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Sequencing and Translational Analysis Revealed Huge Mutation in the N-Terminus End of Leader Proteinase (Lpro) Gene of Foot and Mouth Disease Viruses Isolated From Cattle in Bangladesh

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Abstract: *Foot and Mouth Disease virus (FMDV) comprises four structural and ten nonstructural proteins in its genome. The Leader proteinase (Lpro) is structurally and functionally related to papain-like cysteine proteinase with catalytic cystein and histidine residues which is the first functional component of the Aphthoviral polyprotein. Complete Lpro genes of six Bangladeshi isolates of FMDV of three different serotypes were sequenced and compared with each other as well as with those sequences available in the GenBank to evaluate the extent of mutation in this gene. Out of six isolates investigated a serotype O viruses (BD_SI_5_2013) showed highest level of amino acid (aa) substitution with a critical substitution at L_{10} by V_{10}. The Lpro gene of the investigated viruses showed mutation in 9% (≤) nucleotide and substitution of aa in 11.4% position. A total of 55% variability of aa was seen in the N terminus end between first two conserved initiation codons at 1st and 29th aa positions of Lpro sequences. Methionine at position 1, 29, 126 and 132 are conserved in the Lpro sequences. The L_{ab} form of the Lpro was found more variable than L_b form. Conserved KRLK/R sequence was found at Lpro/VP4 cleavage site at the C terminus end of Lpro in all the isolates. The invariant motif, the catalytic triad and other critical amino acids were totally conserved. Specific clustering of viruses on the basis of serotype as well as geographical origin was not found in the phylogenetic trees constructed but the viruses had lineage specific signature clustered together in Maximum Likelihood (ML) tree.*

Keywords*: Clustering, FMDV, Lpro gene, Sequencing, Substitution of amino acid*

I. Introduction

FMD is a highly contagious viral disease of cloven hoofed ruminants caused by FMD virus (FMDV) showing symptoms like fever, formation of vesicles on the mouth, tongue, interdigital spaces and teats (Shanmugam et al., 2015, APHIS, 2007). The disease is endemic in Bangladesh and frequently confer epidemic tremor with a gap of few years. Annual losses due to FMD in Bangladesh were estimated at about US$62 million (FAO/OIE, 2012). Direct loss in lactating animals is 15% of the total yield. Moreover, the disease is a cause of infertility, abortion, calf mortality and reduced efficiency of work animals. Affected animals hardly attain their original production potential (Rubina et al., 2002). The etiological agent is a single stranded RNA virus of the Aphthovirus genus, family Picornaviridae, occurring in seven serotypes (O, A, C, Asia-1, SAT1, SAT2, and SAT3) and more than 65 subtypes (Saiz *et al*., 2002; Brown, 2003). FMDV consists of a single-stranded, plus sense RNA genome of approximately 8,500 bases surrounded by four structural proteins that form an icosahedral capsid. The genome contains 5′ UTR (untranslated region), 3′UTR and a long open reading frame (ORF) which can be translated into a single polyprotein, that can be cleaved into four (VP4, VP2, VP3 and VP1) structural proteins and 10 (L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D) non-structural proteins (Feng, 2004).

The Leader proteinase (Lpro) is a structurally and functionally related papain-like cysteine proteinase gene with catalytic cystein and histidine residues which is the first functional component of the FMDV polyprotein that plays a significant role in the cleavage of the viral polyprotein at the L/P1 junction. In the cleavage of host protein initiation factors e1F-4F Lpro involved in translation of capped mRNAs, and evasion of the host innate immune response (Shanmugam et al., 2015; Piccone et al., 2011; Piccone et al., 2010; Guarne et al., 2000; Medina et al., 1993; Strebel and Beck, 1986). The Lpro gene is situated at the extreme 5′ end of the coding region of FMDV RNA that contains the first functional initiation codon (AUG) for the whole ORF at position 01. Another AUG codon is present at 84 bases apart in the same ORF (Sanger et al., 1987; Belsham 1992).The N terminus end of the Lpro sequences showed increased amino acid variations with 2 conserved start codons at 1st and 29th site. Lpro gene showed its high variability in the nucleotide sequences as comparable to that of the structural proteins (George et al., 2001; Carrillo et al., 2005). Among the sequences of two forms of Lpro, the L_{ab} showed a high variability than the L_b form. All the critical amino acid residues of the active cleft site are conserved. The phylogenetic analysis of the Lpro region sequences showed a difference in clustering of isolates as observed with the VP1 capsid coding region sequencing. Thus, the Lpro region phylogeny could be

