

Emergence of new variants in foot and mouth disease virus serotype 'O' in Khyber Pakhtunkhwa-Pakistan, 2012 to 2015

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Abstract

Foot and mouth disease (FMD) is a highly contagious disease mostly caused by serotype A, O, and Asia-1 of virus that is endemic in Pakistan. FMD Serotype 'O' is responsible for more than 60% of outbreaks in Khyber Pakhtunkhwa Pakistan. During the present study, a total of 50 samples of FMDV serotype 'O' virus were selected from field outbreaks samples preserved at the center. The nucleotide sequences of the VP1 coding region from all the virus samples were determined. The phylogenetic analysis of FMDV serotype 'O' viruses circulating in different areas of Khyber Pakhtunkhwa Pakistan was determined. This indicated continuous mutation from 2012 to 2015 due to the presence of two different sub lineages within 'O' PanAsia-II lineage. The current study reveals that the presences of two different sub lineages of FMDV O PanAsia-II were identified which are circulating in different regions of Khyber Pakhtunkhwa Pakistan. The emergence of new variants are possibly responsible for the failure of vaccine in the country and creates hindrances in the control of disease in the region.

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Introduction

Foot and mouth disease (FMD) is highly contagious and fatal viral disease of all the cloven hooved animals endemic in Pakistan (Alexanderson and Mowat, 2005; Jamal et al., 2011). FMD is highly transmissible disease and causes substantial financial losses to the livestock sector in the form of high morbidity and mortality. FMD causes acute agalactia which lead to huge losses in milk production especially in dairy animals. This disease causes high mortality in young stocks and lameness in draught animals in the hilly areas leads to high economic losses. Due to the enzootic nature of the FMD, there is continuous international trade embargo in terms of Livestock and

livestock products i.e. meat and milk products, hides, skin and their products. The virus which causes the FMD belongs to the genus *Aphthovirus* with in the family of *Picornaviridae*, the smallest animal origin virus (Belsham, G.J, 2005). FMD virus is non-enveloped single stranded RNA having size of about 8.3K genome. The genome, RNA is surrounded by spherical capsid proteins consisting of about 60 units of the four structurally important components of polypeptides i.e. VP-1, VP-2, VP-3, and VP-4. The VP-1 coding region is the most important variable capsid polypeptide of virus possesses the immunogenic properties (Fox et al., 1989; Jackson et al., 1997). The nucleotide sequences' of Virus Protein 1(VP-1) coding region is routinely using for genetic



characterization of the FMDV strains because this polypeptide is mostly used by virus for adherence and entry to the host cell. The host also raised protective immunity against this, that's why the VP-1 polypeptide is known as highly immunogenic portion of the FMDV virus structure (Belsham, G.J, 2005). The causative agent of the disease is present in seven different serotypes i.e. A, O, C, Asia-1, SAT-1 SAT-2 and SAT-3. These different serotypes do not provide any cross immunity among each other after immunization or infection (Bachrach1968; Domingo et al., 2003). Even within inside the single serotype several subtypes exist which lack cross protection against each other. Furthermore the adequate control and prevention of the FMD might not be possible due to the appearance of new strains within the serotypes. Different strain of the virus is geographically distributed, which is mostly used for identification of the virus origin. These Topotypes label and define the geographical array of genetically different strains within the FMD serotypes (Knowles and Samuel 2003). In some geographical zones the FMD outbreak is caused by the presence of more than one serotype and subtypes of the FMD virus. However it is obvious that the distribution of the serotype and subtype is not specific to any area around the globe. The presence of FMDV is varies from large geographical areas of Asia, South America and Africa, which can simply crosses the borders and causes heavy epizootics in free zones. The serotype 'O' of FMDV is a unique virus serotype which is responsible for more than half of the total outbreaks among the rest of serotypes globally (Rweyemamu et al., 2008) and also reported in some disease free states (Knowles et al., 2001, Bruckner et al., 2002, Sakamoto et al., 2002, Shin et al., 2003, Tsutsui and Yamamoto 2010). In Pakistan the FMD is enzootic and common, interestingly the 'O' serotype has been identified and found to be accountable for almost more than 60 % of the outbreaks in Pakistan. To design the strategies for proper control and prevention of this devastating and economically important disease particularly in Khyber Pakhtunkhwa and in Pakistan. It is required to screen out the different strains of FMD serotypes 'O' circulating in the region as more than 60 % of the outbreaks are occurred due to this serotype. This will also help in the selection of the most suitable vaccine candidate and to incorporate them in the formulation of trivalent vaccine preparation in the province. Due to which it may help in the control of FMD outbreaks in the country and entire glob respectively.

