

Development of a new RT-PCR with multiple primers for detecting Southern African Territories foot-and-mouth disease viruses

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Abstract

Introduction: The extremely high genetic variation and the continuously emerging variants of foot-and-mouth disease virus (FMDV) of Southern African Territory (SAT) serotypes including SAT1, SAT2, and SAT3 make it necessary to develop a new RT-PCR for general use for monitoring viruses based on the updated genome information. **Material and Methods:** A FMDV SAT-D8 one-step RT-PCR was established based on the 1D2A2B genes of the SAT serotype viruses with a multiplex primer set. FMDV A, O, C, and Asia 1 serotypes, other vesicular disease viruses, inactivated SAT viruses, and 125 bovine, ovine, caprine and porcine tissue samples collected from the Chinese mainland were included for evaluating the assay. **Results:** The new RT-PCR was proven to be specific without cross-reactions with Eurasian FMDV, swine vesicular disease virus (SVDV), Seneca valley virus (SVV), or other common viral pathogens of cattle, sheep, goat, and pig. An around 257 bp-sized amplicon clearly appeared when the inactivated SAT viruses were detected. However, all 125 samples collected from FMDV-susceptible animals from the Chinese mainland which has not known SAT epidemics showed negative results. **Conclusions:** A FMDV SAT-D8 one-step RT-PCR is a promising method for primary screening for FMDV SAT serotypes.

Keywords: Southern African Territory foot-and-mouth disease, RT-PCR, diagnosis, specificity, sensitivity.

Introduction

Foot-and-mouth disease (FMD), caused by the FMD virus (FMDV), is a highly infectious disease of cloven-hoofed animals such as cattle, buffalo, sheep, goats, and pigs (2, 10). The disease in infected animals is characterised by high fever, lameness, and vesicular lesions on the tongue, lips, teats, nares, and feet. At the present time, FMD is still endemic in more than 100 countries worldwide, and it has become one of the most important livestock diseases in terms of economic impact due to a dramatic decline in productivity and restrictions on international trades in animal products to enforce FMD control (9, 17).

FMDV is a single-stranded RNA virus belonging to the genus *Aphthovirus* of the family *Picornaviridae*.

The virus can be divided into seven serotypes, namely A, O, C, Asia 1, and Southern African Territories (SAT) 1, SAT2, and SAT3 according to the genetic and antigenic divergence. Generally, the seven serotypes can be classified into two divisions: a Eurasian group consisting of A, O, C, and Asia 1 serotypes, and a SAT group including SAT1, SAT2, and SAT3 (11). Each serotype is characterised by a diversity of topotype, genetic lineages, and strains. There is no cross-protection among these serotypes (7). The seven serotypes are not distributed evenly geographically. Serotypes A, O, and Asia 1 are widespread around the world, especially A and O (13, 14) whereas serotype C has nearly disappeared and was last detected in Brazil and Kenya in 2004 (9, 15). Serotypes SAT1, SAT2, and SAT3 are usually endemic in Southern Africa (5, 8).

However, FMDV SAT serotypes have broken through the geographic barriers and spread beyond Africa. For example, FMDV SAT1 spread to the Middle East during 1962–1965 and 1969–1970. FMDV SAT2 was found in Yemen in 1990 and in Kuwait and Saudi Arabia in 2000 (8). Afterwards, in 2012, there were several SAT2 outbreaks in Egypt, the Palestinian Administered Territories (Gaza Strip), and Bahrain, which were not endemic areas for FMDV SATs (1, 16). FMDV SATs are characterised by high genetic variation, especially for serotype SAT2 which is sub-classified into at least 14 geographically restricted topotypes, and defined as having 80% nucleotide identity in the VP1 segment. This variability further complicates the epidemiology of the disease (3, 4). Hence, it is of great significance to establish accurate molecular detection methods for FMDV SATs to prevent and control the disease, especially in the regions where there have been no of FMDV SATs epidemics. Although some molecular detection methods targeting FMDV SATs have been established previously, these methods are less sensitive and specific due to the great propensity for mutations of FMDV SATs, especially in SAT2 (1, 4). For example, Callens and De Clercq (6) designed a universal reverse primer P1 and multi-forward primers to detect SAT1 (P126 and P151–153), SAT2 (P168–170), and SAT3 (P130 and P158–161), respectively. However, the multiplex mix for FMDV SATs typing could not differentiate between the SAT1 and Asia 1 strains, and it had cross reaction among the three SAT serotypes. Reid *et al.* (12) also developed an RT-PCR assay for the detection of FMDV SATs with SAT-1D209F/FMDV-2B208R primers. However, results showed that this primer set was less efficient in detecting FMDV SATs, and it also gave false positive results for other serotypes. Therefore, it is necessary to redesign the primers to develop a new RT-PCR assay to improve its sensitivity and specificity for detecting FMDV SATs. The availability of a growing set of full genome sequences has now made it possible. The aim of this study was to strengthen and improve RT-PCR for fast, reliable, and specific diagnosis of the FMD caused by SATs by redesigning multiple sets of primers targeting the 1D2A2B gene. This can guarantee the efficiency of primary detection of SAT serotypes in

order to meet the requirements for FMD epidemics to be monitored, especially in the countries with no such cases ever in their history.

