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Research Article

Molecular Characterization and Phylogenetic Analysis of Field Isolates of Foot-and-Mouth Disease Virus Serotype A from Uttar Pradesh, India

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Abstract

The rapid response to the economically important livestock diseases is greatly aided by the surveillance for the circulating etiological agent. Among these, foot-and-mouth disease in India is considered an important disease caused by Apthovirus of picornaviridae family. Continuous studies on the genetic and antigenic characterization of the circulating FMDV serotypes are absolutely essential due to the high mutation rate of the virus. The present study was conducted to determine the molecular characterization and phylogenetic analysis of a FMDV serotype A circulating in the western Uttar Pradesh through partial sequencing of VP1 gene. Vesicular epithelium samples were collected from different outbreaks of FMD and processed for extraction of viral RNA and RT-PCR. DNA sequence analysis was performed and phylogenetic tree was constructed to determine the phylogenetic relationship. Out of 25 tongue epithelium samples analyzed, the amplicons (1301 bp and 865 bp) could be amplified from 20 tongue epithelium (12 FMD serotype O and 8 FMD serotype A). Sequence and phylogenetic analyses of partial 1D gene of two FMDV serotype A samples showed the genetic variation from the previously reported FMDV in India. In conclusion, there is need of continuous monitoring of FMDV mutations and evaluation of the FMD vaccines strains currently used in India to avoid the recurrence of FMDV outbreaks.

Keywords: Bovine; Buffalo; Cattle; FMD; Phylogenetic Analysis; Sequencing

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that leads to severe economic losses in terms of reduction in milk and meat production, death of calves or young ones, treatment cost and trade restriction from endemic areas [1-4]. Foot-and-mouth disease virus (FMDV) is small, non-enveloped, positive-sense, single stranded, non-segmented RNA virus that belongs to the genus Aphthovirus of the Picornaviridae family having four structural viral proteins namely VP1, VP2, VP3, and VP4 [5]. The virus has seven immunologically distinct serotypes O, A, C, Asia-1, Sothern African territories (SAT) 1, SAT 2, SAT 3, but in India only four serotypes viz., O, A, C and Asia-1 are endemic [6]. Among these, FMD serotype O is causing the outbreaks in more than 90% cases followed by A and Asia-1 [7]. These FMD serotypes have more than 65 diversities of topotypes, genetic lineages and strains [5,7,8]. Since, the FMDV is RNA virus, so the replication process is prone to high level of replication errors that cause high level of genetic diversity between virus serotypes [9,10]. Among four structural viral proteins, VP1 is considered as the highly variable protein containing the major antigenic epitopes and plays an important role in the molecular epidemiology investigations of the virus, worldwide [11].

The phylogenetic analyses through multiple sequences alignments of this VP1 help in study of the variation, genetic relationship and geographical circulation among different FMDV serotypes [12,13]. The epidemiology of FMD in India is complicated because of the circulation of endemic FMDV viruses and incursions of exotic viral strains from the neighbouring countries like Pakistan, Nepal, Bangladesh and Bhutan [14]. India has been considered one of the FMDV endemic south asian countries with multiple FMD outbreaks since 1864 [15]. FMDV serotypes O, A and Asia-1 are circulating in the bovine population of India. Among these, serotype A is genetically and antigenically more diverse and classified into three continental topotypes, namely Africa, Asia, and Europe-South America (Euro-SA). Within these three topotypes, 26 global genotypes have been identified differing at VP1 nucleotide sequence level by > 15% [16].

Continuous studies on the surveillance, genetic and antigenic characterization of the circulating FMDV serotypes are absolutely essential due to the high mutation rate of the virus and the potential for the emergence of novel FMDV strains. This will be helpful in rapid response to control the outbreaks of the disease. The present study was conducted to determine the molecular and genetic characterization of a FMDV serotype A circulating in the western Uttar Pradesh through partial sequencing of VP1 gene.

