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VP1 sequencing protocol for foot and mouth disease virus molecular epidemiology

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Summary

Nucleotide sequences of field strains of foot and mouth disease virus (FMDV) contribute to our understanding of the distribution and evolution of viral lineages that circulate in different regions of the world. This paper outlines a practical reverse-transcription polymerase chain reaction (RT-PCR) and sequencing strategy that can be used to generate RNA sequences encoding the VP1 (1D) region of FMDV. The protocol contains a panel of PCR and sequencing primers that can be selected to characterise genetically diverse isolates representing all seven FMDV serotypes. A list of sequences is also described, comprising prototype sequences for all proposed FMDV topotypes, in order to provide a framework for phylogenetic analysis. The technical details and prototype sequences provided in this paper can be employed by FMD Reference Laboratories and others in an approach to harmonise the molecular epidemiology of FMDV.

Keywords

Foot and mouth disease virus – Phylogenetics – Prototype – Reference – Reverse-transcription polymerase chain reaction – Sequence – Sequencing.

Introduction

Foot and mouth disease (FMD) is an economically important veterinary disease affecting multiple livestock species and leading to trade restrictions on animals and their products. Transmission of FMD between livestock can occur by a variety of mechanisms, including animal movement, through contaminated animal products (meat, milk, semen), mechanically by people and via other sources or fomites, and by the air (1). Together, these factors contribute to the ease by which FMD can be spread across country borders and circumvent measures used for its control.

Foot-and-mouth disease virus (FMDV) is the type species in the genus *Aphthovirus* (family *Picornaviridae*) and the virus exists as seven immunologically distinct serotypes: O, A, C, Asia 1, Southern African Territories (SAT) 1, SAT 2 and SAT 3 (2). The seven serotypes are not uniformly distributed around the world: serotypes O and A have the broadest distribution; historically they occurred in Europe and South America and they currently occur in many parts of Africa and Asia (3). In contrast, the SAT and Asia 1 serotypes are normally geographically restricted, either to sub-Saharan Africa, or to southern and central Asia, respectively, while FMD outbreaks due to serotype C have not been reported since 2004 (3, 4).

During the last 20 years, there have been significant advances in the understanding of FMD epidemiology (3, 5, 6). These improvements have arisen largely due to the application of molecular techniques such as polymerase chain reaction (PCR) amplification and nucleotide sequencing. Data generated from these studies can be used to place FMDV field isolates into topotypes and to define the genetic relationships between FMD viruses causing outbreaks (for examples, see references 7, 8 and 9). FMD Reference Laboratories are tasked with monitoring the spread and global distribution of FMDV (10): to do this, the genomic region encoding the VP1 capsid protein (1D gene) is the most frequently studied (3, 11, 12, 13). The development of robust laboratory sequencing protocols poses technical challenges due to the wide sequence variability that exists across (and within) the seven FMDV serotypes. The aim of this study is to provide protocols for the amplification and sequencing of VP1 regions of diverse FMD viruses.

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Materials and methods

Samples

For this study, The Pirbright Institute in the United Kingdom (UK), which is a World Organisation for Animal Health (OIE) Reference Laboratory for FMD and has been designated by the Food and Agriculture Organization of the United Nations (FAO) as the World Reference Laboratory for FMD (WRLFMD), sequenced cell-culture-passaged isolates. However, samples that are rich in FMDV are also suitable for VP1 sequencing; these can be comprised of primary clinical material (such as vesicular fluid and vesicular epithelium). Recommended protocols for the collection of vesicular samples and the transport of this material to the laboratory have been described previously (14).

RNA Extraction

Total RNA was extracted from 460 µl of sample using the RNeasy kit (Qiagen, Crawley, West Sussex, UK), according to the manufacturer's instructions. The RNA was eluted in 50 µl of nuclease-free water and stored at –20°C (or on ice if the RT-PCR was performed immediately). Alternative methods, such as the manual TRIzol protocols (outlined in [15]), other commercial spin-column kits or automated extraction platforms, can also be used to prepare RNA template suitable for RT-PCR.

One-step reverse-transcription polymerase chain reaction

Serotype information derived by antigen-detection enzyme-linked immunosorbent assay (ELISA) (16) can provide valuable supporting data to aid the selection of primers for RT-PCR. Based on this knowledge, primer sets specific to the respective serotype can be selected from a list provided in Table I and Figure 1. These are designed to anneal within the VP3 coding region (forward primers) and the 2B coding region (reverse primers) to amplify the full length of the FMDV VP1 coding region. To accommodate the sequence variability that can occur in the target region within respective serotypes, two primer sets for serotypes A, C, Asia 1, SAT 2 and SAT 3 and three primer sets for serotypes O and SAT 1 were designed. Amplifying test samples with two RT-PCR primer sets is recommended; if the amplification does not produce a product of the expected size, the third primer sets for serotypes O and SAT 1 should be used.

Table I
List of oligonucleotide primers used for reverse-transcription polymerase chain reaction