used for the comparison of the FMDV isolates (Shanmugam et al., 2015). Therefore, to analyze the extent of mutation present in Lpro genes and as well as to evaluate phylogenetic position of Bangladeshi FMDV, six FMDV isolates were sequenced and the sequenced data were compared with other Lpro sequences available in GenBank.

II. Materials And Methods

2.1 Viruses

Six virus isolates collected from cattle of FMD outbreak areas (3 O, 2 Asia1 and 1 A type) were used in this study (Table 1). A number (N= 18) of Lpro sequences available in GenBank were included in this study for comparison and analyses.

Table 1: The FMD viral serotypes isolated from field outbreaks. The Lpro genes were amplified and sequenced. Following editing the gene sequences was submitted in GenBank

Virus Isolate	Serotype	Collection time	Place (district/ country)	Host	GenBank Accession No.
BD_SI_5_2013	O	September,2013	Tangail, Bangladesh	Cattle	KR814825
BD_Gh_2_2013	O	September,2013	Tangail, Bangladesh	Cattle	KR814826
BD_SI_6_2013	O	September,2013	Tangail, Bangladesh	Cattle	KR814827
BD_SI_2_2013	Asia1	September,2013	Tangail, Bangladesh	Cattle	KR814824
BD_2M_3_2013	Asia1	September,2013	Tangail, Bangladesh	Cattle	KR814828
BD_SI_16_2013	A	September,2013	Pabna, Bangladesh	Cattle	KR814829

2.2 RNA Extraction and RT-PCR

The total RNA was extracted from the oral/padal epithelial tissue homogenates using Viral Nucleic Acid Extraction Kit II (Geneaid Biotech Ltd., Taiwan) as per manufacturer's instructions. The purity and concentration of extracted viral RNA were measured by using spectophotometry (A^{260}/A^{280}). Extracted RNA was then subjected to RT-PCR amplification of full length Lpro gene of FMDV using designed primer pairs. FMD LproF (5'-cttctacgcctgaataagcg-3') and FMD LproR (5'-gatgatacttcccgtgttgc-3') primers were designed using the sequence downloaded from GenBank. The RT-PCR was conducted by using SuperScript III one step RT-PCR kit with Platinum Taq (Invitrogen). The RT-PCR was carried out in 50µl volume containing of 2X reaction mixture, forward and reverse primers (20pmol in each), Taq polymerase (1 µl), RNAse out (1 µl), nuclease free water (16 µl) and l RNA template (150-200ng in 5 µl/reaction).The reverse transcription (RT) was carried out in a thermocycler (eppendorf, Germany) at 45°c for 45 minutes. A total of 35 cycle of PCR amplification was carried out with an initial denaturation at 94°c for 5 minutes. The cycling condition consisting of denaturation at 94°c for 1 minute, annealing at 52°c for 1 minute and extension at 72°c for 2 minutes. After final extension at 72°c for 7 minutes, the reaction was hold at 4°c. The PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide with a transilluminator (Alpha imager, USA).

2.3 Sequencing of PCR products

The amplified PCR products/gel of Lpro segment were cleaned with Wizard SV gel and PCR clean-up system (Promega). The gene cleaned PCR products were then sequenced commercially from 1st Base, Malaysia.

2.4 Sequence Analysis

The raw sequence data were first checked for its quality and then edited and assembled with the programmes Chromas Lite, EditSeq and MegAlign. Edited sequences were aligned with MegAlign or MEGA6 programmes. Multiple alignments were done with Clustal W algorithm and Neighbour-Joining as well as Maximum Likelihood phylogenetic trees were constructed with MEGA6 programme. The stability of the nodes in the phylogenetic trees was tested by bootstrapping with 1000 replications. Translational analysis was carried out using MegAlign program to evaluate the level of mutation in Lpro genes and for comparison.

