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Identification of Foot and Mouth Disease Virus Isolates Using Vp1 Gene Sequencing

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Abstract

Foot-and-mouth disease (FMD) is a severely infectious and economically devastating viral disease worldwide, which affects domestic animals with cloven hooves (artiodactyls) and more than 70 species of wild animals. The virus is highly variable with 7 serotypes and numerous subtypes. VP1 is the main immunogenic viral protein of FMDV and using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and nucleotide sequencing, it can be used to characterize type, subtype and determine genetic distances among circulating FMD viruses. The aim of the study was to use VP1 gene sequencing to identify FMDV isolates obtained from the Central Veterinary Laboratory, Harare, Zimbabwe. A total of 35 probang samples were collected from the virology section at CVL. The samples consisted of stored probangs collected from FMDV infected cattle in 2014 and 2015. The VP1 gene was successfully amplified by RT-PCR in16 samples. Only 9 samples that had strong PCR bands were sequenced and used to identify FMD viruses using similarity based annotation approaches. Out of the 9 PCR products sequenced, 7 VP1 sequences were submitted to GenBank and assigned the following accession numbers: KT879751, KT879752, KT879753, KT879754, KT879755, KT879756 and KT879757. Similarity based annotation using BLAST analysis revealed that 5 of the isolates (KT879751, KT879752, KT879753, KT879754 and KT879755) belonged to the FMDV SAT 2 serotype. The remaining 2 isolates (KT879756 and KT879757) belonged to the FMDV SAT1 serotype. Phylogenetic analysis of these sequences using MEGA 7 showed that the viruses formed 3 clusters based on the VP1 gene sequences, 1 for SAT1 and 2 for SAT2, which implied that the SAT 2 isolates belong to 2 distinct topotypes. Our findings indicate that both the SAT2 and SAT1 serotypes are in circulation in Zimbabwe and that VP1 gene based sequencing is a useful tool for detection and identification of FMDV isolates.

Key words: Foot and mouth disease virus, Zimbabwe, VP1, phylogenetic, sequencing

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1. INTRODUCTION

Foot-and-mouth disease (FMD) is a severely infectious and economically devastating viral disease worldwide, which affects domestic animals with cloven hooves (artiodactyls), such as cattle, pigs, goats and sheep as well as more than 70 species of wild animals, including the African buffalo (*Syncerus caffer*) (Kanwal et al., 2014). FMD is characterized by fever, lameness, and vesicular lesions on the tongue, feet, snout, and teats (Abubakar, Kanwal and Saeed 2012).The global impact of foot and mouth disease (FMD) is colossal due to the huge numbers of animals affected. This impact can be separated into two components: (i) direct losses due to high morbidity in adult animals and mortality in young stock, reduced milk production, treatment costs, loss of work efficiency in draught animals and changes in herd structure; and (ii) indirect losses caused by costs of FMD control, poor access to markets and limited use of improved production technologies (Knight-Jones and Rushton 2013).

Foot and mouth disease virus (FMDV) belongs to the genus *Aphthovirus* in the family *Picornaviridae* and possesses a single strand of positive-sense RNA genome of approximately 8500 bases surrounded by four structural proteins to form an icosahedral capsid which is non-enveloped. It contains a single open reading frame (ORF) which is flanked by 5′and 3′ untranslated regions (UTRs) and encodes the four structural proteins which form the capsid [1A (also known as VP4); 1B (VP2); 1C (VP3) and 1D (VP1)], and ten non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1-3, 3C, and 3D) (Domingo et al.,

2002). It has a high mutation rate shown to be between 10^{-5} and 10^{-4} base mis-incorporations per nucleotide site per genome replication because the viral RNA–dependent RNA polymerase lacks proofreading ability, this has resulted in 7 immunogenically distinct serotypes (O, A, C, Southern African Territories [SAT] 1, SAT 2, SAT 3, and Asia 1) and numerous constantly evolving variants showing a spectrum of antigenic diversity (Ayelet et al., 2009).

