TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2018) 42: 335-345

doi:10.3906/vet-1801-17

Evaluation of PCR primers targeting the VP2 region of the foot-and-mouth disease virus for improved serotype detection

Waqas ALI*, Mudasser HABIB, Muhammad SALAH UD DIN SHAH

Department of Biological Sciences, Nuclear Institute for Agriculture and Biology-Faisalabad, affiliated with Pakistan Institute of Engineering and Applied Sciences, Islamabad, Pakistan

Received: 05.01.2018 • **Accepted/Published Online:** 22.04.2018 • **Final Version:** 09.08.2018

Abstract: Frequently, a sequence from a single strain of a virus is used to design primers for PCR-based virus detection. However, high mutation rates in RNA viruses lead to failure of microorganism detection in clinical samples. Therefore, it is essential to find conserved regions within sequences to design primers for the detection of pathogen genomes in suspected clinical samples. The aim of the study was to find conserved regions of the genome for improved serotype detection. In the present study, primers were designed for serotype detection based on the VP2 coding region of foot-and-mouth disease virus (FMDV) after alignment of full genome (n = 375) sequences. These primer pairs and previously reported primer pairs were then compared using positive samples (n = 91) collected during 2010–2016 from Faisalabad District, Pakistan. The detection rate of newly designed primer pairs O-Wqs-F/Rev-Wqs (96.5%), As-Wqs-F/Rev-Wqs (90.4%), and A-Wqs-F/Rev-Wqs (100%) was better compared with previously reported primer pairs P38/P33 (81%), P74–77/P33 (47.6%), and P87–92/P33 (41.6%) in detecting FMDV serotypes O, Asia1, and A, respectively. The higher detection rates of the newly designed primer pairs appeared to be due to the selection of highly conserved sequences within the serotypes for designing primers. To the best of the authors' knowledge, this is the first study that describes serotype diagnosis of FMDV based on primers targeting the VP2 region. In conclusion, this new method offers an improved approach for the serotyping of FMDV compared to previous methods.

Key words: Serotyping, foot-and-mouth disease, Pakistan

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious and economically important viral disease of cloven-footed animals and is a significant barrier for international trade of animals and their products (1,2). The etiological agent, the FMD virus (FMDV), belongs to the family *Picornaviridae* and genus *Aphthovirus* (3). This virus exists as 7 immunogenically different serotypes, O, A, Asia1, C, SAT1, SAT2, and SAT3, with multiple subtypes within each serotype (4,5). Infection with one serotype does not offer cross-protection against other serotypes (6). These serotypes are not uniformly distributed throughout the world, as serotypes O, A, and C have broad distribution in Asia, Africa, the Americas, and Europe; serotype Asia1 is associated with Asia; and the SAT serotypes are confined to Africa (7). The positive-sense single-stranded RNA genome of this virus is approximately 8.2 kb long and encodes structural and nonstructural proteins (8).

The control of FMD relies on the early diagnosis of the virus in infected animals with the help of rapid and sensitive diagnostic techniques (9). Primarily, the diagnosis is made on the basis of clinical signs exhibited by infected animals such as excessive salivation, high temperature, and vesicular lesions in the buccal mucosa and interdigital spaces (10).

Currently, the general detection of all 7 serotypes is well established, and FMDV can be detected with high diagnostic sensitivity and specificity because these assays amplify highly conserved sequences within the 5'-untranslated region (UTR) or the 3D-coding region (RNA polymerase) (11–15). However, the typing of FMDV is performed on the variable region (VP1) of the genome to differentiate the serotypes (16–23). The FMDV serotypes exhibit 86% nucleotide identity with each other across the whole genome (24), but the VP1 region is the most variable, showing 50%–70% identity (25). Development of universal serotype-specific assays using VP1 coding nucleotide sequences is difficult due to intraserotype variation in the VP1 region (26). Due to this high variability in the VP1 coding region, serotype detection is very challenging and often limited to regional assays for respective circulating serotypes (19,27–29).

* Correspondence: drwaqaasali@gamil.com

As incursion of strains from different pools (29–32) and new lineages occurs in every endemic region (33–36), the specificity of these assays will become questionable in the near future due to the highly variable nature of the VP1 coding nucleotide sequences. Therefore, there is a need for the development of a serotype detection assay targeting the conserved region of the FMDV genome instead of VP1, which can enable researchers to detect the FMDV serotypes universally instead of regionally. Therefore, this study was designed to offer a better method for the serotyping of FMDV.

2. Materials and methods

2.1. Sample collection from 2010 to 2016

A total of 109 samples [saliva (n = 26) and epithelial tissue (n = 83)] were collected from different locations in Faisalabad District [Faisalabad (n = 54), Samundri (n = 30), Tandlianwala (n = 25)] based on clinical signs and symptoms. The saliva and epithelial tissues were taken as routine surveillance from different species (Table 1). Samples were transported to the laboratory in glycerolized buffer saline and stored at –80 °C until further processing.

2.2. Oligonucleotide primers

The nucleotide sequences of complete genomes of FMDV serotypes O (n = 100), A (n = 75), and Asia1 (n = 100) belonging to different regional pools of FMDV were downloaded from GenBank and used for designing oligonucleotide primers for the typing of FMDV. Multiple primer options were initially chosen, which were then reduced to three primer pairs finally. The positive strand primers O-Wqs-F, As-Wqs-F, and A-Wqs-F for serotypes O, Asia1, and A respectively were designed on the VP2 coding regions. However, a minus-strand primer (Rev-Wqs) was designed on the VP3 coding region using complete genome alignment of all three serotypes (n = 100) downloaded from GenBank (Figure 1). BioEdit (version 2.7.5) (37) was used for alignment of the sequences using the Clustal W method (Figure 2), and Primer Select software (Lasergene 7.1.0, DNAStar, Madison, WI, USA) was used to calculate melting temperatures, GC content, and primer attachment sites. Some nucleotides were altered from the alignment consensus to maintain internal stability of the primers (GC content and Tm difference between primer pairs), such as primer O-Wqs-F at position 15; primer As-Wqs-F at positions 8 and 14, and primer Rev-Wqs at positions 1, 2, and 14 (Figure 3). The NCBI database (www.ncbi.nlm.nih.gov/blast) was used to check the specificity of the designed primers. All of the primers were finally synthesized commercially by Eurofins Genomics (USA) to analyze their performance against previously reported typing primers (17).

