

NOVEL STRATEGY FOR THE RAPID DETECTION AND CHARACTERIZATION FOOT-AND-MOUTH DISEASE VIRUS (FMDV) SEROTYPES O, A, AND ASIA 1

W. Ali^{1*}, M. Habib¹, R. S. A. Khan¹, S. Sajid^{1,2}, M. A. Zia¹, M. U. Mazhar¹, I. U. Khan¹, A. Javed¹, M. Farooq¹, M. S. U. D. Shah^{1,3}

¹Department of Biological Sciences, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, affiliated with Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad, Pakistan

²Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan

³Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT, United States

*Corresponding Author: drwaqaasali@gmail.com

ABSTRACT

A one-step RT-PCR assay was developed successfully for universal detection of Pakistani FMDV serotypes A, O and Asia1 using newly designed universal primers MF5/MR3 that amplified complete VP1 coding gene and its PCR products can be used for direct sequencing. The assay amplified 995 bp region of the FMDV serotypes A, O and Asia1 directly from field samples including whole VP1 capsid protein-coding gene. Remarkably, PCR products acquired from this technique can be used in second PCR as DNA template for typing of serotypes O, A and Asia1 using serotype-specific primers. This second PCR enhanced the detection sensitivity of the assay evidently. In this study, this assay was used to identify and characterize FMDV isolates (n=34) collected from different outbreaks in Pakistan from 2014 to 2017. All the isolates (n=34) were successfully detected and categorized into serotype O (n=14), serotype A (n=5) and serotype Asia1 (n=15). This new assay will help in general detection in first PCR reaction (products of this PCR can be used for direct sequencing) while typing in second multiplex PCR. Therefore, amplified product can be used for detection, serotyping, sequencing, cloning, and other molecular studies of Pakistani, West Eurasian as well as universal FMDV's strains.

Key words: RT-PCR, serotyping, Pakistan, FMD.

INTRODUCTION

Foot-and-mouth disease (FMD) is one of the most economically important and highly contagious viral diseases of domesticated and wild cloven-hoofed animals i.e. cattle, sheep, goats, and pigs (Jamal and Belsham 2013). This disease is endemic in Asia, Africa and South America wherein huge economic losses occur due to low milk production, deprived growth rate, mortality in young calf and trade restrictions to FMD-free countries (Bachanek-Bankowska *et al.*, 2016; Ambagala *et al.*, 2017). Moreover, FMD-free countries have also incursions of disease outbreaks causing considerable damage to livestock i.e. outbreaks in Japan, Korea (2000 and 2010), Netherlands (2001), United Kingdom (2001 and 2007), and Bulgaria (2010-11) (Reid *et al.*, 2014).

The etiological agent of FMD is Foot-and-mouth disease virus (FMDV) that belongs to genus *Aphthovirus* and family *picornaviridae*. This virus has single-stranded, positive sense, 8400 base pairs (bp) long genome that is surrounded by small non enveloped capsid made up of four structural proteins (VP1, VP2, VP3 and VP4) (Belsham 2005; Carrillo *et al.*, 2005). The virus exists in seven immunologically distinct serotypes i.e. O, A, Asia1, C, SAT (South African territories) I, SAT II and SAT III. These serotypes are widely distributed

worldwide such as serotype O and A are distributed throughout the world, Asia1 mostly in Asia, SAT serotypes are restricted to African countries and serotype C to Europe (Davies 2002; Reid *et al.*, 2014). Interestingly, immunity against one serotype does not give protection against other serotypes and also sometimes fail to protect against different viral strains within same serotype (Paton *et al.*, 2005). VP1 protein present on capsid surface has a significant role in protective immunity and specificity of serotypes due to the presence of important immunological sites in this protein such as G-H loop and C-terminus (Jackson *et al.*, 2002; Jamal *et al.*, 2011c). Furthermore, the VP1 nucleotide sequence is also employed to assess the evolution and movement of viral strains in the majority of outbreaks (Kasanga *et al.*, 2015). Phylogenetic analysis of VP1 gene sequences helped to categorize each serotype of FMDV into different subtypes (Lee *et al.*, 2012). The disease is characterized clinically by the presence of vesicular lesions in the mouth, tongue, feet, and coronary band. However, this disease cannot be differentiated only on the basis of clinical signs due to the resemblance with other vesicular diseases i.e. vesicular stomatitis. Therefore, laboratory confirmation is essential to validate the presence of virus (Ranjan *et al.*, 2014).

Control of FMD outbreaks is reliant on the rapid detection of clinical cases of the disease, for which fast

and sensitive diagnostic technique is required (Hoffmann *et al.*, 2009). In the routine, FMD is diagnosed based on clinical signs, followed by viral isolation and its identification by targeting the highly conserved regions of the genome such as 5'UTR and 3D in RT-PCR assay (Callahan *et al.*, 2002; Reid *et al.*, 2002; Moniwa *et al.*, 2007). After this serotyping is carried out using different PCR formats that detect the virus serotypes by targeting VP1 (1D) region (Meyer *et al.*, 1991; Laor *et al.*, 1992; Rodríguez *et al.*, 1992; Rodríguez *et al.*, 1994; Vangrysterpe and De Clercq 1996; Callens and De Clercq 1997; Habib *et al.*, 2014). This whole process is very lengthy and time-consuming (Suryanarayana *et al.*, 1999).