Materials and Methods

Sample collection

During the study, a total of 25 tongue epithelium samples were collected from clinically diagnosed 19 cattle and 06 buffaloes of different districts of Uttar Pradesh. The animals were showing clinical signs (tongue and mouth vesicles appearance, pyrexia, salivation, loss of appetite and depression) of Foot-and-Mouth disease. Vesicular epithelial tissues were collected from the unruptured or recently ruptured vesicles preferably on tongue or dental pad and was placed in a 50% PBS glycerol. The collected samples were transported to the laboratory in ice containers containing ice packs to maintain cold chain temperature.

Viral Nucleic acid (RNA) extraction

Viral nucleic acid (RNA) was extracted using RNeasy mini-Kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, approximately 150 µL of sample was taken RNA was eluted in a final volume of 50 µl nuclease free water. RNA was reverse transcribed using MMLV RT (Bangalore GeNei) and negative sense primer NK61 (5'-GACATGTCCTCCTGCATCTG-3') [17]. Briefly, in an eppendorof tube, 2.5 µl of 10 mM dNTPs, 5 µl 5X RT Buffer (with DTT), 0.5 µl of 200 U/µl MMLV, 1 µl of RNase inhibitor (3.3 U/µl), 25 pmol primer and 1 µl Diethyl Pyrocarbonate (DEPC) water were taken and total volume of 25 µl was prepared by adding 11 µl of above mixture and 14 µl of RNA tempelate. It was flash spun and then heated at 42°C for 60 minutes and at 94°C for 10 minutes in the thermocycler and thus cDNA (RT products) was obtained. Polymerase chain reaction (PCR) was done using primers NK61 and A-1C562 (virus sense, 5'-TACCAAATTACACACGGGAA) as per method described by Knowles and Samuel (1998). For PCR 3.0 µl of 1.5 mM 25 mM $MgCl_2$, 1.00 µM of 200 µM 10 mM dNTPs, 5.00 µl of 1X 10X buffer, 1.00 µl of 0.2-0.5 (pmol/µl) Forward primer (10-25 pmol/µl), 1.00 µl of 0.2-0.5 (pmol/µl) Reverse primer (10-25 pmol/µl), 0.50 µl of 2.5 U Taq DNA polymerase (5 U/µl), 5.0 µl of RT product (template) and 33.5 µl of DEPC-H_2O and make total volume 50.00 µl. The thermal profile for PCR was 94°C for 05 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Following the amplification of PCR products, the PCR products were analyzed on 1.5% agarose gel electrophoresis system using Tris Borate EDTA buffer (1X) stained with ethidium bromide. A 5 µL of the PCR products and DNA ladder were loaded into the preformed wells. The fragments of positive samples were excised from the agarose gel and the amplified RT-PCR products were purified from the gel using AxyPrep DNA gel extraction Kit (Axygen, USA) according to the manufacturer's protocols and DNA was eluted in a final volume of 30 µl.

DNA sequencing and Phylogenetic analysis

The purified PCR products were sequenced in both directions using the dideoxy chain termination method using the same primers through outsourcing. To determine the substitution rates and construction of phylogenetic tree, computational and bioinformatics analyses were used [18,19]. The similarities in the nucleic acid were determined using the Basic Local Alignment Search Tool (BLAST) available at NCBI using default parameters. The results obtained from local alignment were used to construct the phylogenetic tree by the neighbor-joining method [18], using MEGA version X [19,20]. Phylogenetic trees were constructed from ClustalW-aligned sequences on MEGA-X (http://www.megasoftware.net/mega.php), using the Maximum-Likelihood method with 1000 bootstrap replications [19,20]. The pair-wise sequence identity studies of FMDV were performed using Clustal W in Sequence Demarcation Tool (SDT v1.2) [21].

Results and Discussion

Out of 25 tongue epithelium samples analyzed with RT-PCR, the amplicons (1301 bp and 865 bp) could amplified from 20 tongue epithelium (12 FMD serotype O and 08 FMD serotype A). Out of

08 FMD serotypes A, the 1D gene of two FMDV isolates were sequenced at DNA sequencing facility of Ocimum Biosolutions Ltd., Hyderabad. The sequences were analyzed in EditSeq programme of (DNASTAR) for further analysis. The nucleotide sequence of 1D gene of two isolates revealed a coding region of 865 bp, which was in agreement with the length of the amplified products observed in the gel (Table 1).

