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RESEARCH ARTICLE

Investigations of Foot-and-Mouth Disease Outbreaks in Faisalabad District of Punjab, Pakistan during the Year 2013

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ABSTRACT

Sampling was done from various outbreaks of foot-and-mouth disease (FMD) that occurred during the year 2013 in district Faisalabad, Punjab, Pakistan. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used for typing of foot-and-mouth disease virus (FMDV). A total of 110 clinical samples were received from different administrative regions of Faisalabad; 86 were found FMDV genome positive when tested with 1F and 1R consensus primers. Out of these genome positive samples, 73 were successfully typed into serotypes A (19 samples), O (30 samples) and Asia1 (24 samples). Complementary DNAs (cDNAs) were stored at -80˚C for further sequencing studies. Serotype A FMDV was detected from samples received from outbreaks that occurred in Sumandri, Jaranwala and Tandlianwala tehsils, serotype O from Tandlianwala and Sadhar, and serotype Asia 1 outbreaks were reported from Chak Jhumra and Sadhar.

INTRODUCTION

Foot-and-mouth disease (FMD) is an acute and one of the highly contagious diseases of domestic animals, mostly affecting cattle, swine, sheep, goats, and many species of wild ungulates (Longjam et al., 2011). The disease is characterized by vesicle formation in and around the mouth, on the feet, and has a very high morbidity but low mortality rate in adults (Callens and De Clercq, 1997). In young animals the disease can cause severe lesion in the myocardium, leading to high mortality rates (Sharma and Das, 1984). Due to its aggressive nature, FMD causes huge losses in terms of livestock productivity and trade.

According to World Organization for Animal Health (OIE), FMD ranks first among notifiable infectious diseases of animals (OIE, 2000). The disease is difficult to control due to high contagiousness, wide geographical distribution, broad host range, ability to establish carrier status, antigenic diversity leading to poor cross-immunity, and relatively short duration of immunity (Mdetele et al., 2014; Longjam et al., 2011; Garland, 1999). Poor surveillance and diagnostic facilities as well as inadequate control programs are major constraints in control of this disease (Garland, 2003).

The etiological agent, foot-and-mouth disease virus (FMDV) is classified within the genus *Aphthovirus* in the family *Picornaviridae (*Racaniello, 2001). The virus exists in the form of seven serologically and genetically distinguishable types, namely, O, A, C, Asia1, SAT1, SAT2, and SAT3, and a number of subtypes within each serotype (Jamal and Belsham, 2013). The Serotype C is no more reported in the world; the last reported outbreak due to serotype C FMDV was in Ethiopia during 2005 (WRL-FMD, 2006). The serotypes O, A and Asia 1 are responsible for FMD in Pakistan round the year. Among many other milestones the effective control of the disease requires that outbreaks should be detected at an early stage and

persistent infections should be recognized to prevent further transmission.

In present study we employed multi-primer RT-PCR mixes that were used in conjunction with a consensus RT-PCR for the rapid detection of FMDV from the clinical samples received from Faisalabad district during the year 2013.

MATERIALS AND METHODS

Sample collection

Total 200 plastic vials of 15 ml capacity, each containing 8-10 ml glycerolized buffered saline, were distributed among veterinary practitioners in various administrative regions of Faisalabad, Punjab, Pakistan. Field samples were also collected from nearby areas on reporting of the disease by the authors. Following this approach a total of 110 clinical samples were collected/ received from various towns including Chak Jhumra, Sadhar, Jaranwala, Sumandri and Tandlianwala.

RNA extraction

Total RNA was extracted from the samples using GF-1 Viral Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia) according to manufacturer's protocol. Total RNA was extracted in ~ 40 µl of elution buffer.

Reverse transcription/ cDNA synthesis

Complementary DNA (cDNA) was synthesized using Fermentas Revert Aid First Strand cDNA Kit. The RNA (5 µl) from FMD suspected clinical specimens was heated at 65°C for 5 min with 1 µl of a random hexanucleotide mix (0.2µg/µl conc.), chilled on ice and reverse transcribed at 42°C for 1 hr. in a 20 µl reaction mixture containing 5µl of 5X RT buffer, 1µl of Moloney-murine leukemia virus (M-MuLV) reverse transcriptase (200 U), 1µl RNase inhibitor (20U) and 2µl of 10mM dNTP mix.

Synthetic oligonucleotide primers

The oligonucleotide primers for PCR amplification including 1F, 1R, P38, P40, P74, P75, P76, P77, P87, P88, P89, P90, P91, P92 , P33 designed from 5' UTR, 1D and 2B regions of viral genome as described previously (Vangrysperre and De Clercq, 1996; Reid et al., 2000) were got synthesized from Gene Link, USA.

