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Genetic diversity of FMD virus from outbreak cases in cattle of Kashmir

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Abstract
Foot-and-mouth disease virus (FMDV) is a highly variable virus and is prevalent in the form of several serotypes. In India, where FMDV type 'O' serotype is typically the most prevalent, the disease is endemic and is responsible for the outbreak in the country. In the present study cattle from four districts of Kashmir valley were screened for the presence of FMDV and phylogenetic nature of the FMDV based on VP1 gene was determined. Serotype 'O' was found to be predominant in districts of Kashmir. The sequencing and phylogenetic analysis based on VP1 gene revealed that sequences from the districts of Kashmir showed close relation with each other and with the sequences KJ831717 and KJ831678 from porcine and KR265076 and EU552188 from *Bos Taurus* reported in United Kingdom as all of them shared a more common recent ancestor in their evolutionary history.

Keywords: Foot and mouth disease, 'O' serotype, VP1 gene, phylogenetic analysis

Introduction

The Foot-and-Mouth Disease Virus (FMDV) is a member of the family Picornaviridae and belongs to the genus Apthovirus. It has a linear, single-stranded, positive sense RNA genome that is around 8.5 kb long and is not enclosed. The FMD virus is extremely contagious and infectious in animals with cloven feet, including cattle, pigs, sheep, buffalo, camels, yaks, and many kinds of wild animals. India currently has a high prevalence of the serotypes A, O, and Asia 1, with Serotype O accounting for nearly 85% of FMD incidence in the nation. (Subramaniam *et al*., 2013) [5].

VP1, VP2, VP3, and VP4 are the four main capsid proteins (polypeptides) that the virus produces. VP1, VP2 and VP3 are particularly antigenic and implicated in cell invasion (Sobrino *et al*., 2004) [4]. The VP1 coding region of the FMDV genome has been extensively employed in phylogenetic research to describe the history, transmission, and molecular evolution of the virus (Gurumurthy *et al*., 2002) [2]. Viral RNA-dependent RNA polymerase's lack proofreading capability hence FMDV like the majority of RNA viruses, exhibits a significant amount of genetic variation. The FMD virus can quickly adapt to the selection pressures from the host immune responses according to its genetic heterogeneity, which results in the creation of new variants. The high rates of mutation during RNA replication lead to antigenic variations, which enable FMD viruses to continuously evolve and adapt to new surroundings. Further at specific "hot spot" sections of the genome; genetic recombination between two separate FMDV strains can take place, producing unique variants. Due to FMDV's extreme diversity, it has been divided into serotypes, protectotypes, and genotypes.

Due to severe mortality, morbidity, and productivity losses the disease is proven to be extremely contagious and has a significant economic impact, particularly in cattle and buffaloes. Vesicles, particularly in the epithelia surrounding the mouth, foot, and mammary glands, emerge as a defining feature of the illness. The lesions surrounding the mouth, notably in the lips and tongue, cause the cattle to smack characteristically with the onset of fever (Yoon *et al*., 2012) [8], making them appear dull and dejected. According to Salt *et al*. (1993) [3], the disease's acute phase lasts for around a week before progressively fading away.

The present study was aimed to investigate the genetic diversity of currently prevalent strains of FMD virus in Kashmir and the genotype and serotypes of the FMD virus involved in the FMD outbreaks along with its phylogenetic characterization based on VP1 gene sequence of FMDV. Currently there is lack of scientific information regarding the status of FMD among cattle of Kashmir, its prevalence, epidemiology, economic impact and genomic characterization of different FMD variants important to contemplate the use of vaccines against the disease.

Materials and Methods

Sample collection and processing

A total of fifty samples of FMDV-infected cattle were collected from the districts of Ganderbal, Kupwara, Srinagar, and Pulwama. The samples consisted of vesicular tissue from the lips, foot, and hard palate. Clinical symptoms of the infected cattle included high fever, excessive salivation, and typical fluid-filled vesicular lesions in the mouth, nostrils, feet, and teats, as well as limping, prostration, and death in young animals.