For this purpose phylogenetic analysis based on the VP-1 coding region is mostly use to determine the subtypes of different FMDV serotypes (Tosh et al., 2002, Knowles and Samuel 2003 and Knowles 2005). In Pakistan very limited research work has been conducted in the isolation and characterization of FMDV (Waheed et al., 2010). During the present study field samples from 2012 to 2015 were selected which belongs to different districts of Khyber Pakhtunkhwa Pakistan, have been sequenced for VP-1 coding region and characterized for FMD serotype 'O'.

Material and Methods

Nature of sample

The epithelial samples were previously collected from Animals i.e. cattle and buffalo belongs to different districts of Khyber Pakhtunkhwa under the Project "Progressive control of FMD in Pakistan" funded by FAO from 2012 to 2015 (Table 1). The major objective of the project was to establish a provincial FMD surveillance Laboratory at Foot and Mouth Disease Research Center, Veterinary Research Institute Peshawar, where the samples received from different districts were tested for the diagnosis and serotyping of the FMDV. In continuation with afore mentioned project, another ADP project "Use of Molecular Techniques in Livestock Research at Khyber Pakhtunkhwa" from the provincial government was approved in 2013-14. The major objective of The Project was to strengthen the Foot and Mouth Disease center especially in molecular techniques. For this purpose samples were selected randomly from the previously tested samples and were further subjected to process for genetic characterization. The selected FMDV serotype 'O' samples were processed for genetic characterization of VP-1 coding region to determine the range of virus circulating in the clinically suspected animals. The ELISA positive samples were sent to the Genomics Laboratory previously established, in Phosphate Buffer Saline (PBS) in ice box, where the samples were store at -40°C till further processing. The sample data regarding the species, district, date, year, and serotype were also recorded for onward processing (Jamal et al., 2011). The data regarding the nucleotide sequence for the VP-1 coding region of FMDV serotype 'O' were obtained from Gen Bank data base (www.ncbi.nlm.nih.gov).



Sample preparation and Nucleic Acid extraction

The samples were grinded in 1ml PBS using pestle and motor, and then transferred to 1.5ml Eppendorf tube under ice. The samples were then subjected to centrifugation where it was centrifuged at 1000rpm for 15 minutes at 4°C using a high speed refrigerated centrifuge machine (Z 32 Hermle Germany). The supernatant was collected in another sterile 1.5ml tube and total RNA was extracted from all the samples using Viral Nucleic acid Extraction Kit GF-1 Vivantis following the procedure as mentioned by the manufacturer. The final volume of the viral RNA extracted was 50µl eluted in elution buffer supplied with the kit and store at -40°C till further processing as described by Jamal et al., (2011).

Quantification of the Viral RNA

The extracted viral RNA of 2.0 µl was taken and quantified for concentration and purity using Nanodrop spectrophotometer (Titertek Berthold Germany).

CDNA Amplification

All the quantified viral RNA was then converted to cDNA following the protocol provided by the manufacturer with CDNA amplification kit. All the reagents were brought to room temperature by thawing and flicking.

Then the genomic DNA elimination reaction on ice was made as mentioned in the protocol i.e. 2 µl of gDNA wipeout buffer was taken in a sterile 0.1ml tube and mix with 10µl of template RNA, the total volume of the reaction was made 14µl by adding nuclease free water. The tube content was properly mixed and incubated for two minutes at 42 °C using thermal cycler T100 BioRad.

Then prepared the reverse transcription master mix on ice by adding 1 µl of ‘Quantiscript Reverse Transcriptase’, 4 µl of ‘Quantiscript RT Buffer’, 1 µl ‘RT primer Mix’ and the entire genomic cDNA elimination reaction, the final volume was made 20 µl in 0.1ml sterile tube. The tube was incubated for 15 minutes at 42 °C as previously mentioned. The tube was then again incubated for another 3 minutes at 95 °C to inactivate the Quantiscript Reverse Transcriptase inside the Thermal Cycler. The cDNA was then stored at -40°C till further processing.