III. Result And Discussion

A total of six FMD viral isolates belonging to serotypes O (N=3), Asia1 (N=2) and A (N=1) were investigated in this study to know the extent of variation in the Lpro gene and phylogenetic analysis based on the base sequences in Lpro gene. Results of RT-PCR showed amplification of 730bp product (Figure 1) specific to full length sequence of Lpro gene. FMDVs alone encode a functional Leader proteinase as the first component of the viral polyprotein among the picornaviruses. The Lpro gene of FMDV comprised 603 nucleotides and deduced amino acid sequence consisted of 201 residues (George et al., 2001). The Lpro gene of FMDVs of this study also comprised of 603 nucleotides and 201 amino acid residues in each (Figure 3-4). NCBI (BLAST/Nucleotide) sequence study of full length Lpro gene of different FMDV serotypes also supported this finding. Results of sequencing of Lpro gene showed more than 91% sequence identity among the studied

isolates against 89% sequence identity at nucleotide level with the isolates studied earlier in Bangladeshi and Indian (George et al., 2001; Shanmugam et al., 2015).

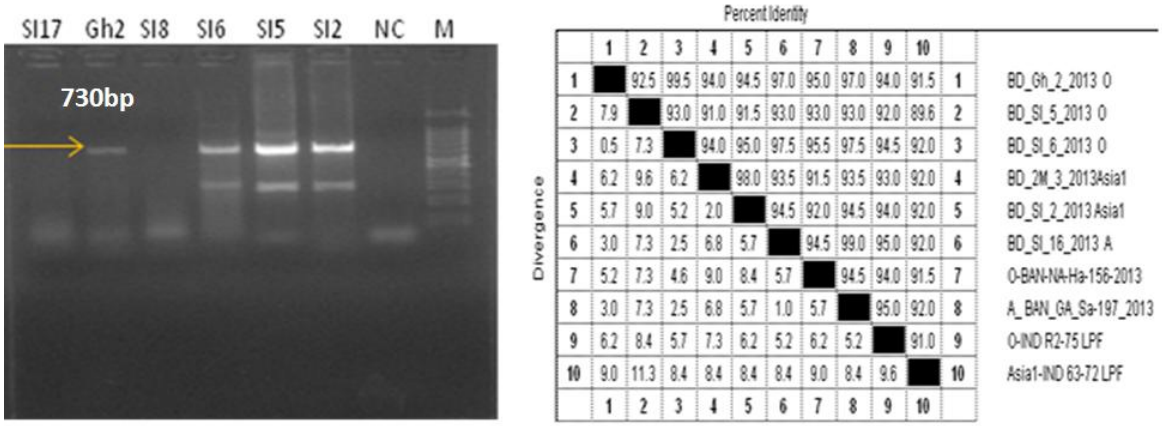

Figure 1: Amplification of Lpro gene (left) by RT-PCR and evaluate percent identity and divergence of the nucleotide in Lpro gene (right).

Among the six isolates, BD_SI_5_2013 showed the highest substitutions of amino acid (N=12). A conserved position in M_1, C_6, A_9 and L_{10} was reported earlier (Mohapatra et al., 2009) which was also invariant in the studied isolates except BD_SI_5_2013 where L_{10} was replaced by $V_{10.}$ Out of 201 amino acid residue in Lpro gene, 23 positions (11.4%) were capable of tolerating residue replacements (Table-2) which was less than the findings (39.8%) of George et al. (2001); this could be due to less number of isolates compared in this study.

