 sec annealing , 72°C extension. These three steps were repeated for 35 cycles and a subsequent hold temperature of 12°C was used.

i) NSP ELISA assay

All sera were screened for antibodies against FMDV nonstructural proteins using Prio CHECK®FMDV NS kit (PriomicsLelystad B.V, The Netherlands). The Prio CHECK®FMDV NS kit is a blocking ELISA that detects antibodies against the non-structural 3ABC protein of FMDV of all the seven serotypes. The test plates are coated with 3ABC specific monoclonal antibody (mAb) followed by incubation with antigen (3ABC protein). Hence test plates of the kit contain FMDV NS antigen captured by the coated mAb. The Prio CHECK®FMDV NS kit detects FMDV infected animals independent of the serotype that has caused the infection and independent of the fact that the animal is vaccinated or not.

Standard protocols and procedures were followed according to manufacturer's instructions. Briefly, 80 µl of ELISA buffer were dispensed to all wells, 20µl of Negative Control to wells A1 and B1, 20µ l of Weak Positive Control to wells C1 and D1, 20µl of Positive Control to wells E1 and F1 and 20µl of test samples to the remaining wells. Test Plate was sealed using the enclosed plate sealers and shaken gently then incubated overnight (16hours) at room temperature (25°C).The Test Plate were emptied after the incubation period and washed 6 times with 250µl washing solution (200x) made to a working solution (1x) with de-mineralized water using a micro plate washer (Mrc scientific, Marty Enterprises ltd, Nairobi, Kenya). 100 µl of diluted conjugate was dispensed to all wells and incubated at room temperature for 60minutes at room temperature (25°C).The Test Plates were emptied after the incubation period and washed 6 times with 250µl washing solution using the plate washer as previously described. Then100 µl of Chromogen; tetra methyl benzidine (TMB) Substrate were dispensed to each of the wells and incubated for 20 minutes at room temperature (25°C) .Then 100µl of Stop Solution was dispensed to each of the all wells.

j) Measurement of the optical density (OD) of the samples

The optical densities (OD) of the wells at 450nm were measured within 15minutes after colour development stopped using Multiskan Ascent spectrophotometer (Thermo lab systems OY UK).

The mean OD_{450} value of wells A1 and B1 (OD450 max) for negative control was calculated as;

$$\left(\frac{\text{ODA 1}\times\text{ODB 1}}{2}\right) = OD_{450}max$$

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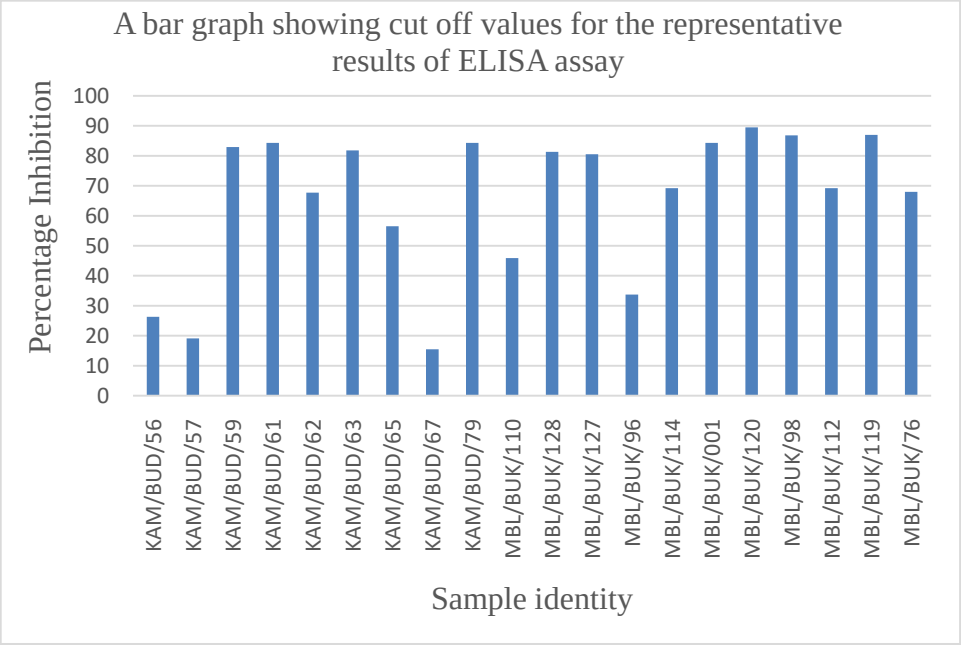

