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Detection of foot-and-mouth disease virus from culture and clinical samples by reverse transcription-PCR coupled to restriction enzyme and sequence analysis

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Abstract – A reverse transcription-PCR (RT-PCR) method is presented for the highly sensitive and specific detection of foot-and-mouth disease virus (FMDV). A primer pair flanking a region of the viral polymerase gene (3D) corresponding to the C-terminus of the protein was designed and a single step RT-PCR reaction was developed. The assay allowed the detection of viral RNA from a variety of animal samples and from a wide range of FMDV isolates of different origins and serotypes. The presence of an *Ahd* I restriction site within the amplicon in 96% of the isolates analyzed allowed an additional confirmation step of the positive reactions by a simple digestion yielding characteristic fragment sizes. The set of primers described here was suitable for direct sequencing of the PCR product (290 bp), and the nucleotide sequences corresponding to the SAT 1 and SAT 3 strains were determined. The segment amplified, when used in phylogenetic studies, allowed the clustering of SAT isolates and the rest of FMDV strains as two separate lineages.

foot-and-mouth disease / direct RT-PCR / restriction enzyme analysis / sequence analysis

1. INTRODUCTION

Foot-and-mouth disease virus (FMDV) is the causal agent of an extremely contagious, widespread and economically devastating vesicular disease affecting cloven-hoofed animals (mainly cattle, pigs, sheep, goats and buffalo) [5, 19]. As a member of the *Picornaviridae* family, genus

Aphthovirus [17], FMDV has a positive single-stranded RNA genome coding for the viral proteins which are expressed as a single polyprotein, subsequently processed by several viral proteases into the functional products. There are seven serotypes of the virus. Serotypes A, O and C, are widely distributed; serotypes SAT 1, SAT 2 and SAT 3, are normally restricted to

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sub-Saharan Africa, and serotype Asia 1 is restricted to Asia. There is considerable heterogeneity within each serotype and no cross-immunity has been found between the serotypes.

Effective control of the foot-and-mouth disease requires the early detection of infected animals even before the onset of the classical signs of the disease. This enables to discriminate between infected and uninfected herds and the subsequent elimination of infectious livestock. Tests based on RT-PCR are therefore assuming an important role for the laboratory detection of FMDV as an alternative to virus isolation tests which are time-consuming (up to 5 days) and require cell culture of the infectious virus, involving work at high-security laboratories. An effective distinction is as well required between FMDV and the two other viruses causing major indistinguishable vesicular diseases affecting swine [11]: swine vesicular disease virus (SVDV) [21] included in the *Picornaviridae* family, and vesicular stomatitis virus (VSV), a rhabdovirus restricted to the Americas [23]. Bovine viral diarrhoea virus (BVDV), a pestivirus of the *Flaviviridae* family can induce a mucosal form of the disease in cattle which might also be confused with FMD [6].

In this study we present a highly sensitive and specific RT-PCR assay for the detection of FMDV isolates performed in a single step reaction. The primer set designed for the amplification brackets a segment of the viral RNA polymerase 3D gene and includes an *Ahd I* restriction site highly conserved among isolates that can be used as an additional confirmation step in a rapid digestion reaction following amplification. No cross-reactions were detected with SVDV, VSV and BVDV isolates. In order to explore the range of detection of our RT-PCR method, we tested 48 different FMDV isolates, and successfully detected the virus in samples from different fluids, tissues and organs from experimentally infected animals. Sequencing of the RT-PCR products using

the same primers allowed reliable nucleotide sequence determination, and we report here new 3D sequence information obtained from SAT 1 and SAT 3 strains. In phylogenetic analysis, the 3D amplicon proved to contain an informative value, clustering SAT isolates independently from the rest of the FMDV strains.

2. MATERIALS AND METHODS

2.1. Viruses

The FMDV viral isolates used in this study are listed in Table I. SAT 1-3 and LEB/88 isolates were provided by the World Reference Laboratory for FMD, Institute for Animal Health (IAH), Pirbright Laboratory, UK. Except FMDV A5w (GER/48), A22 (IRQ 24/64), O1k (GER/60) and CS8 (SPA/70), all FMDV isolates were provided as vesicular epithelium samples from experimental infections and supernatant obtained after passage of the sample in cell culture by Scott Reid from IAH, Pirbright, UK. Other related viruses were used: SVDV isolates IT/66, UK/72, Alfort/73, SP1/93, SP2/93, ROM2/73, Italy 1083/92, HKN 4/72 and HKN 25/91; the last four SVDV isolates were kindly provided by Emilian Brocchi from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "B. Ubertini", Brescia, Italy. New Jersey and Indiana VSV serotypes, and the NADL BVDV strain were also included in the study.