The best known FMDV protein is the VP1 (1D), its coding sequence is the most variable region of the FMDV genome; only 26% of its amino acids are universally conserved between serotypes (Jackson et al., 2007). Many of the residues known or suspected to be critical for cleavage or other functions are located within invariant sequence motifs, indicating that the critical function of those residues may be contextual and require other specific residues. VP1 is responsible for virus attachment and entry, protective immunity and serotype specificity. A major, non-essential immune-dominant site is located within the G–H loop of VP1 (Jackson et al., 2007). The VP1 is also the main immunogenic viral protein and using RT-PCR and nucleotide sequencing, it can be used to characterize type, subtype and antigenic variants among circulating FMD viruses (Knowles et al., 2007). Sequence analysis of the VP1 could be used to determine the relatedness of FMDVs and thus evaluate the likelihood that a vaccine will provide protective immunity to the vaccinated animals since most FMDV vaccines are developed targeting the VP1 (Chenwen et al., 2007). Vaccination is an effective way to control FMD; however, the protection conferred by vaccination or infection is usually serotype specific and sometimes incomplete within a serotype (Ayelet et al., 2009)

Knowledge of molecular structure and phylogenetic relationships of the FMDVs is paramount in defining their origins, their host associations and more so their ecology. This set of information has very important implications in designing future control strategies for the disease and even in determining transmission pathways (Cottam 2007).

FMD is endemic to sub-Saharan Africa; widespread outbreaks of clinical disease occur frequently (Vosloo et al., 2004). Of the seven serotypes (except Asia 1), six have reportedly occurred on the continent, and disease control becomes more complicated because of marked regional differences in the distribution and prevalence of various serotypes and intratypic variants (Knowles and Samuel 2003). In southern Africa, two distinct molecular epidemiological forms of the disease exist, which are the Southern African Territories (SAT) serotypes and the Eurasian or South American (A and O) serotypes (Ayelet et al., 2009).

FMD remains an important disease in Zimbabwe and Southern Africa and its economic impact is well noted (Perry et al., 2003). However little work has been done in the country on molecular characterisation of FMDV isolates, thus the study sought to add to the available literature and to provide valuable information to understand the genetics of the circulating FMDV strains. Comparison of VP1 coding sequences from isolates obtained during outbreaks provides evidence of relatedness between individual FMDV strains and hence the tracing of the spread and transmission of the virus from one region to another or across national borders. VP1 is the preferred genomic region for comparison of FMDV strains because of its significance in the analyses of serotype specificity.

Identification of the virus is crucial as such knowledge is fundamental for future control strategies such as specific and rapid diagnosis, and possible use in the development of recombinant vaccines. The aim of the study was to characterise the VP1 genes of FMDV isolates in Zimbabwe stored from the Central Veterinary Laboratory, Harare.

2. METHODOLOGY

2.1 Sample collection

A total of 35 stored probang samples that had been confirmed to be positive for FMDV were collected from the Central Veterinary Laboratory (CVL), virology section in Harare, Zimbabwe. The samples had been collected from different localities in Zimbabwe, including Bulawayo, Gweru, Gokwe, Mberengwa, Chipinge and Masvingo. All samples were from cattle and had been collected in 2014 (21 isolates) and 2015 (14 isolates). The samples were stored in phosphate buffer solution at -20°C until RNA extraction was performed.

2.2 RNA extraction

Total RNA was extracted from the probangs using the ZR Viral RNA extraction kit (ZYMO Research, USA) following the Direct-Zol RNA mini prep protocol according to the manufacturer's instructions after each sample (50 -100 mg) had been initially homogenized with a pestle and mortar under liquid nitrogen and placed in a 1.5 ml microcentrifuge tube.

2.3 RT-PCR amplification

Single tube RT-PCR was performed. The oligonucleotide primers for RT PCR and subsequent PCR amplification of the VP1 gene were; forward primer: CCACATACTACTTTTTGTGACCTGG, reverse primer: ACAGCGGCCATGCACGACAG. (Balinda et al., 2009).