PCR reaction

Two micro litres of freshly synthesized cDNA was added to 48µl of PCR reaction mixture consisting of: 35µl double distilled deionized water; 5µl, 10XPCR buffer; 5µl $MgCl_2$ (25mM), 0.2µl *Taq* DNA polymerase (5U/µl), 1µl dNTP mix (10mM each), 1µl Forward primer (25 p moles/µl), 1µl Reverse primer (25 p moles/µl).

The RT product from each sample was tested with the primer pair 1F/1R for the diagnosis of FMD virus. Forward primers P38, P74-77 and P87-92 were used in a multiplex format (cocktail of primer sets) for the serotype specific diagnosis of types O, Asia1 and A, respectively with the downstream primer P33.

The programme used for diagnosis of FMD viruses with primers 1F/1R was: 94°C for 5 min, 1 cycle; 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, 30 cycles; 72°C for10 min, 1 cycle. The programme used for the evaluation of O, A and Asia 1 serotype specific primers was: 94°C for 5 min, 1 cycle; 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 30 cycles; 72°C For10 min, 1 cycle.

RESULTS

Consensus PCR for general FMDV detection

Total RNA extracted from clinical samples that comprised mouth epithelium (vesicular/necrotic tissue), vesicular epithelium from inter-digital cleft, saliva and sera, were submitted to RT-PCR using the consensus primers 1F/1R. FMDV genome positive samples gave rise to 328 bp amplified DNA products (Fig.1).

Multiplex PCR for O-A-Asia 1 typing

This multiplex PCR has been adapted with modifications from the one published by Vangrysperre and De Clercq (1996). The multiplex mix of primers was prepared by dilution and mixing to 25 p moles/µl of each forward primer and multiple forward primers within each type were mixed in equal concentrations, and finally forward primer mixtures for each O, A and Asia1 serotypes were mixed in equal ratios, while final concentration of this forward primer mix remained 25 p moles/µl. Using multiplex PCR with primer mix P33-P(Asia1-O-A) the DNA products of 732, 402 and 292 bp representing types Asia1 ,O and A respectively were obtained depending upon serotype of virus causing infection (Fig. 2). All the FMDV positive samples that were successfully amplified with 1F/1R primers were subjected to amplification with Asia1-O-A primer mix. Not all the samples which were detected using 1F/1R primers were successfully typed using multiplex typing RT-PCR. Out of 110 clinical samples received, 86 were found FMDV genome positive when tested with 1F and 1R consensus primers and 73 could be typed into serotypes A (19 samples), O (30 samples) and Asia 1 (24 samples). There was no cross reactivity observed within serotypes using this multiplex reaction. Out of 73 typed samples 30 samples were sent to Foot-and-Mouth Disease Research Centre (FMDRC), Lahore for confirmation by indirect sandwich ELISA. All except one were confirmed in agreement with our testing. The one sample that was serotype 'A' in our testing could not be confirmed by indirect sandwich ELISA.

FMDV serotype distribution in Faisalabad district

A total of 42 outbreaks were investigated. Serotype A FMDV was detected from samples received from outbreaks that occurred in Sumandri, Jaranwala and Tandlianwala tehsils, serotype O from Tandlianwala and Sadhar, and Asia 1 outbreaks were reported from Chak Jhumra and Sadhar (Table 2).

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

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

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

Table 2: FMD viruses and serotypes detected from samples received from various administrative units of Faisalabad

Administrative region	No. of outbreaks	No. of samples	FMDV detected	FMDV typed
Chak Jhumra	10	23	17	Asia1(14)
Jaranwala	6	24	19	A (10)
Sadhar	9	35	28	O(17), Asia1(10)
Sumandri	10	10	8	A (8)
Tandlianwala	7	18	14	A(1),O(13)
Total	42	110	86	73

DISCUSSION

Early diagnosis of diseases is very important for control and eradication programs of infectious diseases. There are a number of techniques in practice for quick diagnosis of foot-and-mouth disease and other transboundary animal diseases. Routine diagnosis of FMD is made at several laboratories by the combined use of enzyme-linked immunosorbent assay (ELISA), virus isolation techniques, supplemented by reverse transcriptase PCR (RT-PCR) (Reid et al., 2000; Vangrysperre and De Clercq, 1996). ELISA assays are being used in many laboratories for antigen detection but still there is scope for improvement in antigen sensitivity, theoretically this ultimate sensitivity can be achieved by polymerase chain reaction (PCR) however, RT-PCR protocols although perform satisfactorily in many instances yet they are insufficiently sensitive and exhibit a narrower spectrum of reactivity compared with ELISA procedures coupled with virus isolation in cell culture (Reid et al., 1999). It is suggested that RT-PCR assay is valuable tool to assist the protocol of antigen detection by ELISA.