Primer name	Sequence (5′ – 3′)	Genome direction*	Gene	Product size**
Serotype O				
O–1C272F	TBGCRGGNCTYGCCCAGTACTAC	+	VP3	1,135
O–1C244F	GCAGCAAAACACATGTCAAACACCTT	+	VP3	1,165
O–1C283F	GCCCAGTACTACACACAGTACAG	+	VP3	1,124
Serotype A				
A–1C562F	TACCAAATTACACACGGGAA	+	VP3	866
A–1C612F	TAGCGCCGGCAAAGACTTTGA	+	VP3	814
Serotype C				
C–1C536F	TACAGGGATGGGTCTGTGTGTACC	+	VP3	883
C–1C616F	AAAGACTTTGAGCTCCGGCTACC	+	VP3	802
Serotype Asia 1				
As1–1C530F	CCACRAGTGTGCARGGATGGGT	+	VP3	886
As1–1C613F	GCCGGCAARGAYTTTGAGTTYCG	+	VP3	803
Serotypes O/A/C/Asia 1				
EUR–2B52R	GACATGTCCTCCTGCATCTGGTTGAT	–	2B	
Serotype SAT 1				
SAT1–1C559F	GTGTATCAGATCACAGACACACA	+	VP3	1,043
SAT1U–OS	GTGTACCAGATCACTGACAC	+	VP3	1,043
SAT1–P1–1228F	AACCTGCACTTCATGTACAC	+	VP3	1,286
Serotype SAT 2				
SAT2–1C445F	TGGGACACMGGIYTGAACTC	+	VP3	1,145
SAT2–P1–1223F	TGAACTACCACTTCATGTACACAG	+	VP3	1,279
Serotype SAT 3				
SAT3–1C559F	CTGTACCAAATYACAGACAC	+	VP3	1,034
SAT3–P1–1222F	AATCTGCATTTCATGTACAC	+	VP3	1,277
Serotypes SAT 1–3				
SAT–2B208R	ACAGCGGCCATGCACGACAG	–	2B	

* (+) relates to forward primer; (–) relates to reverse primer
** approximate size (may vary by up to six base pairs depending on virus strain)

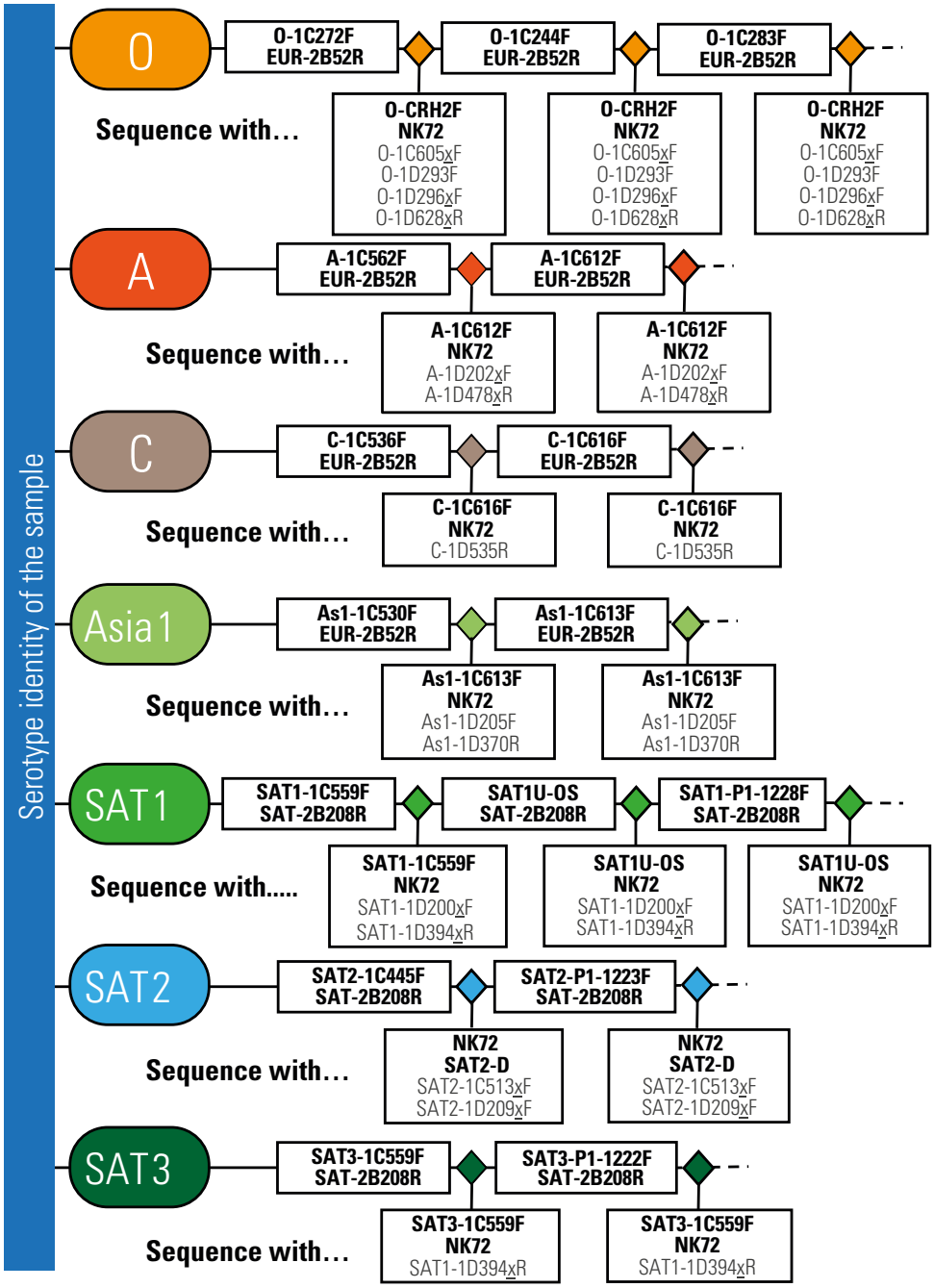

Fig. 1

Decision tree to select primers used for reverse-transcription polymerase chain reaction amplification and sequencing of diverse foot and mouth disease viruses

The first line for each FMDV serotype indicates the primers used for PCR, while the lower boxes denote corresponding oligonucleotides that can be used in the sequencing reactions. Primer sequences are shown in either Table I (PCR) or Table III (sequencing). 'x' in the primer name means one of a set of related primers, dependant on specific sequences generated by NK72 (see Table III)

