INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 5, p. 346-356, 2017

RESEARCH PAPER OPEN ACCESS

Foot-and-mouth disease outbreaks investigations at Sindh, Punjab and Islamabad, Pakistan during the year 2012-2013

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Key words: Foot-and-mouth disease, Sero-types, LFBK, Indirect sandwich ELISA, Lineage, Sequence, Phylogeny, Pakistan

http://dx.doi.org/10.12692/ijb/11.5.346-356 Article published on November 30, 2017

Abstract

This study reports characterization of Foot-and-Mouth Disease Virus (FMDV) from clinical suspected cases collected from Sindh, Punjab and Islamabad, Pakistan during the year 2012-2013. The attempts were made for the recovery FMDV in LFBK cell line and the viruses were confirmed by real time PCR and serotyped by indirect sandwich ELISA. Overall ELISA confirmed 51.2% FMD outbreaks. A total of 39 viruses (45.4%) were isolated from outbreaks which include sero-type O (35.9%), A (28.2%) and Asia-1 (35.9%). FMDV sero-type O isolates were characterized within the PanAsia2 and the sub lineage identified was PanAsia2 $^{ANT-10}$ & PanAsia2 $^{BAL-09}$. FMDV sero-type A was grouped within the lineage A/Asia/Iran-05. The sub lineage identified within FMDV Iran-05 was HER-10, FAR-11 & ESF-10. FMDV sero-type Asia-1 was classified in group VII (Sindh-08). The diagnostic sensitivity of ELISA was found to be 61.54% (95% CI; 44.62% to 76.64%) and specificity was 57.45 % (95% CI; 42.18% to 71.74%).

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Introduction

Foot-and-Mouth Disease (FMD) is a highly contagious viral disease of both domesticated and wild cloven footed animals (Carrillo *et al.*, 2005) caused by an *Aphthovirus* of family *Picornaviridae* (Belsham, 1993). This disease is a constant and a continuous threat to global livestock industries and small-holder farming. The clinical manifestation of this disease is fever, in appetence and lameness in association with characteristic vesicular lesions in the oral cavity, on feet and udders. The severity of clinical FMD varies greatly depending on virus strain and animal species, as well as previous exposure or vaccination history of the animal (Alexandersen and Mowat, 2005; Alexandersen *et al.*, 2003; Artz *et al.*, 2011 a & b).

In countries like Pakistan where the nature of the disease is endemic, it causes huge production losses and compromises food security due to decreased milk yields and growth rates besides the direct costs and logistical requisites of vaccination campaigns for control and prospective eradication in agricultural systems of many developing countries. The disease is havoc for the FMD free countries like Europe, Australia and North America and a possible spread of the disease would lead to an immediate crisis of the agricultural sector due to drastic restrictions on transportation, trade and export of animals and animal products, as well as vast expenses of imposed sanitary measures (Stenfeldt *et al.*, 2016).

South Asia is among largest livestock-producing regions of the world. Geographically this region comprises of Afghanistan, Bangladesh, Bhutan, India, Maldives, Nepal, Sri Lanka and Pakistan (Morzaria *et al.*, 2012). West Eurasia and the Middle East are considered the two sub-regions that maintain an independent pool (Pool 3) of related FMD viruses of serotypes A, Asia-1 and O (Zahur *et al.*, 2006; Abubakar *et al.*, 2015). Epidemics emerging within the region frequently reach neighboring countries, as observed in recent years with the O Panasia-2, type A Iran-05 and Asia-1. These epidemics extended from Pakistan and Afghanistan to Turkey, with occasional incursions into Central Asian and Middle East countries. Since 1990, 11 incursions of FMD into the FMD-free countries of Europe have occurred, involving nine countries, most of which were associated with entry from FMD virus Pool 3, the endemic countries in 'West Eurasia', of which Turkey shares land borders with FMD-free European countries; the most recent incursion being in 2010–2011 in Bulgaria. This pool involves at least 14 countries from Pakistan in the east, to Kazakhstan in the north, and Turkey in the west, and regional epidemics ('pandemics') sweep through the population at 1-3 years intervals (Sumption *et al.*, 2012). In the perspective of the conjectured global status of FMDV circulation Pakistan falls into virus Pool 3, indicating the dominance serotypes O, A and Asia 1. Of these, serotype O is most prevalent, followed by Asia 1 and A. The distribution of serotypes varies from region to region. Circulation of different genotypes/lineages within serotypes O, A and Asia 1 in the country was evident in molecular epidemiological analysis based on 1D/VP1 region sequence data. Emergence and re-emergence of genotypes/lineage occur in the field as part of this evolutionary process (Morzaria *et al.*, 2012).

