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A Reverse transcription-polymerase chain reaction (RT-PCR) based detection of foot and mouth disease in District Faisalabad, Pakistan during the Year 2016

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ABSTRACT

Foot and mouth disease is an economically devastating disease of livestock that mainly effect cloven-hoofed animals i.e. sheep, goat, cattle, pig, buffalo, deer etc. The aim of this study was to determine the serotypes circulating in the region during 2016. Sampling was done from different outbreaks initially on the basis of clinical signs and later reverse transcriptase-polymerase chain reaction (RT-PCR) was employed for the confirmation of FMDV genome. Out of total 72 samples, 65 were found positive which were then serotyped into type O (n=30), Asia1 (n=19) and A (n=5). Some samples (n=5) were found positive for more than one serotype that were subjected to reverse transcriptase loop-mediated isothermal amplification assay (RT-LAMP) for serotype determination.

1. Introduction

Foot-and-mouth disease (FMD), a major disease of cloven-footed animals is prevalent in Pakistan where three virus serotypes are present (O, A and Asia1). A single-stranded, positive-sense RNA virus is responsible for this disease, which belongs to the Picornaviridae family with genus Aphthovirus, referred as FMD virus (FMDV).

The FMDV genome (8400 nucleotides) includes a single large open reading frame (ORF), which is approx. 7 000 nucleotides long. It encodes a polyprotein that is processed into the leader (L) protein, four structural proteins: VP4 (1A), VP2 (1B), VP3 (1C) and VP1 (1D), plus the 2A peptide, as well as non-structural proteins from the P2 (2B and 2C) and P3 (3A, 3B1-3,3C and 3D) precursors [1-4]. Seven serotypes referred to as A, O, C, SAT 1, SAT 2, SAT 3 and Asia 1, have been described based on the antigenic characteristics of the virus [5]. Serotypes O, A and Asia 1 are prevalent among the ruminant population of Pakistan [6-9]. FMDV antigenic variation has been ascribed to amino acid substitutions in major antigenic sites located on exposed regions of the capsid proteins VP1, VP2 and VP3 [10-12].

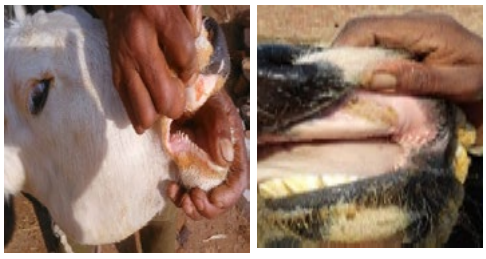

Figure.1. Showing lesions on the hoof and mouth epithelium during a FMD outbreak in District Faisalabad.

Livestock population in Pakistan comprises of 31.8, 29, 27.1, and 56.7 million cattle, buffalo, sheep and goats respectively, the majority reside in Punjab Province, i.e. >43% of the cattle and >61% of the water buffalo [13]. FMD is endemic in Pakistan and epidemics frequently arise causing heavy economic losses in cattle and water buffalo [14]. The severity and prevalence of disease in animal populations in Pakistan is high because of the importation of exotic breeds and cross-breeding of local breeds [13]. Previously, FMD was a seasonal problem, but now the disease remains in the field in a mild or acute form in some parts of Pakistan throughout the year [13]. The direct and indirect losses because of FMD in Pakistan are not accurately defined in detail, but in 2003 approximate losses were US$18 million [15]. Reported prevalence rates for the three FMD serotypes in Pakistan are 70% for type O, 25% for type Asia 1 and 4.7% for type A [15]. At present, vaccination is the only way to control the disease in Pakistan [16], as preformed policy is not reasonable because of the inability to control animal movements within the country and across the lengthy land borders with neighbouring FMD-infected countries. The vaccines available in Pakistan derive either from Veterinary Research Institutes at Lahore, Peshawar and Quetta (monovalent FMDV O) or are imported from abroad (trivalent FMDV O, A and Asia 1). The vaccine production capacity of the public sector institutes can satisfy only about 10% of the livestock population of Pakistan.

However, largely small-scale farmers in Pakistan do not vaccinate their livestock because of the costs involved. As the public sector formalin-inactivated vaccines are less expensive than those that are imported, these are often used in preference. The animals in the areas under study were mostly unvaccinated or if vaccinated, then only against type O. There is also no regional FMD control programme to coordinate actions amongst neighbouring countries.