In this study, reaction master-mix was prepared in a dedicated PCR clean room (to avoid possible contamination) by mixing 8 µl of nuclease-free water, 2.5 µl of selected forward primer (4 pmol/µl), 5 µl of selected reverse primer (4 pmol/µl) and the following components of the QIAGEN OneStep RT-PCR kit (Qiagen, Germany): 5 µl of 5× buffer (containing 12.5 mM $MgCl_2$), 1 µl of dNTPs mix and 1 µl of Qiagen OneStep RT-PCR enzyme mix. In a dedicated template room, 2.5 µl of the viral RNA was added to the RT-PCR tube. For each primer set, template-free amplification controls were performed in parallel to the RNA samples to monitor for cross-contamination. These controls comprised tubes where nuclease-free water was substituted into the reaction instead of the RNA sample. Once assembled, the

reactions containing the PCRs were placed in a thermocycler (Bio-Rad, USA) and the relevant PCR cycling programme was selected (Table II). A heated lid was used in order to minimise evaporation of the reaction liquid; alternatively, 50 µl of mineral oil (Promega) can be used to overlay the liquid in each tube. After the cycling programme was completed the tubes were held at 12°C until processed further.

Post polymerase chain reaction manipulations

The PCR products were analysed by electrophoresis on a 1.5% agarose-Tris-borate-EDTA gel containing 1× GelRed nucleic acid stain (Biotium Inc., USA). DNA size markers (GeneRuler 100 bp DNA Ladder Plus, Fermentas Inc., USA) were run alongside the samples to quantify and confirm the correct size of the product (Table I). Post-PCR removal of unincorporated nucleotides and primers was achieved using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, UK) according to the manufacturer's instructions. These products were eluted using 25 µl of elution buffer and the DNA content determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, UK). In the event that multiple PCR amplicons were generated that could interfere with the sequencing, bands of the correct size were excised and purified (Qiagen).

Sequencing reactions

DNA sequencing of the PCR products was carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). In a total volume of 10 µl, 2 µl 5× sequencing buffer was mixed with 0.5 µl BigDye® Terminator v3.1 (both reagents are supplied with the kit), 3 µl of appropriate sequencing primer (1.6 pmol) (Table III) and target template (RT-PCR products of VP1 specific reactions) diluted to contain 5–20 ng complementary DNA (cDNA). To minimise the possibility of cross-contamination, the reaction master-mix was prepared in a dedicated clean room (no template room), while the previously amplified VP1 template was added in a separate template room. Sequencing PCR reactions were carried out in duplicates for each of the primers, either in individual 0.2 ml thin-walled tubes or in 96-well format in a 0.2 ml PCR plate, following a programme of 96°C for 1 min and 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min (Bio-Rad). The heated lid was activated to eliminate evaporation of samples. If a heated lid was not available, at least 50 µl of mineral oil overlay was added to each tube. The thermocycler was set to hold the tubes at 12°C after the cycling programme had finished.

The choice of sequencing primers depends on the serotype (and often topotype) of the sample, and Table III lists possible sequencing primers for different serotypes (although others may need to be designed for specific geographical areas). A universal reverse sequencing primer (NK72) is used for all serotypes, while a number of different forward primers may be used depending on the serotype. The strategy for selecting sequencing primers is outlined in Figure 1. These anneal to sequences outside the VP1 coding region (i.e. external primers). If necessary, internal forward and reverse primers may be used to ensure full coverage on both cDNA strands. The sequence obtained using the external primers can be used to guide the choice of the internal primers. It is recommended that each strand of cDNA is sequenced at least once (without any ambiguity) to ensure the quality of the consensus sequence.

Following the cycle-sequencing protocol, the reactions were cleaned up by ethanol precipitation. In this procedure, 5 µl of freshly prepared 125 mM EDTA and 60 µl of 100% ethanol were added to each reaction tube containing the products of sequencing PCR reaction, mixed by vortexing and incubated for at least 15 min at room temperature to precipitate the extension products. As the BigDye® reagent is light-sensitive, the precipitation was carried out in the dark. Following precipitation, the tubes were centrifuged at 3,000 × *g* for 30 min and the supernatant was discarded without disturbing the pellet. Subsequently, the pellets were washed with 60 µl of 70% ethanol and centrifuged

Table II
Standard thermocycling protocols used for reverse-transcription polymerase chain reaction amplification of the VP1 region of foot and mouth disease virus

Step	Temperature	Time	Number of cycles
1. Reverse transcription	50°C	30 min	1
2. Inactivation	95°C	15 min	1
3. Denaturation	95°C	60 sec	Repeat steps
4. Primer annealing	50 – 55 – 60°C*	60 sec	3 to 5
5. Extension	72°C	120 sec	35 times
6. Final extension	72°C	5 min	1

* Primer annealing temperatures for the different foot and mouth disease virus serotypes are as follows: serotype O at 60°C; serotypes A, C, Asia 1 at 55°C and the 3 SAT serotypes at 50°C