Total RNA was extracted from the samples using Trizol reagent (Sigma, San Diego, USA) as per the manufacturer's instructions. cDNA was made from the RNA by using a Revert Aid cDNA synthesis kit (Thermofisher, USA) and screened for the presence of the FMDV genome by RT-PCR using universal primers to identify the serotype that were responsible for the outbreaks, targeting the VP1 region. The samples that corresponded to the full length VP1 gene fragment amplified the DNA fragments of the predicted size (1200 bp). Sequencing was done on the amplicons. Software applications ClustalW and Mega 6.0 were used to analyze sequence chromatograms. The Sequences were matched using NCBI BLAST software, aligned and compared with published FMDV Serotype-O VP1 sequences available in the gene bank database. The VP1 gene sequences were submitted to GenBank.

RNA extraction

RNA was extracted by using TRIzol Reagent (Sigma, San Diego, USA), from the tissue samples as per the manufacturer's instructions. The tissue samples were further homogenized using a homogenizer in 1.5 ml nuclease-free tubes with 0.75 ml of TRIzol reagent and incubated at room temperature for 5 min. About 0.2 ml of chloroform was mixed with the suspension and vortexed for 15 seconds and then incubated for 10 to 15 minutes at room temperature. The tube was centrifuged at 12,000 g for 15 minutes after being incubated. Without disrupting the interphase, the top aqueous phase was collected, transferred, and incubated on ice for 10 minutes in new tubes containing 0.5 ml of isopropyl alcohol. The quality and quantity of extracted RNA was checked using Nano Drop 2000 spectrophotometer. RNA samples with A280/A260 ratio in range of 1.8 to 2.0 and A260/A230 ratio of 2.0 to 2.2 were deemed pure to be used for cDNA synthesis.

cDNA synthesis

Complementary DNA (cDNA) was synthesized by using Revert-Aid First-Strand cDNA Synthesis Kit (Thermofisher, USA) in a total of 20 µl volume using random hexamer primers. For each reaction; n µl RNA (2µg) and 1 µl of random hexamer primer (0.5 µM) were added. The volume was then adjusted with 12µl of DEPC-treated water. The reaction mixture was subjected to incubation at 65 °C for 5 minute followed by cooling at 4 °C in a thermal cycler. The reaction mixture was chilled on ice and spinned for a brief period of time. 8 µl of cDNA Synthesis Mix was added then centrifuged and collected. The reaction mixture consisted of 40µ 1 of RT buffer, 20 µl of 10 mM DNTP mix, 10 µl of Ribolock RNAase Inhibitor (40 U/µl), and 10 µl of Revert Aid M-Mul V RT (200 U/ µl). The tubes were incubated in thermal cycler at 25 °C for 10 min, followed by incubation for 1hr at 45 °C and finally the reaction was terminated at 95 °C for 5 min. The cDNA synthesized was collected by brief centrifugation and stored at -70 °C for further use.

Amplification of cDNA

The samples were screened initially by PCR in a standard 25µl reaction, containing 10mM dNTPs, 0.5 µl of each primer (Concentration), 2.5µl of 10x PCR buffer, 2 µl of $MgCl_2$, 0.3 µl of Taq polymerase and 3µl of cDNA. PCR was performed using Universal primers (5'-GCC TGG TCT TTC CAG GTC T /3'- CCA GTC CCC TTC TCA GAT C) and then with 'O' primer (5'-ACC AAC CTC CTT GAT GTG GCT 3'-GACATGTCCTCCTGCATCTG) in a thermal cycler. The cycling conditions with universal primers consisted of initial denaturation at 94 ºC for 5 min followed by denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min, followed by extension at 72 ºC for 2 min and final extension at 72 ºC for 10 min. The cyclic conditions with O primer set consisted of initial denaturation at 94 ºC for 5 min followed by denaturation at 94 ºC for 1 min, annealing at 60 ºC for 45 sec, extension at 72 ºC for 2 min and with final extension at 72 ºC for 10 min. The PCR products were analyzed by agarose gel electrophoresis and were purified using the MinElute Gel Extraction Kit from Qiagen (USA) and about 20 µl was sent for sequencing (Ahemdabad, Gujarat's Exceliris laboratory).