In Pakistan, FMD is endemic and outbreaks due to serotype O, A and Asia1 occurs on regular basis (Klein *et al.*, 2008; Jamal *et al.*, 2010; Jamal *et al.*, 2011a; Jamal *et al.*, 2011b; Jamal *et al.*, 2011c; Waheed *et al.*, 2011; Brito *et al.*, 2013; Jamal and Belsham 2013; Knowles *et al.*, 2015; Ali *et al.*, 2017; Ullah *et al.*, 2017). Different studies are conducted in the country to control the disease through vaccination (Akram *et al.*, 2013; Muhammad *et al.*, 2013).

In countries like Pakistan, where FMD is endemic and multiple serotypes are in circulation development of molecular-based methods for early detection are of utmost importance. In the present study, new primer pair (MF-5 and MR-3) was designed in the

conserved regions (1C and 2B) for universal detection of Pakistani FMDV strains using clinical samples regardless of the serotype A, O and Asia1. This new RT-PCR method will help in general detection in first PCR reaction (products of this PCR can be used for direct sequencing) while typing in second multiplex PCR. Therefore, amplified product can be used for detection, serotyping, sequencing, cloning, and other molecular studies of Pakistani, West Eurasian as well as universal FMDV's strains. This RT-PCR method may act as an effective tool for molecular characterization studies of FMD in Pakistan.

MATERIALS AND METHODS

Samples and RNA extraction: Total 34 samples from clinically infected animals were collected from different outbreaks. Along with clinical samples, three pandemic strains (O/NIAB/PUN/PAK/01/2014, A/NIAB/PUN/PAK/17/2014, ASIA/ NIAB/PUN/PAK/154/2016) were also used for the optimization of the test. Clinical samples were placed in glycerolized buffered saline. RNA was extracted from clinical samples by using Favor Prep® viral nucleic acid extraction kit (Favorgen, Biotech Corporation, Taiwan) according to manufacturer's protocol. Total RNA was eluted in 40 µl of elution buffer and stored at -70°C.

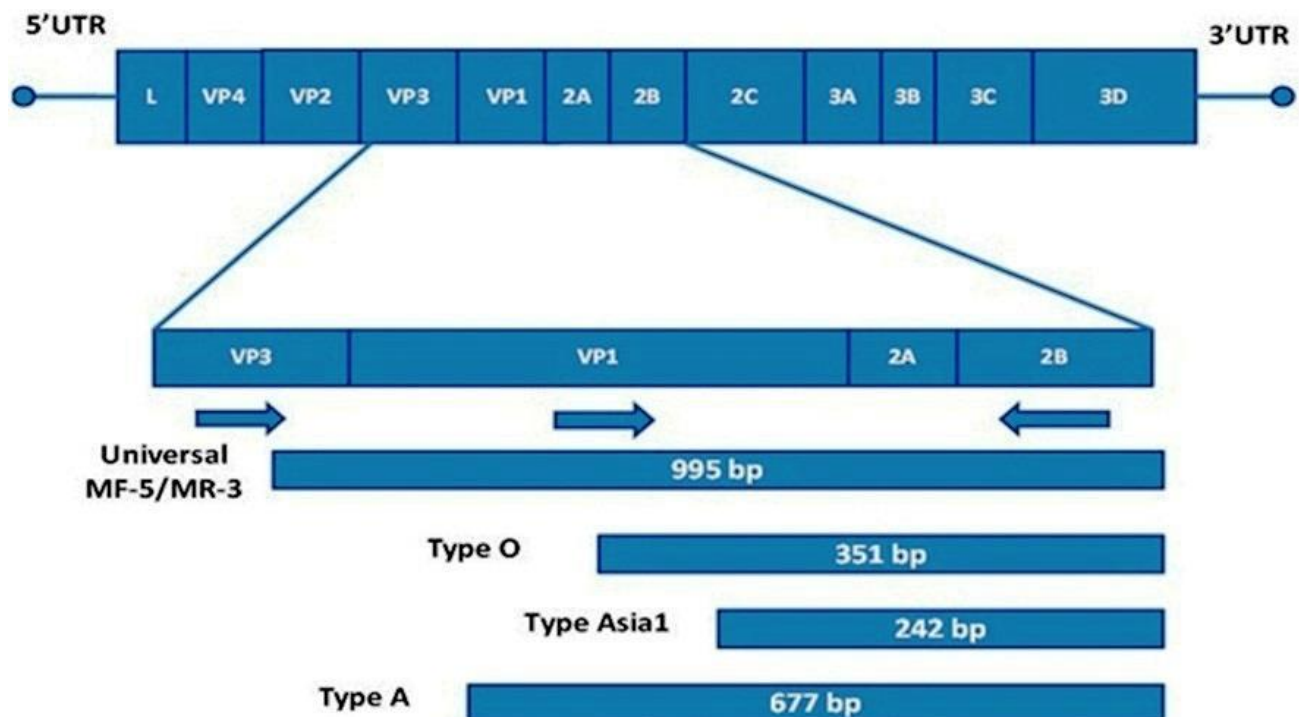


Figure 1. The schematic layout of FMDV genome. PCR amplification of universal (MF-5/MR-3), as well as serotype-specific primers, were schematically shown alongwith their expected product sizes.

Oligonucleotide primers: New universal primers MF-5 (5'-GCTGCTGACTACGCGTACAC-3') and MR-3(5'-CCAGTGGCCAGTTCCTCAA -3') were designed on conserved genome regions 1C and 2B by the alignment of FMDV sequences (n=100) of serotypes O, A and Asia1. Conserved regions among all the selected strains (n=100) were selected for primer design (Figure 2, Figure

3). These primers cover the entire VP1 region amplifying 995bp double standard DNA product of serotypes O, A and Asia1 (Figure 1). The primers were commercially synthesized by Eurofins genomics, USA. Moreover, previously reported serotype-specific primers were used in typing PCR for serotypes O, A and Asia1 (Table 1) (Vangrypeperre and De Clercq 1996) for typing of FMDV.