Table III
List of additional oligonucleotide primers used for sequencing

Primer name	Sequence (5′ – 3′)	Genome direction*	Gene
All serotypes			
NK72	GAAGGGCCCAGGGTTGGACTC	–	2A/2B
Serotype O			
O–CRH2F	GAYTACGCSTACACSGCGTC	+	VP3
O–1C605aF	TGGCTAGTGCTGGTAAAGACTTTGAG	+	VP3
O–1C605bF	TGGCTAGTGCCGGCAAGGACTTTGAG	+	VP3
O–1C605cF	TGGCTAGCGCCGGCAAGGACTTTGAG	+	VP3
O–1C605dF	TGGCTAGCGCCGGAAAGGACTTTGAG	+	VP3
O–1D293F	TGGAYAACACCACYAAYCCAAC	+	VP1
O–1D296F	ACAACACCACCAACCCAAC	+	VP1
O–1D296aF	ATAACACCACTAATCCAAC	+	VP1
O–1D296bF	ACAACACCACCAATCCAAC	+	VP1
O–1D296cF	ATAACACCACCAATCCAAC	+	VP1
O–1D628R	GTTGGGTTGGTGGTGTTGT	–	VP1
O–1D628aR	GTTGGATTAGTGGTGTTAT	–	VP1
O–1D628bR	GTTGGGTTGGTGGTGTTTT	–	VP1
Serotype A			
A–1C612F	TAGCGCCGGCAAAGACTTTGA	+	VP3
A–1D202aF	TCAGCCACCTACTATTTCTCTGA	+	VP1
A–1D202bF	GCAGCAACATACTACTTCTCTGA	+	VP1
A–1D202cF	GCAGCAACCTACTATTTCTCTGA	+	VP1
A–1D202dF	GCGGCCACTTACTACTTCTCTGA	+	VP1
A–1D202eF	GCGGCCACCTACTATTTTTCTGA	+	VP1
A–1D202fF	GCGGCCACCTACTACTTTTCTGA	+	VP1
A–1D478aR	CAGTGCTCCGTAGTTAAAGGATGA	–	VP1
A–1D478bR	AATTGCACCGTAATTGAAGGATGC	–	VP1
A–1D478cR	GAGTGCACCATAGTTGAAAGACGC	–	VP1
A–1D478dR	AATCGCACCAAAGTTGAAGGAAGT	–	VP1
A–1D478eR	AACTGCGCCGTAGTTGAAGGAGGC	–	VP1
A–1D478fR	AATTGCGCCGTAGTTGAAGGATGC	–	VP1
Serotype C			
C–1C616F	AAAGACTTTGAGCTCCGGCTACC	+	VP3
C–1D535R	ARAGYTCIGCICGYTTCAT	–	VP1
Serotype Asia 1			
As1–1C613F	GCCGGCAARGAYTTTGAGTTYCG	+	VP3
As1–1D205F	GCRACGTACTACTTYTCRGACCT	+	VP1
As1–1D370R	GTTGTAYACTGTYGCCAGCACACG	–	VP1
Serotype SAT 1			
SAT1–1C559F	GTGTATCAGATCACAGACACACA	+	VP3
SAT1–1D200F	TGCGYGCIGCCACGTACTAYTTCTC	+	VP1
SAT1–1D200aF	TGCGTGCGGCCACGTATTATTTCTC	+	VP1
SAT1–1D200bF	TGCGCGCTGCTACGTACTACTTCTC	+	VP1
SAT1–1D200cF	TGCGYGCIGCCACGTACTAYTTCTC	+	VP1
SAT1–1D200dF	TGCGTGCTTCCACGTACTACTTCTC	+	VP1
SAT1–1D200eF	TGCGYGCGGCCACGTACTACTTCTC	+	VP1
SAT1–1D394R	GGYTTGTACTTRCARTCACCGTTGTA	–	VP1
SAT1–1D394aR	GGTTTGTAYTTGCAGTYGCCRTTGTA	–	VP1
SAT1–1D394bR	GGCTTGTACTTACAGTCACCATTGTA	–	VP1
SAT1–1D394cR	GGTTTGTAYTTGCAGTTGCCRTTGTA	–	VP1
SAT1–1D394dR	GGCYTGTACTTRCAGTCACCGTTGTA	–	VP1
SAT1–1D394eR	GGTTTGTAYTTGCARTCACCGTTGTA	–	VP1

Continued...

Table III (Contd)

Primer name	Sequence (5′– 3′)	Genome direction*	Gene
Serotype SAT 2			
SAT2–D	GGTGCGCCGTTGGGTTGCCA	–	VP1
SAT2–1C513aF	CACACACACAGACACACCCGCGATGGC	+	VP3
SAT2–1C513bF	CACACACACTGACACACCTGCGATGGC	+	VP3
SAT2–1C513cF	CACCCACACAGACACACCGGCCATGGC	+	VP3
SAT2–1C513dF	CACGCACACAGACACCCCGGCCATGGC	+	VP3
SAT2–1C513dG	CACGCACACGGACACTCCCGCGATGGC	+	VP3
SAT2–1C513dH	CTCTCACACGGACACTCGCGCGAAGGC	+	VP3
SAT2–1D209aF	CCACTTACTACTTTTGTGACCTTGA	+	VP1
SAT2–1D209bF	CCACCTACTACTTTTGTGACCTTGA	+	VP1
SAT2–1D209cF	CCACCTACTATTTCTGTGACCTGGA	+	VP1
SAT2–1D209dF	CCACGTACTACTTCTGTGACCTGGA	+	VP1
Serotype SAT 3			
SAT3–1C559F	CTGTACCAAATYACAGACAC	+	VP3

* (+) relates to forward primer; (–) relates to reverse primer

at 1,650 × *g* for 15 min. After the supernatant had been removed, the pellets were shaded from direct light and dried in a vacuum drier until no ethanol was present. Before loading onto the ABI 3730 DNA Analyser, the samples were re-suspended in 20 µl of HiDi Formamide (Life Technologies) and the sequencing reactions were run according to the manufacturer's instructions.

Phylogenetic analysis

VP1 sequences were assembled from multiple reads using SeqMan Pro (Lasergene package, DNAstar Inc., Madison, Wisconsin, USA). The nucleotide sequences were then used to prepare multiple sequence alignments employing BioEdit v7.2.5 (17), which in turn uses CLUSTAL W 1.83 (18). Midpoint-rooted Neighbor-joining phylogenetic trees, employing the Kimura 2-parameter nucleotide substitution model (19), were constructed and visualised using MEGA 6.06 (20). The robustness of the tree topology was assessed with 1,000 bootstrap replicates as implemented in the program.

Results and discussion

Sequencing and analysis of the VP1 coding region of the FMDV is becoming an increasingly more accessible laboratory tool, providing information that aids our understanding of the spread and global epidemiology of the virus. Despite the advent of next-generation sequencing that allows the relatively fast generation of large parts of the genome, and even full-genome sequences, VP1 sequence data comparisons remain the main method for classification of FMD viruses.