Phylogenetic analysis

The four FMDV Serotype-O VP1 sequences (JK1, JK2, JK3 and JK4) from the present study were compared with the three serotype-O VP1 sequences previously reported in India from Hyderabad (accession numbers JX070591), West Bengal (accession numbers AF390726), NDRI, Karnal (accession numbers X99069) and also with the 19 other database sequences reported from the different parts of the world (Gene Bank accession numbers KJ831717, KJ831678, KR265076, EU552188, DQ164978, AJ318850, AJ318833, GU082480, AJ318847, AJ318839, DQ165026, AJ318826, DQ164930, DQ164903, AJ004652, AJ004662, KJ831684, DQ164982 and DQ164874). A distance-based analysis was conducted and phylogenetic tree was generated using Mega 6.0 software. The evolutionary history was inferred by using the Maximum Likelihood algorithm based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyze.

Results

Out of 50 tissue samples obtained, 46 samples were found to be positive, including 17 samples from Pulwama, 8 samples from Ganderbal, 15 samples from Srinagar and 6 samples from Kupwara. A 328 bp segment was amplified in the samples which confirmed the presence of FMDV genome in the samples. Among the four districts Pulwama was found to be highly affected with most number of positive samples and the screening from the four outbreaks revealed serotype O to be predominant and mainly responsible for outbreaks in the valley and none of the samples were detected positive for serotype A or Asia-1 due to small radius of the outbreaks within the districts of Kashmir valley. Though the vesicular samples in the present study originated from the clinically affected animals, the lack of absolute (100%) detection rate may be attributed to the problems in the samples or poor extractions of RNA or cDNA synthesis or inherent sensitivity of the RT-PCR. Figure 1 shows the amplification of a 328 bp segment which confirmed the presence of FMDV genome in

the samples. Figure 2 shows fragments of the expected size (1200bp) amplified in FMDV positive samples with 'O' primer for VP1 gene.

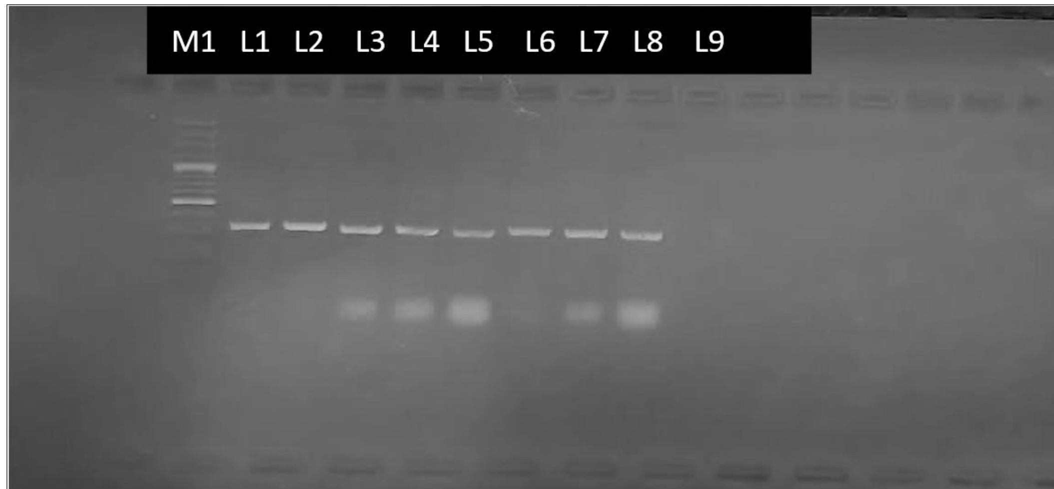

Fig 1: Detection of FMDV serotype in the samples from the cattle: Agarose gel-electrophoresis of PCR products showed general amplification of VP1 gene segment (328bp). Lane M1: Molecular weight marker, Lane 1to 8: Amplification of 328 bp product in samples confirmed the presence of FMDV genome. Lane 9: Negative control