Translational analysis of Lpro gene containing two initiation codons (AUG) at position 1 and 29 in all six Lpro gene sequences of Bangladeshi isolates which is a characteristic feature of FMD viruses (Sanger et al., 1987). The percent identity and divergence among the studied isolates were estimated as 91-99.5% and 0.5-9% respectively. While compared with other Bangladeshi and Indian isolates, those were 89.6-99.5% and 0.5-11.3% respectively (Figure 2). Lpro is known to exist in L_{ab} and L_b form which are both identical except a difference in the start codons at the N terminus end that is used to initiate translation (Medina et al., 1993; George et al., 2001). Lpro region shows high variability in the nucleotide sequences as comparable to that of the structural proteins (George et al., 2001; Carrillo et al., 2005). A high amount of aa (70-87%) variability was seen in the N terminus end between the two initiation codons among the isolates (George et al., 2001; Mohapatra et al., 2009; Shanmugam et al., 2015). In this study, between the first two initiation codons, 43% amino acid positions were variable that represented about 55% amino acid variability in the complete Lpro sequences among six isolates, but when compared with other downloaded Lpro sequences (N=16) it showed 82% amino acid variability. The L_{ab} form of the leader protein was found more variable than Lb form. Zhu et al. (2010) also reported that the both L_{ab} and L_b form of Lpro showed amino acid variability with high mutation rates. The L_{ab} form of the leader protein was relatively more variable than that of L_b form (Sanger et al., 1987; George et al., 2001). The invariant motifs ($H_{148}AVF_{151}$, $P_{182}YD_{184}$ and $N_{50}CWLN_{54}$) between FMDV and equine rhinitis A virus reported earlier by Hinton et al. (2002) remain unchanged in the Lpro gene of FMDV in this study.

An identical amino acid sequence of KRLK/R ↓GAG at the Lpro/VP4 cleavage site at the C terminus end of Lpro was observed previously and found conserved among the serotypes (Seipelt at al., 1999; George et al., 2001). Conserved KRLK/R (R in BD_SI_2_2013) sequence was found at this site in all the isolates used in this study. By the molecular modeling and energy optimization studies Mayer et al. (2008) found that either lysine or methionine at the 143rd amino acid residue was critical for the restricted specificity of Lpro at the Lpro/VP4 cleavage site. Lysine was found as 143rd amino acid residue in all the six Lpro sequences studied. Three isolates (SI_2, SI_5 & 2M_3) contained Aspartic acid (D) at 157th amino acid position where as other contained Asparagine (N) which is the lineage specific signature in the amino acid position 157 described earlier by Mohapatra et al. (2009)

The catalytic triad formed by cysteine, histidine and aspartic acid residues at the 51st, 148th and 163rd amino acid sites respectively is critical for Lpro activity (Roberts and Belsham, 1995). The orientation of H_{148} with respect to C_{51} is maintained by the hydrogen bonds of D_{163} (Piccone et al., 1995; Seipelt et al., 1999; Guarne et al., 1998). Any mutation of amino acid at these residues results the loss of activity of Lpro (Roberts and Belsham, 1995). C_{51}, H_{148} and D_{163} amino acid residues were found conserved in all the FMDV isolates analyzed in this study. E_{76} that is involved in Lpro autocatalysis (Piccone et al., 1995) was althrough invariant in this study. E_{96} makes electrostatic interaction with K_{201} and G_{98}, E_{147} residues involved in hydrogen bonding with L_{200} and K_{201} respectively which is a conserved feature in substrate binding whereas the environment of catalytic residue D_{163} includes a continuous pattern of H-bonds involving D_{163},H_{148}, Y_{168} and K_{144} (Mohapatra et

al., 2009). In this study E_{76}, E_{96}, G_{98}, K_{144}, E_{147} and Y_{168} residues were found conserved. The side chain of L_{200} is completely buried in a hydrophobic pocket formed by the residue W_{52}, $G_{97}GPP_{100}$, $E_{147}HA_{149}$ and L_{178}. The conserved acidic cluster in the S-binding region formed by $D_{163}DED_{166}$ residues was an agreement with the findings of Mohapatra et al. (2009). Guarne et al. (1998) reported that the presence of N_{46} along with D_{49}, N_{54} and D_{164} are also essential for the structural stability and activity of the Lpro enzyme. The side chain of N_{46} is fixed by H-bonding with D_{49} and D_{164} residues and D_{164} also interacts with P1 arginine in e1F-4G for its cleavage (Mohapatra et al., 2009). All the aa residues (N_{46}, D_{49}, N_{54} and D_{164}) were found conserved in the Lpro sequences compared in this study. C_{133} involved in substrate recognition along with C terminus extension (CTE) of L_b^{pro} (183-195) and D_{164} modulated the electrostatic charge at the above catalytic site. Cysteine at 6^{th}, 133^{rd}, 153^{rd} and histidine at 109^{th}, 138^{th} (crucial for e1F-4G cleavage), 148^{th} amino acid position respectively were