In this study, 35 FMDV isolates were selected from the WRLFMD/OIE Reference Laboratory for FMD sample collection held at The Pirbright Institute for VP1 sequencing. They represented all seven serotypes (O: *n* = 11; A: *n* = 11; C: *n* = 4; Asia 1: *n* = 1; SAT 1: *n* = 2; SAT 2: *n* = 2 and SAT 3: *n* = 4). These, together with 61 previously published sequences determined using the same protocol, and another 50 sequences selected from publicly available sources (GenBank), constitute a list of 146 reference sequences for all seven serotypes (O: *n* = 46; A: *n* = 34; C: *n* = 10; Asia 1: *n* = 11; SAT 1: *n* = 13; SAT 2: *n* = 24 and SAT 3: *n* = 8) (Table IV). The criteria for selecting and designating prototype sequences for this list were principally, *i)* being the earliest example of each topotype and/or *ii)* being a laboratory reference strain.

Both FASTA and MEGA format files containing these 146 prototype VP1 sequences are available online (www.wrlfmd.org/fmd_genotyping/prototypes.htm). This list of sequences and sequence data is reviewed regularly and updated as necessary as new FMDV lineages arise or further detail is required for certain serotypes.

The technical protocol described provides a starting point for the development of tailored methods for VP1 sequencing that can be deployed in local FMDV reference laboratories, while the panel of 146 prototype sequences is defined to help standardise the analysis of VP1 sequence data. Using freely available software, robust phylogenetic trees can be prepared to compare FMDV field viruses to the set of reference sequences (Fig. 2). Due to the complexity of the analysis and nucleotide diversity, it is difficult to predict the exact topology of the tree when new sequences are being compared. It is most likely that a sequence of a

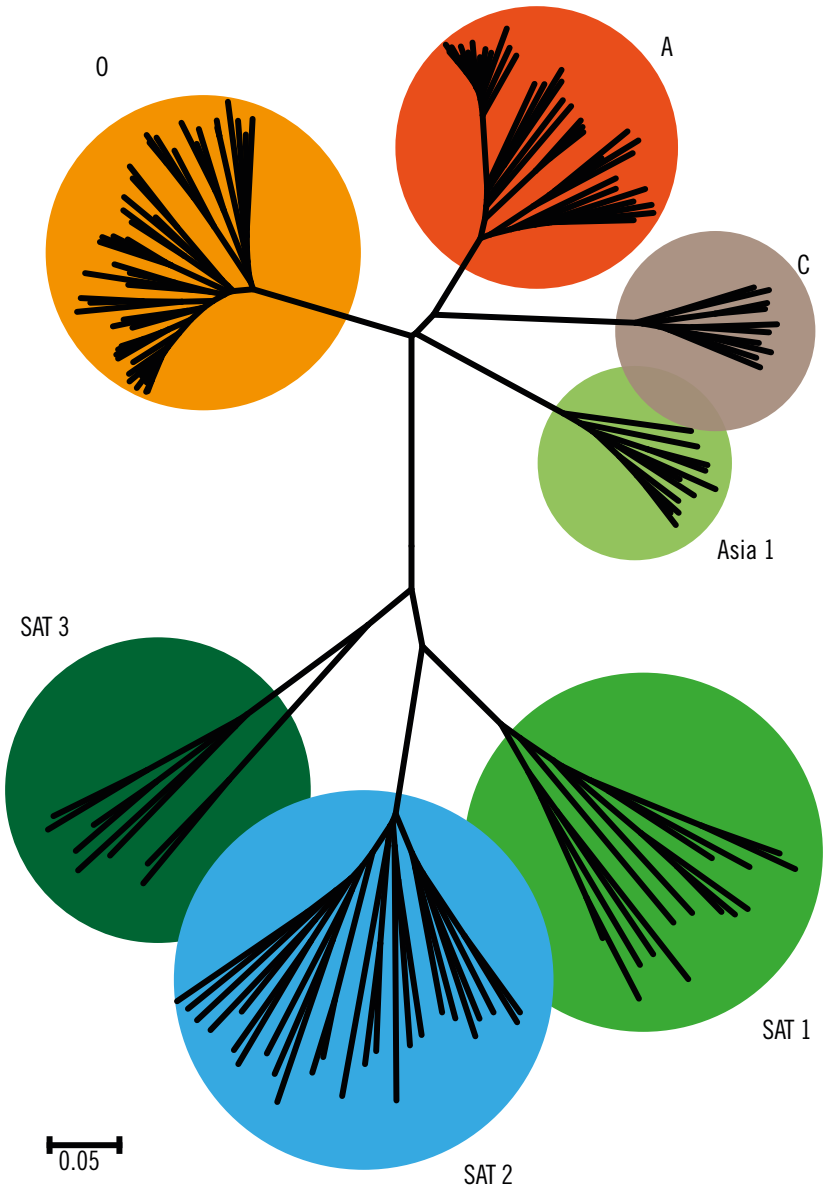

Fig. 2
Phylogenetic tree of all seven serotypes
Framework phylogenetic tree for FMDV representing 146 reference and prototype sequences (Table IV). Figure shows a Neighbor-joining tree that distinguishes sequences from the different FMDV serotypes

field isolate will be most related to one of the prototype sequences, thus identifying the topotype to which the virus belongs. If, however, the sequence cannot be classified into one of the existing topotypes, the authors recommend that it should be submitted to the FMD Virus Nomenclature Working Group of the OIE/FAO FMD Laboratory Network (www.foot-and-mouth.org/foot-and-mouth-disease-virus-nomenclature-working-group) for formal classification.

Over the past ten years, the technical protocol for RT-PCR amplification and subsequent sequencing of the VP1 (1D) region of FMDV used at the FAO WRLFMD has generated sequence data for more than 4,500 virus isolates and field samples (individual reports can be accessed at www.wrlfmd.org/fmd_genotyping/). In all cases, at least one of the RT-PCR primer sets produced a specific amplicon from which the VP1 sequence was determined.