2.2. Experimental infection and sampling of the animals

Two four month-old Landrace × Large White pigs were inoculated in the coronary matrix with 10^5 PFU of FMDV A₂₂ Iraq (IRQ 24/64). Sampling of nasal and pharynx secretions using cotton swabs and blood (sera) was done at 0, 1, 2 and 3 days post infection (pi). At 4 days pi the animals were anaesthetized for slaughtering and several tissues and organs were collected.

Table I. Summary of the results obtained by RT-PCR using 3D-1/3D-2 or A/B primer sets on epithelial suspensions (ES) and supernatant fluid from the passage of ES in cell culture with the FMDV isolates used in this study.

Serotype	Isolate	3D-1/3D-2		A/B	
		Epithelial suspension	Cell culture fluid	Epithelial suspension	Cell culture fluid
A	TUR 4/99	+	+	+	+
	IRN 1/97	+	+	+	+
	BRA 11/93	-	+	+	+
	SAU 19/92	+	+	+	+
	GAM 46/98	+	+	+	+
	BRA 10/93	-	-	+	+
	SAU 17/92	+	+	+	+
	TUR 10/88	+	+	+	+
	ERI 3/98	-	-	+	+
	MCD 6/96	+	+	+	+
	IRQ 24/64	+	+	+	+
	GER/48	NA	+	NA	+
	O	SAU 72/94	+	+	+
TUR 3/87		+	+	+	+
PHI 13/95		+	+	+	+
GRE 21/94		+	+	+	+
TUR 3/94		+	+	+	+
KUW 4/97		+	+	+	+
BRA 4/94		+	+	+	+
SAU 8/88		+	+	+	+
IRN 15/97		+	+	+	+
KUW 3/88		-	-	+	+
GER/60	NA	+	NA	+	
C	PHI 2/90	-	+	+	+
	ITL 2/88	+	+	+	+
	PHI 3/87	+	+	+	+
	GER 26	+	+	+	+
	BEL 1/69	+	+	+	+
	LEB 9/69	+	+	+	+
	BEL 53	+	+	+	+
	SRL 4/88	+	+	+	+
	ANG 3/73 *	-	+	-	+
	BRA 55	+	+	+	+
SPA/70	NA	+	NA	+	
Asia 1	BUR 12/77	+	+	+	+
	CAM 3/93	+	+	+	+
	PAK 2/98	+	+	+	+
	IRN 58/99 *	+	+	+	+
	IND 10/82	+	+	+	+
	PAK 3/98	+	+	+	+
	SAU 39/94	+	+	+	+
	TUR 15/73	+	+	+	+
	KUW 2/81	+	+	+	+
	NEP 58/88	+	+	+	+
LEB/88	+	+	+	+	
SAT 1	2/54	NA	-	NA	+
SAT 2	RHO 1/48	NA	-	NA	+
SAT 3	RV 7/34	NA	-	NA	+

NA: not available.

* Isolate yielding A/B RT-PCR product resistant to *Ahd* I digestion.

2.3. Virus titration

Virus infectivity was determined by titration of tissue culture FMDV isolates O1k (GER/60) and SAT 1 (2/54) in IBRS-2 monolayers. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% foetal bovine serum. Basically, serial dilutions of the isolates in a 500- μ L volume were added to 2×10^6 cells, incubated for 1 h at 37 °C and vigorously shaken every 15 min. Then, the inoculum was removed and the cells were kept at 37 °C under 0.6% agar overlay. Monolayers were fixed and stained 24 hpi. The infections were performed on duplicates. Resulting plaques were scored and referred to the volume of the inoculum (PFU/mL).