To analyze the sequence variation among annealing regions of both the newly designed and previously reported primers (11,17), downloaded sequences of FMDV serotypes A, O, and Asia1 were aligned (Figure 4) with BioEdit software version 2.7.5 (37). Moreover, 33 clinical samples were sequenced to determine the subtypes (Table 1).

2.3. RNA extraction and reverse transcription

Collected samples were subjected to a FavorPrep viral nucleic acid extraction kit (Favorgen Biotech Corporation, Taiwan) to extract RNA by following the manufacturer's protocol except for the last step, where 30 µL of elution buffer was utilized to elute the RNA. Complementary DNA (cDNA) synthesis was carried out using the Fermentas Revert Aid First Strand cDNA kit (Thermo Fisher Scientific, USA) using extracted RNA. Briefly, 11 µL of template RNA was added to 1 µL of random hexamer primer (0.2 µg/µL), followed by incubation at 65 °C for 5 min and brief spinning. Subsequently, 5 µL of RT buffer (5X), 0.8 µL of Moloney-murine leukemia virus (M-MuLV) reverse transcriptase (200 U/µL), 0.8 µL of RNase inhibitor (20 U/µL), and 1.4 µL of dNTPs mix (10 mM each) were added to make the reaction volume 20 µL. RNA was finally reverse-transcribed at 42 °C for 1 h. The prepared cDNA was stored at –80 °C until further use in PCR. Positive (FMDV serotype O PanAsia II) and negative (water) extraction control samples were included in each group of RNA extractions.

2.4. PCR amplification

A total PCR mixture of 50 µL was prepared containing 2 µL of cDNA, 33 µL of ddH_2O, 5 µL of Taq polymerase buffer (10X), 5 µL of $MgCl_2$ (25 mM), 1 µL of dNTPs (10 mM each), 0.2 µL of Taq plus DNA polymerase (5 U/µL) (Thermo Fisher Scientific), and 2 µL each of forward and reverse primers (10 pmol). PCR amplification was performed using the Touchgene gradient PCR system TC-512 (Techne, Cambridge, UK). PCR for the new primers was achieved using the following thermal cycling conditions in separate PCR tubes for each serotype: initial denaturation at 94 °C for 5 min; 30 cycles each at 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 45 s; and final extension at 72 °C for 10 min. However, the amplification with consensus (1F/1R) and serotype-specific primers (P38, P74–77, P87–92, and P33) was carried out as described earlier (17). The PCR products were run on 1.5% (w/v) agarose gel electrophoresis prepared in Tris-borate-EDTA (TBE) buffer and analyzed under ultraviolet transmission (Figure 1).

2.5. Primer quality score

Primer quality score was calculated by the formula:

Tm = 81.5 + 0.41(%GC) – 675/N – % mismatch,

where N is the total number of bases.

This score was calculated by using the tool available at http://depts.washington.edu/bakerpg/primertemp/.

Table 1. Strain IDs of 33 samples with their GenBank accession numbers.

S. no.	Strain	Serotype	Species	GenBank accession
1	Asia1/NIAB/PUN/PAK/197/2016	Asia1	Camel	MF167435
2	Asia1/NIAB/PUN/PAK/142/2016	Asia1	Buffalo	MF115987
3	Asia1/NIAB/PUN/PAK/148/2016	Asia1	Cattle	MF115988
4	Asia1/NIAB/PUN/PAK/152/2016	Asia1	-do-	MF115989
5	Asia1/NIAB/PUN/PAK/156/2016	Asia1	-do-	MF115991
6	Asia1/NIAB/PUN/PAK/160/2016	Asia1	Buffalo	MF140439
7	Asia1/NIAB/PUN/PAK/181/2016	Asia1	Cattle	MF140441
8	Asia1/NIAB/PUN/PAK/190/2016	Asia1	Buffalo	MF140438
9	Asia1/NIAB/PUN/PAK/192/2016	Asia1	Cattle	MF140442
10	Asia1/NIAB/PUN/PAK/195/2016	Asia1	Camel	MF140443
11	Asia1/NIAB/PUN/PAK/203/2017	Asia1	Buffalo	MF115990
12	Asia1/NIAB/PUN/PAK/209/2017	Asia1	Goat	MF140444
13	ASIA/NIAB/PUN/PAK/144/2016	Asia1	Cattle	KY659570
14	ASIA/NIAB/PUN/PAK/145/2016	Asia1	-do-	KY659571
15	ASIA/NIAB/PUN/PAK/146/2016	Asia1	-do-	KY659572
16	ASIA/NIAB/PUN/PAK/147/2016	Asia1	-do-	KY659573
17	ASIA/NIAB/PUN/PAK/149/2016	Asia1	-do-	KY659574
18	ASIA/NIAB/PUN/PAK/154/2016	Asia1	-do-	KY659575
19	Asia1/NIAB/PUN/PAK/207/2017	Asia1	-do-	MF140437
20	O/NIAB/PUN/PAK/143/2017	O	-do-	MF115992
21	O/NIAB/PUN/PAK/115/2017	O	-do-	MF150913
22	O/NIAB/PUN/PAK/205/2017	O	Buffalo	MF140436
23	O/NIAB/PUN/PAK/90/2014	O	-do-	MF374986
24	O/NIAB/SIN/PAK/46/2014	O	Cattle	MF374989
25	O/NIAB/SIN/PAK/35/2014	O	-do-	MF374990
26	O/NIAB/PUN/PAK/104/2014	O	-do-	MF374988
27	O/NIAB/PUN/PAK/18/2014	O	-do-	MF374991
28	O/NIAB/PUN/PAK/150/2016	O	-do-	KY659576
29	O/NIAB/PUN/PAK/120/2015	O	-do-	MF374987
30	A/NIAB/PUN/PAK/119/2015	A	-do-	MF374993
31	A/NIAB/PUN/PAK/16/2014	A	-do-	MF374994
32	A/NIAB/PUN/PAK/162/2015	A	-do-	MF140440
33	A/NIAB/PUN/PAK/17/2014	A	-do-	MF374992

2.6. Sequencing analysis

Samples were subjected to sequencing RT-PCR assay to achieve complete VP1 coding region sequences. Successfully amplified PCR purified products (n = 33) were subjected to direct sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and the ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems). The sequences obtained were assembled and aligned using CLUSTAL X v. 1.81 (38) in the BioEdit alignment editor (37). Sequences were submitted to GenBank for accession numbers (Table 1). Moreover, sequences (n = 90) corresponding to the amplified typing products of serotype Asia1 (n = 48), serotype O (n = 22), and serotype A (n = 20) were downloaded from GenBank, and a UPGMA phylogenetic tree was drawn using MEGA 6 (39) software (Figure 2).