The disease is also endemic in Pakistan and occurs throughout the year (Ahmad *et al.*, 2002; Klein *et al.*, 2008) in all parts of the country. In Pakistan the most prevalent serotypes are O (70%), Asia 1 (25%) and A (4.67%) (Anjum *et al.*, 2006). Another study reported prevalence of FMD sero-type O in 2010 was 61% (Saeed *et al.*, 2011). This trend has continued in 2010 (Jamal *et al.*, 2011). Type C was reported for the first time in 1954 and for the last time in 1995. Phylogenetic analysis of FMD virus serotype O strain, isolated between the years 1997 and 2009, identified three different lineages within the ME-SA (Middle East–South Asia) topotype, namely Pak98, Iran2001 and PanAsia, the latter being predominant. Distinct variants such as PanAsia-II and PanAsia-III are also co-circulating (Jamal *et al.*, 2011). A recent study has shown that the majority of serotype O isolates belong to the PanAsia-2 lineage, whereas serotype A virus isolates belong to the Asia topotype. Pakistan's isolates of serotype O were very much similar

genetically to the virus circulating in neighboring countries (Sri Lanka, India, Iran, Iraq and the People's Republic of China) and belong to PanAsia 1 lineage (Jamal *et al.*, 2011). Sero-type O detected in Punjab province during 2006 and 2007 was genetically much similar on the basis of sequence of VP1 gene to neighboring countries (Sri Lanka, India, Iran, Iraq and China) and it belonged to the Pan-Asia I lineage which caused outbreaks in UK during 2001 and in Saudi Arabia during 1994 (Saeed *et al.*, 2011). Phylogenetic analysis of serotype Asia 1 isolates of 1998–2009 revealed the presence of three different genetic groups circulating in Pakistan, namely group II and VI and a novel group VII. Complete genome sequences of Pakistan serotypes Asia 1 and A isolates revealed inter-serotypic recombination with VP2-VP3-VP1-2A coding sequences derived from a group VII Asia 1 virus and the remainder of the genome from a serotype A virus of the A-Iran05 (AFG-07) sub-lineage.

Therefore the present study was conducted to observe the circulation pattern of the FMDV during the year 2012-13 in Sindh, Punjab and Islamabad. Furthermore, the aim of the study was to identify circulating FMDV sero-types and subtypes which will help to predict and detect the prevailing circulating FMDV strains and in devising an effective control strategy against FMD in Pakistan.

Materials and methods

Outbreak Investigations

A total of 86 outbreaks were investigated in Islamabad Capital Territory, Punjab and Sindh provinces during the year 2012 and 2013. Of 86 outbreaks 23 were reported in 2012 and 63 in 2013. Epithelial samples were collected from 16 districts of the country. In Punjab the samples were collected from from 12 districts namely Bhakar, Chakwal, Chechawatni, Dera Ghazi Khan, Dera Nawab, Faisalabad, Jhang, Lahore, Layyah, Rajanpur, Sargodha and Shorkot. In Sindh province, samples were collected from 3 districts namely Karachi, Larkana and Nawab Shah. The samples were also collected form district Islamabad.

Epithelial tissue was collected from an un-ruptured or recently ruptured vesicle, usually from the tongue and buccal mucosa. Epithelial samples were placed in a transport medium (composed of equal amounts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6, preferably with added antibiotics (penicillin [1000 International Units (IU)], neomycin sulphate [100 IU], polymyxin B sulphate [50 IU], mycostatin [100 IU]). Field data/epidemiological information like disease triggering factors, type of vaccine, husbandry practices, clinical signs, mortality and morbidity were recorded on a purposely designed pro-forma. One outbreak was considered as one epidemiological unit. All the collected samples were properly labelled and transported to Animal Health Laboratories (AHL), Animal Sciences Institute (ASI), National Agricultural Research Center (NARC), Islamabad, under cold conditions and stored at -70°C.

Analysis of samples

Epithelial tissue (1gm) was drawn from the original sample and washed twice with Phosphate Buffered Saline (PBS). The sample was triturated in 1ml PBS using sterilized pestle and mortar. The suspension was collected in 2ml Eppendorf tube and centrifuged at 2000g for 10 minutes. The clear supernatant was collected in a fresh Eppendorf tube and was used for ELISA and virus isolation. An indirect sandwich ELISA kit (BDSL, UK) was used for the identification of FMDV sero-types (Ferris and Dawson, 1988; Roeder and Le Blanc Smith, 1987) in epithelial preparations.