2.4. RNA extraction

Total RNA was extracted with Tri Reagent (Sigma-Aldrich, Steinheim, Germany) from supernatants of virus-infected cells (BHK-21 and IBRS-2) according to the manufacturer's recommendations. Pharynx and nose swabs were solubilized in 1 mL of phosphate-buffered saline (PBS) prior to RNA extraction. Sera samples were extracted without any further manipulation. Alternatively, 10-fold dilutions in PBS of homogenates from different tissues and organs analyzed were extracted.

2.5. Primers

Primers A (5'-CACACGGCGTTCA CCCA(A/T)CGC-3') and B (5'-GACAAA GGTTTTGTCTTGGTC-3') were designed to bracket a 290-bp region in the 3D polymerase gene, based on an alignment of the available FMDV sequences using CLUSTALW 1.8 software (Fig. 1). The EMBL accession numbers for the sequences shown in Figure 1 were as follows: CS8, M11027; C rp146, AJ133359; O Chu-pei, AF026168; O Taiwan, AF154271; O1 Geshur, AF189157; A10-

61, X00429; O1k, X00871; C3 Arg 85, AJ007572; A12, M10975; A5Ww, AJ010871; A22-645, X85493; A22/550, X74812, Asia 1, AF207520, and SAT 2, AJ251473. Sequences in the region analyzed for SAT 1 and SAT 3 were not available and were determined following RT-PCR amplification and were included in the alignment shown in Figure 1.

The previously described primers 3D-1 and 3D-2 [15] were used in comparative detection tests.

2.6. RT-PCR

Aliquots of viral RNA (2 μ L) were added to a single step RT-PCR reaction containing 2.5 μ L of 10 \times PCR buffer II (Perkin-Elmer, Branchburg, NJ, USA), 2 μ L of 25 mM MgCl₂, 0.5 μ L of a desoxynucleotide triphosphate mixture (each at 10 mM; Roche, Indianapolis, IN, USA), 0.25 μ L (5 U) of RNase Inhibitor (Perkin-Elmer, Branchburg, NJ, USA), 0.25 μ L (12.5 U) of MuLV Reverse Transcriptase (Perkin-Elmer, Branchburg, NJ, USA Branchburg, NJ, USA), 0.125 μ L (0.6 U) of AmpliTaq Gold (Perkin-Elmer, Branchburg, NJ, USA), 5–10 pmol of each primer and RNase-free water to a final volume of 25 μ L. Amplification was performed on a PTC-100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA).

The cycling parameters were initial incubation at 48 °C for 30 min and 95 °C for 10 min; then 35 cycles consisting of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 45 s, and then a final incubation at 72 °C for 7 min. Following RT-PCR, a 12- μ L volume of each reaction was subjected to electrophoresis on a 2% agarose gel containing ethidium bromide.

RT-PCR assays using 3D-1 and 3D-2 primers were performed as described [15].