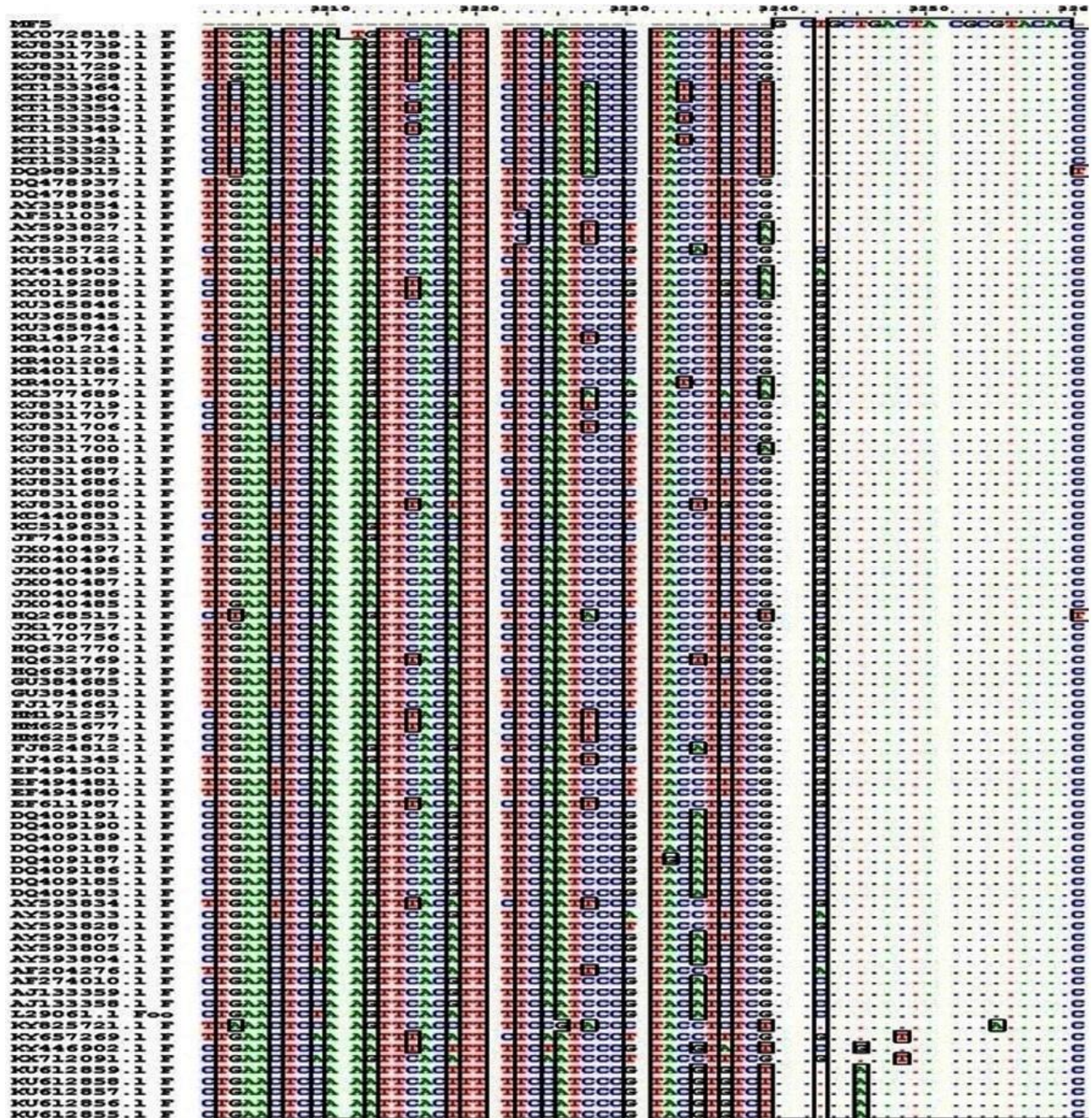


Figure 2. Multiple alignment of complete genome sequences of 100 strains of FMDV obtained from GenBank against newly designed primer MF5. Dots represents matching of the sequences at primer binding site.



Figure 3. Multiple alignment of complete genome sequences of 100 strains of FMDV obtained from GenBank against newly designed primer MR3. Dots represents matching of the sequences at primer binding site.

Table 1. Sequence of oligonucleotides used in this study.

Primer	Sequence (5'-3')	Specificity	Product size (bp)	References
1F	GCCTGGTCTTTCCAGGTCT	All Types	328	(Reid <i>et al.</i> , 2000)
1R	CCAGTCCCCTTCTCAGATC			
P38	GCTGCCTACCTCCTTCAA	Type O, Forward	351	
P74	GACACCACTCAGGACCGCCG	Type Asia1, Forward	242	(Vangrysperre and De Clercq 1996)
P75	GACACCACCCAGGACCGCCG			
P76	GACACCACACAAGACCGCCG			
P77	GACACGACTCAGAACCGCCG			
P87	GTCATTGACCTCATGCAGACCCAC	Type A, Forward	677	
P88	GTTATTGACCTCATGCAGACCCAC			
P89	GTCATTGACCTCATGCACACCCAC			
P90	GTCATTGACCTCATGCAGACTCAC			
P91	GTCATTGACCTCATGCAAACCCAC			
P92	GTCATTGACCTTATGCAGACTCAC			
MF-5	GCTGCTGACTACGCGTACAC	Type O, A, Asia1 Forward	995	This study
MR-3	CCAGTGGCCAGTTCCTCAAA	Type O, A, Asia1 Reverse		

One-step RT-PCR: One-step RT-PCR was performed on RNA extracted from clinical samples by using Verso 1-step RT-PCR Hot-Start Kit (Thermo Scientific, USA). Briefly, 25 µl reaction mixture was prepared to contain 0.5 µl Verso enzyme mix, 12.5 µl 1-step PCR Hot-Start Master Mix (2X), 1.25 µl RT enhancer, 0.5 µl universal MF5/MR3 primers (10 mM each). In this premix, 5 µl of RNA template and 4.75 µl nuclease-free water was added. It was incubated at 56° C for 15 min, followed by 95° C for 15 minutes. Then 30 cycles of 95° C for 20 sec, 55° C for 30 sec and 72° C for 1 min were repeated. Final extension was carried out at 72° C for 10 min.