2.MATERIALS AND METHODS

2.2. Sample collection

72 samples were gathered from the 28 outbreaks (Fig. 4) during this study from district Faisalabad and transferred to the lab in 15ml capacity autoclaved plastic vials containing 10 ml glycerolized buffer saline, where they were stored at -70C until further processing. Detail about the collected samples is given in the table no. 2 along with respective administrative areas.

2.3. RNA extraction

Pavor Prep Viral Nucleic Acid Extraction Kit (Favorgen, China) was used in RNA extraction according to Manufacturer's protocol. Briefly, 570µl of lysis buffer was added to 150 µl of sample (serum/epithelial tissue supernatant) and incubated for 10 minutes at room temperature. Then ethanol (750 µl) precipitation is carried out after which RNA is transferred to silica based column and centrifuged at 8000g for 1 min. One washing with 500 µl of wash buffer1 and two with wash buffer2 was carried out on 8000g for 1min after every washing. After washing RNA was eluted using 35µl elution buffer in a centrifuge tube for 2 min at 8000g and stored at -70º C until further use.

2.4. Complementary DNA Synthesis (cDNA):

Fermentas Revert Aid First Strand cDNA Kit was used for cDNA preparation. Briefly, 5µl RNA template was incubated with 1µl of random hexamer primer (0.2 ug/µl) and 6µl nuclease-free water at 65º C for 5 minutes than ice chilled and short spin. After that, 5X reaction buffer 4µl, Ribolock 1µl, dNTPs (10mM) 2 µl, revert Aid (reverse transcriptase) 1 µl were added into it and incubated in PCR machine with 25º C for 5 minutes, 42º C for 60 minutes and 70º C for minutes to terminate the reaction. After synthesis, cDNA was stored at -20 C for future use.

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2.5. PCR

DNA was subjected to the general FMD detection primers (1F , 1R) as well as typing primers using following procedure: 1 µl cDNA , 1 µl Forward primer (1F) (10 pM/µl), 1 µl Reverse primer (1R) (10 pM/µl) ,5µl 10X Taq polymerase buffer , 5µl MgCl2 (25mM),1µl 4X dNTP mix (10 mM each), 0.2 µl Taq plus DNA polymerase (5U/µl) and 35 µl Double Deionized water (ddH2O) in a micro PCR tube. Thermal cycling conditions were 94º C for 10 min, followed by 35 cycles at 95º C for 45 s, 55º C for 45 s and 72º C for 45 s. A final extension was carried out at 72º C for 10 min.

Table 1: The oligonucleotide primers used for RT-PCR diagnosis and serotyping

Primer *	Sequence 5' → 3'	Location	Sense	Specificity
1F	GCCTGGTCTTTCCAGGTCT	5'UTR	Forward	All types
1R	CCAGTCCCCTTCTCAGATC	5'UTR	Reverse	All types
P33	AGCTTGTACCAGGGTTTGGC	2B	Reverse	All types
P38	GCTGCCTACCTCCTTCAA	1D	Forward	Type O
P74	GACACCACTCAGGACCGCCG	1D	Forward	Type O
P75	GACACCACCCAGGACCGCCG	1D	Forward	Type O
P90	GTCATTGACCTCATGCAGACTCAC	1D	Forward	Type A
P91	GTCATTGACCTCATGCAAACCCAC	1D	Forward	Type A
P92	GTCATTGACCTTATGCAGACTCAC	1D	Forward	Type A
P76	GACACCACACAAGACCGCCG	1D	Forward	Type Asia1
P77	GACACGACTCAGAACCGCCG	1D	Forward	Type Asia1
P87	GTCATTGACCTCATGCAGACCCAC	1D	Forward	Type Asia1
P88	GTTATTGACCTCATGCAGACCCAC	1D	Forward	Type Asia1
P89	GTCATTGACCTCATGCACACCCAC	1D	Forward	Type Asia1

* Primers were adapted from Vangrysperre and De Clercq, 1996; Reid *et al.*, 2000.

Results

For general detection of FMDV genome, consensus primer pair 1F and 1R were used while for serotype detection primers mentioned in the table No. 1 were used. 72 samples were subjected to these primers collected from different outbreak regions of District Faisalabad. Five samples were found positive for serotype A, 19 for serotype Asia 1 and 30 for serotype O. Five samples were found positive for more than one serotype that were than subjected to RT-LAMP to determine their lineage using previously established protocols [17].