2.7. Restriction enzyme digestion

RT-PCR reactions in which amplicons with primers A/B were detected were

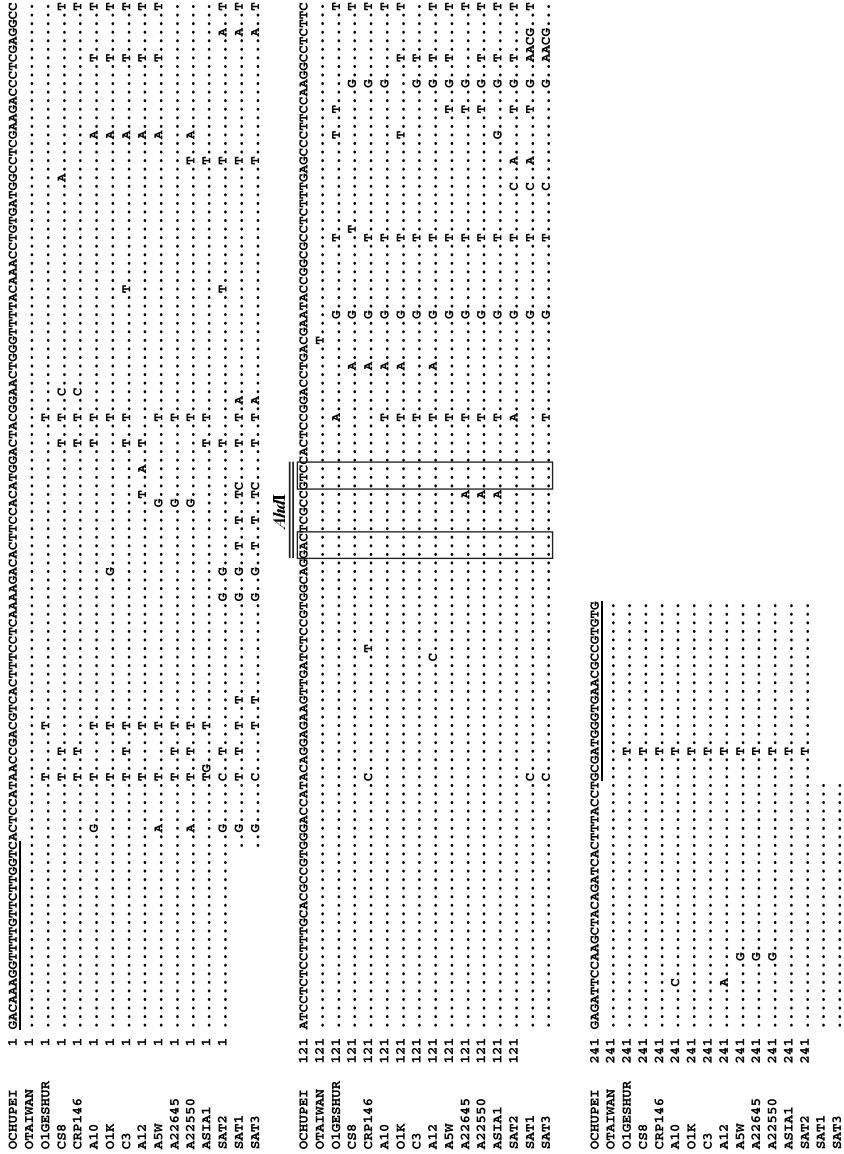


Figure 1. Alignment of FMDV nucleic acid sequences flanked by primers A and B (underlined). The region shown, spans from positions 1111 to 1400 in the 3Dpol gene [7]. SAT 1 and SAT 3 sequences were determined by direct sequencing of the amplicon. The position of the *Aha*I site is indicated by a double line and the recognition regions within the site are boxed.

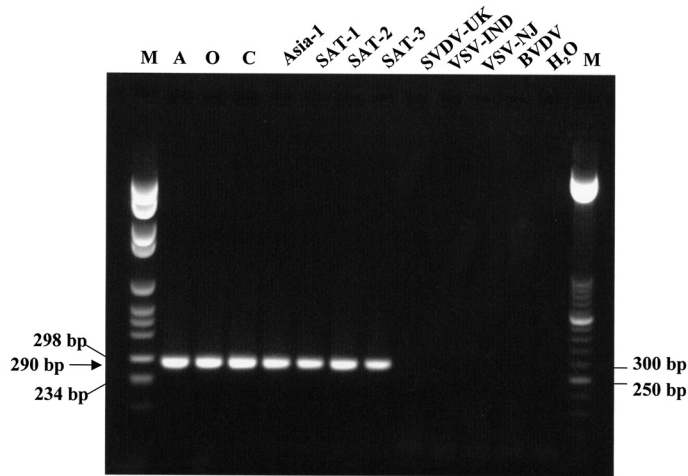


Figure 2. Specificity of primers A/B for FMDV detection. Cell culture isolates representative of each serotype and other related viruses were assayed by RT-PCR and analyzed in 2% agarose gels. The expected size band is indicated by an arrow. M, markers.

subjected to digestion with *Ahd* I (New England Biolabs, Beverly, MA, USA). The digestion mixture consisted of 10 μ L of the RT-PCR reaction and 2 U of the enzyme. The reaction mixtures were incubated for 1 h at 37 $^{\circ}$ C and then loaded onto a 2.5% agarose gel. The expected sizes of the digestion products were 181 and 109 bp, respectively.

2.8. Sequencing

Following RT-PCR, amplicons obtained from SAT 1 and SAT 3 FMDV strains were excised from agarose gel, purified with an Agarose Gel DNA Extraction Kit (Roche, Indianapolis, IN, USA) and subjected to sequencing reactions in both directions with an *fmol* DNA Cycle Sequencing System (Promega, Madison, WI, USA).