Isolation, propagation and confirmation of FMDV isolates

Low passage fetal porcine kidney cell line (LFBK) maintained at virology section of AHL, ASI, NARC, Islamabad was used for the recovery of FMDV isolates. The supernatant collected in epithelium processing was used to infect the cell lines. The presence of FMDV was indicated by the presence of specific CPE such as swelling, rounding, detachment and lysis of cells. The virus was further confirmed using indirect sandwich ELISA and RT-PCR (Alexandersen *et al.*, 2002; Murphy *et al.*, 1999; Zhang and Kitching, 2001).The flasks showing no CPE after third passage on LFBK were declared as negative.

FMDV-specific RNA was extracted from FMDV isolates using a QIAamp Viral RNA Mini kit (Qiagen, Dusseldorf, Germany) following the manufacturer's instructions. The controls (positive and negative) were included in each run. The FMDV were detected from the extracted RNA using standard real time Polymerase Chain Reaction (rRT-PCR) protocols as described by Callahan *et al.* (2002). The core reagents kit (Taq Man®, EZ-RT-PCR CORE REAGENT, N808-0236) was used for rRT-PCR. Briefly, master mix was prepared as, 5µl of TaqMan EZ Buffer, 2.5µl of 25mM $Mn(Oac)_2$, 3µl of dNTPs, 0.1µl (25pm of forward primer, 0.1µl (25pm) of reverse primer, 0.25µl (25pm) of probe, 1µl of rTth DNA Polymerase and 10.5µl of Rnase-free water. Master mix (22.5µl) was added to each well of reaction plate (Micro Amp ™, N801-0560) accordingly. Template RNA (2.5µl) was added to each well to make the final volume of reaction mixture up to 25µl. The plate was then covered with adhesive film (Micro Amp ™ Adhesive film) and spun in refrigerated plate centrifuge at 2500rpm for 1 minute to remove air bubbles.

The reaction plate was loaded on ABI 7500 real time PCR system (Applied BioSystem) using ABI Prism SDS 7500 software. The thermal profile used for rRT-PCR reaction was adjusted. Briefly, the thermal reaction was started initially 60°C for 10 minutes followed by 95°C for 2 seconds and 60°C for 60 seconds and 45 cycles. At the end CT values were analyzed.

Sequencing and Phylogenetic Analysis

The first passage of the cell culture of the field samples that were determined to contain FMDV RNA by rRT-PCR were sequenced following Ludi *et al.* (2016) and de Carvalho Ferreira *et al.* (2017). The PCR products were generated using SuperScript®III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen) and amplification primers designed to amplify the VP1 region of FMDV isolates. The products were visualized by electrophoresis, purified, sequenced, and analyzed following Pauszek *et al.* (2011). Sequenced viruses were queried using BLAST tool (www.ncbi.nlm.nih.gov/blast). Few of the sequences available in GenBank with the highest identity to the study sequences from neighboring countries were included in the phylogenetic reconstruction. Viral classification was carried out using prototype viruses following Knowles *et al.* (2016). Sequences of sero-type O, A, and Asia-1 were aligned using MEGA 6 (Edgar, 2004). The phylogenetic tree was inferred using the Maximum Likelihood method for sero-type O, A, and Asia-1 separately. The analysis was carried out in MEGA7 (Kumar *et al.*, 2016).

Comparison of ELISA and virus isolation

The diagnostic sensitivity and specificity of ELISA was compared with virus isolation assuming later as gold standard. The diagnostic sensitivity represents the probability of identifying true positive samples using the given diagnostic test. The diagnostic specificity on the other hand, is the proportion of truly negative samples that are identified correctly by the diagnostic test (Thrusfield, 2005). This represents the probability of identifying the true negative samples by the diagnostic test.

Results

A total of 86 outbreaks were investigated at Islamabad Capital Territory, Punjab and Sindh provinces during the year 2012 and 2013. Of 86 outbreaks 23 were reported during the year 2012 while the rest 63 were reported during the year 2013. During 2012, of 23 FMD outbreaks 21 were reported from cattle and 2 were reported from buffaloes. During 2013, of 63 FMD outbreaks 40 were reported from cattle and 23 were reported from buffaloes. The data of FMD outbreaks is presented in table 1.

Table 1. FMDV outbreaks confirmed and virus recovery from cattle and buffaloes during 2012-13.