The PCR reaction mixture composed of 10pmol/ µL of one of the forward and reverse primers, 0.2 µL 40 U Reverse trancriptase (ThermoFisher Scientific, USA), 0.2 µL 40U RNase Inhibitor (ThermoFisher Scientific, USA), 12.5 µL 2X TaqMan Universal Master Mix (ThermoFisher Scientific, USA), RNase free water and 5 µL of RNA in a 25 µL reaction volume. Negative controls comprised of a water control. The RT-PCR reactions were done under the following thermal cycling conditions: 48°C for 30 minutes for reverse transcription, 95°C for 10 minutes for TaqGold activation, 94°C for 60 seconds for denaturation, 60°C for 1 minute for annealing, 72°C for 2 minutes for strand extension. The three steps were repeated for 30 cycles followed by a final extension temperature of 72°C for 10 minutes and a subsequent hold temperature of 4°C. A Perkin Elmer GeneAmp PCR System 2400 thermocycler was used for all amplification reactions. The amplified products were then run along a 1.5 % ethidium bromide stained agarose gel with a 100 bp DNA ladder (Invitrogen, USA) in TBE buffer for 1hr at 100V/cm and then viewed and gel image captured using a Uvitec gel documentation system (Uvitec, UK).

2.4 DNA Sequencing

Sequencing of purified PCR products was performed at Inqaba Biotec, Pretoria, South Africa using an automated ABI3500XL Genetic Analyser (Applied Biosystems, Foster City, CA) and BigDye terminator v3.1 cycle sequencing kit (Thermo Fischer, USA according to the manufacturer's instructions. DNA sequence electropherograms were sent back by email for analysis. The sequences were analysed using Basic Local Alignment Search Tool (BLAST) in the NCBI databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was based on the Maximum Likelihood method (supporting bootstrap values from 100 replicates) based on the Tamura-Nei model (Tamura and Nei 1993) using the Molecular Evolutionary Genetics Analysis Software version 7.0 (MEGA 7) (Kumar et al., 2015).

3. RESULTS

3.1 RT-PCR of VP1 genes

A total of 35 FMDV probangs samples were collected from the virology section of the CVL, Harare. After RNA isolation RT-PCR was done to amplify the VP1 genes. RT-PCR of the VP1 gene resulted in 16 samples being successfully amplified. A total of 9 PCR amplicons that showed

strong bands were sent for sequencing. Amplicons with faint bands, though of the expected amplicon size (715bp), were not sent for sequencing. Two of the samples failed quality control checks. Sequencing was done for both the forward and reverse sequences of the genes. In total 14 sequences corresponding to the 7 VP1 amplicons were obtained.

3.2 Molecular characterization of VP1 genes

The 7 forward VP1 sequences were submitted to GenBank which assigned them accession numbers (KT879751 - KT879757). Table 1 shows information relating to the 7 submitted sequences. All sequences were subjected to BLAST search versus the GenBank database. The alignment results confirmed the sequences to be FMDV isolates. Alignment of the 7 sequences using BLASTn and BLASTx resulted in the identification of 2 possible serotypes circulating in Zimbabwe that is SAT 1 and SAT 2. The sequences were each compared against FMDV SAT 2 Zim/7/83 (accession number AF540910) and FMDV SAT 1 Zim/7/99 (accession number AF301439.1). Results of the sequence analysis revealed that KT879756 and KT879757, both samples being from Chipinge (Table 1) were closely related to each other and shared significant similarity at nucleotide level with FMDV SAT 1 Zim/7/99. KT879751, KT879752, KT879753, KT879754 and KT879755, samples from Gokwe, Mberengwa, Masvingo, Gokwe and Gweru respectively (Table 1) were closely related to each other and shared significant similarity at nucleotide level with FMDV SAT 2 Zim/7/83.