Table IV
List of reference sequences
Prototype strains for each topotype are in bold

Topotype	Lineage	Sublineage	Serotype/isolate name	Accession no.	Reference
CATHAY	–	–	**O/HKN/21/70**	AJ294911	(21)
CATHAY	–	–	O/HKN/6/83	AJ294919	(21)
CATHAY	–	–	O/PHI/7/96	AJ294926	(21)
CATHAY	–	–	O/Yunlin/Taiwan/97	AF308157	(22)
EA–1	–	–	**O/K83/79* (Kenya)**	AJ303511	(23)
EA–1	–	–	O/K40/84* (Kenya)	KY091280	This study
EA–1	–	–	O/UGA/5/96	AJ296327	(23)
EA–2	–	–	**O/MAL/1/98**	DQ165074	(7)
EA–2	–	–	O/UGA/3/2002	DQ165077	This study
EA–2	–	–	O/KEN/5/2002	DQ165073	This study
EA–2	–	–	O/TAN/2/2004	KF561679	(24)
EA–3	–	–	**O/SUD/2/86**	DQ165075	(7)
EA–3	–	–	O/ETH/3/2004	FJ798109	(25)
EA–3	–	–	O/ETH/2/2006	FJ798127	(25)
EA–3	–	–	O/ETH/1/2007	FJ798137	(25)
EA–4	–	–	**O/UGA/17/98**	HM211075	(25)
EA–4	–	–	O/ETH/58/2005	FJ798141	(25)
EURO–SA	–	–	**O_1/BFS 1860/UK/67**	AY593815	(26)
EURO–SA	–	–	O_2/Brescia/ITL/47	M55287	(27)
EURO–SA	–	–	O_3/Venezuela/51	AJ004645	(28)
EURO–SA	–	–	O/Corrientes/ARG/06	DQ834727	(29)
ISA–1	–	–	**O_{11}/ISA/1/62**	AJ303500	(23)
ISA–1	–	–	O/ISA/9/74	AJ303502	(23)
ISA–1	–	–	O/ISA/8/83	AJ303503	(23)
ISA–2	–	–	**O/JAV/5/72**	AJ303509	(23)
ISA–2	–	–	O/ISA/1/74	AJ303501	(23)
ME–SA	–	–	**O_1/Manisa/TUR/69**	AY593823	(26)
ME–SA	–	–	O/R2/75* (India)	KP822947	(30)
ME–SA	–	–	O/IND/53/79	AF292107	Hemadri *et al.*, unpublished
ME–SA	Ind–2001	a	O/KUW/3/97	DQ164904	(7)
ME–SA	Ind–2001	b	O/OMN/7/2001	DQ164941	(7)
ME–SA	Ind–2001	c	O/UAE/4/2008	KM921876	(13)
ME–SA	Ind–2001	d	O/BHU/3/2009	KM921814	(13)
ME–SA	PanAsia	–	O/UKG/35/2001	AJ539141	(31)
ME–SA	PanAsia–2	–	O/IRN/8/2005	KY091281	This study
ME–SA	PanAsia–2	ANT–10	O/IRN/88/2009	KY091282	This study
ME–SA	PanAsia–2	BAL–09	O/IRN/18/2010	KY091283	This study
ME–SA	PanAsia–2	FAR–09	O/IRN/31/2009	KY091284	This study
ME–SA	PanAsia–2	PUN–10	O/PAK/16/2010	KY091285	This study
ME–SA	PanAsia–2	SAN–09	O/TUR/2/2009	KY091286	This study
ME–SA	PanAsia–2	TER–08	O/TUR/38/2008	KY091287	This study
SEA	–	–	**O/TAI/189/87***	KY091288	This study
SEA	Cam–94	–	O/CAM/3/98	AJ294910	(21)
SEA	Mya–98	–	O/MYA/7/98	DQ164925	(7)
WA	–	–	**O/GHA/5/93**	AJ303488	(23)
WA	–	–	O/CIV/8/99	AJ303485	(23)
AFRICA	G–I	–	A/KEN/42/66	KF561699	(24)

Continued...

Table IV (Contd)