Year	Animal Species	No. of outbreaks	Indirect Sandwich ELISA				FMDV Isolations			
			A	O	Asia-1	Total	A	O	Asia-1	Total
2012	Buffalo	2				0			2	2
	Cattle	21	6	5	7	18	8	6	6	20
2013	Buffalo	23		3	6	9	1	3	3	7
	Cattle	40	2	9	6	17	2	5	3	10
Total		86	8	17	19	44		11	14	14

During the year 2012 and 2013, 95.7% (22/33) and 38.6% (17/44) FMDV were recovered, respectively. During the year 2012, 100% (2/2) and 95.2% (20/21) FMDV were recovered from cattle and buffaloes, respectively. Whereas, during the year 2013, 30.4% (7/23) and 25% (10/40) were recovered from cattle and buffaloes, respectively. No virus were recovered from 47 samples. The data is shown in table 1. Highest number FMDV 15.4% (6/30) were recovered from Karachi, followed by 12.8% (5/39) from Islamabad, 10.3% (4/39) from Faisalabad, 7.7% (3/39) from Chechawatni, Jahng, Lahore, Layyah, Sargodha, 5.1% (2/39) from Chakwal and Nawab Shah, and 2.6% (1/39) from Bhakkar, Dera Nawab, Larkana, Rajanpur and Shorkot. The detail of FMDV sero-types isolated from 16 districts is presented in table 2.

Table 2. Summary of the recovered FMDV from various locations, their sero-types from cattle and buffalo during the year 2012-2013.

Location	Cattle						Buffalo						Grand Total		
	2012			2013			2012			2013					
	O	A	Asia-1	O	A	Asia-1	O	A	Asia-1	O	A	Asia-1	O	A	Asia-1
Bhakar		1												1	
Chakwal		1	1											1	1
Chechawatni		1		1								1	1	1	1
Dera Ghazi Khan															
DeraNawab				1									1		
Faisalabad		1	1	1		1							1	1	2
Islamabad	1	2							2				1	2	2
Jhang	1		1							1			2		1
Karachi			3			1				1		1	1		5
Lahore	1			1								1	2		1
Larkana					1									1	
Layyah	1	1									1		1	2	
Nawab Shah	2												2		
Rajanpur					1									1	
Sargodha				1		1				1			2		1
Shorkot		1												1	
Total	6	8	6	5	2	3			2	3	1	3	14	11	14

Overall ELISA detected 51.2% in FMD outbreaks (44/86). ELISA detected 57.4% (35/61) and 36% (9/25) FMD outbreaks in cattle and buffalo. During the year 2012, a total of 78.3% outbreaks (18/23) were confirmed by ELISA and during the year 2013 a total of 41.3% outbreaks (26/63) were confirmed by ELISA. A total of 48.8% (42/86) outbreaks were not confirmed by ELISA. The data is shown in table 3. A total of 39 FMDV (45.4%) which were isolated from 86 FMD outbreaks include sero-type O (35.9%), A (28.2%) and Asia-1 (35.9%) during the year 2012-2013. FMDV recovery from cattle and buffaloes was 49.2% (30/61) and 36% (9/25), respectively. The virus recovery data is presented in table 1. The phylogenetic analysis of the FMDV sero-type O, A and Asia-1 is shown in Fig. 1, 2 & 3, respectively.

Table 3. ELISA diagnostic sensitivity & specificity compared to virus isolation.

Test		Virus Isolation (86)							
Status		Positive (39, 45.35%)				Negative (47, 54.65%)			
			A	O	Asia-1	TOTAL	A	O	Asia-1	TOTAL
ELISA (86)	Positive (44, 51.16%)	A	8							
		O		9				8		
		Asia-1			7				12	
		TOTAL				24				20
	Negative (42, 48.84%)	A	3							
		O		5						
		Asia-1			7					
		TOTAL				15				27

The diagnostic sensitivity and specificity of ELISA was compared against virus isolation. The diagnostic sensitivity of ELISA was found to be 61.54% (95% CI; 44.62% to 76.64%) and specificity was 57.45 % (95% CI; 42.18% to 71.74%. The Positive and negative Likelihood Ratio were 1.45 (95% CI; 0.96 to 2.19) and 0.67 (95% CI; 0.42 to 1.07). Both tests showed weak agreement (Kappa= 0.188). The data is presented in table 3.

The spatio-temporal distribution and amino-acid variation among FMDV sero-type O, A and Asia-1 is given in table 4, 5 and 6, respectively.

Table 4. Spatio-temporal distribution and variation among FMDV sero-type O.