2.9. Phylogenetic analysis

Sequences flanked by primers A and B, including all the isolates shown in Figure 1, were used to derive a phylogenetic tree using the neighbor-joining method [18].

3. RESULTS

3.1. Development and specificity of the detection method

By using the available sequence information, we selected primers A and B to amplify a 290-bp fragment of the 3Dpol gene corresponding to the C-terminus of the protein (Fig. 1). The results of the RT-PCR reactions with different isolates corresponding to all seven FMDV serotypes are shown in Figure 2. A unique band of the expected size was observed in the lanes corresponding to FMDV isolates. No amplification product was observed in the samples from SVDV, VSV or BVDV, indicating that the A and B primers did not cross-hybridize with the genomes of these clinically related viruses. Several SVDV isolates (Materials and Methods) were included as control reactions to rule out the possibility of detection interference with this related swine picornavirus, giving all of them negative results (data not shown).

To confirm the positive results of our detection assay, as many as 48 different

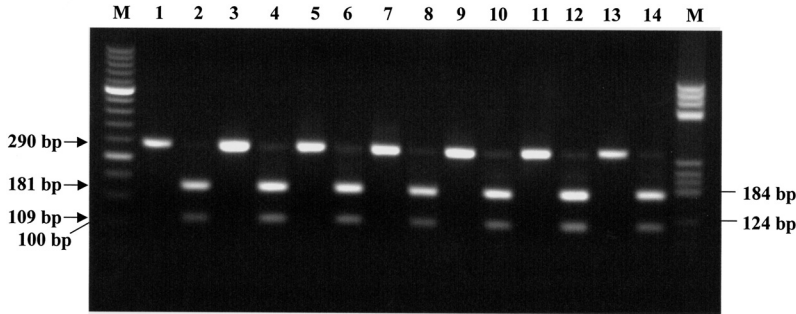


Figure 3. Restriction digestion with *Ahd I* of RT-PCR products from FMDV isolates. Digestion reaction mixtures were electrophoresed on 2.5% agarose gels. Odd number lanes show the undigested RT-PCR products. Even number lanes show the results of incubation of the amplicons with *Ahd I*. Lanes 1 and 2, FMDV A5w (GER/48); lanes 3 and 4, FMDV O1k (GER/60); lanes 5 and 6, FMDV Cs8 (SPA/70); lanes 7 and 8, FMDV Asia 1 (LEB/88); lanes 9 and 10, FMDV SAT 1 (2/54); lanes 11 and 12, FMDV SAT 2 (RHO 1/48); lanes 13 and 14, FMDV SAT 3 (RV 7/34). The undigested RT-PCR product and the expected size of the digestion products are indicated by arrows. M, markers.

FMDV isolates (Tab. I) of very diverse origin and sequences, were tested with primers A/B giving positive results yielding the expected-size amplicon (data not shown). These isolates were assayed as both vesicular epithelium samples and their corresponding cell culture supernatants. Only one of these isolates (ANG 3/73) could not be detected in the epithelium sample but was readily detected in the supernatant resulting from passage in cell culture (Tab. I). Analysis of the FMDV samples with the previously described primers 3D-1/3D-2 failed to detect 6 of the epithelium samples and 3 of the corresponding cell culture supernatants. Moreover, none of the 3 SAT-serotypes isolate could be detected with the 3D-1/3D-2 primers (Tab. I).

3.2. Restriction enzyme digestion

Alignment of the sequences flanked by the A/B primers (Fig. 1) showed the presence of an *Ahd I* site (GACNNN/NNGTC) in the RT-PCR products. The predicted size for the restriction fragments after *Ahd I* digestion were 109 and 181 bp respectively, easily detectable by electrophoresis in agarose gels. Since the

sequence information for the 3Dpol gene was limited among the FMDV isolates, we tested the presence of the *Ahd I* site empirically. Amplicons obtained by RT-PCR from FMDV isolates were subjected to restriction endonuclease digestion with *Ahd I*. After digestion and electrophoresis, viral cDNAs corresponding to all seven serotypes yielded the fragment pattern predicted by sequence analysis, as shown in Figure 3. To assess the degree of conservation of the *Ahd I* site among FMDV isolates, all 48 isolates used in the study, and successfully amplified with primers A/B, were inspected for the presence of the *Ahd I* site in their RT-PCR products. Only 2 isolates (Tab. I) yielded amplicons which remained undigested in the presence of the enzyme (data not shown). Although the absence of the *Ahd I* site sequence remains to be determined, the high conservation of this target (96%) supports its use as a specificity marker. In all the *Ahd I*-digested amplicons the 108/181 bp pattern was confirmed.