Topotype	Lineage	Sublineage	Serotype/isolate name	Accession no.	Reference
AFRICA	G–II	–	A/EGY/1/72	EF208756	(8)
AFRICA	G–III	–	**A_{21}/Lumbwa/KEN/3/64**	KY091289	This study
AFRICA	G–IV	–	A/SUD/3/77	GU566064	(32)
AFRICA	G–V	–	A/NGR/2/73	KF561704	(24)
AFRICA	G–VI	–	A/GHA/16/73	KF561698	(24)
AFRICA	G–VII	–	A/UGA/13/66	KF561705	(24)
ASIA	A22	–	**A_{22}/IRQ/24/64**	AY593763	(26)
ASIA	Irn–87	–	A/IRN/2/87	EF208770	(8)
ASIA	Irn–96	–	A/IRN/1/96	EF208771	(8)
ASIA	Irn–99	–	A/IRN/22/99	EF208772	(8)
ASIA	Irn–05	–	A/IRN/1/2005	EF208769	(8)
ASIA	Iran–05	AFG–07	A/AFG/6/2007	FJ755007	(33)
ASIA	Iran–05	ARD–07	A/TUR/1/2008	FJ755133	(33)
ASIA	Iran–05	BAR–08	A/BAR/6/2008	FJ755010	(33)
ASIA	Iran–05	ESF–10	A/IRN/9/2010	KY091290	This study
ASIA	Iran–05	EZM–07	A/TUR/33/2008	FJ755155	(33)
ASIA	Iran–05	FAR–09	A/IRN/78/2009	KY091291	This study
ASIA	Iran–05	FAR–11	A/IRN/1/2011	KY091292	This study
ASIA	Iran–05	HER–10	A/AFG/10/2010	KY091293	This study
ASIA	Iran–05	KSS–09	A/TUR/3/2010	KY091294	This study
ASIA	Iran–05	QAZ–11	A/IRN/9/2011	KY091295	This study
ASIA	Iran–05	SIS–10	A/IRN/125/2010	KY091296	This study
ASIA	Iran–05	SIS–12	A/IRN/15/2012	KY091297	This study
ASIA	A15	–	A_{15}/Bangkok/TAI/60	AY593755	(26)
ASIA	Sea–97	–	A/TAI/2/97	EF208778	(8)
ASIA	Sea–97	–	A/TAI/7/2003	HQ116312	(12)
ASIA	Thai–87	–	A/TAI/118/87*	EF208777	(8)
EURO–SA	A12	–	**A_{12}/119/Kent/UK/32**	M10975	(34)
EURO–SA	A24	–	A_{24}/Cruzeiro/BRA/55	AY593768	(26)
EURO–SA	A5	–	A_5/FRA/1/68 (1960)	KY091298	This study
EURO–SA	A–81	–	A/Alem/ARG/81	AJ306219	(35)
Unassigned	G–VIII	–	**A_{23}/Kitale/KEN/64 (KEN/46/65)**	KY091299	This study
Unassigned	A11	–	**A_{11}/Germany/c.29 (AGB)**	EU553852	(36)
AFRICA	–	–	**C/K267/67 (KEN/32/70)**	KY091300	This study
AFRICA	–	–	C/ETH/1/71	FJ798151	(25)
ASIA	–	–	**C/N65/Tadjikistan/USSR/67**	KY091302	This study
ASIA	–	–	C/IND/51/79 (1977)	KY091301	This study
EURO–SA	–	–	**C_3/Resende/BRA/55**	M90381	(37)
EURO–SA	–	–	C_1/Santa Pau/Spain/70 (C-S8c1)	AJ133357	(38)
EURO–SA	–	–	C_3/Indaial/BRA/71	M90376	(37)
EURO–SA	–	–	C/PHI/7/84	KY091303	This study
Unassigned	–	–	**C/Germany/c.26 (CGC)**	EU553893	(36)
Unassigned	–	–	**C/UK/149/34**	EU553905	(36)
ASIA	–	–	**Asia1/PAK/1/54**	AY593795	(26)
ASIA	G–I	–	Asia1/AFG/1/2001	DQ121109	(39)
ASIA	G–II	–	Asia1/IRN/10/2004	DQ121119	(39)
ASIA	G–III	–	Asia1/ IND/762/2003*	DQ101240	(39)

Continued...

Table IV (Contd)

Topotype	Lineage	Sublineage	Serotype/isolate name	Accession no.	Reference
ASIA	G–IV	–	Asia1/HKN/19/74	FJ785230	(39)
ASIA	G–V	–	Asia1/IND/18/80	DQ121116	(39)
ASIA	G–VI	–	Asia1/IND/14/95	AF390678	Gurumurthy *et al.*, unpublished
ASIA	Sindh–08	–	Asia1/PAK/8/2008	KY091304	This study
ASIA	–	–	Asia1/Shamir/ISR/89	JF739177	Lee *et al.*, unpublished
ASIA	–	–	Asia1/IND/63/72*	Y09949	(40)
ASIA	–	–	Asia1/YNBS/China/58	AY390432	Chang *et al.*, unpublished
I (NWZ)	–	–	**SAT1/T155/71***	KF561706	(24)
I (NWZ)	–	–	SAT1/ZIM/23/2003	KF219690	This study
II (SEZ)	–	–	**SAT1/RV/11/37**	AY593839	(26)
II (SEZ)	–	–	SAT1/RHO/5/66	AY593846	(26)
III (WZ)	–	–	**SAT1/BEC/1/48**	AY593838	(26)
III (WZ)	–	–	SAT1/BOT/1/68	AY593845	(26)
IV (EA–1)	–	–	**SAT1/UGA BUFF/21/70**	KF219682	This study
V	–	–	**SAT1/NIG/11/75**	AF431711	(41)
V	–	–	SAT1/ETH/3/2007	FJ798154	(25)
VI	–	–	**SAT1/ISR/4/62**	AY593844	(26)
VI	–	–	SAT1/SUD/4/76	AY441997	(42)
VII (EA–2)	–	–	**SAT1/UGA/13/74**	AY442010	(42)
VIII (EA–3)	–	–	**SAT1/UGA/1/97***	AY442012	(42)
I	–	–	**SAT2/SA/106/59**	AY593848	(26)
I	–	–	SAT2/ZIM/14/2002	KF219689	This study
II	–	–	**SAT2/ZIM/7/83***	AF540910	(43)
II	–	–	SAT2/ZIM/5/81	EF134951	(44)
III	–	–	**SAT2/RHO/1/48**	AY593847	(26)
III	–	–	SAT2/ BOT/P3/98 (VUM-29)	KY091305	This study
IV	–	–	**SAT2/KEN/1/84**	AY344505	(45)
IV	–	–	SAT2/ETH/1/90	AY343935	(45)
V	–	–	**SAT2/NIG/2/75**	AF367139	(46)
V	–	–	SAT2/GHA/2/90	AF479415	(46)
VI	–	–	**SAT2/GAM/8/79**	AF479410	(46)
VII	–	–	**SAT2/SAU/6/2000**	AF367135	(46)
VII	–	–	SAT2/CAR/8/2005	JX570616	(47)
VIII	–	–	**SAT2/ZAI/1/74**	DQ009737	Maree *et al.*, unpublished
VIII	–	–	SAT2/RWA/1/00*	AF367134	(46)
IX	–	–	**SAT2/KEN/3/57**	AJ251473	Newman *et al.*, unpublished
IX	–	–	SAT2/KEN/2/84	AY343941	(45)
X	–	–	**SAT2/ZAI/1/82**	AF367100	(46)
X	–	–	SAT2/UGA/19/98	AY343969	(45)
XI	–	–	**SAT2/ANG/4/74**	AF479417	(46)
XII	–	–	**SAT2/UGA/51/75**	AY343963	(45)
XIII	–	–	**SAT2/SUD/6/77**	AY343939	(45)
XIII	–	–	SAT2/ETH/2/2007	FJ798161	(25)
XIV	–	–	**SAT2/ETH/2/91**	FJ798159	(25)
I (SEZ)	–	–	**SAT3/SA/57/59**	AY593850	(26)

Continued...