FMDV Sro-type O												
				Amino acid change and position								
District	Year	animal specie	FMDV Isolates	58/211	96/211	125/211	133/211	135/211	140/211	174/211	195/211	197/211	198/211
Islamabad	2012	Cattle	FMDV_O/ISB-533/Pak_2012	A	I	V	N	R	H	S	H	D	G
Jhang			FMDV_O/JHG12/13/Pak_2012	A	I	V	N	R	H	S	H	D	G
lahore			FMDV_O/LHR12/19/Pak_2012	A	T	V	N	K	S	N	Q	N	E
Layyah			FMDV_O/LAY12/8/Pak_2012	A	T	A	N	K	S	N	Q	N	G
Nawab Shah			FMDV_O/NWB12/11/Pak_2012	A	T	V	H	K	S	N	Q	N	E
Nawab Shah			FMDV_O/NWB12/20/Pak_2012	A	T	V	N	K	S	N	Q	N	E
Chechawatni	2013		FMDV_O/CHE13/48/Pak_2013	A	T	V	H	K	S	N	Q	N	E
Dera Nawab			FMDV_O/DNB13/4/Pak_2013	A	T	V	N	K	S	N	Q	N	E
Faisalabad			FMDV_O/FSD13/29/Pak_2013	A	T	V	N	K	S	N	Q	N	E
Lahore			FMDV_O/LHR13/3/Pak_2013	A	T	V	N	K	S	N	Q	N	E
Sargodha			FMDV_O/SGD13/35/Pak_2013	V	T	V	N	K	S	N	Q	N	E
Jhang		Buffalo	FMDV_O/JHG13/2/Pak_/2013	A	T	V	N	K	S	N	Q	N	E
Karachi			FMDV_O/KHI13/47/Pak_2013	V	T	V	N	K	S	N	Q	N	E
Sargodha			FMDV_O/SGD13/23/Pak_2013	V	T	V	N	K	S	N	Q	N	E

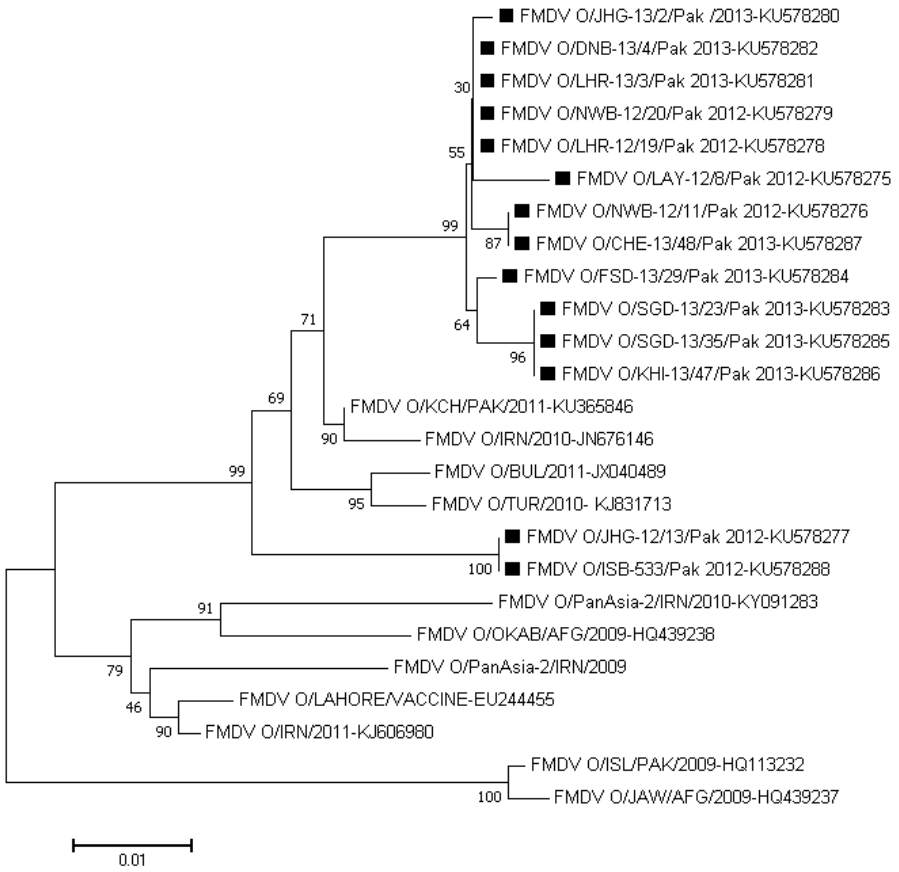

Fig. 1. Phylogenetic analysis of FMDV sero-type O.

Table 5. Spatio-temporal distribution and variation among FMDV sero-type A.