Material and Methods

Recombinant plasmids and viruses. SAT1 strains in a set of 8 (GenBank accession nos. JF749860, AY593846, AY593842, AY593841, AY593840, AY593839, AY593838, and HM067706), 13 SAT2 (GenBank accession nos. HM067705, AF540910, KC440884, JF749862, AY593848, AY593847, AJ251473, JF749861, JF749864, HM067704, KU821592, KM268900, and KR108955), 6 SAT3 (GenBank accession nos. AY593850, AY593852, AY593853, KR108950, KJ820999, and KM268901), and 4 C (GenBank accession nos. DQ409188, AY593809, AY593810, and AJ007347) FMDV representative strains with low homology within the same serotype were selected by the comparative analysis of their full genome sequences deposited in GenBank. Their 1D2A2B genes were synthesised and inserted into the pUC57 vector to be used as a template to develop the assay. Viruses including FMDV A, O, and Asia 1, swine vesicular disease virus (SVDV), Seneca valley virus (SVV), bovine viral diarrhoea virus (BVDV), orf virus (ORFV), sheep pox virus (SPPV), goat pox virus (GTPV), bluetongue virus (BTV), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), porcine pseudorabies virus (PRV), porcine parvovirus (PPV), and porcine circovirus (PCV) stored at -70°C in the Lanzhou Veterinary Research lab were used to determine the specificity of the one-step RT-PCR assay.

Primers of FMDV SAT-D8 one-step RT-PCR assay. The 3' end region of FMDV SATs 1D gene, encoding the variable VP1 structure protein, was selected to design multiple forward primers and the reverse primer was located in the highly conserved 2B gene. The primer set, named SAT-D8, consisted of seven forward primers and one reverse primer (Table 1). The RT-PCR product was ~257 bp for FMDV SATs.

Table 1. Multiple primers of FMDV SAT-D8 RT-PCR assay

Primers	Sequences
P280-F (FMDV-SAT)	5- GGCGTTGAGAAACAACACTGTG-3
P281-F (FMDV-SAT)	5-GGCGTTGAGAAACAGCTGTG-3
P282-F (FMDV-SAT)	5- GGTGTCGAAAAACAGTTGTG-3
P283-F (FMDV-SAT)	5- GGTGTCGAAAAACAGCTGTG-3
P310-F (FMDV-SAT)	5-GGCGTCGAAAGACAGACCCT-3
P329-F (FMDV-SAT)	5-GCACCAGCCAAACAACACTGTG-3
P331-F (FMDV-SAT)	5-AGTGCTGACAAGCAGATGTG-3
P296-R (FMDV-SAT)	5- ACYTTGTACCAGGGYTTGGC-3 (Y = C + T)

RNA/DNA extraction. Viral RNA samples were extracted using a QIAGEN RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's protocol. Viral DNA samples from cells, tissues, and sera for analysis were extracted using a TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Version 4.0 (TaKaRa, China) according to the manufacturer's instructions.

Establishment of FMDV SAT-D8 PCR based on the synthesised 1D2A2B gene and measurement of the sensitivity. The FMDV SAT-D8 PCR method was firstly established after selection rounds for primers based on the synthesised 1D2A2B genes of FMDV SATs. PrimeSTAR GXL DNA Polymerase (TaKaRa, China) was used to prepare the mix solutions according to manufacturer's guidelines. The final 20 μ L volume of PCR reaction solution consisted of: 0.4 μ L of PrimeSTAR GLX enzyme (1.25U/ μ L), 4 μ L of $5 \times$ PS PrimeSTAR Buffer, 1.6 μ L of dNTP mixture (2.5 mM/ μ L), 5 pM of each forward primer, 10 pM of reverse primer, 3–30 ng of DNA template, and Nuclease-free water up to 20 μ L. The optimised PCR amplification condition was 95°C for 5 min, followed by 37 cycles of 94°C for 1 min, 60°C for 30 s, and 68°C for 30 s, and then 68°C for 8 min. The products were detected with 2% agarose gel electrophoresis in $1 \times$ TAE buffer (40 mM Tris-acetate and 1 mM ethylene-diaminetetraacetic acid, pH 8.0).

FMDV SAT-D8 PCR sensitivity was measured by a randomly selected representative of the synthesised DNA of SAT1, SAT2, and SAT3. First, DNA concentration was determined by spectrophotometry and then the molecule numbers were calculated according to the corresponding formulations. Then, the DNA was 10-fold serially diluted from 4×10^8 copies/ μ L to 4×10^0 copies/ μ L and used as the template for amplification. The minimum detectable quantity of DNA template was defined as the analytical sensitivity of the assay.

Establishment of FMDV SAT-D8 one-step RT-PCR based on RNA transcript of 1D2A2B gene and measurement of the sensitivity. After the multiplex primer set, *i.e.* SAT-D8, was selected by the FMDV SAT-D8 PCR method based on the synthesised 1D2A2B DNA, an FMDV SAT-D8 one-step RT-PCR was then developed with the corresponding RNA transcripts. A one-step RT-PCR kit (TaKaRa, China) was used to prepare the mix solutions according to the manufacturer's protocol. Each reaction mixture contained: 0.8 μ L of PrimeScript 1 Step Enzyme Mix, 10 μ L of $2 \times$ 1 step buffer, 0.5 μ L of each of the seven forward primers (10 μ M), 1 μ L of reverse primer (10 μ M), and 1 μ L of RNA transcript template in the final volume of 20 μ L. The amplification conditions were 50°C for 30 min, 35 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s, and then 72°C for 8 min. The RT-PCR products were detected on 2% agarose gel as mentioned above.

To prepare RNA transcripts, the synthesised 1D2A2B genes were amplified by PCR with primers containing T7 promoter by adding the sequence to the N-

terminal of the reverse primer. These primers were as follow: P386-F(SAT2):5-GCCAGTGAATTGACGCG TAT-3; P387-T7-R(SAT2):5-TAATACGACTCACTA TAGGGTTGTGTGGAATTGTGAGCGG-3; P388-F(SAT1/3):5-GTTGTAAAACGACGGCCAGT-3; P389-T7-R(SAT1/3):5-TAATACGACTCACTATAGGGGC TTCCGGCTCGTATGTTGTG-3.