Table 1. **VP1 sequences submitted to GenBank. All FMDV isolates were collected in Zimbabwe**

Specimen number	Accession # (GenBank)	Collection date	Place of Origin	serotype
CVL1/15	KT879751	Jan 2015	Gokwe	SAT2
CVL8/15	KT879752	Jan 2015	Gweru	SAT2
CVL18/15	KT879753	Jan 2015	Gokwe	SAT2
CVL15/14	KT879754	Sept 2014	Mberengwa	SAT2
CVL17/14	KT879755	Oct 2014	Masvingo	SAT2
CVL10/15	KT879756	Feb 2015	Chipinge	SAT1
CVL111/15	KT879757	Feb 2015	Chipinge	SAT1

CVL - Central Veterinary Laboratory FMDV isolate
- number
SAT – Southern African Territories

3.3 Phylogenetic analysis of FMDV based on VP1 region sequencing

Phylogenetic reconstruction of the 7 VP1 sequences was done using MEGA 7 and yielded the phylogenetic tree shown in figure 1. The analysis involved 17 nucleotide sequences (7 from the current study and 10 reference sequences). The 10 reference sequences were obtained from GenBank and were all of FMDV, SAT 1 and SAT 2 isolates obtained in Zimbabwe. The derived phylogenetic tree had 3 main clusters. Cluster I had most of the SAT 2 isolates from the current study, cluster II had a reference SAT 2 isolate (AF540910.1) and one isolate from Gokwe (KT879753.1) and cluster III had all the SAT 1 isolates including those from the current study (Figure 1).