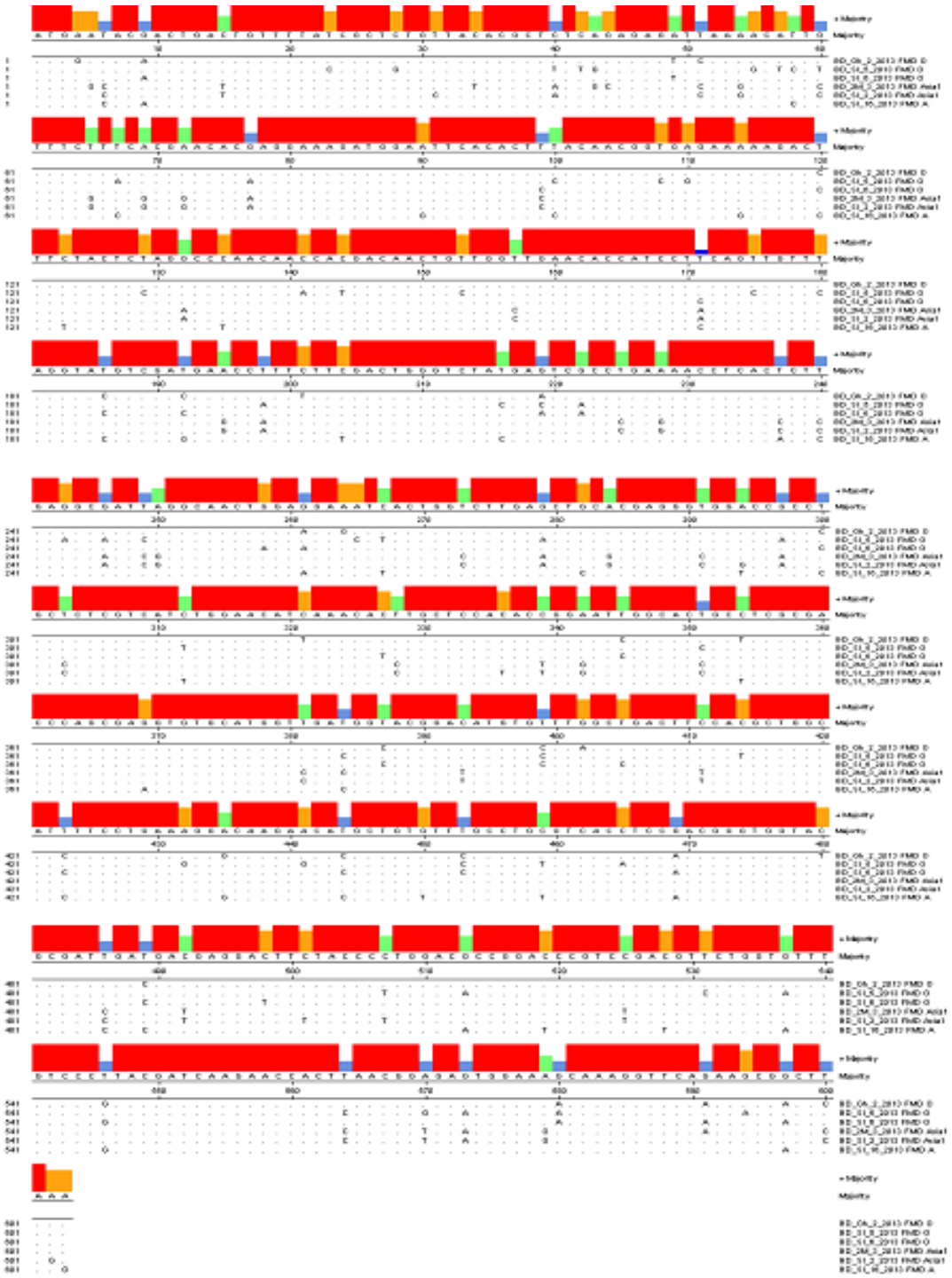

Figure 2: Comparison of complete nucleotide sequences of Lpro gene of six Bangladeshi FMDV isolates.

Figure 3: Comparison of complete deduced amino acid sequences of Lpro gene of six Bangladeshi FMDV isolates.