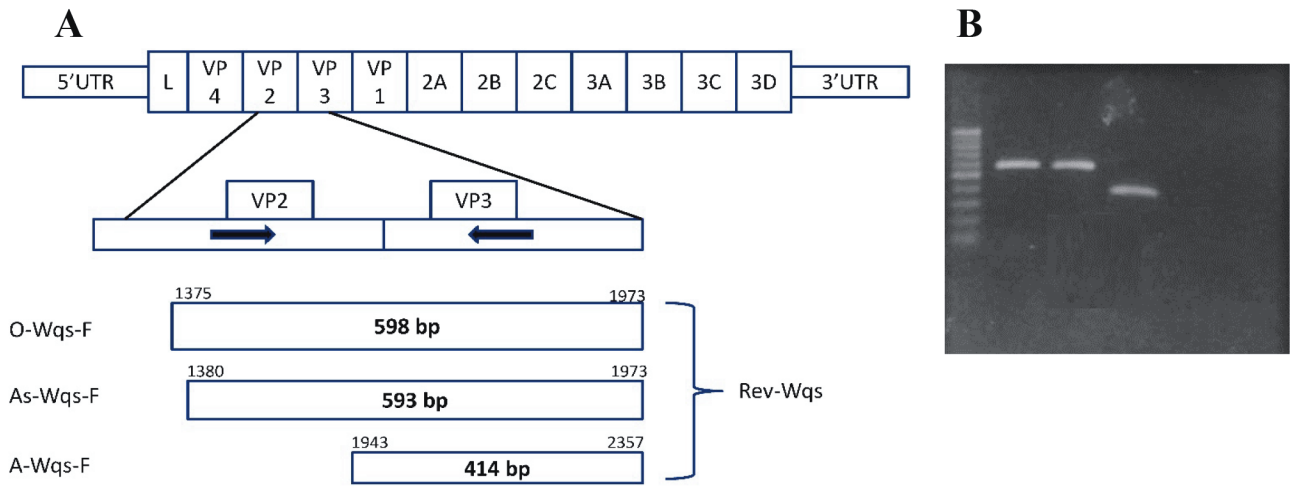

Figure 1. A) Genome organization of FMDV. The figure shows positions and directions of newly designed serotype-specific primers for O (O-Wqs-F), Asia1 (As-Wqs-F), and A (A-Wqs-F) with consensus reverse primer (Reverse-Wqs) with expected product sizes. Positions of the primers are according to the GenBank sequence IDs GU384685.1, JF749849.1, and JN099694.1 for O-Wqs-F, As-Wqs-F, and A-Wqs-F, respectively. B) Lane 1 depicts marker (GeneRuler 100-bp DNA Ladder), while lanes 2, 3, and 4 show the PCR products of type O (598 bp), type Asia1 (593 bp), and type A (414 bp), respectively, with type-specific primers.

2.7. Specificity analysis

Specificity of the assay was achieved using goat pox virus (GPV), peste des petits ruminants virus (PPRV), and infectious bursal disease virus (IBDV). Typing specificity was also assessed using RNA templates of type A against type O and Asia1 primers and vice versa.

3. Results

3.1. Consensus RT-PCR for general FMDV detection

Genomic RNA extracted from clinical samples that comprised mouth epithelium (vesicular/necrotic tissue), vesicular epithelium from the interdigital cleft, and saliva was subjected to RT-PCR using the consensus primers 1F/1R (11). FMDV genome-positive samples gave rise to 328-bp amplified products.

3.2. Serotype detection

Positive samples (n = 91) in consensus RT-PCR that came from different locations of Faisalabad District (40 from Faisalabad, 28 from Sumandri, and 15 from Tandlianwala) were subjected to RT-PCR for serotype detection using previously reported (17) as well as the newly designed pair of primers, as listed in Table 2. The results of typing PCR are summarized in Table 3. In general, among all the primer pairs used in this study, newly designed primer pairs O-Wqs-F/Rev-Wqs (96.5%), As-Wqs-F/Rev-Wqs (90.4%), and A-Wqs-F/Rev-Wqs (100%) performed better than previously reported primer pairs P38/P33 (81%), P74–77/P33 (47.6%), and P87–92/P33 (41.6%) in detecting FMDV serotypes O, Asia1, and A, respectively. Some serotype Asia1 samples (n = 10) gave false positive results with the serotype O-specific primer pair (P38/P33). However, sequencing of these samples revealed that they belonged to serotype Asia1 (subtype group VII$^{Sind-08}$) (data not shown) and, interestingly, alignment of their sequences showed similarity at the primer binding site for serotype O-specific primer P38 (Figure 5).

3.3. Specificity analysis

New primer sets were found specific for FMDV when subjected to RNA templates of other viruses such as GPV, PPRV, and IBDV. The results of typing primers also revealed that all of the primers were type-specific and showed no cross-reactivity for other serotypes.

3.4. Detection efficiency is related with sequence conservation and primer quality score

The difference in detection efficiency among the primer pairs in PCR might be explained by the difference in the conservation of the sequences of VP1 and VP2 coding regions at oligo annealing sites. Therefore, primer quality scores were calculated, and the scores of newly designed primer pairs O-Wqs-F/Rev-Wqs (83.1), As-Wqs-F/Rev-Wqs (84.2), and A-Wqs-F/Rev-Wqs (83.2) were better than previously reported primer pairs P38/P33 (79.8), P74–77/P33 (76.7), and P87–92/P33 (75) designed for the detection of FMDV serotypes O, Asia1, and A, respectively. Analysis of the typing PCR detection rate using clinical samples revealed that the primer pair with the higher quality score was, in general, able to detect the samples that were not detected by the primer pair with the lower score.