S. No.	FMDV serotype A isolates	Nucleotide sequence of 1D region
1	UP1	GCAAGACTTTGAGCTGCGCCTCCCGATTGACCCCCGCACACAAACCACCTCAGCCGGGGAGTCCG-CAGACCCAGTCACCACCACTGTTGAGAACTACGGTGGTGAGACACAAGTCCAGCGACGCCACCACACT-GACGTCGGCTTTATAATGGACAGATTTGTGAAAATTGGAAACACCAGCCCCACACATGTCATTGACCT-CATGCAAACCCACCAACACGGGTTAGTGGGCGCCTTGTTGCGTGCCGCCACGTACTACTTCTCCGACCTG-GAGATTGTGGTTCGCCACGACGGCAACCTGACATGGGTACCCAATGGAGCACCCGAGGCAGCCCTATC-CAACACAGGAAACCCCACAGCCTACAACAAAGCGCCGTTCACGAGGCTTGCACTTCCCTACACCGCGCCA-CACCGCGTGTTGGCAACAGTGTACAACGGGACGAACAAGTACTCCGCGGCTAGTGGGCGCACGCGGGGT-GACCTGGGACAGCTTGCGGCGCGCGTCGCCGCCCAACTTCCTGCCTCTTTTAACTTTGGTGCGGTTC-GGGCCACTACCATCCACGAGCTGCTTGTGCGCATGAAGCGTGCCGAACTCTACTGCCCCAGACCGCT-GTTGGCGGTGGAGGTCACGTCGCAAGACAGACACAAACAGAAGATCATTGCGCCAGCTAAACAGCTCCT-GAACTTCGACCTGCTCAAGTTGGCAGGAGACGTTGAGTCCAACCCTGGGCCCTTCTTCTTCTCTGACGT-GAGGTCGAACTTTTCCAAACTGGTGGAAACCATCAACCAGATGC
2	Brl	TCAGAGAAGAAGAAGGGCCCAGGGTTGGACTCAACGTCTCCCGCCAACTTGAGCAGGTCGAAGTTCAG-GAGCTGTTTCACAGGTGCCACAATCTTTTGTTTGTGTCTGGCTTGGCTCGGGTGAACGGCCAACAGAG-GCCGAGGACAGTATGTTTCGGCCCTCTTCATGCGGTAAAGCAGTTCAGTCACCCGGGTAGCCTTGATG-GCACCATAGTTGAAGGAGGTGGGCAGCGTTCTTGCTGCCTTCTGGGCCAACACTTGCAGGTCACCCCTCA-CATTGGTCACATCGCTTTCGCCATACTTGCAGTTCCCGTTGTAAACGGTAGCCAGAACACGGTGTGGTGC-CGTGTAGGGCAGCGCAAGTCGGGTGAGCGGTGCCTTGTGGTAGGCCGTTGGGTTGGTGGTGTTATCCAAC-GCCGCCTCGGGCGCCCCGTTTGGGACCCAGGTGAGGTTCCCCTCGTGCTTCACCGCCACTTCTAAATCT-GCGAAGTAGTAAGTAGCGGTGCGGAGGAGCGCGCCCACCAAAGTGTGAGCAGGGGTTTGCATCAGGTC-CAACACGTTGATTTGGTCTTTTGGTGTCACTTTCACAAATCTGTCCAAAATGAACGAAACGTCGGTGT-GCTGACGTCTCTGGACCTGTGTCTCTCCACCGTAGTTCTCAACGGTGGTGGTCACGGGATCAGCAGACTC-GCCTGTGGAGGTGGTCTGTGTGCGGGCGTCAACCGGCAAACGCAGCTCAAAGTCCTTACCGGCGCTAGC-CAGAATGACCAGTGCGTCACCGTCAGCTTTCCCGTGTGA

Table 1: Nucleotide sequence of 1D region for two FMD virus serotype A isolate.