FMDV Sro-type A											
District	Year	animal specie	FMDV Isolates	Amino acid change and position							
				24/211	35/211	45/211	92/211	109/211	140/211	160/211	168/211
Bhakkar	2012	Cattle	FMDV_A//BHK-12/7/Pak-2012	A	I	V	V	K	S	G	K
Chakwal			FMDV_A/CHK-12/5/Pak-2012	T	I	A	E	R	G	S	R
Chechawatni			FMDV_A/CHE-12/16/Pak-2012	A	V	V	V	K	S	G	K
Faisalabad			FMDV_A/FSD-12/17/Pak-2012	A	V	V	V	K	S	G	K
Islamabad			FMDV_A/ISB-12/9/Pak-2012	T	I	A	E	R	G	S	R
Islamabad			FMDV_A/ISB-12/12/Pak-2012	A	V	V	V	K	S	G	K
Layyah			FMDV_A/LAY-12/4/Pak-2012	A	V	V	V	K	S	G	K
Shorkot			FMDV_A/SHO-12/14/Pak-2012	T	I	A	E	R	G	S	R
larkana	2013		FMDV_A/LAR-13/52/Pak-2013	A	V	V	V	K	S	G	K
Rajanpur			FMDV_A/RAJ-13/43/Pak-2013	A	V	V	V	K	S	G	K
Layyah		Buffalo	FMDV_A/LAY-13/27/Pak-2013	A	V	V	V	K	S	G	K

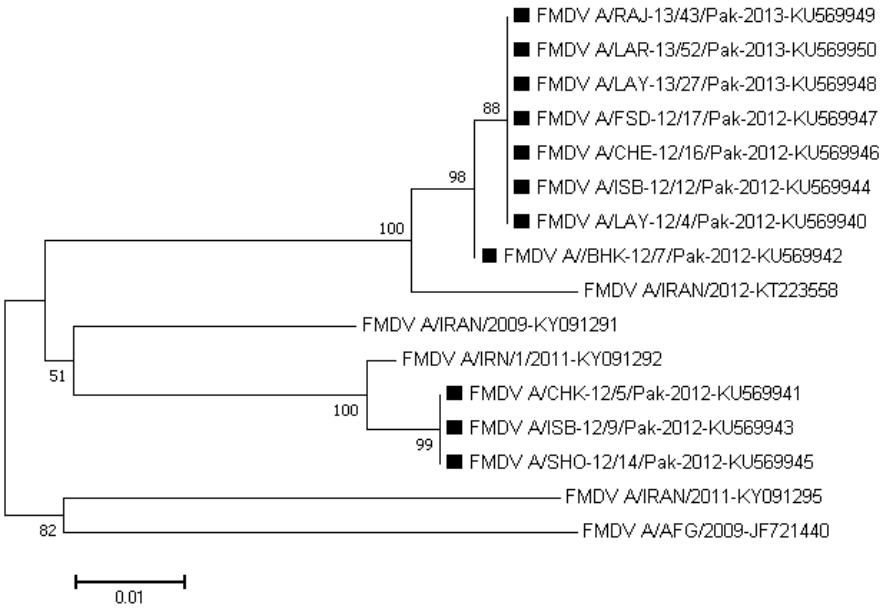

Fig. 2. Phylogenetic analysis of FMDV sero-type A.

Table 6. Spatio-temporal distribution & variation among FMDV sero-type Asia-1.

FMDV Sro-type ASIA-1																					
District	Year	Animal	FMDV Isolates	Amino acid change and position																	
				4/209	11/209	24/209	28/209	42/209	44/209	47/209	55/209	59/209	83/209	138/209	140/209	153/209	156/209	169/209	192/209
Faisalabad	2012	Cattle	FMDV_Asia-1/FSD-12/1/Pak-2012	V	V	A	L	L	N	A	Q	Y	T	P	A	V	S	E	L
Chakwal			FMDV_Asia-1/CHK-12/3/Pak-2012	T	V	T	L	F	N	A	Q	H	T	P	A	A	S	D	L
Jhang			FMDV_Asia-1/JHG-12/15/Pak-2012	T	V	T	L	F	N	A	Q	H	T	P	A	A	S	D	L
Karachi			FMDV_Asia-1/KHI-12/2/Pak-2012	V	V	A	F	L	N	A	Q	H	A	Q	T	V	S	D	L
Karachi			FMDV_Asia-1/KHI-12/6/Pak-2012	V	V	A	F	L	N	A	Q	H	A	Q	T	V	S	D	L
Karachi			FMDV_Asia-1/KHI-12/10/Pak-2012	V	V	A	F	L	N	A	Q	H	A	Q	T	V	S	D	L
Islamabad		Buffalo	FMDV_Asia-1/ISB-12/21/Pak-2012	T	V	T	L	F	N	A	Q	H	T	P	A	A	S	D	L
Islamabad			FMDV_Asia-1/ISB-591/Pak-2012	V	T	A	L	L	N	A	Q	H	T	Q	A	V	S	D	L
Faisalabad	2013	Cattle	FMDV_Asia-1/FSD-13/34/Pak-2013	A	V	A	L	L	S	T	Q	H	T	P	A	V	S	E	L
Karachi			FMDV_Asia-1/KHI/13/53/Pak-2013	V	V	A	L	L	N	A	R	H	T	Q	T	V	N	D	L
Sargodha			FMDV_Asia-1/SGD-13/24/Pak-2013	A	V	A	L	L	S	A	Q	H	T	P	A	V	S	E	L
Chechawatni		Buffalo	FMDV_Asia-1/CHE-13/49/Pak-2013	A	V	A	L	L	S	T	Q	H	T	P	A	V	S	E	L
Karachi			FMDV_Asia-1/KHI-13/62/Pak-2013	V	V	A	L	L	N	A	R	H	T	Q	T	V	N	D	F
lahore			FMDV_Asia-1/LHR-13/1/Pak-2013	V	V	A	L	L	N	A	Q	H	T	Q	T	V	N	D	L