The P386 and P387 primers were used for SAT2 RNA transcript preparation, while P388 and P389 served for SAT1 and SAT3. The PCR was performed using TaKaRa Premix Taq (TaKaRa, China). After purification with DNA Gel Extraction Kit (Axygen, China), PCR products with T7 promoter were transcribed using an mMESSAGE mMACHINE Kit (Invitrogen, USA). Afterwards, the transcribed RNA was purified with an RNeasy MinElute Cleanup Kit (Invitrogen, USA). The RNA transcript was 10-fold serially diluted from 4×10^9 copies/ μ L to 4×10^0 copies/ μ L and served as the template for the one-step RT-PCR sensitive measurement assay. The minimum detectable amount of RNA transcript template was defined as the analytical sensitivity of the assay.

Specificity of FMDV SAT-D8 one-step RT-PCR assay. The specificity of the FMDV SAT-D8 one-step RT-PCR assay was determined with the variety of other viruses mentioned above. Those viruses were firstly verified by RT-PCR or PCR using their specific primers under appropriate amplification conditions, and then they were used to verify the specificity of the newly established assay.

Clinical specimen. To test the efficiency of the new assay, 128 samples were subjected to an FMDV SAT-D8 one-step RT-PCR. FMDV SAT1, SAT2, and SAT3 inactivated antigens were obtained from Pirbright Laboratory, United Kingdom. The other 125 field samples including organ tissues (heart, liver, spleen, lung, kidney and tongue) and sera of sheep, goats, cattle and pigs were collected on the Chinese mainland. Either RNA or DNA was extracted using the TaKaRa MiniBEST Viral RNA/DNA Extraction Kit as mentioned above.

Results

Establishment of FMDV SAT-D8 PCR based on the synthesised 1D2A2B gene and sensitivity measurement. The FMDV SAT-D8 PCR assay was first established based on the synthesised 1D2A2B genes. The 8 SAT1, 13 SAT2, 6 SAT3, and 4 C of FMDV representative strains with complete genome sequences deposited in GenBank were selected by their low homology (around or less than 85%) of the 1D gene within the same serotype. Fig. 1 shows the nucleotide identity of the 1D gene of all 31 selected strains. Their 1D2A2B genes were synthesised and then inserted into the pUC57 vector to be used as the templates to develop the assay. The 3' end regions of the FMDV SATs 1D genes encoding the variable VP1 structure protein were

selected to design the multiple forward primers and the reverse primer was located in the highly conserved 2B gene. The multiplex primers composed of seven forward primers and one reverse primer were selected because

they were found to generate the expected amplicon of ~257 bp on the 2% agarose gel for all the 27 SAT1, SAT2, and SAT3 representative strains (Fig. 2).