Typing of Pakistani FMDV serotypes O, A and Asia:

A second PCR was performed using 995bp PCR product as template (amplified using universal primer pair MF5/MR3) as a template for serotype-specific PCR reaction using serotype-specific forward primers (P38-P92) (Vangrysperre and De Clercq 1996) and universal MR-3 as reverse primer for typing of FMDV serotypes O, A and Asia1 (Table 1). PCR conditions were the same as published in our previous study (Habib *et al.*, 2014).

Sensitivity and specificity analysis: The sensitivity of MF-5/MR-3 primer pair was compared with previously reported universal consensus primer pair (1F/1R) (Reid *et al.*, 2000) on three pandemic strains (O/NiAB/ PUN/ PAK/ 01/2014, A/NiAB/PUN/PAK/17/2014, ASIA/NiAB/PUN/PAK/154/2016) by making a ten-fold serial dilution of the templates in PCR assay.

For specificity analysis, primer sets were subjected to other viruses such as Infectious Bursal disease virus (IBDV) and Peste Des Petites Ruminants virus (PPRV) in RT-PCR assay.

Sequence Analysis: PCR products were subjected to direct sequencing using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) and ABI PRISM 3730xl Genetic Analyzer developed by Applied Biosystems, USA. The sequences obtained were assembled and aligned using Bioedit alignment editor (Hall 1999) against downloaded FMDV sequences.

RESULTS

Optimization of one-step RT-PCR was done by using three Pandemic strains (O/NiAB/ PUN/ PAK/ 01/2014, A/NiAB/ PUN/ PAK/ 17/2014, Asia1/ NiAB/ PUN/ PAK/ 154/ 2016). In all three strains, amplification of entire VP1 region was successful, yielding 995bp intense band.

The second multiplex typing PCR resulted in three different band sizes of 351, 242 and 677bp for serotype O, A and Asia1 respectively. Furthermore, detection sensitivity was increased as we performed the second PCR for typing of FMDV and strong bands were found for all the dilutions (Figure 4 C of I-III). Total 34 clinical samples collected from infected animals and three Pandemic strains were subjected to present one-step RT-PCR assay. Out of total 34 samples, 14 was of serotype O, 5 of serotype A and 15 of serotype Asia1 (data not shown).

The detection limit and sensitivity of newly designed primers and consensus primer pair 1F/1R (Reid *et al.*, 2000) were found same in the one-step RT-PCR assay. Both assays were able to detect viral genome up to the dilution of 10⁻³, 10⁻², 10⁻² for pandemic strains of serotype O (O/NiAB/PUN/PAK/01/2014), serotype A (A/NiAB/PUN/PAK/17/2014) and serotype Asia1

(Asia1/NIAB/PUN/PAK/154/2016) respectively (Figure 4 A and B of I-III).

The present RT-PCR assay was found specific for FMDV, as it was unable to detect IBDV and PPRV viruses.