Affordability of diagnostic assays is important for endemic resource-poor countries and the possibility to cut the cost of diagnosis through using inexpensive or low-cost reagents could improve the diagnosis. The RT-PCR was established and evaluated to save the cost of diagnosis that is about Pakistani Rupees five hundred per test compared with indirect ELISA assay that costs more than one thousand Rupees per test. The RT-PCR is a time saving technique as well; an RT-PCR assay takes eight hours to complete whereas ELISA assay may require preliminary step of inoculation on cell cultures, if the sufficient quantities of antigen are not available and it would take several days before the typing could be done (Ferris and Dawson, 1988).

This study reports investigation of forty two outbreaks of FMD in Faisalabad during 2013. Serotype identification is a fundamental part of FMD diagnosis. The RT-PCR methodology was successfully established in this laboratory for the typing of FMD virus being it cheaper and less resource demanding as compared with ELISA technique. A total of 42 outbreaks were investigated from Chak Jhumra, Jaranwala, Sadhar, Sumandri and Tandlianwala regions of Faisalabad district to determine the status of serotype distribution in these areas. The count of reported outbreaks from these regions was 10, 6, 9, 10 and 7 in respective manner. Seroype A FMDV was detected from samples received from outbreaks that occurred in Sumandri, Jaranwala and Tandlianwala tehsils, serotype O from Tandlianwala and Sadhar, and Asia 1 outbreaks were reported from Chak Jhumra and Sadhar (Table 2). There were samples from two outbreaks in Jaranwala that were consensus PCR positive for FMD but type of virus remained unidentified that possibly could be variants of FMDV or limitation of RT-PCR assay. Thirty samples were sent to Foot-and-Mouth Disease Research Centre (FMDRC), Lahore for confirmation by indirect sandwich ELISA. All except one were confirmed in accordance with our testing. The one sample that was serotype 'A' in our testing could not be typed by indirect ELISA; this indicates that RT-PCR is more sensitive than ELISA.

Distribution pattern of serotypes on virus was indicative that they had a sort geographical boundaries;

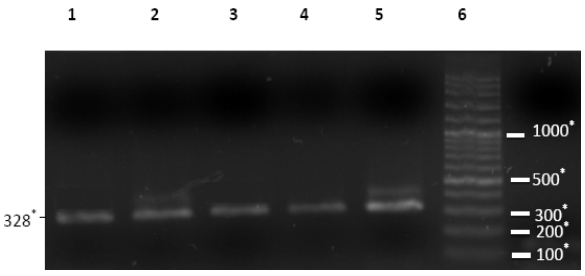

Fig. 1: Detection of Foot-and-Mouth Disease Virus RNA by RT-PCR: Lane1 through Lane5): Agarose gel electrophoresis (1.5%) of PCR product 328 bp obtained with primer pair 1F/1R. Lane 6): 100 bp DNA ladder; *bp

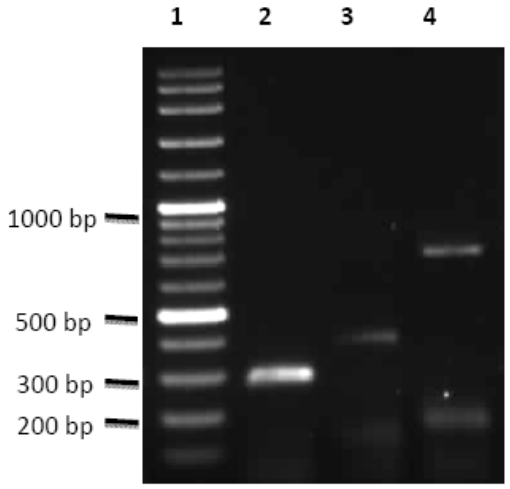

Fig. 2: Agarose gel electrophoresis of the amplified gene products for typing of FMDV for serotypes A, O and Asia 1 using the multi-primer mix. Lane 1, 100 bp DNA ladder. Lanes 2,3,4, multiplex PCR with primer mix P33-P(Asia1-O-A) showing DNA products of 292, 402 and 732 bp representing serotypes A,O and Asia 1, respectively.

serotype Asia 1 was found in outbreaks from north-west of the district. Seroype A was circulating in eastern Faisalabad whereas all three endemic types 'O, A, and Asia 1' were found from outbreaks in south west of the district. Results indicate that it may not be the air only that was main medium for transport of virus from one place to the other but based on history of outbreaks we could say that it was the transport of animals and then movement of livestock holders and veterinary staff that may have played a role in disease spread. We had conducted some outbreak investigations in 2012 and some most recently in 2014. In 2012 the predominant type in Faisalabad region was Asia 1 while in recent out breaks in 2014 all the outbreaks typed were of serotype 'O'. This indicates that patterns of type specific outbreaks keep changing every time and well organized integrated campaigns of extensive vaccination along with regulation and control of movement of animals can be helpful in controlling this disease.

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