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Molecular characterization of serotype O foot-and-mouth disease virus from pigs: Implications for multi-species approach to disease control in Uganda

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In Uganda, where foot-and-mouth disease (FMD) control is mainly done through vaccination, constant monitoring of outbreaks and phylogenetic studies are important in designing effective disease control strategies. These efforts however, have mainly concentrated on cattle since they are the main visible disease hosts. In this study, the molecular characteristics of the VP1 coding sequences of the pig-derived FMD virus and its phylogenetic relationship with other historical Ugandan FMD virus sequences are determined. Sixty seven samples were collected from three districts of south western Uganda and subjected to RT-PCR. Partial VP1 capsid protein coding sequences of 12 positive samples were amplified and sequenced using serotype specific primers. Eight sequences were successfully obtained, all of which were from domestic pigs. All samples were confirmed as belonging to serotype O using BLAST search. Phylogenetic analysis of the test sequences with selected sequences, showed a close relatedness (average of 3.77% pair-wise distance) to viruses isolated from central and western Uganda in the years 2005 and 2006 from cattle. These results show that domestic pigs in the western region of Uganda have been infected with the same circulating strain of FMDV and play a potentially important role in FMD maintenance and spread.

Key words: Foot-and-mouth disease (FMD), pig-derived FMDV sequence, persistence, domestic pigs.

INTRODUCTION

Foot-and-mouth disease is an acute vesicular disease in cattle, sheep, goats including pigs and all cloven-hooved animals, both wild and domestic (Thompson, 1995). The disease is caused by the foot-and-mouth disease virus (FMDV) which is a picornavirus belonging to the genus Apthovirus. The virus is a single stranded RNA positive sense RNA genome of about 8.3 kb in size (Quian et al., 2003). FMD causes an enormous burden on the livestock economy causing deaths in young animals and low productivity in adults (James et al., 2002). Its consequences were realised in 2010 in Korea where over 3.5 million animals were culled due to an out break of FMD and approximately 2.7 billion U.S dollars spent on control strategies (Insung et al., 2012). Other cases of economic burden

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caused by FMD were witnessed in the 2001 outbreak of FMD in the United Kingdom, which resulted in losses estimated at 3 billion Great Britain Pounds sterling (Thompson et al., 2002). In Uganda, FMD outbreaks still continue to ravage the country, with spontaneous annual outbreaks according to the Ministry of Agriculture Animal Industry and Fisheries (MAAIF). The 2007/08 and 2008/ 2009 Ugandan national budgets reported that a total of over 41 billion was spent on FMD control strategies for those years. Out of the seven FMDV serotypes known (O, A, C, Asia1, Southern African Territories (SAT) 1, 2, and 3), the most prevalent serotypes that have been recorded in Uganda are O and SAT2 (Balinda et al., 2010; Mwiine et al., 2010; Kasambula et al., 2011). Despite the enormous work that has been carried out on FMD in Uganda, there is still inadequate information on the FMD status in other domestic species, especially pigs. Most of the reported outbreaks on FMD have involved cattle and buffalos (Ayebazibwe et al., 2010) with a few studies having been carried out on sheep and goats (Balinda et al., 2009; Vosloo et al., 2002). In addition, during FMD outbreaks, quarantines and ring vaccinations which are used to limit the spread of FMD are enforced and emphasised mainly on cattle and cattle products while other domestic species and their products are left to move freely. Goats and sheep have been implicated in FMD epidemiology as they may act as FMDV carriers and if infected may not show clear signs and symptoms of the disease (Balinda et al., 2009). Notwithstanding, pigs have also been implicated in FMD spread, as they are said to exhale the largest amount of the virus under certain climatic conditions (Alexandersen et al., 2002). This paper therefore provides the first description of the partial VP1 nucleotide sequence (the most antigenic and important for vaccine design) of the pig-derived FMD viruses in Uganda. It also compares the test sequence with other sequences previously obtained from East Afri-ca and other neighbouring countries.

MATERIALS AND METHODS

Study area

The study was carried out in western Uganda in the districts of Kasese, Rakai and Mbarara. A total of 67 samples were collected comprising of 18 from Rakai, 22 from Mbarara and 27 from Kasese. Samples included oropharyngeal tissue from cattle, blood from pigs and lesion swabs from both pigs and cattle. The samples were collected in RNAlater (Qiagen, Germany), placed on ice and transported to the laboratory where they were then stored at -80°C until RNA extraction.