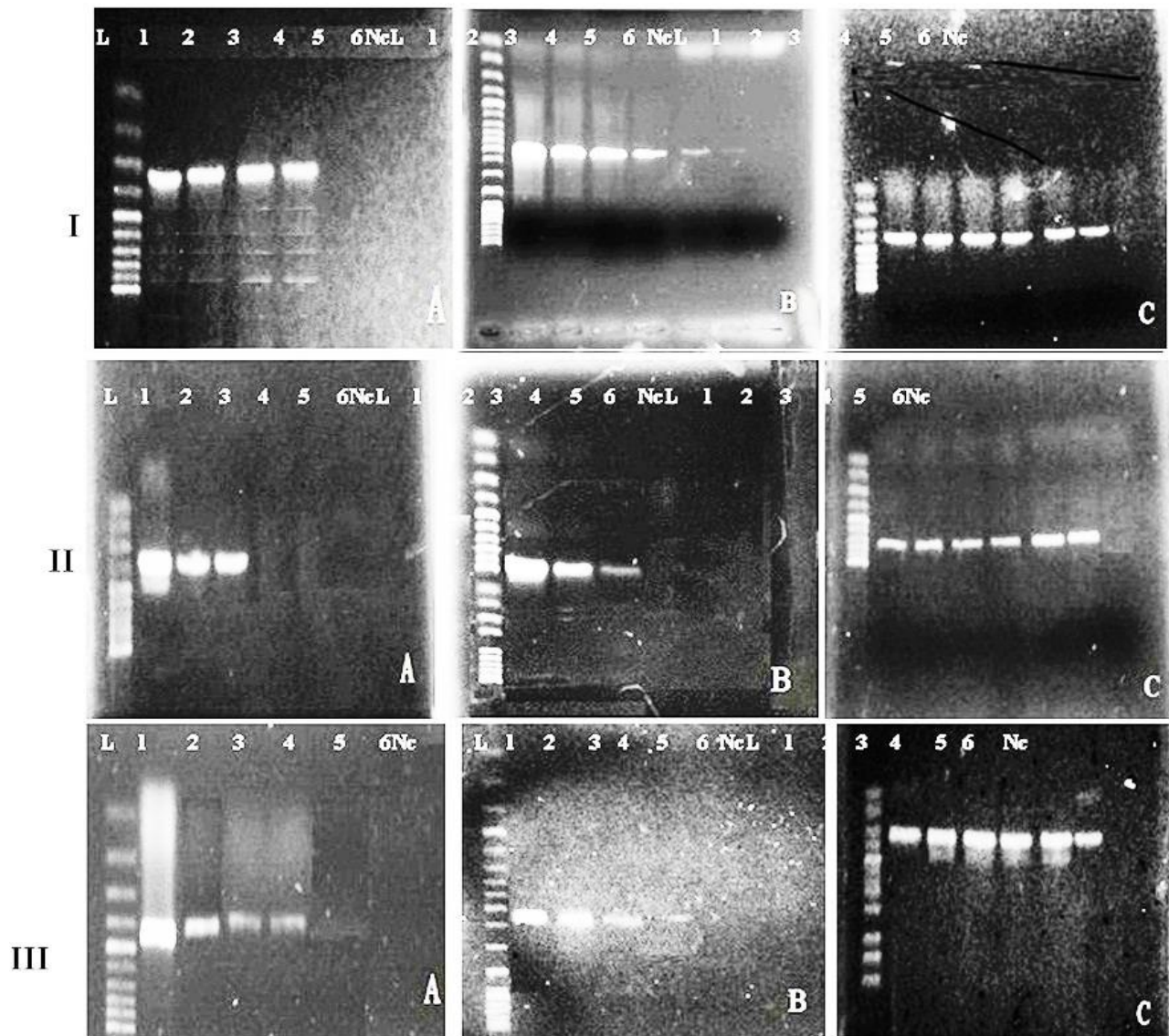


Figure 4. Sensitivity of one-step RT-PCR methods for FMDV strains O/NIAB/PUN/PAK/01/2014 (I), A/NIAB/PUN/PAK/17/2014 (II) and ASIA/NIAB/PUN/PAK/154/2016 (III). (Left to right) (A) Lane M shows 100bp DNA ladder (VC 100bp Plus Vivantis®), while lane 1, 2, 3, 4, 5, 6 and Nc depicts the amplification (320 bp) of 1, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} dilutions of RNA and negative control respectively using consensus primer pair (1F/1R) (Reid *et al.*, 2000) in one-step RT-PCR for FMDV. (B) Lane M shows DNA ladder (GeneRuler DNA Ladder Mix, Thermo Scientific), while lane 1, 2, 3, 4, 5, 6 and Nc depicts the amplification (1274 bp) of 1, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} dilutions of RNA and negative control respectively using universal primer pair (MF6/MR3) in one-step RT-PCR for FMDV. (C) Second PCR amplification using the previously reported serotype-specific primers (Vangryspere and De Clercq 1996) for serotyping of FMDV serotypes O, A, and Asia 1 (0.5 μ l of the PCR products of the first one-step RT-PCR was used as template for second amplification).

Sequencing of the complete VP1 region using RT-PCR products: Sequencing of the RT-PCR products was performed to confirm the detection ability of the assay for

universal detection of Pakistani FMDV serotypes A, O and Asia1 using new primers MF5/MR3.ABI PRISM 3730xl Genetic Analyzer developed by Applied Bio

systems, USA was used for the sequencing of the purified PCR products. To confirm the results nucleotide sequences were analyzed using BLAST website (<http://www.ncbi.nlm.nih.gov>). Results revealed that serotype O strains belonged to topo type ME-SA, serotype A viruses to Iran-05 lineage and serotype Asia1 viruses to Group-VII (Sindh-08)(paper submitted).

DISCUSSION

FMD is endemic in Pakistan and viruses belonging to serotype O, A and Asia1 are continuously circulating in the region as reported in recent studies (Jamal *et al.*, 2010; Jamal *et al.*, 2011a; Jamal *et al.*, 2011b; Jamal *et al.*, 2011c; Waheed *et al.*, 2011; Abubakar *et al.*, 2015; Brito *et al.*, 2017; Mahapatra *et al.*, 2017). For containment and emergency vaccine, quick detection is necessary as it helps in tracking the emergence and spread of an outbreak. In present study one-step, RT-PCR method was developed and optimized for the detection of FMDV serotype O, A and Asia1 circulating in Pakistan directly from clinical samples. Classification of the serotype A, O and Asia1 into subtypes is determined on the basis variation in the VP1 region nucleotide sequences (Tosh *et al.*, 2002; Knowles and Samuel 2003; Kitching *et al.*, 2005; Knowles *et al.*, 2005; Valarcher *et al.*, 2009). To amplify the entire VP1 region we designed two universal primers in the conserved regions (1C and 2B) of serotype O, A and Asia1. These primers, when used in one-step RT-PCR, allowed the rapid, sensitive and specific amplification of entire VP1 region by directly using clinical samples.