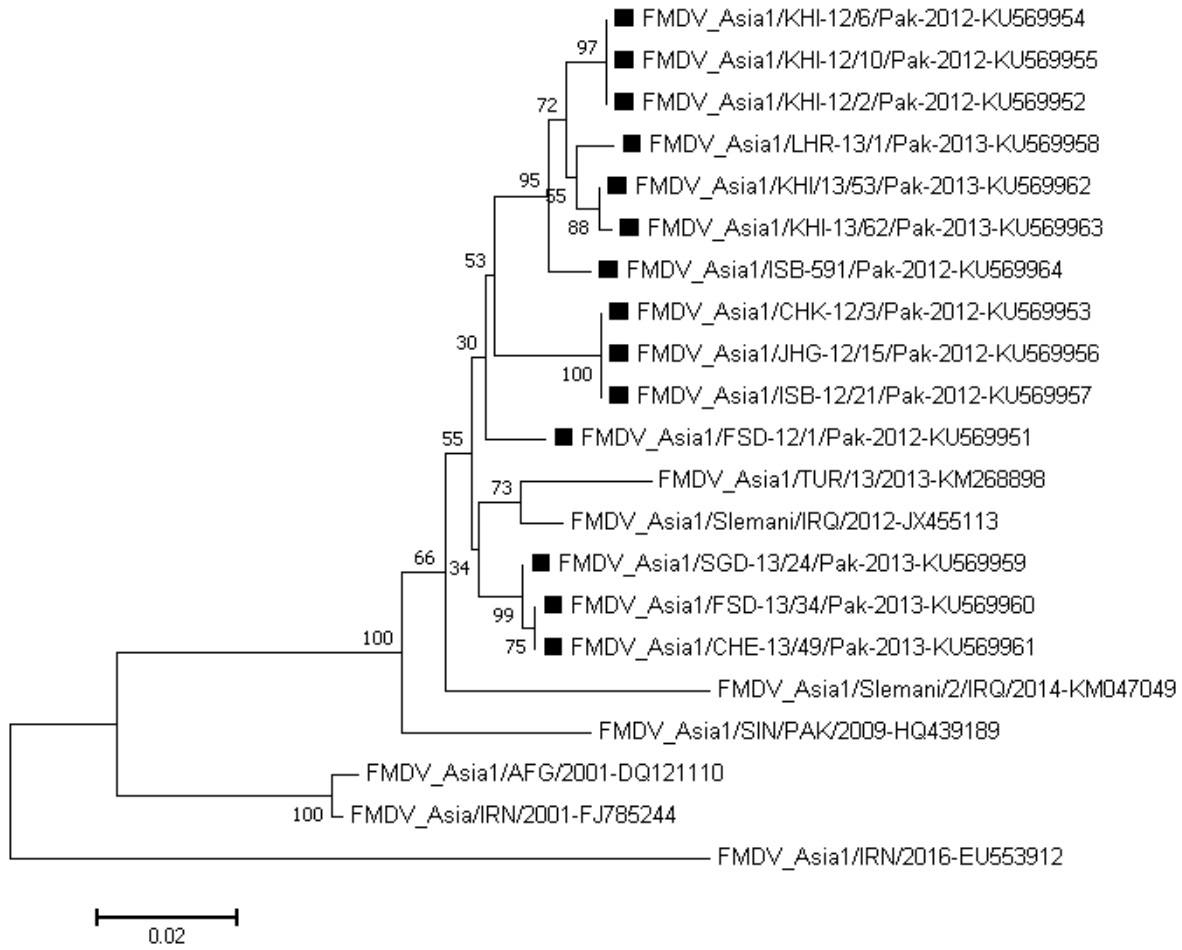

Fig. 3. Phylogenetic analysis of FMDV sero-type Asia-1.