Sequencing

For sequencing of entire VP-1 coding region of FMDV serotype ‘O’ all the amplified cDNA samples

along with corresponding extracted RNA were packed in dry ice according to the International export rules and regulation for biologics and sent to Shanghai ZJ Bio-Tech Co, Ltd China. The sequencing was performed using both forward and reverse primers for every single sample by the lab.

Genome Sequence Analysis

The sequences obtained were edited after comparing the peaks of nucleotides basis using Accelsys Genes Software (Jamal et al., 2011). The VP-1 sequences of Serotype ‘O’ were aligned using Mega 6 software (Tamura et al. 2007). Phylogenetic relationship of the VP-1 sequences for individual sample was determined by constructing Neighbor Joining method using Mega 6 Software (Saitou and Nei, 1987). The strength of the tree topology was assisted with 1000 bootstrap replicate as applied in the program (Felsenstein, 1985). To infer the phylogenetic tree, it was constructed to scale with the same branch length as used in the evolutionary study.

Results

A total of 50 epithelial samples were selected from the FMD cases belong to different districts of Khyber Pakhtunkhwa preserved at the center during 2012 to 2015. All these samples were previously tested in serology Laboratory by using Sandwich ELISA for serotyping the FMDV. The same samples were further reconfirmed on another advance technique i.e. Real Time Polymerase Chain Reaction (RT-PCR) using one step kit specific for serotype ‘O’. The positive samples were processed and analyzed by subjecting to sequencing the VP-1 region of FMD Serotype ‘O’ virus to determine their sub type prevailing in the different areas of Khyber Pakhtunkhwa Pakistan. Out of 50 samples 13 VP1 (26%) sequences were identified and determined using BLAST as serotype ‘O’ (Fig 2). Moreover during the initial serotyping of the samples, two samples one from district Bannu and the other from D.I.Khan were found positive for both serotype (O+A) collected in 2012 while another sample collected during 2013 from District Hazara was also found mixed i.e. (O+A). All these three samples were collected from cattle of the above mentioned districts of Khyber Pakhtunkhwa.

In this study 13 out of 50 samples previously collected during 2012 to 2015 form suspected FMD clinical cases were of that Serotype ‘O’. The FMDV serotype ‘O’ from field outbreak sequenced in this study



belongs to two different sub lineages within O PanAsia II. The vaccinal strain of the serotype 'O' being used for production of vaccine in Pakistan belongs to a separate lineage named earlier as Pak-98 with O/PAK12/2003,(Accession Number DQ165066).

The virus O/PAK/2008, O/PAK/2005, O/IRN/8/2005 and O/IRN/2001 belong to this lineage are responsible for FMD outbreaks in the region of Khyber Pakhtunkhwa Pakistan. There are two different sub lineages of O/PanAsiaII of serotype 'O' has been identified in 26% of the samples selected for the study in Khyber Pakhtunkhwa Pakistan. These sub lineages

of the O Pan Asia-II were identified based on the differences in the VP-1 coding region sequence (Hemadri et al., 2002, Nagendrakumar et al., 2009). The viruses belonging to different two sub linages of FMDV serotype 'O'/Pan Asia-II were identified in Khyber Pakhtunkhwa in 2012 to 2015. These were originated from different districts i.e. Bannu, D.I.Khan, Mardan, Kohat, Hazara, Malakand, and Peshawar respectively (Central , Northern and Southern region of Khyber Pakhtunkhwa Pakistan) (Table 1).

Table-1; Samples collected from different districts of Khyber Pakhtunkhwa-Pakistan used in this study.

S. No.	Sample No.	Origin	Collection Year	Specie	Serotype on ELISA
1	F-1	Bannu	2012	Cattle	O+A
2	F-2	D.I.Khan	2012	Cattle	O+A
5	F-5	Peshawar	2012	Cattle	O
6	F-6	Hazara	2012	Cattle	O
7	F-7	Bannu	2013	Cattle	O
8	F-8	D.I.Khan	2013	Buffalo	O
9	F-9	Kohat	2013	Cattle	O
11	F-11	Malakand	2013	Cattle	O
13	F-13	Mardan	2013	Cattle	O
16	F-16	Peshawar	2013	Cattle	O
18	F-18	Hazara	2013	Cattle	O
20	F-20	Hazara	2013	Cattle	O+A
21	F-21	Bannu	2014	Cattle	O
22	F-22	D.I.Khan	2014	Cattle	O
23	F-23	Kohat	2014	Cattle	O
25	F-25	Malakand	2014	Cattle	O
28	F-28	Mardan	2014	Cattle	O
31	F-31	Peshawar	2014	Cattle	O
34	F-34	Hazara	2014	Cattle	O
47	F-47	Anonymous	2015	Vaccine strain	O
48	F-48	Anonymous	2015	Vaccine strain	O