Table IV (Contd)

Topotype	Lineage	Sublineage	Serotype/isolate name	Accession no.	Reference
I (SEZ)	–	–	SAT3/KNP/10/90/3	AF286347	(48)
II (WZ)	–	–	**SAT3/BEC/1/65**	AY593853	(26)
II (WZ)	–	–	SAT3/BEC/20/61	AY593851	(26)
III (NWZ)	–	–	**SAT3/ZIM/P25/91 (UR-7)**	KY091306	This study
IV	–	–	**SAT3/ZAM/P2/96 (MUL-4)**	KY091307	This study
V (EA)	–	–	**SAT3/UGA BUFF/27/70 (aka UGA/92/70)**	KF219685	This study
V (EA)	–	–	SAT3/UGA/10/97 (UGA/2/97/3)	KY091308	This study

* Not a WRLFMD Ref. No.

The choice of primers for VP1 RT-PCR is usually guided by prior knowledge of the serotype of the samples analysed. However, due to high genetic variability within the capsid-coding sequence of samples belonging to different FMDV serotypes, the primers are designed to target a broad range of genetically distant sequences. Therefore, these primers are not necessarily serotype specific and, due to possible cross-serotype amplification, they cannot be used for virus serotyping. However, sequencing of the amplicon and comparison with the prototype sequences can be used for correct FMDV type identification. As non-homologous primer sets were not normally used, it was not possible to determine the frequency with which cross-serotype amplification occurred.

This framework of prototype strains is a vital first step to harmonising the nomenclature used to describe and classify field strains of FMDV. This list will be reviewed regularly and, if necessary, updated to take into account new FMDV strains and lineages that may arise in different regions of the world. In terms of defining a framework of FMDV topotypes, there is still work to be done, particularly for SAT viruses, where it is a priority to reach a consensus between reference nomenclatures currently used.

The information included here can be used by FMD diagnostic laboratories to supplement the brief recommended procedures for FMDV strain characterisation outlined in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (15). Since these prototype strains encompass inter- and intra-serotypic variability between FMDV isolates, this list can also be used as a rational basis for diagnostic test validation (for strain sensitivity) and may also be useful for standardising antigenic comparisons between FMDV field and vaccine strains.

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Protocole de séquençage de la VP1 pour les études d'épidémiologie moléculaire du virus de la fièvre aphteuse

N.J. Knowles, J. Wadsworth, K. Bachanek-Bankowska & D.P. King

Résumé
Les séquences de nucléotides des souches de terrain du virus de la fièvre aphteuse nous aident à comprendre la distribution et l'évolution des lignées virales présentes dans les différentes régions du monde. Les auteurs décrivent les grandes lignes d'un protocole pratique, basé sur l'amplification en chaîne par polymérase couplée à une transcription inverse (RT-PCR) et sur le séquençage, qui peut être utilisé pour générer des séquences d'ARN codant pour la région VP1 (1D) du virus de la fièvre aphteuse. Le protocole permet de procéder à une sélection parmi un panel de PCR et de marqueurs de séquençage dans le but de caractériser les gènes de divers isolats représentant les sept sérotypes du virus de la fièvre aphteuse. Les auteurs décrivent également une liste de séquences pouvant servir de cadre à l'analyse phylogénétique, dont des séquences prototypes pour tous les topotypes proposés du virus de la fièvre aphteuse. Les données techniques détaillées et les séquences prototypes fournies par les auteurs peuvent être utilisées par les Laboratoires de référence pour la fièvre aphteuse et d'autres institutions, en vue d'harmoniser l'épidémiologie moléculaire du virus de la fièvre aphteuse.

Mots-clés
Amplification en chaîne par polymérase couplée à une transcription inverse – Phylogénétique – Prototype – Référence – Séquençage – Séquence – Virus de la fièvre aphteuse.

Protocolo de secuenciación de la VP1 para estudiar la epidemiología molecular del virus de la fiebre aftosa

N.J. Knowles, J. Wadsworth, K. Bachanek-Bankowska & D.P. King

Resumen
Las secuencias nucleotídicas de las cepas salvajes del virus de la fiebre aftosa nos ayudan a entender la distribución y evolución de los linajes víricos circulantes en distintas regiones del mundo. Los autores exponen sucintamente un práctico procedimiento de reacción en cadena de la polimerasa acoplada a transcripción inversa (RT-PCR) y de secuenciación que se puede utilizar para generar secuencias de ARN que codifican la región VP1 (1D) del virus de la fiebre aftosa. El protocolo ofrece la posibilidad de elegir entre todo un repertorio de cebadores de PCR y de secuenciación en el que están representados los siete serotipos víricos existentes con objeto de caracterizar genéticamente diversas cepas aisladas sobre el terreno. Los autores también presentan una lista de secuencias que comprende secuencias prototípicas de todos los topotipos propuestos del virus, a fin de proporcionar un marco de referencia para el análisis filogenético. Los laboratorios de referencia para la fiebre aftosa, así como otros establecimientos, pueden servirse de las detalladas técnicas y las secuencias prototípicas aquí presentadas para armonizar el estudio de la epidemiología molecular del virus de la fiebre aftosa.

Palabras clave
Filogenética – Prototipo – Reacción en cadena de la polimerasa acoplada a transcripción inversa – Referencia – Secuencia – Secuenciación – Virus de la fiebre aftosa.

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