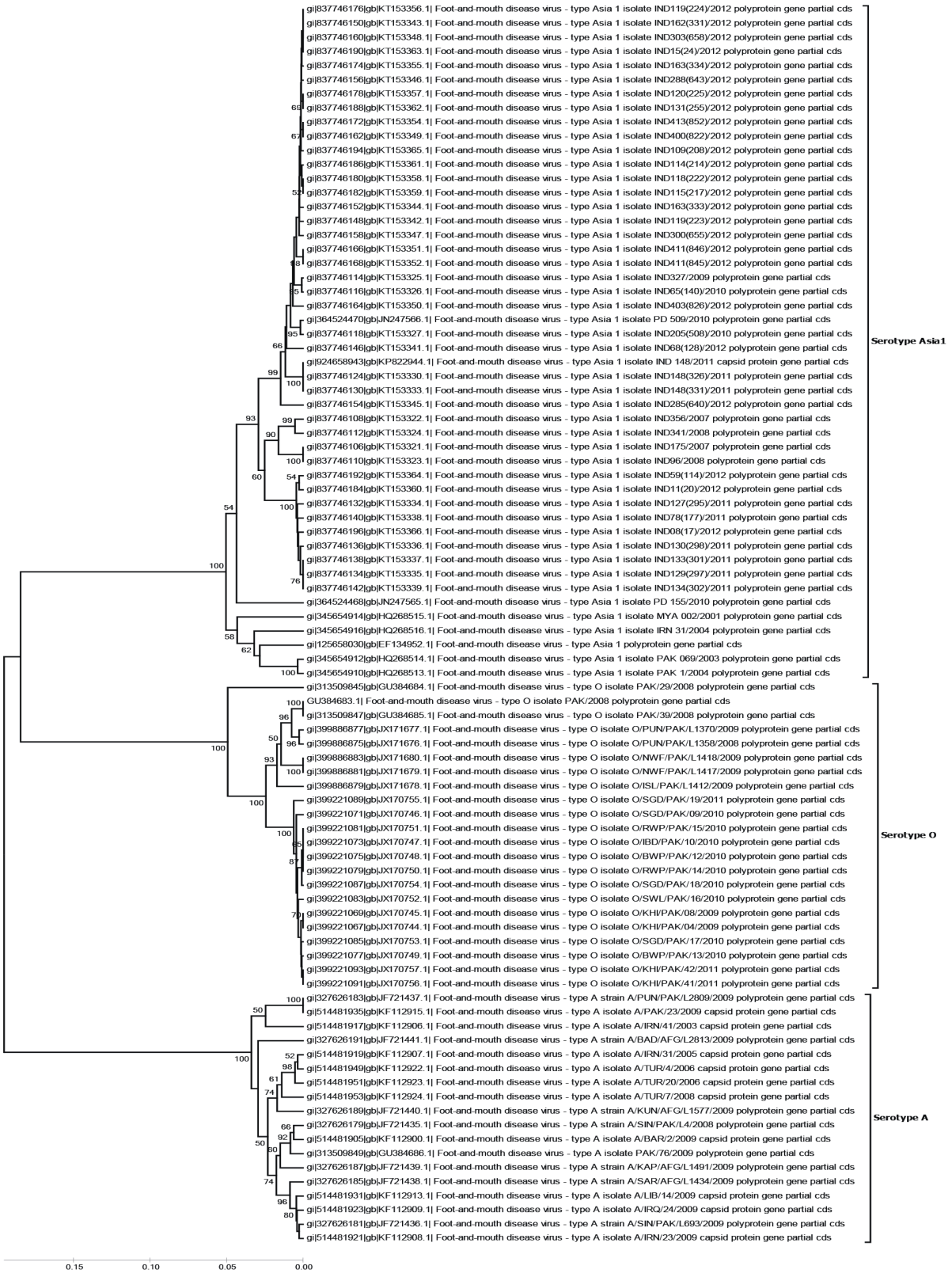

Figure 2. UPGMA phylogenetic tree generated using nucleotide sequences of the VP2 coding region of FMDV serotype Asia1 (n = 48), serotype O (n = 22), and serotype A (n = 20) from different regional pools.