In this method, amplified VP1 coding region PCR products can also be used for direct sequence, serotyping, cloning and other molecular epidemiological investigations without the need for cell culture and virus purification of serotype A, O and Asia1. As the VP1 coding region is routinely used to establish the origin of an outbreak and to determine the genetic relationship between the reference and field strains (Reid *et al.*, 2001), its large quantity is needed for a successful direct sequence of the clinical samples. The present one-step RT-PCR assay gives researchers a robust tool to discover emerging FMDV strains for molecular studies. These handy and convenient properties make present one-step RT-PCR assay based on VP1 coding region amplification, an ideal method that may provide a remarkable edge over the conventional FMDV detection methods (Habib *et al.*, 2014; Reid *et al.*, 2014; Khan *et al.*, 2017). An extra benefit of the present one-step RT-PCR assay is that, because the achieved VP1-PCR products (995 bp) amplify the whole VP1 coding gene, these products can be used as a template for the second PCR for typing FMDVs. This two-step PCR amplification increased the sensitivity of FMDV detection significantly mainly for field diagnosis, where

the concentration of virus in samples is usually low. Negative controls are essential to avoid false-positive results due to contamination during sample handling, as double amplification is very sensitive and minute quantities of impurities can give false positive results. However, false-positive results were not found in this study.

In the present study, three previously characterized pandemic strains for type O (O/NIAB/PUN/PAK/01/2014), type A (A/NIAB/PUN/PAK/17/2014), and type Asia1 (ASIA/NIAB/PUN/PAK/154/2016) was used for optimization and validation of the test. These strains belong to lineage Pan Asia II, Iran-05 and Group VII that were responsible for explosive outbreaks in Pakistan, Afghanistan, Turkey and Iraq during recent years (Knowles *et al.*, 2005; Waheed *et al.*, 2009; Jamal *et al.*, 2011a; Jamal *et al.*, 2011b; Jamal *et al.*, 2011c; Saeed *et al.*, 2011; Waheed *et al.*, 2011; Brito *et al.*, 2013; Jamal and Belsham 2013; Knowles *et al.*, 2015; Ullah *et al.*, 2017). The method used in this study successfully characterized all the three pandemic strains during direct sequencing.

In West Eurasia region FMD is endemic in Kazakhstan, Turkey, Afghanistan, Iran, Pakistan, Syria and Iraq. As the close relationship is found in previous studies among viruses circulating in this region (Schumann *et al.*, 2008; Sumption *et al.*, 2008; Jamal *et al.*, 2010; Jamal *et al.*, 2011a; Jamal *et al.*, 2011b; Jamal *et al.*, 2011c; Waheed *et al.*, 2011; Brito *et al.*, 2013; Knight-Jones *et al.*, 2016; Ullah *et al.*, 2017) the present one-step RT-PCR method may be applied in these West Eurasia countries. Serotype C and SAT I, II and III (South African Territories) was not considered during universal primers (MF5/MR3) designing because they were not found circulating in Pakistan in any of recent studies.

To conclude, the present one-step RT-PCR method is a simple, robust and easy method to characterize and timely detect FMDV serotypes O, A and Asia1 by amplifying full VP1 coding region. This method may provide a quick way to study the molecular evolution of field strains circulating in Pakistan by direct sequencing of amplified DNA. This assay may be used to give understandings in the spread of novel FMDV strains belonging to serotypes O, A and Asia1 at the molecular level that will help in quick response to control their spread.

Declaration of interest: We have no conflict of interest to declare.

Acknowledgment: We want to acknowledge Higher Education Commission (HEC), Islamabad, Pakistan and International Atomic Energy Agency (IAEA) for the provision of funding for this study.