FMD virus RNA extraction

Total viral RNA was extracted using the QIAampviral RNA extraction kit (Qiagen, Germany) utilising the minispin columns. Generation of cDNA was performed using the Ready-To-Go You-Prime First-Strand Beads cDNA synthesis kit using random hexamer primers (Applied Biosystems,USA). In order to detect the presence of FMDV, the synthesised cDNA was subjected to a standard PCR targeting the 5'untranslated region (UTR) of the FMDV genome. The amplification reaction was performed using two specific forward and one reverse primer. The PCR was performed using a Multi-II PCR assay utilising 2X TaqMan Universal PCR Master Mix (Applied Biosystems, USA). Reactions were performed in final volumes of 25 µl using 2 ng of cDNA, 0.2 pmol of each primer. The PCR was carried out in an EppendorfMastercycler PCR machine, under the following conditions; 50°C for 2 min for UNG digestion, 95°C for 10 min for TaqGold activation, 95°C for 15 s for denaturation, 60°C for 60 s for primer annealing. These steps were repeated for 40 cycles and a subsequent holding temperature of 10°C. The PCR products when analysed produced a clear fragment of 96 bp on a 2% agarose gel.

FMDV VP1 amplification

Eight samples of the remaining cDNA that had tested positive in the above experiment were then subjected to another PCR where amplification was performed using serotype specific primers for serotypes O and SAT1 and SAT2, targeting the VP1 coding region of the FMDV genome (Kasambula et al., 2011). The reaction was carried out using aTaqMan Gold PCR kit (Applied Biosystems, Germany) and was performed in final volumes of 50 µl using 2 to 5 ng of cDNA, 0.2 pmol of each primer and 2.5 U AmpliTaq gold DNA polymerase, 200 µM of each dNTP and 1.5 mM $MgCl_2$. Temperature conditions were as for the previous PCR above. The PCR products were analysed on a 2% agarose gel using a molecular-weight marker ϕX174-RT DNA (Promega, Madison).

FMDV VP1 sequencing

The PCR products were purified and sent to Macrogen, Netherlands for automated Sanger sequencing. Sequencing was performed using the same specific PCR primers that were used in the serotype-specific PCR. The cycle sequencing employed the Big Dye Terminator version 3.1 kit (Applied Bio systems, Germany) and was run on an automated DNA sequencer (ABI Prism® 3700) for both the forward and reverse sequences.

Sequence analysis

The individual sequences were analysed using CLC DNA work bench 5.6.1 (CLC bio, Cambridge, MA). Using default parameters, the sequences were examined using BLAST search. Using the Neighbour-Joining tree to infer evolutionary history, a phylogenetic tree was drawn to elucidate the relationships of the sequences. MEGA5 was used to establish the sequence divergences between the study sequence and other FMDV related sequences from the GenBank.

RESULTS

Nucleotide sequence comparison

Eight sequences were successfully generated. All these sequences constituted a single haplotype represented by 18RAKAI (accession number KC987540). The nucleotide and translated amino acid sequences of the pig-derived sequences are shown in Figure 1. The partial VP1 coding region contained 423 nucleotides encoding 138 amino acid protein residues (Figure 1). A nucleotide sequence comparison conducted using the BLAST program with default search parameters indicated that 18RAKAI had the

```
  6 ttgtacctggaactggcagtgaaacacgagggcaatctcacttgg
    L  Y  L  E  L  A  V  K  H  E  G  N  L  T  W
 51 gtcccgaacggagcacccgaagccgcactggacaacaccaccaac
    V  P  N  G  A  P  E  A  A  L  D  N  T  T  N
 96 ccaacggtgtaccacaaggcacctctcactcgccttgcactgcct
    P  T  V  Y  H  K  A  P  L  T  R  L  A  L  P
141 tacaccgcaccacaccgcgtgttggcaaccgtgtacaacgggagc
    Y  T  A  P  H  R  V  L  A  T  V  Y  N  G  S
186 tgcaagtacagtggttccccaaccactaatgtgaggggtgacctc
    C  K  Y  S  G  S  P  T  T  N  V  R  G  D  L
231 caagtgttggcccagaaggctgagagaacactgcccacctccttc
    Q  V  L  A  Q  K  A  E  R  T  L  P  T  S  F
276 aactacggtgccgtcaaggccactcgggtgacagaactgctttac
  N  Y  G  A  V  K  A  T  R  V  T  E  L  L  Y
321 cgcatgaagagggctgaaacatactgcccccggcctcttttggcc
    R  M  K  R  A  E  T  Y  C  P  R  P  L  L  A
366 atccacccgagtgaagctagacacaaacaaaagattgtggcacct
    I  H  P  S  E  A  R  H  K  Q  K  I  V  A  P
411 gtcaaacaact 421
    V  K  Q
```