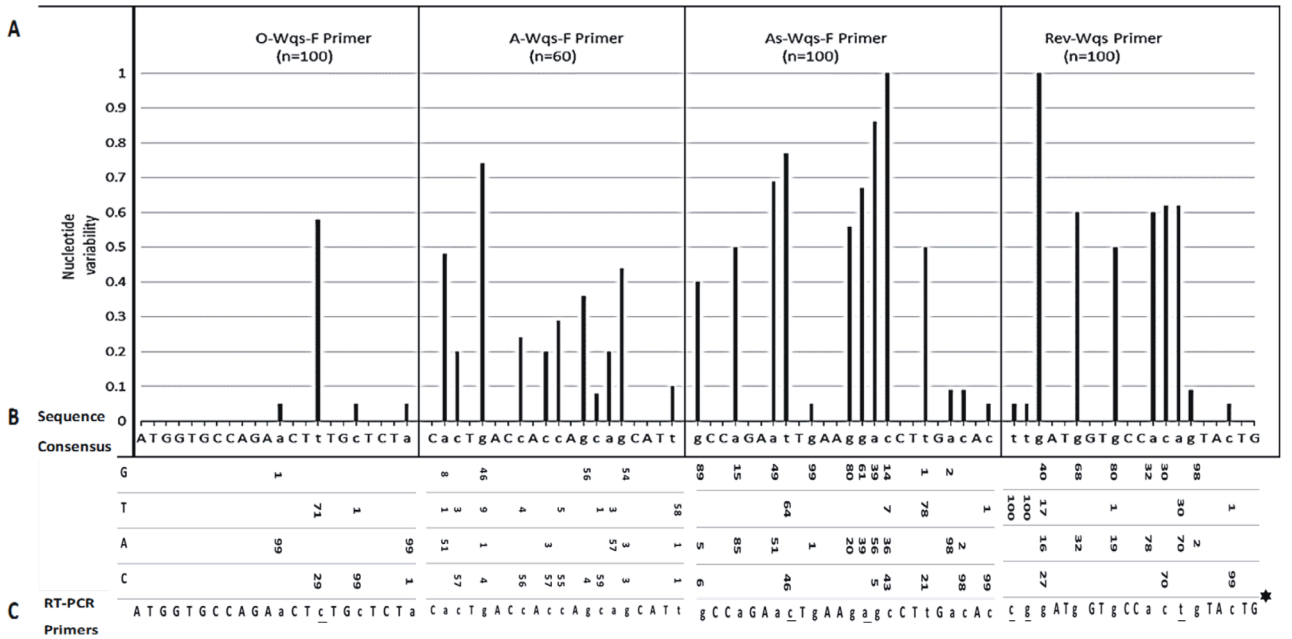

Figure 3. A) Nucleotide variability in multiple alignment of complete genome sequences of 100 strains of FMDV serotype Asia1, 100 of serotype O, 60 of serotype A, and 100 strains of FMDV (all 3 types) at primer binding sites of As-Wqs-F, O-Wqs-F, A-Wqs-F, and Rev-Wqs primers respectively. B) Multiple alignment sequence consensus at primer binding sites and number of mismatched nucleotides. C) RT-PCR oligo sequences comparison with the alignment consensus. *To maintain the Tm difference between the primers below 5 °C, underlined nucleotides were altered from the alignment consensus.

3.5. Mutation at primer binding sites affects detection by PCR

Samples (n = 9) were positive for both serotypes O and Asia1; some (n = 10) gave false positive results for serotype O using old type-specific primers (P38/P33) (P74–77/P33). Analysis of the sequences of these samples revealed that they belonged to serotype Asia1 and mutation at the primer binding site made them detectable with the serotype O-specific primer pair (P38/P33), making the specificity of the previously designed primers questionable (Figure 5).

3.6. Phylogenetic analysis

For in silico phylogenetic analysis of the amplified VP2 region, related sequences (n = 90) were downloaded from the GenBank database for serotypes A, O, and Asia1. MEGA 6 (39) software was used to draw a UPGMA tree (Figure 2), showing that the VP2 region could be used to characterize the FMDV into serotypes A, O, and Asia1 at a 15% nucleotide difference as a cut-off.

4. Discussion

Reliable detection of the FMDV serotypes is essential for diagnosis, epidemiological studies, and planning of control strategies like vaccination, especially for endemic countries (27). Information about the relative abundance of each serotype in the viral population of a region gives knowledge about selection and competition occurring within serotypes.

In the previous decade, increased sequencing data have allowed scientists to study microbes in incredible depth (40). Primers can now be designed in an accurate and unbiased way using computational tools (41). Primers used for universal serotype detection of FMDV were designed more than a decade ago (11,16,17). Therefore, it can be assumed that consensus decay may have occurred at primer binding sites, as previously reported serotype-specific primers were designed in the highly variable region (VP1) of the genome (17,25).

FMDV is a continuously evolving virus, and all the assays developed for serotype detection are designed in the most variable region (VP1) of its genome (19,27–29,42). Therefore, the introduction of new lineages or the finding of new strains in the region with changes within the primer attachment sites limits the detection capability of these assays. It is therefore necessary to discover more conserved regions for global and long-term detection ability of the typing assays for FMDV.

Improved detection of this assay (Table 3) may be due to the high sequence conservation in the VP2 (>80%) (24) region as compared to the VP1 region (50%–70%) (25). This study shows that the higher detection rate is correlated with the higher conservation of the sequences