Phylogenetic analysis revealed that the FMD virus serotypes of UPl were distributed in two major clusters varying at the distance 128 and 8 (Figure 1). Cluster I consists of UPl, FJ755012 and FJ 755013 which shows closer similarity of UPl serotype to FJ (755012 and 755013) serotypes at distance 12. On the basis of phylogenetic tree, the cluster II also consist of some sub-clusters which are having serotypes FJ 617248, FJ617244, FJ617243, FJ617247, FJ617242, FJ617251.1, FJ617249 and FJ617245, which are genetically similar to UPl and FJ (755012 and 755013) but very distantly. It was clearly indicated by the dandogram that the cluster I joined to cluster II at distance 8.

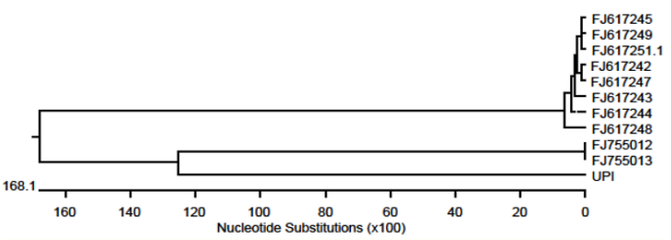

Figure 1: Nucleotide sequence based phylogenetic tree (UPl).

However FMD virus serotypes of Brl were distributed in three major clusters observed in the dandogram varying at the distance of 264.3, 180 and 152 (Figure 2). On the basis of dandogram analysis using 1 serotype (1 original and 10 serotypes from GENEBANK for comparison), the serotype Brl makes the clusters at distances 152 with the serotype EF494503, EF494506 and DQ296501. The serotype Brl joined to the cluster III at distance 152 shows the genetic similarity distantly with the comparable serotypes AJ539139, AY593824, AJ539138, AF506822 and AY333431. On the basis of phylogenetic tree, it was evident that it also showed a genetic similarity at distance 180 with other comparable serotype EF494504 and very distantly (264.3) it also showed similarity with comparable serotype AY312587. The cluster I, II and III also made some sub-clusters at the different distances. The pair-wise identity/divergence of both the isolates was analyzed using the laser gene software (DNASTAR) showing more than 20% homology between them (Table 2-4).

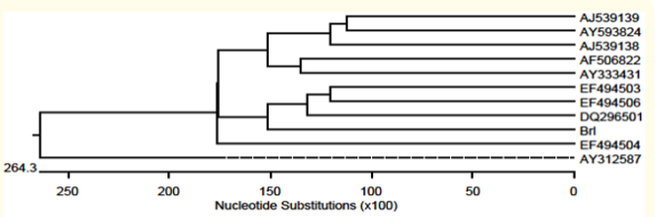

Figure 2: Nucleotide sequence based phylogenetic tree (Brl).

		Percent identity												
		1	2	3	4	5	6	7	8	9	10	11		
Divergence	1		27.7	28.3	24.0	27.1	25.9	24.8	26.1	26.2	26.4	26.5	1	AF506822
	2	254.4		28.2	26.4	25.1	28.1	25.1	25.1	28.1	27.0	24.2	2	AJ539138
	3	233.5	238.5		24.3	26.9	28.8	24.5	24.8	26.6	23.0	25.0	3	AJ539139
	4	350.0	303.4	350.0		25.4	23.8	25.1	23.3	25.7	24.3	23.9	4	AY312587
	5	269.6	350.0	281.5	435.5		24.4	22.7	21.8	25.4	24.3	24.5	5	AY333431
	6	350.0	242.9	223.2	350.0	350.0		24.2	23.3	25.5	25.9	25.9	6	AY593824
	7	350.0	350.0	350.0	350.0	350.0	350.0		26.6	23.8	27.7	27.4	7	Brl
	8	350.0	350.0	350.0	350.0	350.0	350.0	292.3		27.7	25.5	26.9	8	DQ296501
	9	350.0	350.0	286.9	350.0	417.9	350.0	350.0	251.0		25.8	28.3	9	EF494503
	10	350.0	403.0	350.0	350.0	350.0	344.7	350.0	350.0	350.0		26.8	10	EF494504
	11	350.0	350.0	350.0	350.0	350.0	350.0	278.5	274.9	240.9	290.0		11	EF494506
		1	2	3	4	5	6	7	8	9	10	11		

Table 2: Sequence identity among FMD based on nucleotide sequences of 1D using clustal W (Brl).