Figure 1 : Representative NSP 3ABC FMDV ELISA results: Positive samples represented by PI ≥50% while negative samples by PI < 50%

The OD 450 values of all samples were expressed as percentage inhibition (PI) relative to the OD450 max.

$$PI = 100 - \left(\frac{OD450\ test\ sample}{OD450\ max}\right) \times 100$$

PI< 50% was interpreted as negative while P≥ 50% was positive.

k) Detection of amplification products

i. *Real time reverse transcription polymerase chain reaction (rRT-PCR)*

The PCR amplification was carried out in the thermal cycler Rotor- Gene Q (Qiagen, Germany). The successfully amplified target gave an amplification curve and the cycle threshold, Ct at which the target amplicon was initially detected above the background fluorescent levels as determined by the instrument software noted. Each rRT-PCR was perfomed minimally in duplicate and the mean Ct value with standard deviation reported.

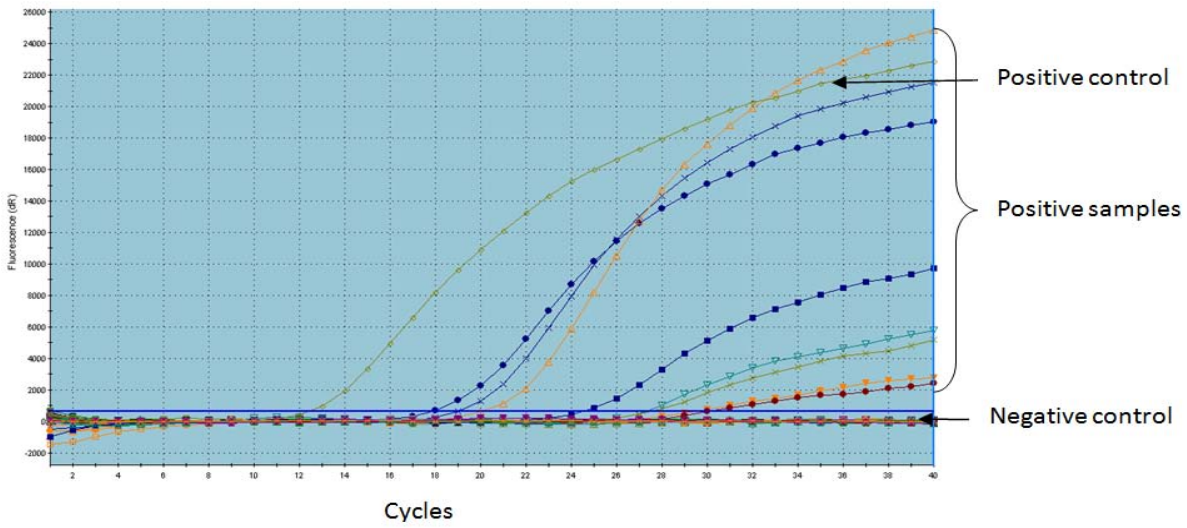

Figure 2 : Real time representative results showing amplification curves: The positive control (field isolate), negative control (Molecular grade water) and positive sample are indicated.

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The Ct values in the range ≥40.0 indicated a negative sample and Ct values < 40 indicated positive sample (Figs 2 & 3). In all cases, the positive control gave the minimum Ct value and the negative control gave no Ct.