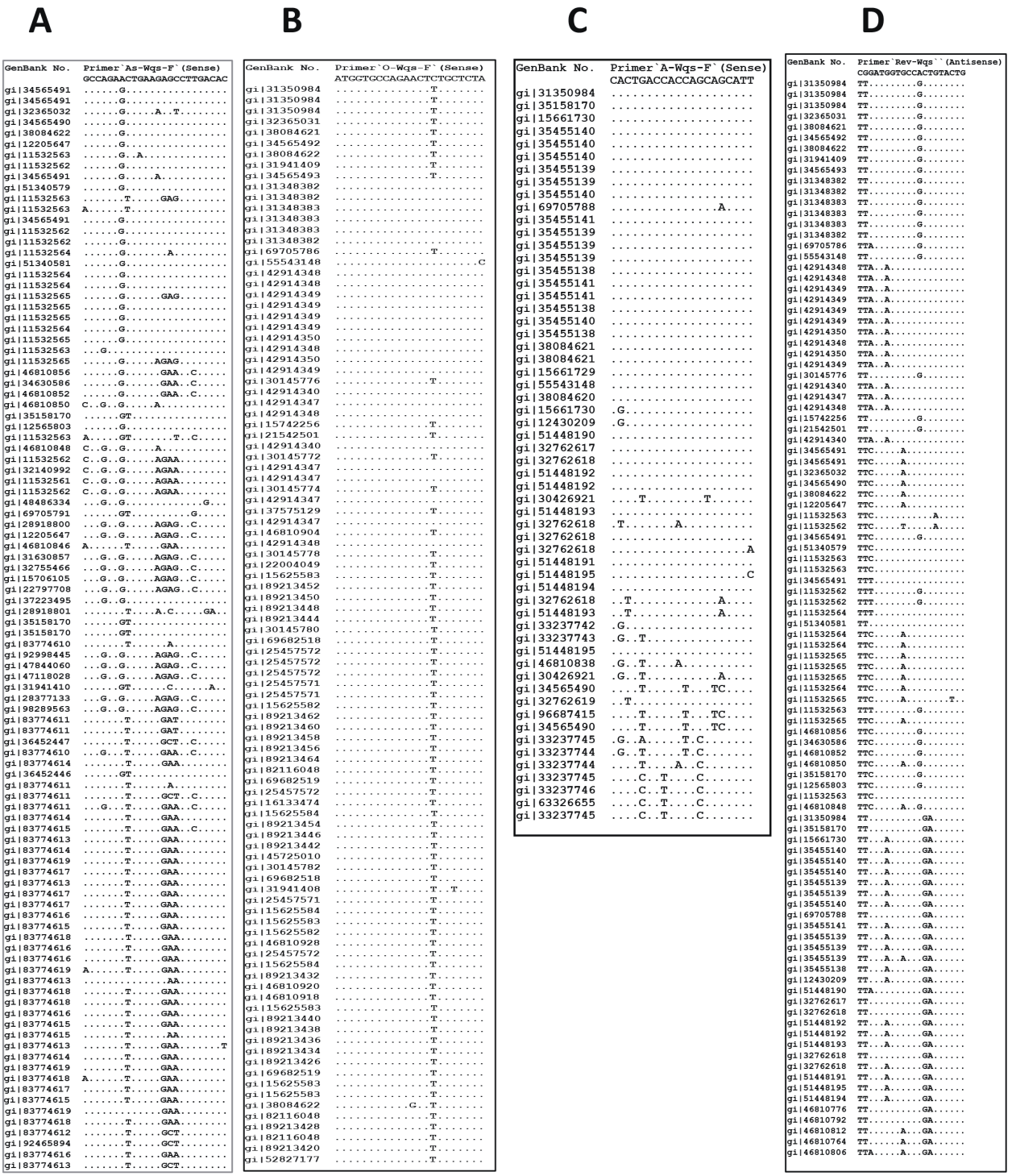

Figure 4. Left to right: A) Multiple alignment of complete genome sequences of 100 strains of FMDV serotype Asia1, B) 100 of serotype O, C) 60 of serotype A, and D) 100 of FMDV; all 3 types (A, O, Asia1) at primer binding sites of As-Wqs-F, O-Wqs-F, A-Wqs-F, and Rev-Wqs primers, respectively.

at the primer binding sites. Detection efficiency of the primers was also calculated using the quality score, which depends on primer binding site dissimilarity, melting temperature, and GC content. The findings of this study suggest that there is a strong correlation between primer quality score and detection rate. Primers with high quality

Table 2. List of primers used in the study with their sequences, polarity, specificity, and product sizes.

Primer	Sequence (5'–3')	Polarity	Gene location	Specificity	Product size (bp)	Reference
1F	GCCTGGTCTTTCCAGGTCT	+	5'UTR	All types	328	(11)
1R	CCAGTCCCCTTCTCAGATC	-				
P33	AGCTTGTACCAGGGTTTGGC	-	2B	All types	402	(17)
P38	GCTGCCTACCTCCTTCAA	+	1D	Type O		
P74	GACACCACTCAGGACCGCCG	+				
P75	GACACCACCCAGGACCGCCG	+	1D	Type Asia1	299	
P76	GACACCACACAAGACCGCCG	+				
P77	GACACGACTCAGAACCGCCG	+				
P87	GTCATTGACCTCATGCAGACCCAC	+	1D	Type A	732	
P88	GTTATTGACCTCATGCAGACCCAC	+				
P89	GTCATTGACCTCATGCACACCCAC	+				
P90	GTCATTGACCTCATGCAGACTCAC	+				
P91	GTCATTGACCTCATGCAAACCCAC	+				
P92	GTCATTGACCTTATGCAGACTCAC	+				
Rev-Wqs	CGGATGGTGCCACTGTACTG	-	1C	All types	598	This study
O-Wqs-F	ATGGTGCCAGAACTCTGCTCTA	+	1B	Type O		
A-Wqs-F	CACTGACCACCAGCAGCATT	+		Type A	414	
As-Wqs-F	GCCAGAACTGAAGAGCCTTGACAC	+		Type Asia1	593	

Table 3. Detection rate of previously designed and newly designed primer pairs by PCR for serotyping of FMDV.

Sample location / number of samples	Number of positive samples with consensus primers (1F/1R)	Detection rate (%)					
		Type O primer pairs		Type Asia1 primer pairs		Type A primer pairs	
		O-Wqs-F+ Rev-Wqs	P38+P33	As-Wqs-F+ Rev-Wqs	P74-77+ P33	A-Wqs-F+ Rev-Wqs	P87-92+ P33
Faisalabad (n = 54)	40	25/25 (100%)	20/25 (80%)	10/10 (100%)	6/10 (60%)	5/5 (100%)	2/5
Sumandri (n = 30)	28	17/18 (94.4%)	15/18 (83.3%)	5/6 (83.3%)	3/6 (50%)	4/4 (100%)	1/4
Tandlianwala (n = 25)	23	14/15 (93.3%)	12/15 (80%)	4/5 (80%)	1/5 (20%)	3/3 (100%)	1/3
Total (n = 109)	91	53/58 (96.5%)	47/58 (81%)	19/21 (90.4%)	10/21 (47.6%)	12/12 (100%)	5/12 (41.6%)

scores performed better in PCR than the primers with lower scores. Although the RT-PCR amplicons have sizes that are almost the same on agarose gel, a separate reaction for each serotype-specific primer pair was carried out instead of multiplex reactions.

In silico phylogenetic analysis (UPGMA) revealed that the VP2 coding region amplified during this study could be used to characterize the FMDV into serotypes A, O, and Asia1 at a 15% nucleotide difference as a cut-off (Figure 2).

One previously reported primer pair (P38/P33) used for serotype O detection was found to have low specificity, as it also detected clinical samples of serotype Asia1. Moreover, sequencing results of these samples revealed that they have similarity at primer binding sites for the

GenBank No.	Primer`P-38`(Sense)
	GC-TGCCTACCTCCTTCAA
KY659570	..T....C..T........
KY659571	..T....C..T........
KY659572	..T....C..T........
KY659573	..T....C..T........
KY659574	..T....C..T........
KY659575	..T....C..T........
MF115987	..T....C..T........
MF115988	..T....C..T........
MF115989	..T....C..T........
MF115990	..T....C..T........
MF115991	..T....C..T........
MF140437	..T....C..T........
MF140438	..T....C..T........
MF140439	..T....C..T........
MF140441	..T....C..T........
MF140442	..C....C..T........
MF140443	..T....C..T........
MF140444	..T....C..T........
MF167435	..T....C..T........

Figure 5. Multiple alignment of serotype Asia1 lab samples (n = 19) at primer binding site of previously reported serotype O-specific primer P38 (17), with their GenBank Accession numbers. Similarities at the primer binding sites of primer P38 for serotype Asia1 lab samples are shown with dots.

serotype O-specific forward primer (P38) (Figure 5). This may be due to the high mutation rate of the VP1 region, as discussed above. Therefore, it can be assumed that consensus decay may have occurred at primer binding sites, because the previously reported serotype-specific primers (17) were designed in the hypervariable region (VP1).