Figure 1. The nucleotide and translated amino acid sequence of the pig-derived sequence (18RAKAI). The amino acid residues are indicated below the nucleotide sequences by their single letter codes.

greatest sequence similarity to FMDV isolates of serotype O with nucleotide identity ranging between 97 and 87%. East African sequences were more similar (maximum identity ranging between 97 and 90%), while sequences from other countries were much less similar (with a modal maximum identity of 88%). It is therefore likely that the 18RAKAI belongs to serotype O.

The partial VP1 nucleotide sequences of selected FMDV-O serotypes isolated previously from Uganda, Kenya, Sudan, Tanzania, Mali, Ethiopia, Russia and Hong Kong were used to construct the FMDV-O partial VP1 sequence based similarity tree. From the analysis, 18RAKAI shared the greatest similarity at nucleotide level with a group of FMDV-O viruses isolated in 2005/2006 from central and western Uganda. Among the other isolates examined, 18RAKAI was less similar in nucleotide level to the current vaccine strain in use (O/K77/78) and formed an outgroup with FMD viruses isolated from Hong Kong and Russia (Table 1 and Figure 2).

DISCUSSION

Knowledge on molecular structure and phylogenetic relationships of the FMDVs is paramount in defining their origins, their host associations and more so their ecology. This cocktail of information has very important implications in designing future control strategies for the disease and even in determining transmission pathways (Cottam, 2007). The VP1 protein coded for by the ID region, is 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 (Bachrach, 1968; Knowles et al., 2007). It is important to note that the DNA sequences obtained in this

Table 1. The selected FMD serotype O virus sequences used for comparison in the study alongside their sequence divergences from the pig-derived FMDV sequence.

Accession number	Virus name	Serotype	Year	Country of origin	Sequence divergence with 18 RAKAI
JN974311.1	O/UGA/2009AMOLATAR	O	2009	UGANDA	0.0791
JN974312.1	O/UGA/2008KAMULI	O	2008	UGANDA	0.0761
JN974310.1	O/UGA/2009DOKOLO	O	2009	UGANDA	0.0703
HM756628.1	U25/06	O	2006	UGANDA	0.0323
EF611987.1	UGA/KSE/06	O	2006	UGANDA	0.0349
AJ296327.1	O/UGA/5/96	O	1996	UGANDA	0.2080
AY349950.1	UGA/11/02	O	2002	UGANDA	0.0875
HM756627.1	U/18/06	O	2006	UGANDA	0.0297
HM756625.1	U12/05	O	2005	UGANDA	0.0481
AY349956.1	UGA/7/03	O	2003	UGANDA	0.0872
HM756620.1	U13B/04	O	2004	UGANDA	0.1161
AY344620.1	UGA/11/00	O	2000	UGANDA	0.0614
FJ461344.1	KUMI	O	?	UGANDA	0.0759
EU919244.1	O/UGA/1/75	O	1975	UGANDA	0.1785
HM756604.1	U97/99	O	1999	UGANDA	0.2516
AY344631.1	UGA/9/78	O	1978	UGANDA	0.1906
AY344625.1	UGA/28/75	O	1975	UGANDA	0.1638
AY344621.1	UGA/12/76	O	1976	UGANDA	0.1681
HM7566607.1	U/20B/04	O	2004	UGANDA	0.1050
AJ296320.1	O/TAN/7/8	O	?	TANZANIA	0.1293
HM756607.1	K48/05	O	2000	KENYA	0.1312
HM776640.1	K32/08	O	2008	KENYA	0.1346
DQ165072.1	O/K77/78	O	1978	KENYA	0.2071
DQ165076.1	O/SUD/1/99	O	1999	SUDAN	0.2009
GU566054.1	O/SUD/26/2004	O	2004	SUDAN	0.2157
U566049.1	O/SUD/12/2004	O	2004	SUDAN	0.2157
FJ798127.1	O/ETH/2/2006	O	2006	ETHOPIA	0.2305
FJ798142.1	O/ETH/59/2005	O	2005	ETHOPIA	0.2388
DQ165074.1	O/MAL/1/98	O	1998	MALI	0.1271
EF614457.1	O/SKR/02	O	2002	RUSSIA	0.1881
AY317098.1	HKN/2002	O	2002	HONG KONG	0.2603