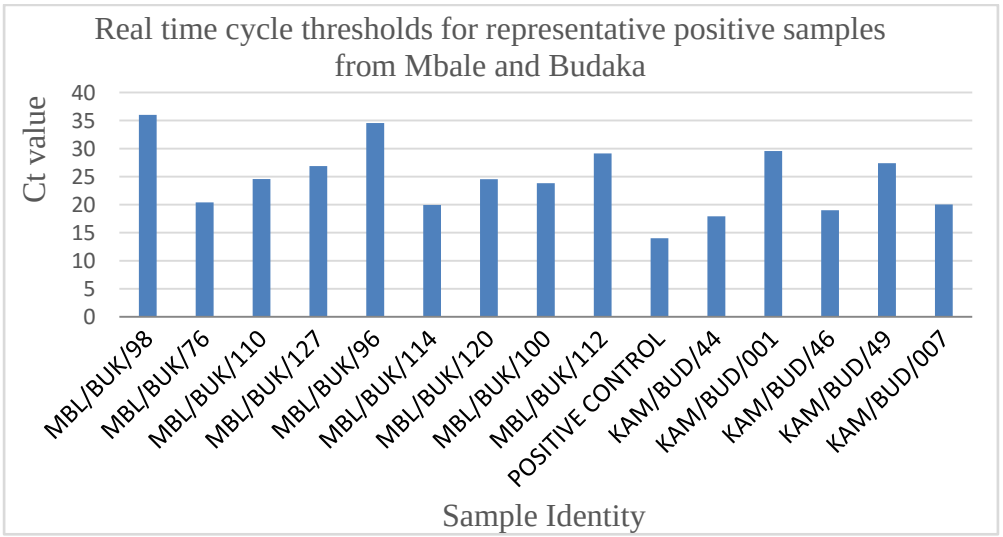

Figure 3 : Representative real time results with Ct values ≤ 40 showing positive animals for FMDV.

ii. *Reverse transcription polymerase chain reaction (RT-PCR)*

The 2 µl of the reaction mixture was electrophoresed on a 2% agarose gel electrophoresis after ethidium bromide staining under UV light using a ΦX174 marker (Amersham Biosciences, UK) to determine the size of the PCR product.