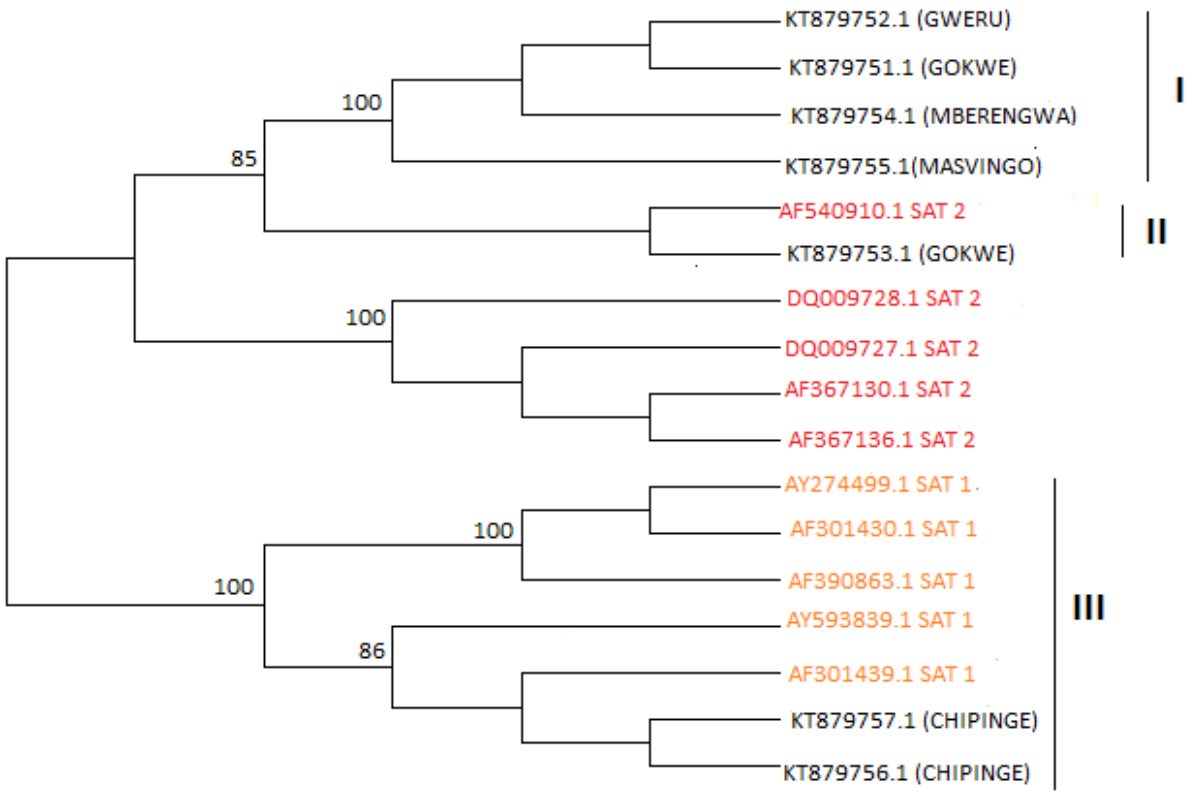

Figure 1. Phylogenetic tree of FMDV VP1 sequences after multiple sequence alignment using the Maximum Likelihood method, supporting bootstrap values from 100 replicates.

4. DISCUSSION

Of the 35 samples collected, only 16 (46%) were successfully amplified by RT-PCR. The low numbers of amplicons obtained through RT-PCR could have been due to several reasons. In this study the samples that were used were brought to the CVL between 2014 and 2015 and they were kept at -20°C instead of the recommended -80°C, in addition power cuts may have had a bearing on the overall quality of the samples. If a sample is collected during the early phase of infection (especially from vesicles), chances of viral amplification by RT-PCR are higher, but if the lesions get invaded by bacteria, or lesions start healing, the probability of obtaining the intact viruses from samples decreases drastically. Some samples could have been collected in the late phase of infection and the viral genome may have been degraded by bacterial RNAses and other degradative enzymes resulting in either weak or no detectable signals by RT-PCR (Al-Rodham 2014). Moreover, we may have missed detection of FMD viruses in some of the samples as conventional end point PCR is less sensitive than the real-time PCR that makes use of fluorescence dyes for detection (Reid et al., 2001).

Phylogenetic analysis of the VP 1 region of FMD viruses has been used extensively to investigate the molecular epidemiology of the disease worldwide, thus it was used to identify the FMDV isolates. These techniques have helped define genetic relationships between FMDV isolates and geographic distribution of lineages and genotypes; they have also helped establish genetically and geographically linked topotypes and to trace the source of outbreaks. FMDV topotypes are defined as geographically clustered viruses that form a single genetic lineage generally sharing >85% (O, A, C, and Asia 1) or >80% (SAT 1, SAT 2, and SAT 3)

nucleotide identity in the VP1-coding region (Knowles et al., 2009)

Nucleotide sequence comparisons conducted using BLAST searches indicated that the sequences obtained from the 2014/2015 FMD outbreaks in Zimbabwe had the greatest sequence similarity to FMDV isolates of serotype SAT 2 and SAT 1. Two samples from the 2015 outbreak mainly KT879756 and KT879757 which were from Chipinge (Table 1) were closely related to FMDV SAT 1 Zim/7/99 (accession number AF301439.1) (Figure 1). According to studies done by Bastos et al., (2001) this SAT 1 isolate originated from African buffalo (*Syncerus caffer*) in Zimbabwe. The SAT 1 Chipinge isolates were also closely related to another SAT 1 isolate (AY593839.1) that originated from kudu (*Tragelaphus strepsiceros)* in Zimbabwe (Carrillo et al., 2005). Although SAT 1 is not frequently reported in Zimbabwe it might be circulating within wildlife and infrequently transmitted to domestic animals. Limited genetic studies of SAT1 viruses have however indicated that these viruses appear to group on the basis of geographical origin (Bastos, 1998). The possibility of this is further supported as some antelope species, particularly impala and kudu have the ability to jump over high fences and to serve as an intermediary between infected buffalo and livestock. Risk analysis of the situation in Zimbabwe has shown that in certain circumstances, fences should be at least 2.4 m high to reduce the risk of such events (Sutmoller et al., 2000) but the majority are only 1.2 m high also over the past years due to economic meltdown and land redistribution some of the conservancy fences have been destroyed which served as a barrier between livestock and wildlife. Studies have shown that outbreaks in impala are derived from buffalo herds as demonstrated by sequence analysis of viruses isolated from outbreaks in impala and persistently infected buffaloes (Vosloo et al., 2001).