		Percent identity												
		1	2	3	4	5	6	7	8	9	10	11		
Divergence	1		93.1	93.0	94.6	97.4	91.9	94.6	94.8	26.0	26.0	26.3	1	FJ617242
	2	7.4		97.5	95.0	93.4	92.4	95.0	95.0	26.6	26.6	27.0	2	FJ617243
	3	7.5	2.6		94.5	93.4	92.4	94.7	94.9	27.2	27.2	26.7	3	FJ617244
	4	5.7	5.2	5.8		95.6	93.2	97.8	97.6	26.3	26.3	26.3	4	FJ617245
	5	2.7	7.0	7.1	4.6		92.2	95.6	95.7	26.1	26.1	26.8	5	FJ617247
	6	8.7	8.0	8.0	7.1	8.2		93.3	93.4	26.4	26.4	27.3	6	FJ617248
	7	5.6	5.2	5.5	2.1	4.5	6.9		98.1	26.6	26.6	26.1	7	FJ617249
	8	5.5	5.2	5.4	2.5	4.4	6.9	1.9		26.4	26.4	26.8	8	FJ617251.1
	9	329.1	309.8	292.2	345.9	328.8	340.0	334.7	340.0		100.0	29.0	9	FJ755012
	10	329.1	309.8	292.2	345.9	328.8	340.0	334.7	340.0	0.0		29.0	10	FJ755013
	11	350.0	350.0	350.0	350.0	350.0	304.3	350.0	350.0	250.7	250.7		11	UPI
		1	2	3	4	5	6	7	8	9	10	11		

Table 3: Sequence identity among FMD based on nucleotide sequences of 1D using clustal W.

	Percent identity		
Divergence		1	2	
	1		20.3	Brl
	2	124.4		UPI
		1	2	

Table 4: Sequence identity between FMD based on nucleotide sequences of 1D using clustal W (UPl and Brl).

UP1 isolate showed 29% homology with FJ (755012 and 755013) and more than 27% homology with FJ (617244 and 617248) strain. With the strain FJ (617247, 617251.1 and 617244), UPl showed about 27% homology. However FJ (617242, 617245 and 617249) strain showed more than 26% homology with UPl isolate. While the pair-wise identity/divergence of Brl isolate showed more than 27% homology with EF (494503, 494504 and 494506). It showed more than 26% homology with DQ296501 and more than 25% homology with AJ539138 and AY312587. Homology was more than 24% with AF506822, AJ539139 and AY593824 while homology was about 23% with AY333431.

The endemicity of foot-and-mouth disease is a major hurdle to the growth of the dairy industry due to its adverse effect on the productivity of livestock, and trade restrictions for livestock and their products. In India, there is control programmes namely National Animal Disease Control Programme through vaccinating

bovine (cattle and buffalo) with inactivated polyvalent vaccine consisting of FMDV serotype O, A and Asia-1 bi-annually is going on but FMD remains an endemic disease in the country. Although, the incidences of FMD outbreaks have been reduced, but FMD virus remain circulating due to the multiple serotypes and subtypes, low vaccine coverage (less than 2%) and unrestricted movement of animals [22,23]. Moreover, the unique geography of the country poses a considerable risk for the occurrence of circulating serotypes from neighboring countries. In the present study, molecular characterization of FMDV from the vesicular epitheliums recovered from the disease outbreaks from western Uttar Pradesh, India was conducted. The VP1 coding sequence of 02 FMD serotype A isolates were determined and by combining the sequences from India and different countries of the globe, the molecular epidemiology of FMDV serotype A was studied using phylogeographic tree and the maximum likelihood method.