Nucleotide variability in multiple alignment of complete genome sequences of all three types (100 strains of FMDV serotype Asia1, 100 of serotype O, 60 of serotype A, and 100 of FMDV) at primer binding sites of As-Wqs-F, O-Wqs-F, A-Wqs-F, and Rev-Wqs primers respectively was analyzed to highlight mismatched nucleotides. All of the primers showed conservation at the last 5 bp of the 3' end at the binding sites. To maintain the Tm difference below 5 °C between respective forward and reverse primers, some nucleotides were altered from the alignment consensus (Figure 3). Results of this study are in agreement with previous ones, which suggests that a high number of dissimilarities among the primer binding sites results in poor or no amplification of the template in PCR (43), especially in the critical last 5 bp of the 3' end of the primers (40,44).

Alignment of complete genome sequences downloaded from the NCBI GenBank database for serotype strains O (n = 100), A (n = 75), and Asia1 (n = 100) (Figure 4) from different regional pools of FMDV was carried out, and primer attachments were analyzed in silico using PrimerSelect software. The newly designed primers showed attachment with all of the strains (data not shown). This shows that the assay may be used for the detection of serotypes A, Asia1, and O worldwide, but further validation on field samples from different regional virus pools is needed.

Due to the limitations in the availability of FMDV strains belonging to other regional pools, the performance of this technique using viruses from other regions needs to be evaluated. RT-PCR is mostly used for the diagnosis of FMDV in Pakistan due to limited resources. However, this method can be optimized for real-time RT-PCR for serotype detection. Furthermore, development of universal RT-PCR assays for the detection of serotypes (i.e. SAT I, SAT II, and SAT III of FMDV) can be achieved by following the methodology used in this manuscript. In conclusion, this novel technique suggests an improved approach for the serotyping of FMDV compared to previous methods.

Acknowledgment
This work was financially supported by the Higher Education Commission, Islamabad, Pakistan.

References

1. Alexandersen S, Mowat N. Foot-and-mouth disease: host range and pathogenesis. In: Mahy BW, editor. Foot-and-Mouth Disease Virus. Current Topics in Microbiology and Immunology, Vol. 288. Berlin, Heidelberg: Springer; 2005. pp. 9-42.
2. Clavijo A, Wright P, Kitching P. Developments in diagnostic techniques for differentiating infection from vaccination in foot-and-mouth disease. Vet J 2004; 167: 9-22.
3. Ali W, Habib M, Khan RSA, Zia MA, Mazhar MU, Javed A, Farooq M, Shah MS. P1 coding region diversity of Group VII (Sind-08) serotype Asia-1 foot-and-mouth disease virus. Kafkas Univ Vet Fak 2018; 24: 341-347.
4. Carrillo C. Foot and mouth disease virus genome. In: Garcia M, editor. Viral Genomes. Rijeka, Croatia: InTech; 2012. pp. 53-68.
5. Lee KN, Nguyen T, Kim SM, Park JH, Do HT, Ngo HT, Mai DT, Lee SY, Nguyen CV, Yoon SH. Direct typing and molecular evolutionary analysis of field samples of foot-and-mouth disease virus collected in Viet Nam between 2006 and 2007. Vet Micro 2011; 147: 244-252.
6. Kitching R, Knowles N, Samuel A, Donaldson A. Development of foot-and-mouth disease virus strain characterisation—a review. Trop Ani Heal Prod 1989; 21: 153-166.
7. Jamal SM, Belsham GJ. Foot-and-mouth disease: past, present and future. Vet Res 2013; 44: 1.
8. Biswal JK, Bisht P, Mohapatra JK, Ranjan R, Sanyal A, Pattnaik B. Application of a recombinant capsid polyprotein (P1) expressed in a prokaryotic system to detect antibodies against foot-and-mouth disease virus serotype O. J Virol Meth 2015; 215: 45-51.
9. Hoffmann B, Beer M, Reid SM, Mertens P, Oura CA, Van Rijn PA, Slomka MJ, Banks J, Brown IH, Alexander DJ. A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Vet Microbiol 2009; 139: 1-23.
10. Kitching R. Clinical variation in foot and mouth disease: cattle. Rev Sci Tech OIE 2002; 21: 499-502.
11. Reid SM, Ferris NP, Hutchings GH, Samuel AR, Knowles NJ. Primary diagnosis of foot-and-mouth disease by reverse transcription polymerase chain reaction. J Virol Met 2000; 89: 167-176.
12. Reid SM, Ebert K, Bachanek-Bankowska K, Batten C, Sanders A, Wright C, Shaw AE, Ryan ED, Hutchings GH, Ferris NP. Performance of real-time reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus during field outbreaks in the United Kingdom in 2007. J Vet Diag Invest 2009; 21: 321-330.
13. Reid SM, Ferris NP, Hutchings GH, Zhang Z, Belsham GJ, Alexandersen S. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. J Virol Meth 2002; 105: 67-80.
14. Callahan JD, Brown F, Osorio FA, Sur JH, Kramer E, Long GW, Lubroth J, Ellis SJ, Shoulars KS, Gaffney KL. Use of a portable real-time reverse transcriptase polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. J Am Vet Med Assoc 2002; 220: 1636-1642.
15. Moniwa M, Clavijo A, Li M, Collignon B, Kitching RP. Performance of a foot-and-mouth disease virus reverse transcription-polymerase chain reaction with amplification controls between three real-time instruments. J Vet Diag Invest 2007; 19: 9-20.
16. Reid S, Ferris N, Hutchings G, De Clercq K, Newman B, Knowles N, Samuel A. Diagnosis of foot-and-mouth disease by RT-PCR: use of phylogenetic data to evaluate primers for the typing of viral RNA in clinical samples. Arch Virol 2001; 146: 2421-2434.
17. Vangrysperre W, De Clercq K. Rapid and sensitive polymerase chain reaction based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolates, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. Arch Virol 1996; 141: 331-344.
18. Rasmussen TB, Uttenthal Å, De Stricker K, Belak S, Storgaard T. Development of a novel quantitative real-time RT-PCR assay for the simultaneous detection of all serotypes of foot-and-mouth disease virus. Arch Virol 2003; 148: 2005-2021.
19. Bachanek-Bankowska K, Mero HR, Wadsworth J, Mioulet V, Sallu R, Belsham GJ, Kasanga CJ, Knowles NJ, King DP. Development and evaluation of tailored specific real-time RT-PCR assays for detection of foot-and-mouth disease virus serotypes circulating in East Africa. J Virol Meth 2016; 237: 114-120.
20. 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.
21. Alexandersen S, Forsyth MA, Reid SM, Belsham GJ. Development of reverse transcription-PCR (oligonucleotide probing) enzyme-linked immunosorbent assays for diagnosis and preliminary typing of foot-and-mouth disease: a new system using simple and aqueous-phase hybridization. J Clin Micro 2000; 38: 4604-4613.
22. Ehizibolo D, Haegeman A, De Vleeschauwer A, Umoh J, Kazeem H, Okolocha E, Van Borm S, De Clercq K. Detection and molecular characterization of foot and mouth disease viruses from outbreaks in some states of northern Nigeria 2013–2015. Transb Emerg Dis 2017; 64: 1979-1990.
23. Ali W, Habib M, Khan R, Zia M, Khan I, Saliha U, Farooq M, Shah M, Muzammil H. Reverse transcription-polymerase chain reaction (RT-PCR) based detection and economic impact of foot-and-mouth disease in District Faisalabad, Pakistan during the year 2015. Iraqi J Vet Sci 2017; 31: 1-6.
24. Fry E, Stuart D, Rowlands D. The structure of foot-and-mouth disease virus. In: Mahy BW, editor. Foot-and-Mouth Disease Virus. Current Topics in Microbiology and Immunology, Vol. 288. Berlin, Heidelberg: Springer; 2005. pp. 71-101.