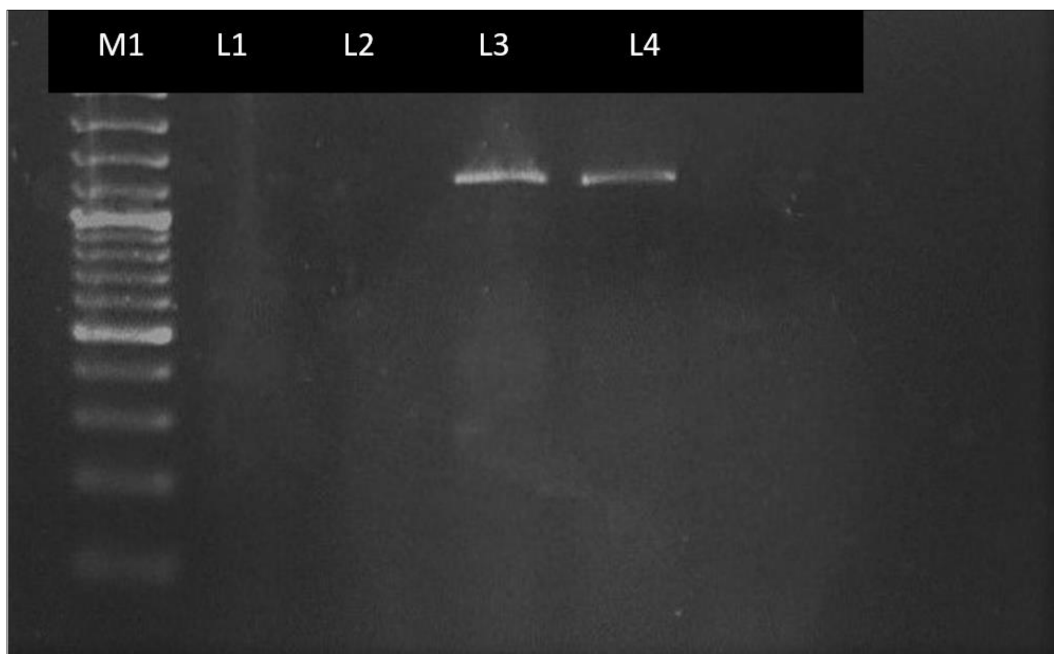

Fig 2: Detection of FMDV serotype in the samples from the cattle: Agarose gel-electrophoresis of PCR products showed characteristic amplification of VP1 gene (1200bp). Lane M1: Molecular weight marker, Lane 1: Negative control. Lane 3 and 4: Amplification of Serotype O (1200 bp) product in positive sample

The NCBI BLAST of all the sequences obtained in the present the study confirmed that all sequences belonged to Serotype-O VP1 sequences of FMD virus sharing 91%-99.95% identity with the database sequences. The phylogenetic tree based alignment of serotype-O VP1 sequences revealed that all the four sequences (JK1, JK2, JK3 and JK4) were sister taxa closely related to each other that were decedent from a more common recent ancestor and were grouped in a single clad. Evolutionary history as inferred from the phylogenetic tree also suggested the close relationship between all the four sequences (JK1, JK2, JK3 and JK4) with the sequences of KJ831717 and KJ831678 from porcine and KR265076 and EU552188 from *Bos Taurus* reported in United Kingdom as all of them shared a more common recent ancestor in their evolutionary history. All the four sequences from this study were distinctly clustered from sequences previously reported in India from Hyderabad (accession numbers JX070591), West Bengal (accession numbers AF390726), NDRI, Karnal (accession numbers X99069) and were not closely related.