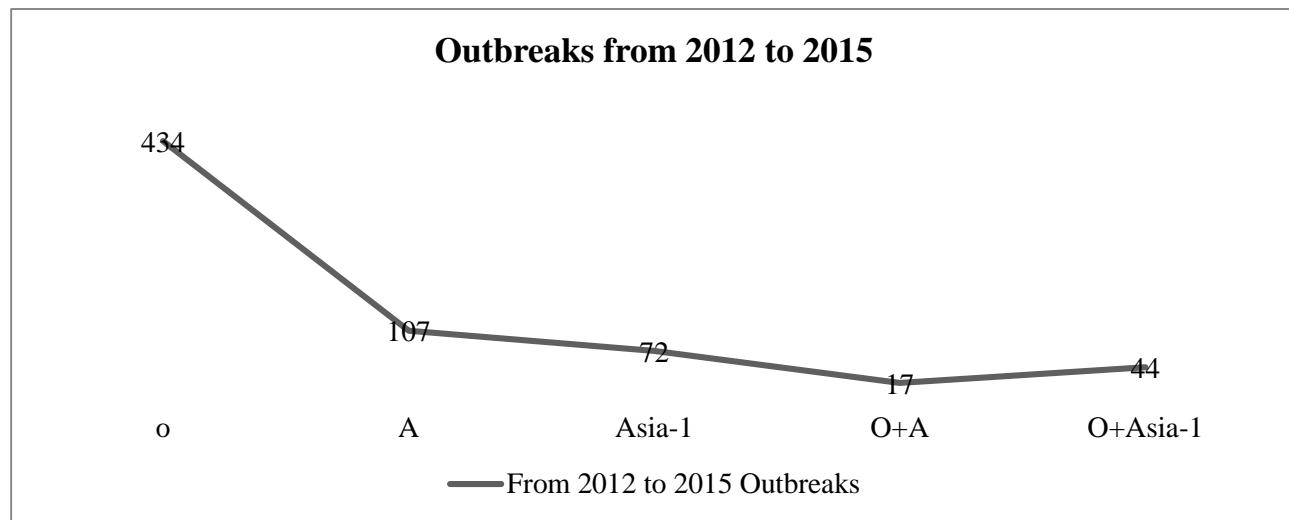


Fig.- 1: The serotype ‘O’ has been identified and found to be accountable for more than 60 % of the outbreaks as compared to serotype A, serotype Asia-1 in Khyber Pakhtunkhwa-Pakistan during 2012-2015.

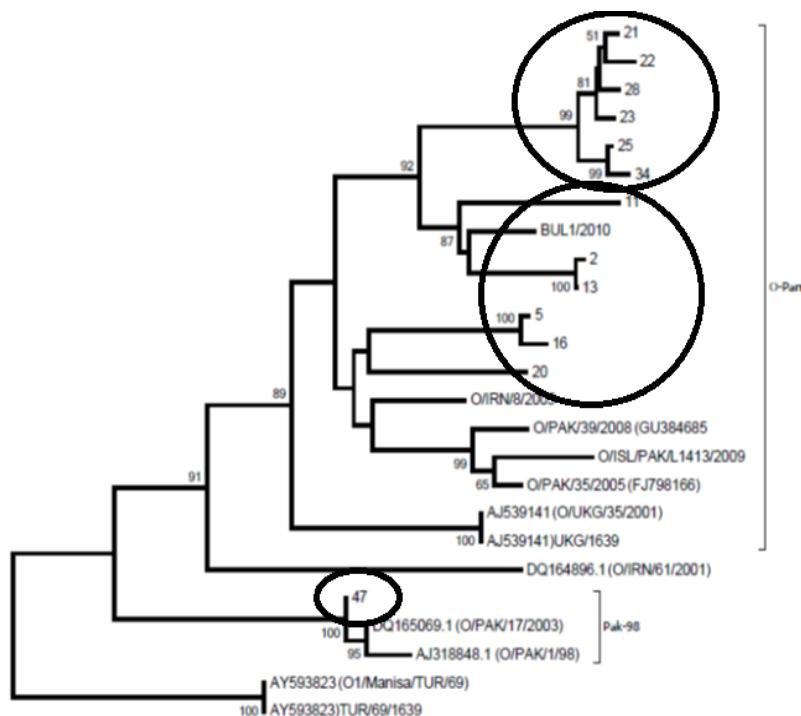


Fig. - 2: Phylogenetic relationship of the FMDV serotype O sequences obtained from different regions of Khyber Pakhtunkhwa -Pakistan in comparison with other available sequences in Genebank.