		Percent Identity																																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31		
Divergence	1	100	77.4	75.9	73.9	73.5	73.0	72.9	72.7	60.2	60.0	61.6	60.0	59.2	59.2	58.3	59.4	60.3	58.6	59.9	57.6	60.4	58.4	59.4	59.8	57.1	57.3	59.2	53.2	52.0	54.5	55.5	1	SAT1(JF749860)
	2	27.1	100	87.9	75.0	74.8	75.1	73.9	73.3	60.1	59.5	61.6	62.5	61.9	61.1	58.9	61.1	61.9	59.4	61.3	58.4	60.9	59.8	59.8	57.0	58.6	58.4	57.5	53.6	53.8	55.3	55.8	2	SAT1(AY593839)
	3	29.4	13.4	100	76.0	76.2	74.7	74.8	72.2	60.4	60.0	61.1	62.6	59.8	58.9	58.1	58.6	60.8	58.1	59.2	57.6	60.7	59.4	60.6	57.7	56.9	58.5	59.3	53.5	52.8	54.9	56.0	3	SAT1(AY593846)
	4	32.3	30.6	29.0	100	85.7	86.6	85.2	70.7	62.5	62.0	61.7	63.1	62.5	61.1	61.6	60.8	62.3	61.6	62.1	61.7	62.4	60.3	59.8	61.1	58.3	59.8	61.0	54.8	53.6	55.1	56.0	4	SAT1(AY593842)
	5	33.0	30.8	28.8	16.3	100	87.6	85.1	71.6	63.0	61.2	60.0	60.0	60.2	59.2	60.2	59.1	60.8	60.0	60.1	57.9	60.4	58.6	60.9	60.4	57.2	59.2	59.0	54.6	54.3	55.4	55.8	5	SAT1(AY593840)
	6	33.8	30.4	31.1	15.1	13.8	100	84.5	70.1	63.3	62.5	60.5	60.9	61.4	60.5	61.5	61.2	61.1	60.2	60.9	58.7	60.6	60.7	60.7	59.7	56.3	59.0	59.5	53.0	54.1	54.9	54.8	6	SAT1(AY593841)
	7	33.9	32.2	30.8	16.8	17.0	17.8	100	70.3	61.9	60.0	59.5	60.9	60.6	59.7	60.5	60.5	60.9	59.5	60.4	59.2	60.6	58.1	59.8	60.9	56.8	59.2	59.9	53.8	53.0	52.8	55.6	7	SAT1(AY593838)
	8	34.2	33.4	35.0	37.3	35.7	39.3	38.2	100	59.5	58.8	62.0	59.7	59.2	59.1	67.2	60.0	59.8	60.0	61.2	59.6	59.3	59.2	59.2	59.8	57.9	60.6	52.2	51.5	52.0	52.6	8	SAT1(HM067706)	
	9	56.6	56.9	56.3	51.8	50.8	50.2	53.1	57.9	100	99.8	77.0	74.4	70.8	70.5	74.7	69.1	74.1	71.8	76.2	74.0	74.3	57.3	58.6	57.5	55.3	57.3	58.1	53.0	52.5	53.0	52.9	9	SAT2(AF540910)
	10	57.2	58.3	57.2	53.0	54.6	52.1	57.3	60.0	38.7	100	71.6	72.7	76.7	77.8	71.1	76.2	70.7	74.7	73.1	70.6	69.2	60.2	61.8	59.8	55.4	60.2	61.8	52.3	51.9	53.3	52.7	10	SAT2(AJ251473)
	11	54.0	54.0	55.0	53.7	57.3	56.3	58.6	53.0	27.4	35.7	100	78.2	69.3	71.8	78.5	69.9	76.4	72.4	83.8	80.1	78.0	57.9	57.9	57.1	56.3	57.7	58.9	52.8	53.7	54.2	54.3	11	SAT2(AY593847)
	12	57.3	52.0	51.7	50.8	57.3	55.3	55.3	57.8	31.4	34.0	25.9	100	73.1	71.6	79.5	72.1	80.4	72.7	79.3	78.2	84.6	60.5	61.8	60.1	56.5	60.3	60.7	53.9	53.4	54.9	55.4	12	SAT2(AY593848)
	13	58.8	53.3	57.5	52.0	56.9	54.2	55.9	58.8	36.8	28.0	39.5	33.3	100	82.6	72.5	83.0	71.1	72.5	71.7	71.0	71.2	59.4	61.2	60.5	58.5	60.2	60.8	51.1	51.1	51.0	50.2	13	SAT2(HM067704)
	14	58.8	54.9	59.5	54.9	59.0	56.2	58.0	59.2	37.4	26.5	35.4	35.8	20.4	100	71.1	90.6	70.8	73.1	70.1	70.9	69.3	59.0	59.3	59.0	58.7	60.2	60.5	51.0	51.0	51.6	51.2	14	SAT2(HM067705)
	15	61.0	59.6	61.4	53.9	56.8	53.9	56.2	63.5	31.0	36.5	25.5	24.1	34.2	36.5	100	70.5	79.5	71.0	80.1	86.9	78.8	58.2	57.7	60.7	57.6	60.2	58.6	52.0	52.1	52.6	52.0	15	SAT2(JF749861)
	16	58.5	54.9	60.3	55.6	55.6	54.9	55.9	57.2	39.7	28.8	38.5	35.0	19.6	10.2	37.5	100	71.9	71.9	70.6	71.5	69.8	58.7	60.5	58.7	56.3	59.9	59.4	50.2	51.1	50.5	50.6	16	SAT2(JF749862)
	17	56.5	53.4	55.6	52.4	55.6	54.9	55.4	57.5	32.0	37.3	28.7	22.9	36.5	37.0	24.1	35.2	100	70.4	77.9	78.0	79.9	58.0	59.9	59.0	55.2	58.6	58.3	52.9	52.1	52.8	52.7	17	SAT2(JF749864)
	18	60.5	58.6	61.5	54.0	57.2	56.9	58.3	57.2	35.3	31.1	34.5	34.2	34.3	33.3	36.8	35.3	38.0	100	73.1	72.0	71.2	57.6	59.9	58.8	57.7	60.5	60.2	54.2	53.6	52.5	53.8	18	SAT2(KC440884)
	19	57.1	54.2	58.9	52.6	56.8	55.1	56.3	54.5	28.5	33.3	18.4	24.2	35.4	38.0	23.1	37.2	26.3	33.3	100	80.5	78.2	58.6	58.7	59.2	58.0	58.7	60.3	55.3	54.4	55.3	55.5	19	SAT2(KU821592)
	20	62.5	60.7	62.6	53.7	61.9	60.1	59.1	57.5	32.1	37.5	23.4	26.0	36.7	36.9	14.6	35.9	26.2	35.3	22.5	100	77.9	57.8	57.0	59.2	56.4	58.7	58.6	52.5	52.5	53.0	53.0	20	SAT2(KM268900)
	21	56.4	55.5	55.7	52.1	56.4	56.1	58.1	58.7	31.5	39.9	26.2	17.5	36.4	39.6	25.1	38.8	23.6	36.6	25.8	26.4	100	59.4	59.8	59.8	56.4	59.7	60.3	53.0	52.8	54.6	54.2	21	SAT2(KR108955)
	22	60.9	58.1	59.0	56.8	60.7	55.7	61.6	59.3	63.0	56.7	61.9	56.2	58.4	59.5	61.1	60.2	61.5	62.8	60.2	62.1	58.5	100	85.8	75.2	59.4	75.0	76.7	55.2	53.9	54.7	52.8	22	SAT3(AY593853)
	23	58.8	58.3	56.4	58.0	55.4	55.7	57.7	59.5	60.3	53.5	62.0	53.6	54.8	59.0	62.3	56.2	57.4	57.4	59.9	64.2	57.6	18.0	100	75.8	60.7	78.6	76.2	55.4	54.3	55.5	53.5	23	SAT3(AY593852)
	24	57.6	64.7	63.3	55.0	56.4	58.0	55.3	57.8	62.9	57.8	63.8	57.1	56.1	59.5	55.7	60.2	59.4	60.1	58.8	59.0	57.5	30.4	29.5	100	62.1	86.2	86.9	54.5	51.9	53.2	52.5	24	SAT3(AY593850)
	25	64.7	60.7	65.5	61.6	64.2	66.4	65.8	62.5	68.2	68.0	65.9	65.3	60.6	60.1	62.7	65.8	68.6	62.5	61.5	65.7	65.5	58.7	56.2	52.9	100	61.2	61.3	60.3	59.5	59.4	60.8	25	SAT3(KJ820999)
	26	63.5	61.4	61.2	57.8	59.2	59.5	59.1	59.1	63.3	56.8	62.4	56.5	56.8	56.8	56.8	57.5	60.2	56.2	59.8	60.0	57.8	29.3	25.3	15.7	55.0	100	83.7	55.1	52.8	54.6	52.1	26	SAT3(KR108950)
	27	59.1	63.5	59.0	55.1	59.6	58.5	57.3	56.1	61.3	53.5	59.5	55.8	55.5	56.1	60.2	58.5	60.9	56.8	56.3	60.4	56.5	28.2	29.0	14.6	54.6	18.8	100	54.8	52.0	54.2	53.1	27	SAT3(KM268901)
	28	75.1	73.0	73.6	70.0	70.7	75.2	73.4	77.2	73.8	76.1	74.6	71.6	79.1	79.7	76.8	82.2	74.4	70.7	67.9	75.3	74.1	68.6	68.4	70.4	57.0	68.4	69.6	87.9	85.7	86.3	28	C(DQ409188)	
	29	78.1	72.3	75.2	72.8	71.1	71.4	75.1	78.5	75.1	77.1	72.0	72.9	79.2	79.8	76.4	79.2	76.9	72.5	70.2	75.4	75.1	72.1	70.9	77.2	58.8	74.4	76.9	13.5	88.2	87.3	29	C(AY593809)	
	30	71.1	68.7	69.7	69.1	68.3	69.4	76.1	77.5	73.9	73.3	70.7	69.0	79.8	77.9	75.0	81.2	75.0	75.6	68.0	74.0	69.8	70.0	68.0	73.7	58.9	69.7	71.1	16.3	13.1	86.4	30	C(AY593810)	
	31	67.9	67.0	66.6	66.7	67.2	69.6	67.7	75.9	73.9	74.8	70.5	67.6	82.0	79.0	76.6	80.8	75.2	71.8	67.3	73.8	70.9	75.3	73.7	76.0	55.9	76.6	74.1	15.6	14.3	15.4	31	C(AJ007347)	