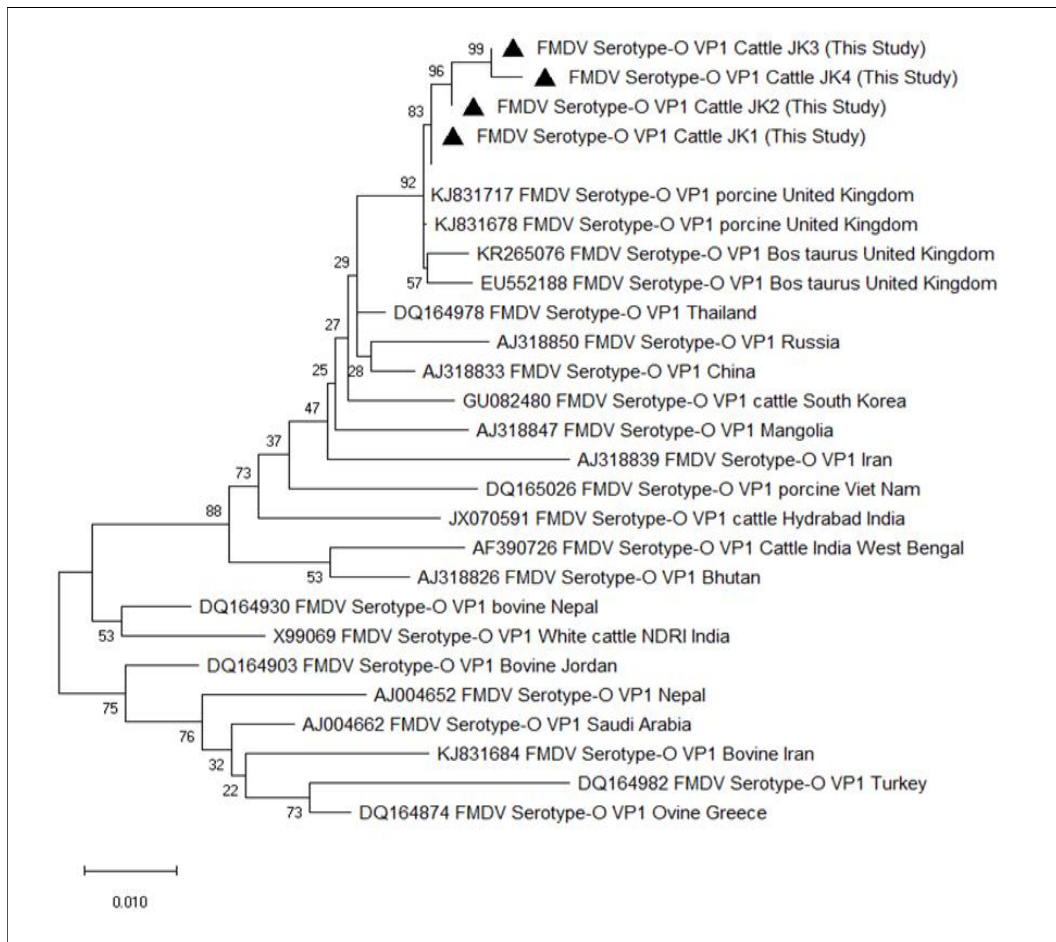

Fig 3: The evolutionary history was inferred by using the Maximum Likelihood algorithm based on the Tamura-Nei model

Discussion

FMD continues to impact more than hundred countries, including India and the disease's distribution closely reflects the economic development of the country. The antigenic diversity of the virus complicates attempts to control FMD by immunization. The persistence of virus in the environment and the quasispecies nature of the RNA genome, which tends to accumulate mutation at greater rates, are the main contributors to the emergence of novel variants (Tully and Fares, 2009) [7].