Discussion

In the current study 13 out of 50 new field isolates of FMDV serotype ‘O’ VP-1 region sequences have been created and analyzed. The sequences of these Serotype O isolates were from different districts including Peshawar, Swat, Malakand, Dir, D.I.Khan,

Hazara, Mardan, Kohat and Bannu etc. in Khyber Pakhtunkhwa Pakistan. In Khyber Pakhtunkhwa more than 60% of the disease outbreak is due to the FMDV Serotype ‘O’ (Fig 1). These different sub group of serotypes ‘O’ of FMDV were found responsible for the disease outbreak in different districts of Khyber Pakhtunkhwa during 2012 to 2015. The sequences



data were compared with already available FMDV sequence in the Data bank specifically those serotypes of the viruses which are circulating in the neighbor countries. These sub lineages of FMDV serotype ‘O’ were emerged and circulating in the different regions of Khyber Pakhtunkhwa. The FMDV serotype ‘O’ virus used in Trivalent Vaccine production in Pakistan also belong to a separate lineage i.e. Pak 98, while the viruses circulating in different regions of Khyber Pakhtunkhwa Pakistan belongs to two different sub lineages of O Pan Asia II. In Pakistan the vaccine strain mostly introduced from foreign organization like the “World Reference Laboratory” (WRL) for “FMD institute for Animal Health”, Pirbright, UK (Zulfiqar, 2005). All the serotype ‘O’ viruses belong to two sub lineages within the O Pan Asia II of O PAN Asia lineage which is outcome of continuous genetic mutation within this strain. This lineage is predominant and mostly circulating in different regions of KPK particularly and in Pakistan generally. According to statements of ‘Schumann et al., 2008 and Klein et al., 2008’ the serotype ‘O’ of the PAN Asia II lineage was first time identified in 2003 in different region of Pakistan and also in the neighboring countries. This PAN Asia II lineage was primarily identified in Nepal in 2003 and Bhutan in 2003-4. Later on it was also recognized in Afghanistan in 2004 and then in Pakistan in 2006-7 (Schumann et al., 2008, Klein et al., 2008).

The current research shows that this sub lineages of ‘O’ PAN Asia II is circulating in Khyber Pakhtunkhwa and in the other provinces of the country respectively. As it was also present since 2005 in different region of Pakistan and afterward also detected in Iran, Turkey and Kazakhstan and Kyrgyzstan in 2007(Scherbakov et al., 2008). This study shows that the subgroups of this sub lineage is circulating in different region of Khyber Pakhtunkhwa, Pakistan is responsible for the disease outbreak. It is worth mentioning here that the presence of new variants of the viruses of this sub lineage circulating in the country and in neighboring states, might be responsible for the wide spread outbreaks. Furthermore the unrestricted movement of animals especially during Eid Ul Adha, across the different provinces of country is also pave way to the dissemination of viruses which lead to the heavy outbreaks in the country. It is pertinent to say here that the concurrent presence of more than one groups of a sub lineage of FMD Serotype ‘O’ virus in different districts of the province, made the situation tough for the surveillance system of this disease in the country,

this statement support our results. The emergence of new variants within the sub lineages of O Pan Asia II viruses lead to the failure of disease control through vaccination campaign in the country.

Conclusion

It is obvious from this research study that two different sub lineages within the FMDV serotype ‘O’ Pan Asia II emerged due to mutation which now circulating in the Khyber Pakhtunkhwa Pakistan. The detection of these two sub lineages within the serotype O Pan Asia II viruses are continuously and rapidly changing in Pakistan. These changes in the virus genome may be due to genetic shift and drift or may be due to the less immunogenicity as a result of past infection, low immunization coverage and either due to non-matching or poorly matching vaccine or inadequate dose. The newly emerging strains of this virus may pose a serious risk to the livestock farming community in the form of heavy economic losses and also due to international trade embargo.

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