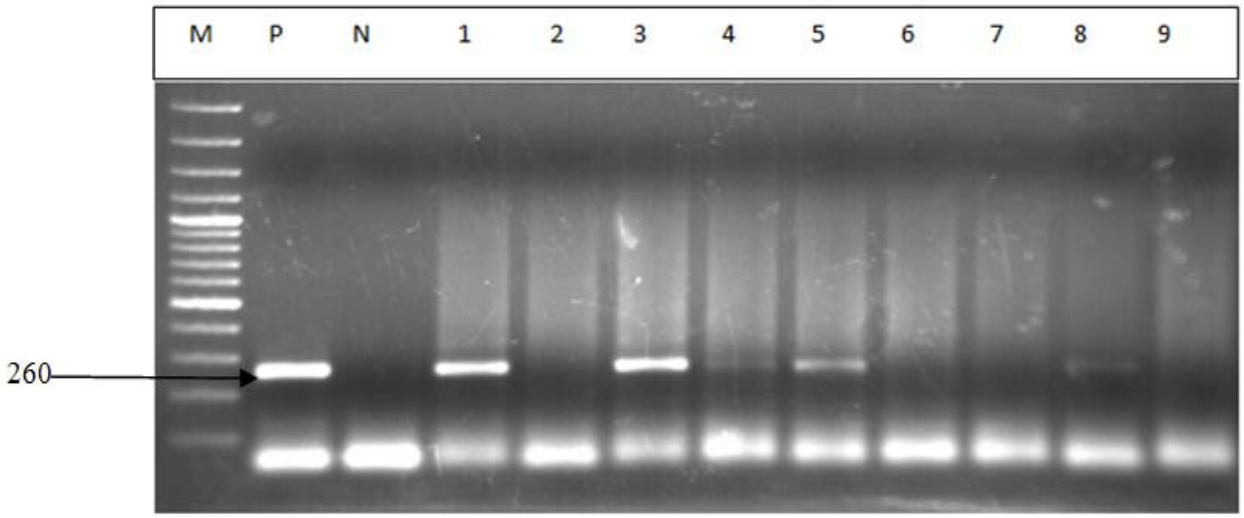

Figure 4 : Conventional PCR representative gel for FMD virus detection

A 2% agarose gel electrophoresis of PCR products: Lane M, DNA 2000bp marker (Invitrogen), lane P is for positive control (field isolate), lane N is negative control (nuclease free water), lanes 1-9 FMDV samples. Lanes 1, 3, 4,5and 8, are positive samples while lanes 2, 6, 7 and 9are negative samples

Positive samples and the positive control gave bands corresponding to the 260bp (Fig. 4) as determined from the marker since it is the size of the 3D pol gene. Negative samples gave no bands.

l) Data analysis

Every sample was tested twice by each of the methods and in case of a disagreement; the test was repeated for all the three assays to come up with the final result. Sensitivity and specificity of each test was then determined as percentages with 95% confidence intervals (CIs). The two tests were then each compared to the reference test/gold standard (rRT-PCR) using Fisher's exact test. The sensitivities and specificities of each test compared to the gold standard were determined. Kappa values to assess the level of test agreement were also determined. All analyses were done at 95% CI.

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III. Results

Table 2 : Summarizes results of two molecular assays of conventional PCR and real time PCR and, the NSP ELISA assay using the Prio CHECK®FMDV NS kit for all the 176 cattle samples.

NSP ELISA	Conventional PCR	Real Time PCR	Number of Cows
Positive	Positive	Positive	24
Positive	Positive	Negative	00
Positive	Negative	Negative	50
Negative	Negative	Negative	92
Positive	Negative	Positive	06
Negative	Positive	Negative	00
Negative	Negative	Positive	02
Negative	Positive	Positive	02

A total of 24 of the 176 cattle tested positive by all the three assays of conventional PCR, real time quantitative PCR and NSP ELISA. A total of 92 cattle tested negative for all the three assays. Real time quantitative PCR identified 34 animals as being positive with FMDV RNA.

The NSP ELISA assay identified 80 out of the 176 animals as positive of which only 30 animals were also positive by the gold standard and 50 negative by the gold standard (Tables 2,3 and Figs 1, 2, 3) giving a diagnostic sensitivity of 37.50% (95% CI=26.92% - 49.04%) and a specificity of 95.83% (95% CI= 89.67% - 98.88%). The RT-PCR assay also identified 24 animals as positive out of the 34 animals identified as positive by real time PCR and missed out 8 animals (Tables 2,3 and Fig. 4) giving a diagnostic sensitivity of 100% (95% CI = 86.77% - 100.00%) and a specifiity of 94.67% (95% CI = 89.76% - 97.67%). These results for both assays NSP ELISA and RT-PCR were statistically significant (P< 0.0001) when analyzed by Fisher's exact test.

Table 3 : Sensitivity and specificity of conventional PCR and one NSP ELISA assays for identification of foot-and-mouth disease virus in serum and swab/ tissue samples from cattle in Budaka and Mbale districts of Eastern Uganda.

Diagnostic Assay	Medium Sensitivity	95% Confidence internal		Medium Specificity	95% Confidence interval	
		Lower	Upper		Lower	Upper
NSP ELISA	37.50%	26.92%	49.04%	95.83%	89.67%	98.85%
RT-PCR	100.00%	86.77%	100.00%	94.67%	89.76%	97.67%