However, the molecular nature of FMDV strains prevalent in Kashmir region has not been characterized so far. This study explains the characteristics of the FMDV strains that circulates in Kashmir Valley and establishes their evolutionary link to other FMDV strains that are widespread around the world. Due to convenience, expense, and time restrictions, PCR-based tests have largely taken the role of conventional diagnostic techniques for FMDV identification. By using a universal primer set and an RT-PCR-based test targeting the VP1 gene region, the presence of FMDV in vesicular lesions was confirmed in the current investigation.

Although VP1 gene is highly variable, we targeted the conserved region of VP1, to enable the detection of any serotype or variant of the virus. Of the 50 samples screened, 46 samples were detected positive with PCR assay accounting for 92% detection rate. Though the vesicular samples in the present study originated from clinically affected animals, the lack of absolute (100%) detection rate may be attributed to the problems in the samples or poor extraction of RNA or cDNA synthesis or inherent sensitivity of the RT-PCR. To determine which serotype was involved in the outbreaks, we carried out RT-PCR based assay using serotype –specific primers targeting VP1 region. All the samples were detected positive for serotype “O” and none of the samples were detected positive for Serotype ‘A’ or ‘Asia -1’ due to small radius of the outbreaks within the districts of Kashmir valley.

To study the evolutionary relationship of the FMDV strains circulating strains in Kashmir region with the strains circulating in other parts of the world, we sequenced the 1200bp VP1 region of FMDV genome. The choice for VP1 region was due to highly polymorphic nature (Carrillo *et al*., 2005) [1]. Phylogenetic analysis was performed and sequence chromatograms were analysed. A distance-based analysis was conducted and phylogenetic tree was generated using Mega 6.0 software.

Evolutionary history inferred from the phylogenetic tree suggested the close relationship between all the four sequences (JK1. JK2, JK3 and JK4) with the sequences KJ831717 and KJ831678 from porcine and KR265076, KR265067 and EU552188 from Bos Taurus reported in United Kingdom as all of them shared a more common recent ancestor in their evolutionary history. All the four sequences from the study were distinctly clustered from the sequences previously reported in India from Hyderabad (accession number X99069), West Bengal (accession number AF390726), NDRI Karnal (accession numbers X99069) and were not closely related. It was inferred from the cladogram that the virus isolates from the present study were more recently evolved while virus sequences previously reported from India (accession numbers JX070591, AF390726, X99069) were diverged far earlier from the common ancestor in the evolutionary past.

Conclusion

FMD has been found to be common as indicated by the consequential outbreaks attended during the study period. The etiology of the disease was confirmed as FMD virus based on the clinical signs, PCR amplification, nucleotide sequencing and serotype detection. This study documents the nature of the circulating FMDV strains in Kashmir valley and determined their phylogenetic relationship with other FMDV strains prevalent around world. Serotype ‘O’ was found to be prevalent within the districts of Kashmir. Furthermore, phylogenetic analysis revealed close relationship between all the four sequences and with other sequences reported worldwide especially with the KJ831717 and KJ831678 sequences from porcine and KR265076, KR265067 and EU552188 sequences from Bos Taurus reported in United Kingdom and with the ones reported in India.

In Kashmir, FMDV outbreaks are being constantly observed in cattle and other susceptible hosts. The occurrence of diseases like FMD is affecting the economic status of the farmers. There is lack of scientific information regarding the status of FMD among cattle of Kashmir, its prevalence, epidemiology, economic impact and genomic characterization of different FMD variants as it is important to contemplate the use of vaccines against the disease. The data generated in the present study provides an insight to transmission and epidemiology of the FMDV virus among the cattle population of Kashmir and shall help in formulating the control strategies against the disease.

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