The genetic divergence analyses were conducted using the Kimura 2-parameter model (Kimura, 1980).

study were directly sequenced from swine tissue without any cell culture propagation. This eliminated any possibility of the virus genome mutation in order to adapt to the cell culture (Sobrino et al., 1983). Thus, the sequences obtained from this study are a true reflection of the FMD outbreaks that occurred during the study period.

The coding sequence of the pig-derived sequence was not in any way significantly different except for slight amino acid variations that were realised. Whereas SAT 2 viruses are characterised by the 'RGDR' motif within the VP G-H loop, the other serotypes (SAT1, SAT3 and Asia 1) including serotype O viruses have 'RGDL' motif where, instead of a positively charged arginine residue, have a non-polar leucineresidue. The intergrin receptor binding site motif 'RGDL' found within the VP1GH loop of the sequence was not affected by the amino acid variation, thus the region was conserved in the test sequence. The genetic divergence between the test sequences and other Ugandan sequences is shown in Table 1. The analyses show that sequences from the west and central isolated in 2005 and 2006 have a lower mean pair-wise sequence divergence (3.39%) which lies between 2 and 5% a value consistent with origin of the virus from within the same epizootic (Samuel et al., 1997). This emphasises that the sequences are very similar to each other and that they belong to the same lineage even after a space in time of four years. The neighbour joining tree further elucidates the closeness between the test sequence and the sequences obtained in 2005/6 since they cluster together forming a sub-clade. This further confirms that the test sequences and the 2005/06 sequences are from the same lineage. This therefore may mean that the emerging