Five samples (KT879751, KT879752, KT879753, KT879754 and KT879755) from Gokwe, Mberengwa, Masvingo, Gokwe and Gweru respectively (Table 1) were closely related to each other as they formed two clusters as shown on Figure 1 and shared significant similarity at nucleotide level with a FMDV SAT 2 clone Zim/7/83 (accession number AF540910) which is a Zimbabwean vaccine strain. However KT879753 appeared more closely related to with the reference isolate (AF540910) and formed a separate cluster from the other SAT 2 isolates from this study. This implies that these samples share a geographically linked topotype since they shared > 80% of nucleotide identity in the VP1-coding region (Figure 1). This is possible since the geographical regions covered were mainly the Midlands and southern parts of Zimbabwe. Our findings indicate that both serotypes SAT 2 and SAT 1 are still present in the country, which is in contrast to CVL reports (unpublished) that only serotype SAT 2 is in circulation, though the study was consistent that SAT 3 is not in circulation since it was last detected in August 1989 (CVL unpublished). Occurrence of SAT 2 in these outbreaks further confirms serotype endemicity in sub-Saharan Africa as SAT 2 is most frequently associated with FMD outbreaks in southern and West Africa livestock especially cattle (Olabode et al., 2014).

The three SAT types differ from each other with regard to geographic distribution, infection rates in wildlife and involvement in FMD outbreaks of livestock (Bastos et al., 2001). This is reflected in the study as the 2 SAT types identified are geographically distributed within Zimbabwe [South-eastern region (SAT1), Midlands and Southern regions (SAT2)]. In addition, SAT 1 has the highest seroprevalence rate in the maintenance host, the African buffalo. Despite the high incidence of SAT 1 type virus in buffalo, this serotype has accounted for only 36% of the SAT-type outbreaks of FMD in cattle in southern Africa this century, with most outbreaks (48%) being caused by SAT 2 type viruses and SAT 3 accounting for only 16% of outbreaks (Bastos et al., 2001). In a study done by Bastos et al., (2003) of the 17 African countries included

in their study, 13 had a single SAT 2 topotype within their borders. However within some countries, notably Botswana, Zimbabwe and Senegal, there were at least two topotypes present. Despite the limited sample size in this study two different SAT 2 topotypes were identified (Figure 1).

4.1 Limitations

Potential biases in our study are related to the relatively small sample size of FMDV isolates used. More samples need to be sequenced to obtain a more holistic picture of the serotypes currently circulating in Zimbabwe.

4.2 Recommendations

As FMDV continues to spread in the country, further collection and analysis of samples, together with the improvement of the local epidemiological investigation of FMD outbreaks is recommended. Intensive phylo-geography studies should be carried out that will locate particular serotypes or topotypes that are responsible for the disease outbreak. This is a very important step in controlling the spread of the disease and also for the production of a specific vaccine which will cater for a specific topotype. The VP1 segment was used simply because it has been the most commonly sequenced section of the genome, but use of a larger part would be more suitable and probably more viable in the era of next-generation sequencing.

5. CONCLUSION

This study provides a glimpse into the molecular epidemiology of FMDV in Zimbabwe. Although only a limited number of *adhoc* samples were available for the sequence analysis, the present study provides a picture of the phylogenetic relationships of FMDV isolates currently in circulation in Zimbabwe. The identification of SAT 1 viruses is very significant, as most cases of FMDV continue to be attributed to only the SAT 2 serotype.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this paper.

Authors' contributions

This work was conducted by B.D, J.M and B.S at the NUST, Bulawayo and at CVL, Harare. B.D (NUST) carried out experimental work in the project and was involved in the project design. J.M (NUST) was responsible for the project design, was the principle supervisor responsible for the management of the project and the guidance of B.D in all aspects of the project. B.S (CVL) acted as co-supervisor and provided assistance with the isolates studied. All three authors contributed to the writing of this article.

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