The study cattle FMDV prevalence (Table. 4) was estimated at 45.45% (95%CI=37.95% - 53.12%) by NSP ELISA and 14.77% (95%CI=9.88%-20.89%). The corresponding medium Positive likelihood ration (Table. 4) was 9.00 with a 95% credible interval of 3.31 to 30 for NSP ELISA and 18.75 for RT-PCR at a 95% credible interval of 9.55 to 36.80. Both likelihood ratios show that the test result is associated with the disease with RT-PCR showing a twice chance of post test probability of the disease (Table.4). The kappa value for agreement between RT-PCR and gold standard, test real time PCR was 0.84 (95% CI=0.733 – 0.947) at a standard error (SE) of kappa of 0. 055 showing a very good agreement between the two assays. On the other hand the kappa value for agreement between NSP ELISA and real time PCR assay was 0.35 (95% CI = 0.231 – 0.469) at a standard error (SE) of kappa of 0.061 showing a fair agreement.

Table 4 : Disease prevalence and positive likelihood ratio of conventional PCR and NSP ELISA assays for foot-and-mouth disease virus in cattle from Budaka and Mbale districts of Eastern Uganda

Diagnostic Assay	Medium Disease Prevalence	95% Confidence interval		Medium Positive likelihood ratio	95% Confidence interval	
		Lower	Upper		Lower	Upper
NSP ELISA	45.45%	37.95%	53.12%	9.00	3.31	24.47
RT-PCR	14.77%	9.88%	20.89	18.75	9.55	36.80

IV. Discussion

The aim of this study was to compare the sensitivity and the specificity of the NSP ELISA and conventional PCR which are the commonly used assays in the detection of FMD virus in Uganda (Mwiine *et al.,* 2010, Kasambula, 2011) using real time PCR as the gold standard (Office International des Epizooties (OIE), 2008). Previous studies by Saliki, 2000 have shown that

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disease recognition is essential for any disease control program. This is again paramount in the control of FMD due to the several serotypes and topotypes causing clinically indistinguishable disease (Vosloo et al., 2002).

In the present study, the results of RT- PCR and NSP ELISA were compared with real time PCR as the gold standard. The ELISA results indicated more infected animals than all the three assays on samples from the same animals. It is noted that 24 (13.64%) of the 176 cattle examined were positive on all the three techniques. However, ELISA positive were 80 (45.46%) and ELISA negative were 96 (54.54%) (Table 2, Fig.1) whereas the RT-PCR positive 26 (14.77%) and RT-PCR negative were 150 cattle (85.23%) (Table 2, Fig.3). This gave FMD virus NSP ELISA sensitivity of 37.50% and specificity of 95.83% as well as the FMD virus RT-PCR sensitivity of 100% and a specificity of 94.67%. The FMD virus NSP ELISA sensitivity in the current study was lower than the sensitivity in the earlier study by Minga et al., (2015) which gave a sensitivity of 64.00%. However the specificity in our study was almost consistent with that identified by Minga et al., (2015) of 99.40%. On the other hand, the FMD virus RT-PCR gave a specificity and a sensitivity of 100.00% and 94.67% respectively consistent with the earlier findings by Moniwa M, Clavijo A, Li M, Collignon B, (2007).

The high ELISA positive in this study is not surprising since it has been explained in earlier studies by Alexandersen *et al.,* (2003). Initial virus multiplication occurs in the vesicular epithelium and mucosal swabs in the five days after infection. Later the antibodies remain in plasma for several weeks, or months sampling could have been done in this time when the antibodies have remained in the plasma. Secondly, the high false positives by antigen ELISA assay been explained in earlier studies by Ma *et al.,* (2011). According to their work on overview of ELISA techniques for FMD diagnosis," no single ELISA technique can differentiate infected from vaccinated animals with confidence. This is aggravated by the use of non-purified vaccines in Eastern Africa which elicit antibodies against NSPs increasing chances of false positive (Ayebazibwe, Mwiine, Balinda, Jornehoj, & Alexandersen, 2012). In addition antibodies agaist NSPs do not appear until 8-9 days after infection (Lu et al., 2007) increasing chances of false negative. Consequently to be effective, NSP ELISA should be used for sera sampled in late sub-acute or even under chronic or persistent FMDV infection. Fortunately or un fortunately the antibodies against NSP persist for long post infection and therefore NSP ELISA cannot be used with absolute confidence to differentiate new and previous infection (Sørensen et al., 1998). This is consistent with the findings of the current study. This posits a challenge for FMD diagnosis in our country where NSP ELISA is the most commonly used assay for routine detection of FMD in cattle and other domestic ungulates (Namatovu et al., 2013) due to its simplicity. Conventional PCR though it has demonstrated higher sensitivity and specificity compared to NSP FMD virus ELISA both in earlier studies by Moniwa M, Clavijo A, Li M, Collignon B, (2007) and in our study. However in our country, the RT-PCR for foot-and-mouth disease is restricted to research institutions but in national reference laboratories NSP ELISA is the most commonly used as underlined in the previous study by Namatovu et al., (2013)