Discussion

During the present study, it was observed that the FMDV sero-type O (35.9%), A (28.2) and Asia-1 (35.9%) continues to be isolated from the clinically infected buffaloes and cattle during the year 2012 and 2013. The sero-type Asia-1 and O were most commonly present in these outbreaks. Circulation of FMDV sero-types O, A and Asia-1 have been reported earlier in the country (Jamal *et al.*, 2010). However, in earlier studies most of the FMD sero-type O outbreaks were reported in Pakistan. A previous study showing FMDV outbreak data from 1952-2007 indicated high prevalence of sero-type O (61%) compared to other FMDV sero-type A (14%) and Asia-1 (24%) (Jamal *et al.* 2010). This difference in prevalence of FMDV sero-types in the clinical samples might be due to shift of circulation pattern of various sero-types.

The occurrence of sero-types O, A and C has long been established in this region. In an earlier study conducted from 1943-47, a total of 42 FMDV isolates were typed from disease outbreaks in various parts of pre-partition Indo-Pakistan using cross protection tests in guinea pigs against the known standard sero-types i.e. O, A and C. Of these, 54.7% (23/42), 26.2% (11/42) and 16.7% (7/42) were sero-typed as O, A and C, respectively. Only 2.4% (1/42) FMDV isolate which could not be sero-typed was atypical isolate. Retrospective testing of this atypical isolate from Rawalpindi (now part of present-day Pakistan) identified was sero-type Asia-1 (Yasin and Huq, 1960). Although Asia-1 was first detected by WRL-FMD in 1954 from a sample which originated from Pakistan (Brooksby and Rogers, 1957), the atypical strain responsible for FMD outbreaks during 1943-1947 is the earliest documented Asia-1 virus isolate.

This shows that sero-type Asia-1 was circulating in Indo-Pakistan subcontinent well before 1954. Valarcher *et al.* (2008) reported the endemic nature of sero-type Asia-1 in this region and its spread to neighboring countries. It was speculated that the distribution of this sero-type within Asian continent is related to the presence of Asian water buffaloes. The sero-type Asia-1 appeared to move in the early 1970s from Pakistan through Afghanistan and Iran to Iraq and Turkey (Firoozi Bandpay *et al.,* 1974). All the FMDV sero-type O isolates during the present study were within the PanAsia2 lineage, already reported in the region (Brito *et al.*, 2017). The sub lineage identified were PanAsia2 $^{ANT-10}$ & PanAsia2 $^{BAL-09}$.

FMDV sero-type A characterized during this study were grouped within the lineage A/Asia/Iran-05, which are endemic and widespread in the region. This sero-type is closely related to the viruses circulating in Iran during 2009, 2011 & 2012 and to Afghanistan during 2009. The sub lineage identified within FMDV Iran-05 belonged to HER-10, FAR-11 & ESF-10. FMDV sero-type Asia-1 isolates in this study were classified in group VII (Sindh-08), which were closely related to viruses circulating in Turkey during 2013, Iraq during 2012 & 2014, Iran during 2001 & 2016 and Afghanistan during 2001.

In present study, LFBK cell line was used for FMDV recovery. Bovines, ovine and porcine derived cell cultures could also be used this purpose. However, the greatest recovery of virus is on primary bovine thyroid cells. Various other cell lines can also be used for FMDV recovery namely IBR-2, MVPK-1 clone, LFBK, BHK-21 and LFBKαVβ6. However, these are less efficient compared to primary bovine thyroid cells. Furthermore, LaRocco *et al.* (2013) reported that LFBK cell line is immortalized line of fetal porcine kidney (LFBK) cells that had high susceptibility to most FMDV sero-types and can be maintained over many passages. In comparison to bovine thyroid cells, LFBK cells had similar susceptibilities to most FMDV sero-types and had equal or better susceptibility than MVPK, IB-RS-2, and fetal bovine kidney cells.

The ELISA used in the present study has an advantage over CFT in term of detecting more positive number which can be further confirmed by inoculating into sensitive cell cultures. Longjam *et al.* (2011) described that the recovered virus can be further confirmed by the ELISA, which require time in days. The rapid diagnostic is very important in disease control strategy. Therefore, there is need of an alternative assay which allows more rapid confirmation of clinical diagnosis with more sensitivity and this has resulted in development of polymerase chain reaction (PCR) or the more recent real-time PCR.

Pakistan has recently launched a FMD control programme and the vaccination is carried only in case of outbreaks. The prediction of circulating strains is very much important for successful vaccine in the field.

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