REFERENCES

- Ambagala, A., M. Fisher, M. Goolia, C. Nfon, T. Furukawa-Stoffer, R. Ortega Polo and O. Lung (2017). Field-deployable Reverse Transcription-insulated Isothermal Pcr (rt-iipcr) Assay for Rapid and Sensitive Detection of Foot-and-mouth Disease Virus. *Trans. and Emerg. Dis.* 64(5): 1610-1623.
- Bachanek-Bankowska, K., H. R. Mero, J. Wadsworth, V. Mioulet, R. Sallu, G. J. Belsham, C. J. Kasanga, N. J. Knowles and D. P. King (2016). 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. of Virol. Meth.* 237: 114-120.
- Belsham, G. (2005). Translation and replication of FMDV RNA. *Curr. Top. in Micro. and Immunol.* 288: 43-70.
- Brito, B., A. Perez, S. Jamal, G. Belsham, S. Pauszek, Z. Ahmed and L. Rodriguez (2013). Foot-and-Mouth Disease Virus Serotype O Phylodynamics: Genetic Variability Associated with Epidemiological Factors in Pakistan. *Trans. and Emerg. Dis.* 60(6): 516-524.
- Brito, B., L. Rodriguez, J. Hammond, J. Pinto and A. Perez (2017). Review of the Global Distribution of Foot-and-Mouth Disease Virus from 2007 to 2014. *Trans. and Emerg. Dis.* 64(2): 316-332.
- Callahan, J. D., F. Brown, F. A. Osorio, J. H. Sur, E. Kramer, G. W. Long, J. Lubroth, S. J. Ellis, K. S. Shoullars and K. L. Gaffney (2002). Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *J. of the Amer. Vet. Med. Assoc.* 220(11): 1636-1642.
- Callens, M. and K. De Clercq (1997). Differentiation of the seven serotypes of foot-and-mouth disease virus by reverse transcriptase polymerase chain reaction. *J. of Virol. Meth.* 67(1): 35-44.
- Carrillo, C., E. Tulman, G. Delhon, Z. Lu, A. Carreno, A. Vagnozzi, G. Kutish and D. Rock (2005). Comparative genomics of foot-and-mouth disease virus. *J. of Virol.* 79(10): 6487-6504.
- Davies, G. (2002). Foot and mouth disease. *Research in veterinary science.* 73(3): 195-199.
- Habib, M., M. S. Shah, H. M. Muzammil, S. Manzoor, R. S. A. Khan, R. Munir, Z. I. Rajput and U. Farooq (2014). Investigations of Foot-and-Mouth Disease Outbreaks in Faisalabad District of Punjab, Pakistan during the Year 2013. *Pak. J. of lif. and soc. sci.* 12(3): 165-169.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series*, [London]: Information Retrieval Ltd., c1979-c2000.
- Hoffmann, B., M. Beer, S. M. Reid, P. Mertens, C. A. Oura, P. A. Van Rijn, M. J. Slomka, J. Banks, I. H. Brown and D. J. Alexander (2009). 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. Micro.* 139(1): 1-23.
- Jackson, T., A. P. Mould, D. Sheppard and A. M. King (2002). Integrin $\alpha\beta 1$ is a receptor for foot-and-mouth disease virus. *J. of Virol.* 76(3): 935-941.
- Jamal, S. M., S. Ahmed, M. Hussain and Q. Ali (2010). Status of the foot-and-mouth disease in Pakistan. *Arch. of Virol.* 155(9): 1487-1491.
- Jamal, S. M. and G. J. Belsham (2013). Foot-and-mouth disease: past, present and future. *Vet. Res.* 44(1): 116.
- Jamal, S. M., G. Ferrari, S. Ahmed, P. Normann and G. J. Belsham (2011a). Genetic diversity of foot-and-mouth disease virus serotype O in Pakistan and Afghanistan, 1997–2009. *Infect. Genet. and Evol.* 11(6): 1229-1238.
- Jamal, S. M., G. Ferrari, S. Ahmed, P. Normann and G. J. Belsham (2011b). Molecular characterization of serotype Asia-1 foot-and-mouth disease viruses in Pakistan and Afghanistan; the emergence of a new genetic Group and evidence for a novel recombinant virus. *Infect. Genet. and Evol.* 11(8): 2049-2062.
- Jamal, S. M., G. Ferrari, S. Ahmed, P. Normann, S. Curry and G. J. Belsham (2011c). Evolutionary analysis of serotype A foot-and-mouth disease viruses circulating in Pakistan and Afghanistan during 2002-2009. *J. of Gen. Virol.* 92(12): 2849-2864.
- Kasanga, C., J. Wadsworth, C. Mpelumbe-ngeleja, R. Sallu, F. Kivaria, P. Wambura, M. Yongolo, M. Rweyemamu, N. Knowles and D. King (2015). Molecular Characterization of Foot-and-mouth Disease Viruses Collected in Tanzania Between 1967 and 2009. *Trans. and Emerg. Dis.* 62(5): e19-29.
- Khan, R. S. A., S. Sajid, M. Habib, W. Ali, M. S.-u.-D. Shah and M. Sarfraz (2017). History of Gumboro (infectious bursal disease) in Pakistan. *Saud. Pharma. J.* 25(4): 453-459.
- Kitching, R. P., A. Hutber and M. Thrusfield (2005). A review of foot-and-mouth disease with special consideration for the clinical and epidemiological factors relevant to predictive modelling of the disease. *The Vet. J.* 169(2): 197-209.
- Klein, J., M. Hussain, M. Ahmad, M. Afzal and S. Alexandersen (2008). Epidemiology of foot-