Highest substitution was observed in BD_SI_5_2013 isolate. Maximum residue substitutions occurred in between the first two (1&29) initiation codons found conserved among all isolates in this study as reported earlier by Piccone et al. (1995), Roberts and Belsham (1995). T_{55} and E_{147} amino acid residues related to cleavage and activity of Lpro (George et al., 2001; Mohapatra et al., 2002) were found conserved in all the Lpro sequences compared in this study.

Table 2: Position wise amino acid substitution in Lpro gene of Bangladeshi isolates. 22 out of 40 substitutions occurred in between 1st and 29th amino acid positions. Highest substitutions (N=12) occurred in BD_SI_5_2013 among six isolates.

aa position	**2**	**8**	**10**	**12**	**14**	**15**	**17**	**19**	**20**	**22**	**23**	**26**	**34**	**37**	**47**	**64**	**73**	**84**	**89**	**95**	**157**	**194**	**201**	**Total**
Majority	**N**	**I**	**L**	**H**	**L**	**R**	**I**	**T**	**L**	**L**	**S**	**Q**	**Y**	**E**	**N**	**D**	**E**	**R**	**I**	**H**	**D**	**A**	**K**	**23**
BD_Gh_2_2013	**D**	**I**	**L**	**H**	**L**	**R**	**F**	**T**	**L**	**L**	**S**	**R**	**Y**	**E**	**N**	**D**	**E**	**R**	**I**	**H**	**N**	**A**	**K**	**4**
BD_SI_5_2013	**N**	**T**	**V**	**H**	**F**	**G**	**I**	**A**	**L**	**L**	**T**	**Q**	**H**	**G**	**K**	**D**	**D**	**R**	**L**	**H**	**D**	**T**	**K**	**12**
BD_SI_6_2013	**N**	**I**	**L**	**Y**	**L**	**R**	**F**	**T**	**L**	**L**	**S**	**R**	**Y**	**E**	**N**	**D**	**E**	**R**	**I**	**H**	**N**	**T**	**K**	**5**
BD_2M_3_2013	**S**	**I**	**L**	**H**	**I**	**A**	**I**	**T**	**F**	**R**	**S**	**Q**	**Y**	**E**	**N**	**D**	**E**	**G**	**I**	**R**	**D**	**T**	**K**	**8**
BD_SI_2_2013	**N**	**I**	**L**	**H**	**I**	**R**	**I**	**T**	**F**	**R**	**P**	**Q**	**Y**	**E**	**N**	**D**	**E**	**G**	**I**	**R**	**D**	**A**	**R**	**7**
BD_SI_16_2013	**N**	**I**	**L**	**H**	**L**	**R**	**I**	**T**	**L**	**L**	**S**	**R**	**H**	**E**	**N**	**E**	**E**	**R**	**I**	**H**	**N**	**A**	**K**	**4**