Fig. 1. The nucleotide identity of 1D genes of all 31 selected representative strains of FMDV SATs and C with complete genomic sequences deposited in GenBank

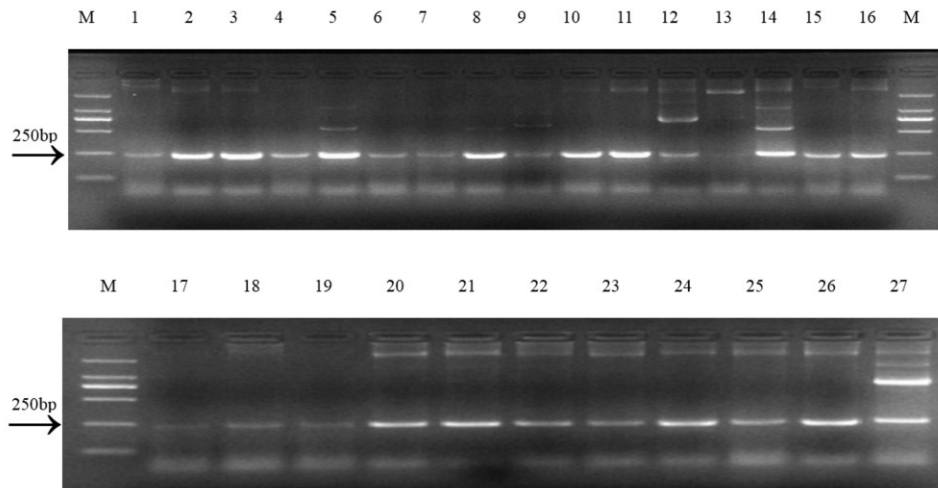


Fig. 2. FMD SAT-D8 PCR results with 27 synthesised 1D2A2B genes (cloned into pUC57 vector) of the selected representative strains of FMDV SATs as the templates. Lanes: M – DL2000 marker (2000, 1000, 750, 500, 250, 100bp), 1 – SAT1-AY593850, 2 – SAT1-AY593852, 3 – SAT1-AY593853, 4 – SAT2-KU821592, 5 – SAT2-KM268900, 6 – SAT2-KR108955, 7 – SAT3-KR108950, 8 – SAT3-KJ820999, 9 – SAT3-KM268901, 10 – SAT1-AY593839, 11 – SAT1-AY593840, 12 – SAT1-AY593841, 13 – SAT1-AY593846, 14 – SAT1-AY593838, 15 – SAT1-HM067706, 16 – SAT1-JF749860, 17 – SAT1-AY593842, 18 – SAT2-JF749864, 19 – SAT2-AF540910, 20 – SAT2-AY593848, 21 – SAT2-JF749861, 22 – SAT2-JF749862, 23 – SAT2-AY593847, 24 – SAT2-HM067705, 25 – SAT2-AJ251473, 26 – SAT2-JHM067704, 27 – SAT2-KC440884