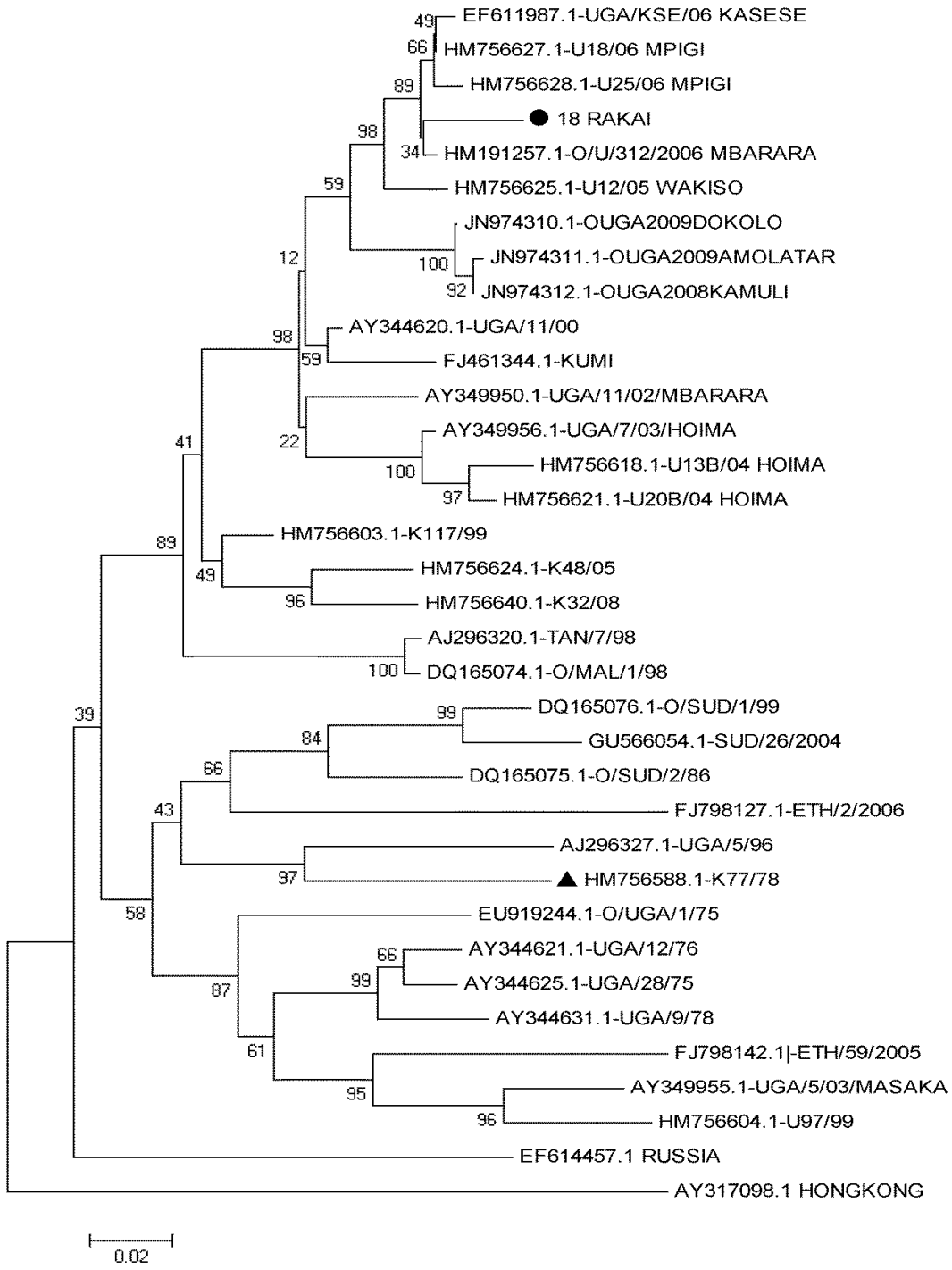

Figure 2. Phylogenetic relationships between the pig-derived sequence 18RAKAI (with round dot), vaccine strain K77/78 (triangle) and other FMD serotype O sequences

viruses in the western and central areas are not entirely new strains, emerging from a different lineage but that the same virus must have caused a spontaneous outbreak of FMD even after four years. The proximity of these districts (Rakai, Mbarara, Kasese, Mpigi and Kasese) to each other explains the re-emergence of a virus from the same lineage. Trade and movement of both people and animals from one district to another, also emphasises the probable explanation of the re-emergence of a virus of the same lineage within the region.

Observations from the sequence similarity tests and the phylogenetic tree also show that the test sequences which

were isolated from swine were not different from the 2005/06 sequences that were isolated from cattle. The closeness of the sequences presented herein means that this virus strain can infect both species. This presents implications in disease control methods especially with regards to vaccination. Therefore, control strategies especially vaccination regimes should cover all the susceptible animal species including pigs. The phylogenetic comparison of the pig-derived virus with the vaccine strain K77/78 reveals that they form entirely different clades and have a sequence divergence of over 20%. The large sequence differences between the vaccine strain and the recently isolated Uganda FMDV strains (from 2005 to 2010) may present implications in FMD control in Uganda (Kasambula et al., 2011). This emphasises the need for further research in testing the efficacy and effectiveness of the vaccine strain against the circulating FMD strains in Uganda.

Conclusion

This is the first report on the nucleotide and amino acid sequences of partial VP1 gene of FMD virus isolated from domestic pigs in Uganda. The findings also show that the pig-derived virus strain is not different from recent circulating FMD viral strains in cattle in the western and central regions of the country. This has implications on strategies used in FMD control. Last but not the least, the results as well highlighted the significant genetic difference between the vaccine strain K77/78 and recently isolated Ugandan strains (2005 to 2010) highlighting a need for further research to be carried out on vaccine efficacy and efficiency. The continued use of vaccination and quarantines should not only cover cattle but should also cut across all other FMD susceptible animal species, including pigs.

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