3.3. Sensitivity

The sensitivity of the RT-PCR assay with the A/B primer pair was assessed for

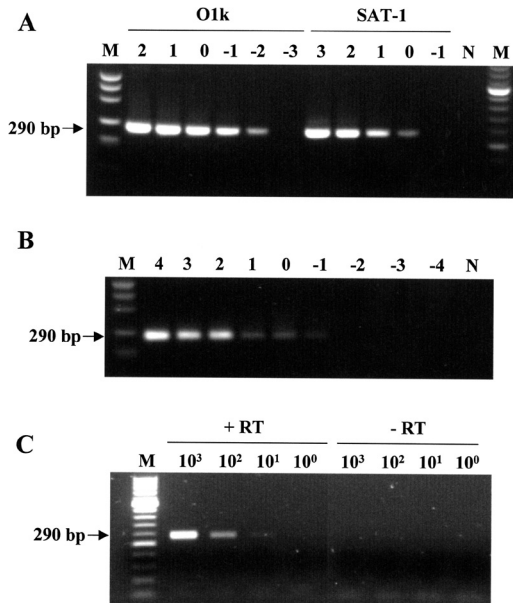


Figure 4. Detection limit of FMDV RNA by RT-PCR with primers A/B. (A) Serial dilutions of RNA extracted from cell culture: 10^2 to 10^{-3} PFU of FMDV O1k and 10^3 to 10^{-1} PFU of FMDV SAT 1 (2/54). (B) FMDV O1k (GER/60) RNA extracted independently from serial dilutions of virus (10^4 to 10^{-4} PFU). (C) Serial dilutions (from 10^3 to 10^0 fg) of full-length FMDV O1k RNA. In (-) RT lanes, reverse transcriptase was not added to the reaction mixture. RT-PCR products were analyzed by 2% agarose gel electrophoresis. N, negative control (uninfected). M, marker.

two FMDV isolates belonging to genetically and geographically divergent groups of isolates. O1k (GER/60) is a well characterized isolate at the molecular and clinical level, while for FMDV SAT 1 (2/54) there is little sequence information available. The sensitivity assay was performed by serial dilutions of RNA initially extracted from the cell culture supernatant. Independently, serial dilutions of the infected cell culture supernatant were used for RNA extractions. For serial dilutions of an initially extracted FMDV RNA, the detection limit corresponded to 10^{-2} PFU for the O1k isolate and 1 PFU for the SAT 1 isolate (Fig. 4A). When FMDV O1k RNA extractions were performed independently for each serial dilution, the detection limit was 10^{-1} PFU (Fig. 4B). The capacity of detection of quantified FMDV RNA was

assessed using full-length O1k RNA transcripts (Fig. 4C). The detection limit was 10 fg of RNA. The absence of template DNA interfering in the amplification was confirmed by (-) RT reactions.

3.4. Detection of FMDV in samples from experimentally infected pigs

Animal samples taken from 0 to 4 days pi were assayed for the presence of FMDV RNA. Table II shows the results of FMDV detection in several tissue samples using both A/B and 3D-1/3D-2 primers. Pigs 1 and 2 developed clinical signs of disease (foot lesions) on days 3 and 4 pi, respectively. We were able to detect the virus in sera as early as 1 day pi with the primers A/B. When using the 3D-1/3D-2 primers, FMDV could only be detected in the sera

Table II. Detection of FMDV by RT-PCR from tissue samples of experimentally infected pigs with the primers 3D-1/3D-2 [15] (a) and A/B (b).