- and-mouth disease in Landhi Dairy Colony, Pakistan, the world largest Buffalo colony. *Virol. J.* 5(1): 53.
- Knight-Jones, T., L. Robinson, B. Charleston, L. Rodriguez, C. Gay, K. Sumption and W. Vosloo (2016). Global Foot-and-Mouth Disease Research Update and Gap Analysis: 4-Diagnostics. *Trans. and Emerg. Dis.* 63(S1): 42-48.
- Knowles, N. and A. Samuel (2003). Molecular epidemiology of foot-and-mouth disease virus. *Vir. Res.* 91(1): 65-80.
- Knowles, N. J., A. R. Samuel, P. R. Davies, R. J. Midgley and J.-F. Valarcher (2005). Pandemic strain of foot-and-mouth disease virus serotype O. *Infect. Dis.* 11(12): 1887-1893.
- Knowles, N. J., J. Wadsworth and K. Bachanek-Bankowska (2015). Genotyping Report FMDV type A, O and Asia 1 Pakistan. FAO World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) 2015.
- Laor, O., H. Torgersen, H. Yadin and Y. Becker (1992). Detection of FMDV RNA amplified by the polymerase chain reaction (PCR). *J. of Virol. Meth.* 36(3): 197-207.
- Lee, K.-N., T. Nguyen, S.-M. Kim, I.-S. Cho, D. D. Khang, N. B. Hien, D. Van Quyen and J.-H. Park (2012). A rapid molecular strategy for early detection and characterization of Vietnamese foot-and-mouth disease virus serotypes O, A, and Asia 1. *J. of virol. meth.* 180(1): 1-6.
- Mahapatra, M., S. Upadhyaya, S. Aviso, A. Babu, G. Hutchings and S. Parida (2017). Selection of vaccine strains for serotype O foot-and-mouth disease viruses (2007–2012) circulating in Southeast Asia, East Asia and the Far East. *Vacc.* 35(51): 7147-7153.
- Meyer, R., C. Brown, C. House, J. House and T. Molitor (1991). Rapid and sensitive detection of foot-and-mouth disease virus in tissues by enzymatic RNA amplification of the polymerase gene. *J. of Virol. Meth.* 34(2): 161-172.
- Moniwa, M., A. Clavijo, M. Li, B. Collignon and R. P. Kitching (2007). Performance of a foot-and-mouth disease virus reverse transcription-polymerase chain reaction with amplification controls between three real-time instruments. *J. of Vet. Diag. Invest.* 19(1): 9-20.
- Muhammad, K., M. Tariq, I. Altaf, M. Rabbani, R. Khawaja, Q. Akram, M. Ali and K. Hanif (2013). Effect of Different Adjuvants Used in Foot and Mouth Disease Virus Vaccine on Antibody Response of Buffalo Calves. *The J. Anim. Plan. Sci.* 23(3): 736-739.
- Paton, D., J. Valarcher, I. Bergmann, O. Matlho, V. Zakharov, E. Palma and G. Thomson (2005). Selection of foot and mouth disease vaccine strains-a review. *Rev.Sci. et technique. intern. des.epic.* 24(3): 981.
- Ranjan, R., M. Kangayan, S. Subramaniam, J. K. Mohapatra, J. K. Biswal, G. K. Sharma, A. Sanyal and B. Pattnaik (2014). Development and evaluation of a one-step reverse transcription-loop-mediated isothermal amplification assay (RT-LAMP) for rapid detection of foot and mouth disease virus in India. *Vir.dis.* 25(3): 358-364.
- Reid, S. M., N. P. Ferris, A. Brüning, G. H. Hutchings, Z. Kowalska and L. Åkerblom (2001). Development of a rapid chromatographic strip test for the pen-side detection of foot-and-mouth disease virus antigen. *J. of Virol. Meth.* 96(2): 189-202.
- Reid, S. M., N. P. Ferris, G. H. Hutchings, A. R. Samuel and N. J. Knowles (2000). Primary diagnosis of foot-and-mouth disease by reverse transcription polymerase chain reaction. *J. of Virol. Meth.* 89(1): 167-176.
- Reid, S. M., N. P. Ferris, G. H. Hutchings, Z. Zhang, G. J. Belsham and S. Alexandersen (2002). Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *J. of Virol. Meth.* 105(1): 67-80.
- Reid, S. M., V. Mioulet, N. J. Knowles, N. Shirazi, G. J. Belsham and D. P. King (2014). 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. of Virol. meth.* 207: 146-153.
- Rodríguez, A., J. Dopazo, J. Saiz and F. Sobrino (1994). Immunogenicity of non-structural proteins of foot-and-mouth disease virus: differences between infected and vaccinated swine. *Arch. of Virol.* 136(1): 123-131.
- Rodríguez, A., E. Martínez-Salas, J. Dopazo, M. Dávila, J. C. Sáiz and F. Sobrino (1992). Primer design for specific diagnosis by PCR of highly variable RNA viruses: typing of foot-and-mouth disease virus. *Virol.* 189(1): 363-367.
- Saeed, A., Q. M. Khan, U. Waheed, M. Arshad, M. Asif and M. Farooq (2011). RT-PCR evaluation for identification and sequence analysis of foot-and-mouth disease serotype O from 2006 to 2007 in Punjab, Pakistan. *Compare. Immunol. micro. and infect. dis.* 34(2): 95-101.
- Schumann, K. R., N. J. Knowles, P. R. Davies, R. J. Midgley, J.-F. Valarcher, A. Q. Raoufi, T. S. McKenna, W. Hurtle, J. P. Burans and B. M. Martin (2008). Genetic characterization and molecular epidemiology of foot-and-mouth

- disease viruses isolated from Afghanistan in 2003–2005. *Vir. Gen.* 36(2): 401-413.
- Sumption, K., M. Rweyemamu and W. Wint (2008). Incidence and Distribution of Foot-and-Mouth Disease in Asia, Africa and South America; Combining Expert Opinion, Official Disease Information and Livestock Populations to Assist Risk Assessment. *Trans. and Emerg. Dis.* 55(1): 5-13.
- Suryanarayana, V., B. Madanamohan, P. Bist, C. Natarajan and J. D. Tratschin (1999). Serotyping of foot-and-mouth disease virus by antigen capture reverse transcriptase/polymerase chain reaction. *J. of Virol. Meth.* 80(1): 45-52.
- Tosh, C., A. Sanyal, D. Hemadri and R. Venkataramanan (2002). Phylogenetic analysis of serotype A foot-and-mouth disease virus isolated in India between 1977 and 2000. *Arch. of Virol.* 147(3): 493-513.
- Ullah, A., S. Jamal, A. Romey, K. Gorna, M. Kakar, F. Abbas, J. Ahmad, S. Zientara and L. Bakkali Kassimi (2017). Genetic Characterization of Serotypes A and Asia-1 Foot-and-mouth Disease Viruses in Balochistan, Pakistan, in 2011. *Trans. and Emerg. Dis.* 64(5): 1569-1578.
- Valarcher, J.-F., N. J. Knowles, V. Zakharov, A. Scherbakov, Z. Zhang, Y.-J. Shang, Z.-X. Liu, X.-T. Liu, A. Sanyal and D. Hemadri (2009). Multiple origins of foot-and-mouth disease virus serotype Asia 1 outbreaks, 2003–2007. *Emerg. Infect. Dis.* 15(7): 1046.
- Vangrysperre, W. and K. De Clercq (1996). Rapid and sensitive polymerase chain reaction-based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolate, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. *Arch. of Virol.* 141(2): 331-344.
- Waheed, U., S. Parida, Q. Khan, M. Hussain, K. Ebert, J. Wadsworth, S. Reid, G. Hutchings, M. Mahapatra and D. King (2011). Molecular Characterisation of Foot-and-Mouth Disease Viruses from Pakistan, 2005–2008. *Trans. and Emerg. Dis.* 58(2): 166-172.
- Waheed, U., A. Saeed, A. Mobeen and Q. M. Khan (2009). The VP1 (Capsid Protein) Gene-Based DNA Sequencing for Epidemiological Analysis of FMDV, Isolated From Buffaloes in Pakistan. *Pak. J. of Zool.* 09: 333-339.