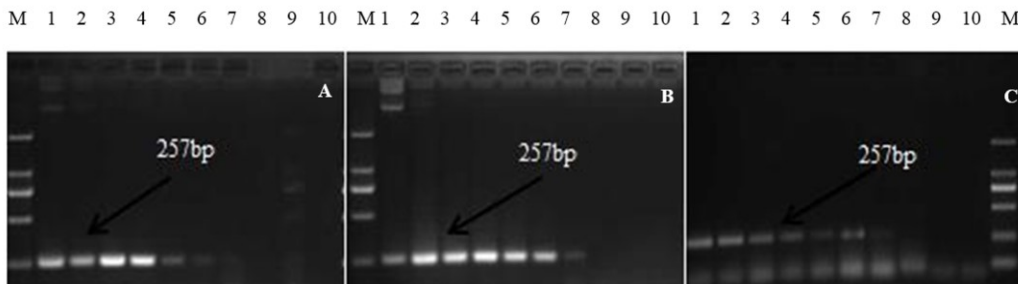


Fig. 3. Analytical sensitivity of FMD SAT-D8 PCR assay with the synthesised 1D2A2B genes of FMDV SATs (cloned into pUC57 vector) as the templates. A – SAT1 strain (GenBank accession number: AY593846), B – SAT2 strain (JF749862), C – SAT3 strain (AY593852). Lanes 1–9 – PCR products of 1D2A2B genes of SATs with concentrations of 4×10^8 – 4×10^0 copies/reaction, lane 10 – negative control

The sensitivity of the FMDV SAT-D8 PCR was assessed with series of 10-fold dilutions of recombinant plasmids containing the synthesised 1D2A2B DNA insert (10^8 – 10^0 copies/ μ L). As shown in Fig. 3, the PCR product was successfully amplified at a minimum concentration of 4×10^2 copies/ μ L of DNA of the SAT1, SAT2, and SAT3 strains. Therefore, the results indicated that the sensitivity of FMDV SAT-D8 PCR assay was 4×10^2 copies/ μ L for DNA.

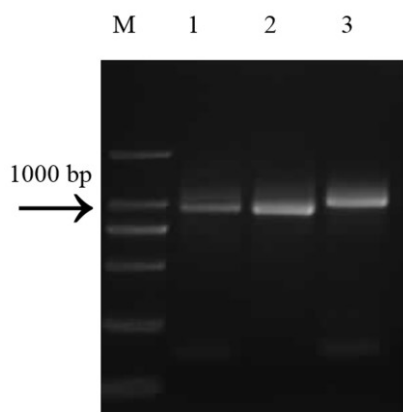


Fig. 4. Electrophoresis of *in vitro* transcription products of the synthesised 1D2A2B genes (cloned into pUC57 vector) of the representative strains of FMDV SATs registered in GenBank. M – DL2000 Marker, 1 – SAT1-AY593846, 2 – SAT2-JF749862, 3 – SAT3-AY593852

Establishment of FMDV SAT-D8 one-step RT-PCR based on 1D2A2B gene RNA transcripts and sensitivity measurement. After the multiplex primer set was selected *via* the synthesised 1D2A2B DNA, an FMDV SAT-D8 one-step RT-PCR was successfully established based on RNA transcripts of the synthesised 1D2A2B genes of the 8 SAT1, 13 SAT2, and 6 SAT3 representative strains. First, 27 synthesised 1D2A2B genes of FMDV SATs were amplified by PCR with primers containing T7 promoter which was added to the N-terminal of the reverse primer and then the transcribed RNA was purified with the kit. Transcription products of approximately 1,280 bp for FMDV SAT1 and SAT2, and 1,300 bp for FMDV

SAT3 were in accordance with expectations (Fig. 4). Thus, the RNA transcripts were well suited to serve as the templates for further establishing a one-step RT-PCR assay to detect FMDV SATs. Clear ~257 bp-sized bands were obtained as expected for all the 27 RNA transcript templates (data not shown).

When the sensitivity was assessed with a series of 10-fold dilutions of RNA transcript samples (10^9 – 10^0 copies/reaction), the results showed that the sensitivity was 4×10^3 copies/reaction (Fig. 5). In comparison with the detection limit at the DNA level, the sensitivity was 10-fold lower at the RNA transcript level.

Specificity of FMDV SAT-D8 one-step RT-PCR. Specificity of the assay was confirmed by using RNA extracted from vesicular disease viruses comprising FMDV O, A, and Asia 1, SVDV and SVV, and RNA or DNA extracted from other common bovine, ovine, caprine, or porcine pathogens including BVDV, ORFV, SPPV, GTPV, BTV, PRRSV, CSFV, PRV, PPV, and porcine circovirus. As for FMDV serotype C, four synthesised 1D2A2B genes from the representative strains were used as a template to further evaluate the assay specificity. These viruses were first verified by the RT-PCR or PCR method using their specific primers under appropriate amplification conditions and then by sequencing the amplicons. The results indicated that there was no cross-reaction with all these virus samples nor even with the synthesised 1D2A2B genes of the four FMDV C strains (data not shown).

Detection of clinical samples. To test the efficiency of the FMDV SAT-D8 one-step RT-PCR assay, 128 samples were subjected to the specific amplification. All FMDV SAT1, SAT2, and SAT3 inactivated antigen samples showed the expected positive results (Fig. 6), while the other 125 field samples including organ tissues (such as heart, liver, spleen, lung, kidney, and tongue) and sera of sheep, goat, cattle, and pigs collected on the Chinese mainland were FMDV SATs-negative (electrophoresis pictures not shown). There have been no FMDV SATs cases in China up to now.