Sample		Days Post Infection									
		Pig 1					Pig 2				
		0	1	2	3	4	0	1	2	3	4
Sera	(a)	-	-	+	+	-	-	-	+	+	
	(b)	-	+	+	+	+	-	+	+	+	
Pharynx Swab	(a)	-	-	-	+	+	-	-	-	+	+
	(b)	-	-	-	+	+	-	-	-	+	+
Nose Swab	(a)	-	-	+	+	+	-	-	-	+	+
	(b)	-	-	+	+	+	-	-	+	+	+
Liver	(a)					-					-
	(b)					-					-
Spleen	(a)					+					-
	(b)					+					+
Heart	(a)					+					NA
	(b)					+					NA
Lymph Nodes	(a)					NA					-
	(b)					NA					+
Kidney	(a)					-					-
	(b)					-					-
Tonsil	(a)					+					-
	(b)					+					+
Vesicular Epithelium	(a)					+					+
	(b)					+					+

NA: not available.

after 2 or 3 dpi depending on the animal. Nose swab samples gave positive results on day 2 pi and pharynx swabs on day 3 pi with the A/B primers. In pig 2, the virus was detected in nose swab samples on day 3 pi with the 3D-1/3D-2 primers. When the pigs were slaughtered on day 4 pi, a variety of samples were taken from different organs to test the presence of the virus. As shown in Table II, FMDV was detected in the spleen, heart, lymph nodes, tonsils and vesicular epithelium from foot lesions. Primers 3D-1/3D-2 failed to detect FMDV in the spleen, lymph nodes and tonsils from pig 2. The virus was not detected in the liver and kidney.

3.5. Sequence and phylogenetic analysis

RT-PCR with primers A/B enabled the direct sequencing of SAT 1 and SAT 3 amplicons. The new sequences were included in the alignment performed previously using the available sequence data (Fig. 1). All sequences shown in this alignment, immediately adjacent to the sequence of primers A and B, and corresponding to different FMDV isolates and strains, were subjected to phylogenetic analysis. The dendrogram obtained is depicted in Figure 5. Two different clusters, including the SAT isolates and the rest

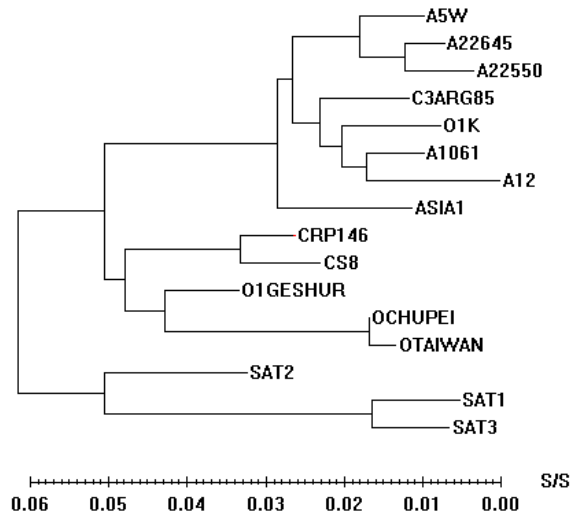


Figure 5. A phylogenetic tree derived from nucleotide sequences flanked by the primer pair A/B corresponding to FMDV isolates shown in Figure 1. Vertical distances are arbitrary and horizontal distances represent the number of substitutions per site (S/S).

of the FMDV isolates, respectively, can be clearly identified. This proves the divergent nature of the South African FMDV strains, showing marked genomic and antigenic variation.

4. DISCUSSION

Early and specific diagnosis of FMD represents an essential tool for the control of the disease. As shown in the recent UK outbreak [10], this highly contagious disease can spread rapidly in an area where infected animals have been introduced, travelling easily to other regions and countries through direct or indirect contact between livestock. The threat represented by the disease now in Europe illustrates how an old-known disease considered basically eradicated can strike back with devastating economical consequences, involving massive slaughtering of herds and introducing trade restrictions on animals and livestock products.