25. Knowles N, Samuel A. Molecular epidemiology of foot-and-mouth disease virus. Virus Research 2003; 91: 65-80.

26. Reid SM, Hutchings GH, Ferris NP, De Clercq K. Diagnosis of foot-and-mouth disease by RT-PCR: evaluation of primers for serotypic characterisation of viral RNA in clinical samples. J Virol Meth 1999; 83: 113-123.

27. Reid SM, Mioulet V, Knowles NJ, Shirazi N, Belsham GJ, King DP. Development of tailored real-time RT-PCR assays for the detection and differentiation of serotype O, A and Asia-1 foot-and-mouth disease virus lineages circulating in the Middle East. J Virol Meth 2014; 207: 146-153.

28. Ahmed H, Salem S, Habashi A, Arafa A, Aggour M, Salem G, Gaber A, Selem O, Abdelkader S, Knowles N. Emergence of foot-and-mouth disease virus SAT 2 in Egypt during 2012. Transb Emerg Dis 2012; 59: 476-481.

29. Knowles N, Bachanek-Bankowska K, Wadsworth J, Mioulet V, Valdazo-González B, Eldaghayes I, Dayhum A, Kammon A, Sharif M, Waight S. Outbreaks of foot-and-mouth disease in Libya and Saudi Arabia during 2013 due to an exotic O/me-sa/ind-2001 lineage virus. Transb Emerg Dis 2016; 63: e431-e435.

30. Samuel A, Knowles N. Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). J Gen Virol 2001; 82: 609-621.

31. Knowles NJ, He J, Shang Y, Wadsworth J, Valdazo-González B, Onosato H, Fukai K, Morioka K, Yoshida K, Cho IS. Southeast Asian foot-and-mouth disease viruses in Eastern Asia. Emerg Infect Dis 2012; 18: 499.

32. Valdazo-González B, Timina A, Scherbakov A, Abdul-Hamid NF, Knowles NJ, King DP. Multiple introductions of serotype O foot-and-mouth disease viruses into East Asia in 2010–2011. Vet Res 2013; 44: 76.

33. Knowles N, Nazem Shirazi M, Wadsworth J, Swabey K, Stirling J, Statham R, Li Y, Hutchings G, Ferris N, Parlak Ü. Recent spread of a new strain (A-Iran-05) of foot-and-mouth disease virus type A in the Middle East. Transb Emerg Dis 2009; 56: 157-169.

34. Jamal SM, Ferrari G, Ahmed S, Normann P, Belsham GJ. Genetic diversity of foot-and-mouth disease virus serotype O in Pakistan and Afghanistan, 1997–2009. Infect Genet Evol 2011; 11: 1229-1238.

35. Jamal SM, Ferrari G, Ahmed S, Normann P, Belsham GJ. Molecular characterization of serotype Asia-1 foot-and-mouth disease viruses in Pakistan and Afghanistan; emergence of a new genetic group and evidence for a novel recombinant virus. Infect Genet Evol 2011; 11: 2049-2062.

36. Jamal SM, Ferrari G, Ahmed S, Normann P, Curry S, Belsham GJ. Evolutionary analysis of serotype A foot-and-mouth disease viruses circulating in Pakistan and Afghanistan during 2002–2009. J Gen Virol 2011; 92: 2849-2864.

37. Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999; 41: 95-98.

38. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25: 4876-4882.

39. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013; 30: 2725-2729.

40. Waud M, Busschaert P, Ruyters S, Jacquemyn H, Lievens B. Impact of primer choice on characterization of orchid mycorrhizal communities using 454 pyrosequencing. Mol Eco Res 2014; 14: 679-699.

41. Logan G, Freimanis GL, King DJ, Valdazo-González B, Bachanek-Bankowska K, Sanderson ND, Knowles NJ, King DP, Cottam EM. A universal protocol to generate consensus level genome sequences for foot-and-mouth disease virus and other positive-sense polyadenylated RNA viruses using the Illumina MiSeq. BMC Genomics 2014; 15: 828.

42. Jamal SM, Belsham GJ. Development and characterization of probe-based real time quantitative RT-PCR assays for detection and serotyping of foot-and-mouth disease viruses circulating in West Eurasia. PLoS One 2015; 10: e0135559.

43. Ibarbalz FM, Pérez MV, Figuerola EL, Erijman L. The bias associated with amplicon sequencing does not affect the quantitative assessment of bacterial community dynamics. PLoS One 2014; 9: e99722.

44. Lefever S, Pattyn F, Hellemans J, Vandesompele J. Single-nucleotide polymorphisms and other mismatches reduce performance of quantitative PCR assays. Clin Chem 2013; 2013: 203653.