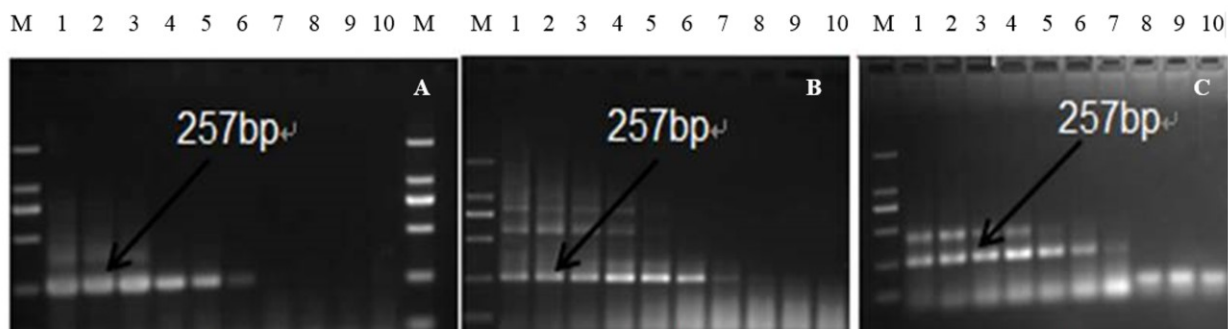


Fig. 5. Analytical sensitivity of FMD SAT-D8 one-step RT-PCR assay with RNA transcripts of 1D2A2B genes of the representative strains of FMDV SATs as the templates. Lanes: 1–10 – RT-PCR products of RNA transcripts of 1D2A2B genes of FMDV SATs with concentrations of 4×10^9 – 4×10^0 copies/reaction. A – SAT1 strain (GenBank accession no. AY593846); B – SAT2 strain (JF749862); C – SAT3 strain (AY593852)

Therefore, all the results were consistent with the facts. The sequencing results of RT-PCR products of SAT1, SAT2, and SAT3 were logged in to BLAST in GenBank. The results showed that the amplified products belonged to FMDV SAT1, SAT2, and SAT3, correspondingly. The RT-PCR assay coupled with nucleotide sequencing further demonstrated the efficiency of the developed method. More accredited FMDV SATs samples will be needed for further assessing the new RT-PCR methods.

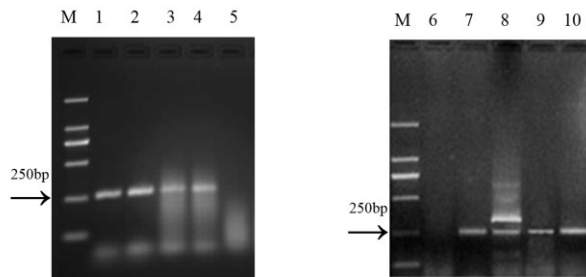


Fig. 6. FMDV SAT-D8 one-step RT-PCR products of RNA samples extracted from FMDV SATs inactivated antigens and RNA transcripts of the correspondingly synthesised 1D2A2B genes of the reference strains of FMDV SATs registered in GenBank as positive controls. Lanes: M – DL2000 marker, 1 – SAT1-AY593839, 2 – SAT1-AY593841, 3, 4 – SAT1 inactivated antigen, 5, 6 – Puc57 plasmid as negative control, 7 – SAT2 inactivated antigen, 8 – SAT3 inactivated antigen, 9 – SAT2-AY593861, 10 – SAT3-AY593850

Discussion

In this study, a new one-step RT-PCR was successfully established for the primary diagnosis of FMDV SATs with multiplex SAT-D8 primers and the amplification conditions were optimised based on the synthesised 1D2A2B genes and the corresponding RNA transcripts. Although some RT-PCR methods have been developed for detecting FMDV SATs, there is much room left for improvement of the efficiency due to the problems of cross-reaction and less than optimal sensitivity caused by high genetic variation and the limited availability of genome sequences at the time of development of those PCRs (6, 12). In recent years, more complete genome sequences have been released and make it possible to redesign the primers for good RT-PCR diagnosis of FMDV SATs. In the present study, all the FMDV SATs strains with full-length genome sequences available in GenBank were analysed. Finally, 1D2A2B genes of 8 SAT1, 13 SAT2, and 6 SAT3 representative strains characterised by $\leq 85\%$ nucleotide identity of the 1D gene encoding VP1 structural protein were selected and artificially synthesised to develop the new RT-PCR. We firstly tested those primer sets developed previously (6, 12) using the 27 synthesised 1D2A2B genes and confirmed their defects (data not shown). Then the primers were redesigned and after several selection rounds, the SAT-

D8 set was proven to be an excellent candidate by producing the expected amplicon of ~ 257 bp for all the synthesised 1D2A2B genes of FMDV SATs and their corresponding RNA transcripts. However, some non-specific bands did appear in the gels. The seven forward primers of the multiplex SAT-D8 primer set are all located in the 3' end of the 1D gene which is the foundation of FMDV serotype classification and the only reverse primer matches the 2B gene which encodes the nonstructural protein and is highly conserved (11). In regard to the 10-fold lower sensitivity at RNA transcript level compared with that at the synthesised DNA level, we speculate that the reverse transcription process was not efficient enough to turn all RNA into cDNA, leading to a lower amount of template for the later PCR reaction. There were no cross-reactions with Eurasian FMDV, *i.e.* serotypes A, O, C, or Asia 1, or other vesicular disease viruses including SVDV and SVV. Furthermore, BVDV, ORFV, SPPV, GTPV, SVDV, BTV, PRRSV, CSFV, PRV, PPV, and PCV also showed negative results when they were subjected to the FMDV SAT-D8 one-step RT-PCR. These results showed that the newly established SAT FMDV RT-PCR is promising. As for the clinical sample testing, the inactivated SAT1, SAT2, and SAT3 antigens obtained from Pirbright Laboratory (U.K.) were appropriately identified by further sequencing the amplicons after amplification. The RT-PCR assay coupled with nucleotide sequencing can serotype FMDV SATs. All the 125 samples including organ tissues and sera collected from sheep, goats, cattle, and pigs in China showed negative results which were in accordance with expectations due to the freedom of China from FMD SATs cases up to now. More accredited FMDV SATs samples will be applied in continuation work for fully assessing the specificity and sensitivity of the new RT-PCR method. The newly established FMDV SAT-D8 one-step RT-PCR will also lay the foundation for an alternative PCR assay, such as real-time PCR or nested PCR.