New strategies based on RT-PCR assays are being applied to develop rapid

and sensitive detection tests for FMDV [1, 2, 8, 12]. These protocols are an alternative or are complementary to the classical serological and viral isolation assays due to their higher sensitivity and speed and the fact that the handling of infectious viruses is not required. The main challenge found for the design of primers suitable for diagnosis has been the genetic diversity of the virus, which complicates both the efficient universal amplification of all isolates and their serotyping using specific primers, and requiring complex combinations of oligonucleotides and protocols. Various serotype-specific primer pairs have been used for the amplification of genomic regions in order to type FMDV isolates [3, 15]. RT-PCR protocols with immunocapture techniques [20] or preceded by direct adsorption of the virus to microtiter plates [16] have also been developed. Universal primers based on the 2B gene sequence have been reported [14, 22], and, in order to match virus isolation sensitivity, a nested RT-PCR was developed for FMDV detection in clinical samples [9]. Non-coding regions of the viral genome have also

been used as targets for primer design with serotype-dependent efficiency [13].

We describe here a single-tube RT-PCR assay, coupled with digestion with a restriction enzyme, for sensitive and reliable amplification of FMDV isolates. The primers designed are anchored in highly conserved regions of the 3D polymerase gene, less susceptible to nucleotide changes than capsid protein coding regions, subjected to immunological pressure and host selection [15]. Primers A/B were able to amplify a 290-bp fragment in FMDV samples. It was indeed shown that viral RNA from every FMDV isolate tested (as many as 48) was amplified and no cross-hybridization was observed with the viruses genetically or clinically related to FMDV. Detection of the virus from diverse clinical samples at early times postinfection suggest the suitability of the assay as a valuable tool for FMDV detection.

Compared with primers A/B, previously described primers 3D-1/3D-2 [15], designed to amplify a 209-bp fragment of the 3Dpol gene corresponding to the C-terminus of the protein, exhibited an impaired capacity for FMDV detection. Among the FMDV-positive samples tested, 6 epithelia and 3 cell culture supernatants failed to be detected, including the 3 SAT-serotype isolates. These results were confirmed by the detection analysis from clinical samples, since 3D-1/3D-2 primers failed to detect the virus in some organs and showed a 1 to 2 day delay in detection from the sera.

The amplification reaction with A/B primers exhibited a high detection limit, 10 fg of viral RNA, and 10^{-2} PFU for a O1k isolate. A remarkable improvement in sensitivity has been achieved with the assay described here as compared to the ELISA detection limit of 1.8×10^4 PFU for serotype O isolates in our hands, using the OIE/FAO recommended method, and PCR detection limit of 3 PFU with primers 3D-1/3D-2 [15]. However, some differences in

sensitivity were found among the two isolates analyzed (1 PFU for the SAT 1 isolate). Since they belong to genetically divergent groups, and the sequences corresponding to the primer annealing regions for SAT 1 and SAT 3 are not available, the presence of mismatches in these regions, as well as differences in the efficiency of RNA extraction and/or titration of the isolates, cannot be ruled out and contribute to the lower sensitivity in detection of the SAT 1 isolate as compared to that of O1k. Additionally, the ratio between infectious particles (measured as PFU) and amplifiable RNA may vary among different isolates and samples. Inter-serotype differences in the sensitivity of detection are as well found using ELISA (2.3×10^3 PFU for C serotype isolates, data not shown).

The possibility of sequence confirmation by restriction digestion with the *Ahd* I enzyme, which works well without having to purify the amplicon from the RT-PCR mixture, and resulting in two fragments easy to identify by gel electrophoresis, is an additional advantage to the method. Moreover, nucleotide sequence data are easy and rapid to obtain from the amplicons using the same set of primers. SAT 1 and SAT 3 sequences corresponding to the amplified 3D fragment were determined within one day.

Sequences flanked by primer pair A/B including all seven FMDV serotypes were used to derive a phylogenetic tree. The dendrogram obtained shows the segregation of the SAT isolates into an independent group, with SAT 1 and SAT 3 being more closely related among them than the SAT 2 strain. It is remarkable that a 290 nt window of a relatively conserved gene (3D) contains an informative value to allow the independent clustering of the two main, geographically and genetically divergent viral isolates. A similar cluster for SAT isolates was found when the highly variable VP1 gene (capsid protein component) sequences were used in the phylogenetic analysis [4].

In summary, we have described a rapid, sensitive and specific method for FMDV detection, well-suited for clinical samples and isolate clustering. Future work in the lab will be focused on the development of real time PCR technology with primers A/ B and analysis of sheep samples.

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