V. Conclusions and Recommendations

Our study compared the sensitivity and specificity of the two commonly used assays of NSP ELISA and gel based PCR for the detection of FMD in our country using real time PCR as the gold standard. The NSP ELISA assay has demonstrated a high false positive rate compared to gel based PCR using real time PCR which is recommended as the gold standard in countries whose biosafety levels do not permit them to perform virus isolation including Uganda. The conventional PCR demonstrated a higher sensitivity and specificity as compared to NSP ELISA but it uses sophisticated equipment and requires special training of the laboratory staff, its use for routine screening is not practical. So in Uganda, focal screening of FMD is based on NSP ELISA nearly in all regional and national reference labs due to its simplicity and its ability to screen large volumes of samples. This puts FMD diagnosis in our country in an empirical dilemma yet FMD is a highly contagious disease and its management is contingent upon accurate and timely diagnosis. The high frequency of the misclassification of cattle when using NSP ELISA suggest that FMD prevalence estimates based on NSP ELISA may be inflated, therefore confirmation by nucleic acid techniques should be the priority in national referral laboratories. We recommend the use of RT-PCR in the national reference laboratories for foot-and-mouth disease virus for confirmation, genotyping and to justify fresh infection, otherwise the NSP ELISA can be used for routine screening. We further recommend that more studies be done using large samples to improve on the accuracy of the findings. The scope of the sample types can also be extended to oral pharyngeal fluids in asymptomatic animals. Finally we recommend that vaccine strains should be matched with field strains and purified vaccines should be used to reduce on the false positive rates and hence more reliable results.

Conflict of interest

We declare that we have no competing interests in regards to the authorship of this article or its publication.

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References Références Referencias