In this study, a new one-step RT-PCR with multiplex primers targeting the 1D2A2B genes of FMDV SATs was successfully established using synthesised DNA and the corresponding RNA transcript. The assay is specific and shows no cross-reaction with Eurasian FMDV, SVDV or SVV or other common pathogens of pig, cattle, sheep, or goats. This new sensitive and specific RT-PCR can be adapted to use as a tool for molecular epidemiological studies for rapidly detecting FMDV SATs and making primary diagnosis. In addition, this assay can also be used to serotype SAT1, SAT2, and SAT3 by further sequencing the amplified products and online alignment.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The studies were approved by the Local Bioethics Committee.

References

- Ahmed H.A., Salem S.A., Habashi A.R., Arafa A.A., Aggour M.G., Salem G.H., Gaber A.S., Selem O., Abdelkader S.H., Knowles N.J., Madi M., Valdazo-Gonzalez B., Wadsworth J., Hutchings G.H., Mioulet V., Hammond J.M., King D.P.: Emergence of foot-and-mouth disease virus SAT 2 in Egypt during 2012. *Transbound Emerg Dis* 2012, 59, 476–481.
- Alexandersen S., Mowat N.: Foot-and-mouth disease. host range and pathogenesis. *Curr Top Microbiol* 2005, 288, 9–42.
- Ayelet G., Mahapatra M., Gelaye E., Egziabher B.G., Rufeal T., Sahle M., Ferris N.P., Wadsworth J., Hutchings G.H., Knowles N.J.: Genetic characterization of foot-and-mouth disease viruses, Ethiopia, 1981–2007. *Emerg Infect Dis* 2009, 15, 1409–1417.
- Bastos A.D., Haydon D.T., Sangare O., Boshoff C.I., Edrich J.L., Thomson G.R.: The implications of virus diversity within the SAT 2 serotype for control of foot-and-mouth disease in sub-Saharan Africa. *J Gen Virol* 2003, 84, 1595–1606.
- Brito B.P., Jori F., Dwarka R., Maree F.F., Heath L., Perez A.M.: Transmission of foot-and-mouth disease SAT2 viruses at the wildlife-livestock interface of two major transfrontier conservation areas in Southern Africa. *Front Microbiol* 2016, 7, 528.
- Callens M., De Clercq K.: Differentiation of the seven serotypes of foot-and-mouth disease virus by reverse transcriptase polymerase chain reaction. *J Virol Meth* 1997, 67, 35–44.
- Cao Y., Lu Z., Li D., Fan P., Sun P., Bao H., Fu Y., Li P., Bai X., Chen Y., Xie B., Liu Z.: Evaluation of cross-protection against three topotypes of serotype O foot-and-mouth disease virus in pigs vaccinated with multi-epitope protein vaccine incorporated with poly(I:C). *Vet Microbiol* 2014, 168, 294–301.
- Grubman M.J., Baxt B.: Foot-and-mouth disease. *Clin Microbiol Rev* 2004, 17, 465–493.
- Jamal S.M., Belsham G.J.: Foot-and-mouth disease. past, present, and future. *Vet Res* 2013, 44, 116.
- Kitching P., Hammond J., Jeggo M., Charleston B., Paton D., Rodriguez L., Heckert R.: Global FMD control is it an option? *Vaccine* 2007, 25, 5660–5664.
- Knowles N.J., Samuel A.R.: Molecular epidemiology of foot-and-mouth disease virus. *Virus Res* 2003, 91, 65–80.
- Reid S.M., Ferris N.P., Hutchings G.H., Samuel A.R., Knowles N.J.: Primary diagnosis of foot-and-mouth disease by reverse transcription polymerase chain reaction. *J Virol Meth* 2000, 89, 167–176.
- Rweyemamu M., Roeder P., MacKay D., Sumption K., Brownlie J., Leforban Y.: Planning for the progressive control of foot-and-mouth disease worldwide. *Transbound Emerg Dis* 2008, 55, 73–87.
- Rweyemamu M., Roeder P., Mackay D., Sumption K., Brownlie J., Leforban Y., Valarcher J.F., Knowles N.J., Saraiva V.: Epidemiological patterns of foot-and-mouth disease worldwide. *Transbound Emerg Dis* 2008, 55, 57–72.
- Sangula A.K., Siegismund H.R., Belsham G.J., Balinda S.N., Masembe C., Muwanika V.B.: Low diversity of foot-and-mouth disease serotype C virus in Kenya: evidence for probable vaccine strain re-introductions in the field. *Epidemiologic Infect* 2011, 139, 189–196.
- Valdazo-Gonzalez B., Knowles N.J., Hammond J., King D.P.: Genome sequences of SAT 2 foot-and-mouth disease viruses from Egypt and Palestinian Autonomous Territories (Gaza Strip). *J Virol* 2012, 86, 8901–8902.
- Yoon H., Yoon S.S., Wee S.H., Kim Y.J., Kim B.: Clinical manifestations of foot-and-mouth disease during the 2010/2011 epidemic in the Republic of Korea. *Transbound Emerg Dis* 2012, 59, 517–525.