Based on 1D sequence analysis, the FMD virus type A from different parts of world could be divided into 10 distinct genotypes [24]. Out of that, four genotypes viz. I, IV, VI and VII have been reported from India [24]. Further, endemic co-circulation of genotype VI, VII and disappearance of genotype I and IV since 1990 has been observed with the recent dominance of genotype VII in the field [25]. Since 2001, genotype VII has out competed other genotypes and has been associated with all the outbreaks in the recent years [26]. The present study sheds some lights on molecular epidemiological situation of the disease in the western parts of Uttar Pradesh, India due to serotype A which is considered to be antigenically and genetically most diverse. Such continuous monitoring of the field strains help in selection of vaccine strains and evolving a proper control strategy for the disease in the future. Nucleotide sequence analysis is now considered as a method of choice for strain identification and epidemiological investigation. In order to study the molecular alterations associated with genetic diversity in the field isolates, the genome of 1D gene of two recently collected isolates of type A were sequenced. Sequences obtained from two isolates revealed more than 20% homology between them. Further alignment and phylogenetic analysis studies of UPl sequence with published sequences of other FMD virus strains confirmed that it was more closer (29%) to FJ755013 and FJ755012 and revealed low level of homology (about 26%) with FJ617249. However alignment and phylogenetic analysis of Brl sequence with other published sequence of FMD virus strains showed close relation (about 28%) to EF494504 and EF494506, and revealed low level of homology (23%) with AY333431. The alignment report indicates that most of the substitutions are at VP1 positions and these residues have been identified to be critical by neutralization-escape mutants of FMD virus serotype A [27,28]. The nucleotide sequence of the two isolates (UPl and Brl) of FMD virus serotype A showed distant relation and a low level of homology to other published sequences of FMD virus strains which may be due to the recombination [29], geographical differences, mutation and illegitimate recombination. The findings are in concurrence with the results of previous workers. The genetic heterogenecity of type O FMD virus in animal population over different regions may be due to unrestricted livestock movement [30]. The endemic co-circulation of multiple genotypes of type A virus, which may lead to recombination, if multiple genotypes of closely related strains co-infect the host [24]. RNA viruses have genomes that replicate in the absence of repair mechanisms, they evolve very rapidly with a mutation frequency per nucleotide site of 10-3 to 10-5 substitutions per year [31]. Each FMD virus population such as field isolate is genetically and antigenically heterogenous, consisting of a spectrum of variants and termed them quasispecies [32]. The main immunogenic site of FMD viruses is located on the 1D region, which makes the VP1 region, the gene of choice to study variation and relationships between the different isolates [33,34]. However, a regular mass vaccination and the achievement and maintenance of an FMD free status have major benefits for International trade. In many situations, regular vaccination is an essential part of the disease control strategy. However, due to the high number of virus strains, vaccination provides only limited protection. These measures may have to be supported by emergency vaccination in order to limit the spread of the disease. This was emphasized during the 2001 epidemic in Europe, when massive culling met strong criticism [35]. The evolution of FMDV is usually by point mutation, and the size of this mutant plays crucial role in the virus's ability to adapt, spread, and ability to cause disease [7,36]. These evolutions of new strain of the virus may challenge the efforts in disease control strategies especially in endemic areas by failure of the in-use vaccine strain to provide the cross-protection [37]. However, the current vaccines do not prevent the development of a carrier state in the infected animals. Infected cattle and buffaloes can excrete virus for years, regardless whether they had been vaccinated before infection. Animal afflicted with FMD usually do not die, but the disease is debilitating and animals do not recover. The vaccine for FMD only reduces the severity of clinical signs; it does not prevent infection or completely stop transmission of disease [38].

Conclusion

The findings of the present study demonstrated that the circulating FMD virus serotypes in western Uttar Pradesh belong to FMD virus serotype O and A. The FMDV serotype A showed a significant genetic variation from the previously reported FMDV in India. To the authors' knowledge, the circulation of FMDV may pose risk of future FMD outbreaks. The findings of this study recommend

the continuous monitoring of FMDV mutations and evaluation of the FMD vaccines strains currently used in India to avoid the recurrence of FMDV outbreaks

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Conflict of Interest

The authors declare no conflict of interest.

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