Bands of 328 bp with consensus primer pair and of 292, 432 and 702 bp (fig. 2) for serotype O, Asia1 and A respectively with typing primers were achieved when electrophoresed by 1.5% agarose gel (Invitrogen, Carlsbad, USA), prepared in 1×Tris–borate–EDTA buffer and stained with 0.5 g/ml ethidium bromide. The PCR products were visualized using a transilluminator at 302 nm. Out of total 72 cases detected positive for FMD based on clinical signs in the field, 65 were found positive in the laboratory. This indicates an accurate clinical diagnosis by field veterinarians in 90% cases, which shows sufficient skills of field staff of Livestock Department of District, Faisalabad may be due to the prominent signs of the disesase. We were able to type 54 (83%) samples using previously reported typing primers.

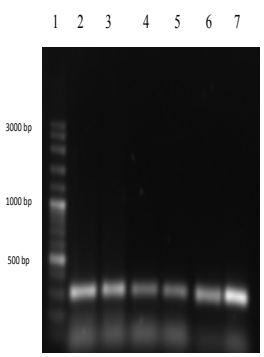

Figure 2. Lane 1 depicts marker (GeneRuler™ 100 bp Plus DNA Ladder). Lane 2, 3, 4, 5, 6 and 7 shows the PCR product (328 bp) with consensus primer.

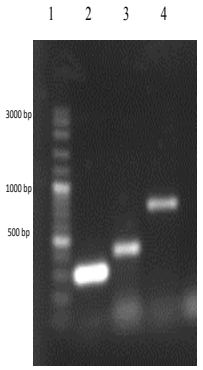

Figure 3. Lane 1 depicts marker (GeneRuler™ 100 bp Plus DNA Ladder), while lane 2, 3, and 4 shows the PCR products of type Asia1 (292 bp), type O (402 bp) and type A (732 bp) respectively with type specific primers.

Table 2. FMDV outbreak locations and results of Faisalabad district

Administrative regions	No. of outbreaks	No. of samples	FMDV detected	FMDV typed		
				A	Asia1	O
Chak Jhumra	04	10	09	-	01	06
Jaranwala	02	07	05	02	-	02
Sadhar	16	26	24	03	10	05
Sumandri	04	09	08	-	03	04
Tandlianwala	02	20	19		05	13
Total	28	72	65	05 (10%)	19 (35%)	30 (55%)

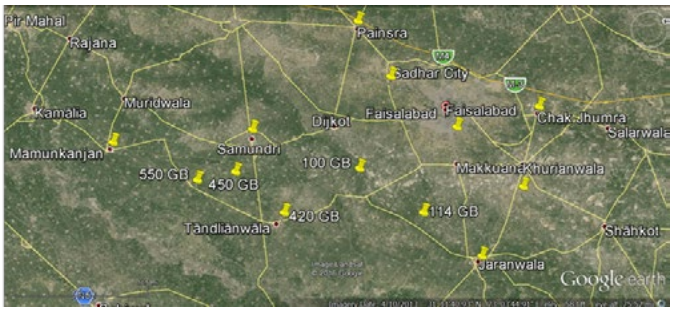

Figure 4. FMD outbreak location attended during this study are shown with place mark

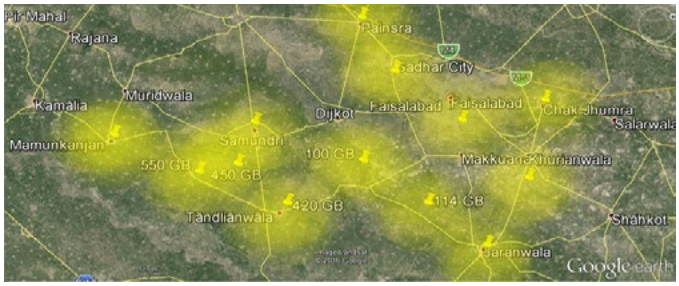

Figure 5. Yellow circles depicts zones of 10 Km radius that were generated using Google earth software.

Discussion

Among 54 samples that were successfully serotyped, serotype O was found most frequently (55%) in the outbreaks studied area, followed by serotype Asia 1 (35%) and then serotype A (10%). Nine samples were not successfully serotyped which were found positive with consensus primers may be due to the low concentration of the genomic RNA in the samples. Moreover, primer specificity may be a reason and regional primers are needed to overcome this issue because virus differs from region to region. Different parts of the world have already designed their own primers to tackle this issue. [18]. Buffer zones for FMD ring vaccination are kept of 5-10 km radius [19] because FMDV spread in an outbreak scenario is predicted to cover a distance of 10 km [20]. This finding was used to predict the zones of 10 km radius (Fig. 4) in Faisalabad district that illustrate that zones are intermingling with one another and it is very difficult to distinguish their boundaries indicating that almost whole study area is in the reach of FMDV because of these reported outbreaks.

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