1. Alexandersen, S., & Mowat, N. (2005). Foot-and-mouth disease: host range and pathogenesis. *Curr Top Microbiol Immunol*, *288*, 9–42.
2. Alexandersen, S., Quan, M., Murphy, C., Knight, J., & Zhang, Z. (2003). Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentary infected with foot-and-mouth virus. *Journal of Comparative Pathology*, *129*, 268–282.
3. Anderson, E., Anderson, J., Doughty, W., & Drevmo, S. (1975). The pathogenicity of bonine strains of foot-and-mouth disease virus for impala nd wildbeast. *J Wild Dis*, *11*(2), 248–255.
4. Atuhaire, D., Afayo, M., Ochwo, S., Katiti, D., Mwiine, F., Nanteza, A., Ojok, L. (2014). Comparative detection of African swine fever virus by loop-mediated isothermal amplification assay and polymerase chain reaction in do mestic pigs in Uganda. *African Journal of Microbiology*, *8*(23), 2322–2328.
5. Ayebazibwe, C., Kirsten, T., Mwine, F., Muwanika, V., Ademun Okurut, AR Siegismund, H., Belsham, G., Alexandersen, S. (2010). The role of the African buffalos (Suncerus caffer) in the mantainance of foot-and-mouth disease in Uganda. *Veterinary Research*, (6), 54.
6. Ayebazibwe, C., Mwiine, F., Balinda, S., Jornehoj, K., & Alexandersen, S. (2012). Application of the ceditest (R) FMDV type O and FMDV-NS enzyme linked immunosorbent assay for detection of antibodies against foo-and-mouth disease virus in selected livestock and wild life species in Uganda. *J Vet Diagn Invest*, *24*, 270–276.
7. Balinda, S., Sangula, A., Heller, R., Muwanika, V., Belsham, G., Masembe, C., & Seigismond, H. (2010). Diversity and transboundary mobility of serotype O foot-and-mouth disease virus in East Africa: Implications for vaccination policies. *Infect Genet Evol*, *10*, 1058–1065.
8. Boothroyd, J., Highfield, P., Cross, G., Rowlands, D., & Lowe, P. (1981). Molecular cloning of foot and mouth disease virus genome and nucleotide sequences in the structural protein genes. Nature 290: 800–802. *Nature*, *290*, 800–802.
9. Buderer, N. (1996a). Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity. *Acad Emerg Med*, *3*, 895–900.
10. Buderer, N. (1996b). Statistical methodology: I. Incorporating the prevelance of disease into the sample size calculation for sensitivity and specificity. *Acad Emerg Med.*, *3* (9), 895–900.
11. Burrows, R Mann, JA and Garland, A. (1981). "The pathogenesis of natural and simulated natural foot-and-mouth disease infection in cattle." *Journal of Comparative Pathology*, *91*(4), 599–609.
12. Diego, D. M. E., Brocchi, D., Mackay, & De Simone, F. (1997). The nonstructural polyprotein 3ABC of foot and mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. *Arch. Virol*, (142), 2021–2033.
13. Hopkins, H., González, I. J., Polley, S. D., Angutoko, P., Ategeka, J., Asiimwe, C., … Bell, D. (2013). Highly Sensitive Detection of Malaria Parasitemia in a Malaria-Endemic Setting : Performance of a New Loop-Mediated Isothermal Ampli fi cation Kit in a Remote Clinic in Uganda, *208*, 645–652. http://doi.org/10.1093/infdis/jit184
14. Kafeero, H., Frank, M., Mwiine, N., Kalenzi, D., Sylvester, A., & Nanteza, O. A. (2016). " International Journal of Biotechnology and Food Science" Comparative detection of foot-and-mouth disease virus by reverse transcription loop-mediated isothermal amplification assay and real time polymerase chain reaction in Uganda, *4* (March), 22–33.
15. Kasambula etal (2012). Inter-epidemiologic molecular characterization of foot-and-mouth disease virus in Eastern and Northern Uganda between 2008 and 2009. *Un Published*, 11–12.
16. Kasambula, L., Belsham, G., Siegismund, H.R Muwanika1, V., & C, A.-O. A. M. (2012). Serotype Identification and VP1 Coding Sequence Analysis of Foot-and-Mouth Disease Viruses from Outbreaks in Eastern and Northern Uganda in 2008/9. *Transboundary and Emerging Diseases*, *29*(4), 323–330.
17. Lu, Z., Cao, Y., Guo, J., Qi, S., Li, D., Zhang, Q., Xie, Q. (2007). Development and validation of a 3ABC indirect ELISA for differentiation of foot-and-mouth disease virus infected from vaccinated animals. *Vet Microbiol*, *125*, 157–169.
18. Ma, L., Zhang, J., Chen, H., Zhou, J., & Yong, S. (2011). An overview on ELISA techniques for FMD. *Virology Journal*, *8*.
19. Margo, E Chase-Topping Handel, I., Bartlomiej, M Bankowski Juleff, N., Deb, G., Cox, S. J., Windsor, M. A., Reid, E., Woolhouse, M. E. (2013). Understanding foot-and-mouth disease virus transmission biology: identification of the indicators of infectiousness. *Veterinary Research*, *44*, 48.