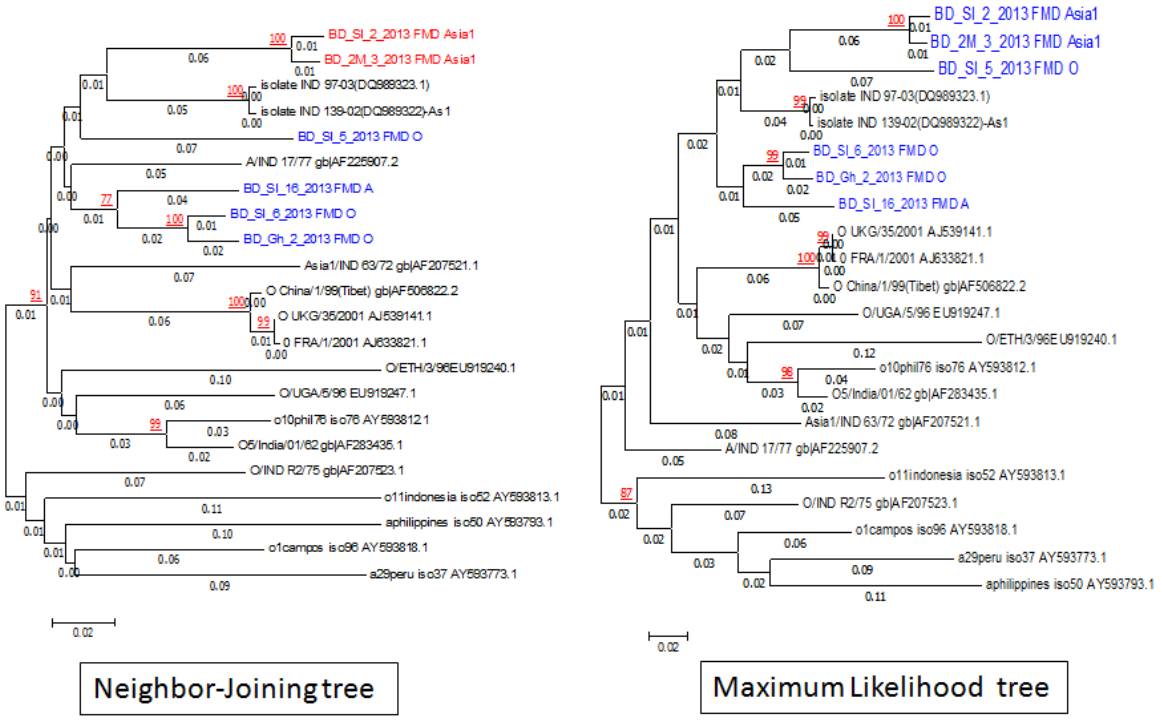

Figure 4: The Phylogenetic trees showing relationship among Lpro genes of FMDVs. The branch lengths presented next to branches indicate the number of nucleotide substitutions per site as well as the evolutionary distances used to infer the phylogenetic tree (N-J tree). The percentage (above 70%) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

The Lpro gene sequences of the study viruses (N=6) and other viruses (N=16) downloaded from GenBank was used to construct Neighbour-Joining (N-J) and Maximum Likelihood (M-L) phylogenetic trees (Figure 5). Absence of specific clustering of viruses on the basis of serotype as well as geographical origin found in the phylogenetic trees constructed in this study was an agreement with George et al. (2001), Shanmugam et al. (2015) and Chitray et al. (2014). Two Asia1 isolates clustered together with some Indian Asia1 isolates. FMDV A and two FMDV O isolates clustered together with some Middle East-South Asian (ME-SA) isolates irrespective to serotype. On the other hand, one FMDV O (BD_SI_5_2013) isolate took separate place apart from other two FMDV O isolates in N-J tree but in M-L tree it clustered together with Asia1 isolates. In the ML tree, two Asia1 and one O (BD_SI_5_2013) isolates clustered together and on the other hand, two O and one A isolates clustered together due to have the lineage specific signature D and N respectively in the amino acid position 157 that was described earlier by Mohapatra et al. (2009).

IV. Conclusion

L^{pro} isoforms have indistinguishable activities and specificities and played a role in virulence through the regulation of host interferon responses. The L^{pro} gene also contributed in the phylogenetic analysis of the viruses. There are two methionines in the L^{pro} sequence of the virus (aa position 1 and 29) are invariant, indicating that two methionine in L^{pro} isoforms are significant for aspects of FMD viral biology. The catalytic triad formed by cysteine, histidine and aspartic acid residues at the 51^{st}, 148^{th} and 163^{rd} amino acid sites respectively is critical for Lpro activity and found conserved. Results of position wise aa substitution in Lpro gene of Bangladeshi isolates showed that 22 out of 40 substitutions occurred in between 1^{st} and 29^{th} aa positions. Highest substitutions (N=12) was seen in BD_SI_5_2013 among six isolates. The aa residues N_{46}, D_{49}, N_{54} and D_{164} were conserved in the Lpro sequences in the studied viruses. Cysteine at 6^{th}, 133^{rd}, 153^{rd} and histidine at 109^{th}, 138^{th}, 148^{th} aa position was also conserved among all isolates. These data indicate that despite a wide range aa substitution in L^{pro} sequence in the FMDV isolates examined, functional elements such as the catalytic sites and cleavage sites and previously described motifs are invariant. The variability observed at the N and C termini of L^{pro} sequence may affect specific host-virus interactions, including ribosomal recognition of alternative start codons and virulence of the viruses, required further study.

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