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20. Matovu, E., Kuepfer, I., Boobo, A., Kibona, S., & Burri, C. (2010). Comperative detection of Trypanasomal DNA by Loop-mediated Isothermal Amplification and PCR from Flinders Technology Associates Cards Spotted with patient blood. *Journal of Clinical Microbiology*, *48*(6), 2087–2090.
21. Minga, Y., Satya, P., Tim, S., Kate, H., Lauro, V.-S., & Alfonso, C. (2015). Development of a competitive ELISA for the detection of antibodies against the 3B protein of foot-and-mouth disease virus. *Vaccine Immunol*.
22. Moniwa M, Clavijo A, Li M, Collignon B, K. P. (2007a). Perfomance of a foot-and-mouth disease virus reverse transcription polymerase chain reaction with amplification controls between three real-time instruction. *J Vet Diagn Invest*, *19*, 9–20.
23. Moniwa M, Clavijo A, Li M, Collignon B, K. P. (2007b). Performance of a foot-and-mouth disease virus reverse transcription-polymerase chain reaction with amplification controls between three real-time instruments. *J Vet Diagn Invest*, *19*, 9–20.
24. Mori, Y., Nagamine, K., Tomita, N., & Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Biophys, Res. Commun*, *289*, 150–154.
25. Mwiine, F., Ayebazibwe, C., Olaho-Mukani, W Alexandersen, S., Balinda, S., Masembe, C., Ademun Okurut, A., Tjornehoj, K. (2010). Serotype specificity of antibodies against foot-and-mouth disease virus in cattle in selected districts in Uganda. *Transboundary and Emerging Diseases*, *57*, 365–374.
26. Nagamine, K., & Hase, T Notomi, T. (2002). Accelerated reaction by loop mediated isothermal amplification using loop primers. *Mol.cell Probes*, *16*, 223–229.
27. Namatovu, A., Sebenzia, N., Tjornehoj, K., Dhikusooka, M., Muwanika, V., Siegismund, H., & Ayebazibwe, C. (2013). Laboratory Capacity for dianosis of foot-and-mouth disease in Eastern Africa: Implications for the progressive control pathway. *BMV Veterinary Research*, *9*, 19.
28. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated Isothermal amplification of DNA. *Nucleic Acids Res*, *28*, 63.
29. Office International des Epizooties (OIE). (2008). Manual of diagnostic tests and vaccines for terrestrial animals.
30. OIE. (2009). Foot-and-mouth disease. *In Mannual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Edn, Paris*.
31. OIE(2008). (2008). Foot-and-mouth disease, OIE mannual 2008. *In Mannual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals. Paris*, *6th editio*.
32. Paixao, T., Neta, A., Paiva, N., Reis, J., Barbosa, M., Serra, C., … Clarke, N. (2008). *Diagnosis of foot-and mouth disease by real time reverse transcription polymerase chain reaction under field conditions in Brazil. BMC Vet Res*.
33. Rweyemamu, M. (1982). Developments in the biochemical and immunoassays assessment of foot and mouth disease antigen, in proceedings of the international conference on the impact of viral disease on the development of Latin America Countries and Caribbean region. *Riode Janeiro Brazil*, *2*, 78–79.
34. Saliki, J. (2000). The role of diagnostic laboratories in disease control. *Ann NY Acad SCI*, *916*, 134–138.
35. Sørensen, K., Madsen, K., Madsen, E., Salt, J., Nqindi, J., & MACKAY, D. (1998). Differentiation of infection from vaccination in foot-and-mouth disease by detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. *Arch Virol*, *8*, 1461–1476.
36. Sutmoller. (1992). *Vesicular diseases in foreign Animal diseases, Foreign Animal diseases of the United states,*.
37. Syed, M. J., & Graham, B. (2013). Foot-and-mouth disease: past, present anf future. *Veterinary Research*, *44*, 116.
38. Vosloo, W., Bastos, A., Sangare, O., Hargreaves, S., & Thomson, G. (2002). Review of status and control of foot-and-mouth disease in sub-Saharan Africa. *Rev Sci Tech*, *21*, 437–449.

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