Phylogenetic analysis of the leader proteinase (L^{pro}) region of Indian foot and mouth disease serotype O isolates

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Foot-and-mouth disease virus,
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Phylogenetic analysis,
Serotype O.

Summary
In this study, the nucleotide sequences of the complete leader proteinase (L^{pro}) region of 21 isolates of foot-and-mouth disease virus (FMDV) serotype O collected during various outbreaks in India were sequenced and compared with vaccine strains. The phylogenetic analysis of these L^{pro} sequences showed a difference in the clustering of the isolates based on the VP1 capsid coding region sequences. The comparison of amino acid sequences at the N terminus end of the L^{pro} region showed very high variability, although 2 conserved start codons (AUG) at 1st and 29th sites. Furthermore, all the amino acid residues that formed the active cleft site of the L^{pro} sequences of this study were conserved. These results suggest that L^{pro} sequences could also be used for phylogenetic comparison of FMDV isolates.

Studio filogenetico di ceppi indiani del virus dell'Afta epizootica Sierotipo O mediante analisi della regione proteinasi leader (L^{pro})

Parole chiave
Analisi filogenetica,
Regione proteinasi leader (L^{pro}),
Sierotipo O,
Virus dell'afta epizootica.

Riassunto
In questo studio è stato effettuato il sequenziamento completo della regione proteinasi leader (L^{pro}) di 21 ceppi del virus dell'afta epizootica, sierotipo O, isolati in India nel corso di diversi focolai. Le sequenze nucleotidiche della regione L^{pro} sono state confrontate con le sequenze omologhe dei ceppi vaccinali. L'analisi filogenetica condotta sulle sequenze L^{pro} ha rivelato una differente distribuzione dei ceppi nei cluster rispetto a quella ottenuta dall'analisi della sequenza nucleotidica codificante la proteina del capside VP1. Il confronto fra le sequenze amminoacidiche della porzione N terminale della regione L^{pro} ha mostrato un'elevata variabilità con la sola presenza di due codoni AUG conservati in posizione 1 e 29. È importante notare che tutti i residui amminoacidici che formano il sito attivo di taglio delle sequenze L^{pro} analizzate in questo studio risultano conservati. I risultati ottenuti suggeriscono che la sequenza L^{pro} potrebbe essere utilizzata per effettuare studi filogenetici sugli isolati del virus dell'Afta epizootica.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven foot animals caused by FMD virus (FMDV) belonging to the genus *Aphthovirus* in the family *Picornaviridae*. The disease is characterized by vesicular lesions on the mouth, tongue, teats, and inter-digital spaces. This disease is economically important as its incidence causes severe financial losses due to the national and international trade restrictions (Depa *et al.* 2012). There exist 7 serotypes of FMDV: O, A, C, Asia1, Southern African Territories (SAT) 1, SAT2 and SAT3. Among these serotypes, serotype O has been reported in all the FMDV prevalent

continents, along with serotype A (Rweyemamu *et al.* 2008); whereas, the other serotypes are restricted to certain geographical boundaries at least until recently. Since 2000, occasional spillovers of SAT1 and SAT2 viruses have been found in Egypt, Libya and other countries in the Middle East (Ahmed *et al.* 2012, Valdazo-González *et al.* 2012).

The genome of FMD consists of a positive sense RNA (8.2 kb) that codes for a single polyprotein, which undergoes sequential cleavage to yield 4 structural proteins, *i.e.*: viral protein1 (VP1), VP2, VP3, and VP4 along with 8 non-structural proteins: (NSPs), L(a)b, 2A, 2B, 2C, 3A, 3B, 3C, and 3D. The structural proteins assemble to form an icosahedral capsid of the virion enclosing the positive sense RNA. Antigenic variants arise during replication, due to its error prone viral replication cycle (Domingo and Holland 1977), inside the host cell.

The processing of the polyprotein starts with the self-cleavage of leader proteinase (L^{pro}) and $3C^{pro}$ from the FMDV polyprotein (Strebel and Beck 1986). The L^{pro} plays a significant role in the cleavage of host protein initiation factors and evasion of the host innate immune response (Medina *et al.* 1993, Piccone *et al.* 2010, Piccone *et al.* 1995, *et al.* 2011); L^{pro} is known to exist in 2 forms, L^{ab} and L^{b} (Sangar *et al.* 1987), which were both found to be active with indistinguishable deeds. Both forms of proteins are identical except at the N terminus end with a difference in the start codons used to initiate translation (Medina *et al.* 1993). The FMDV L^{pro} is structurally and functionally related to papain-like cysteine proteinase, with catalytic cysteine and histidine residues (Skern *et al.* 1998, Guarne*et al.* 2000, Guarne *et al.* 1998). L^{pro} region has also shown high variability in the nucleotide sequences as comparable to that of the structural proteins (Carrillo *et al.* 2005, George *et al.* 2001).

Epidemiological characterization of the FMDV isolates is frequently reported by the sequencing of complete or partial capsid coding regions of the genome (Jamal *et al.* 2011, Waheed*et al.* 2011, Yuvaraj*et al.* 2013). Use of the NSP region, L^{pro} for epidemiological studies had been reported earlier in the literature, as well as phylogenetic analysis of the L^{pro} region in Indian serotype A and Asia1 isolates (Mohapatra *et al.* 2009, Mohapatra *et al.* 2008, Mohapatra *et al.* 2002). This paper describes the genetic characterization of Indian type O isolates with the L^{pro} region sequences.

Materials and methods

Viruses

This study focused on serotype O FMD field strains (n = 21) that were isolated from various outbreaks in India between 2002-2010 and maintained as master banks in the virus repository of FMDV Laboratory, Research and Development Centre, Indian Immunologicals Limited (IIL), Hyderabad, Telengana. Details of the field viruses used in this study are shown in Table I. For analysis, some of the VP1 and L^{pro} sequences available in GenBank were included in the study (Nagendrakumar *et al.* 2009, Yuvaraj *et al.* 2013).

RNA extraction and PCR

The RNA was extracted from the master bank stock with RNeasy kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The primer pair L01F (GTGCCCCAGTTTAAAAAGCTT) and DH6 (TTGTTCTGAGTGTTGGTTGTGTG) was used for the amplification of L^{pro} region of the FMDV genome (Piccone *et al.* 1995, Sabarinath 2001) using OneStep RT-PCT kit (Qiagen, Hilden, Germany). The extraction process was conducted following the same conditions of the RT-PCR reaction, 1 cycle of reverse transcription conditions of 50 °C for 30 minutes, and 95 °C for 15 minutes followed by 30 cycles of 94 °C for 1 minute, 50 °C for 1 minute and extension of 72 °C for 2 minutes and finally 1 cycle of final extension of 72 °C for 10 minutes.

Sequencing and analysis of genome

Amplified PCR products were used in direct DNA sequencing using BigDye Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems, Grand Island, NY, USA) as per the manufacturer's instruction. The PCR products were purified with Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequencing was carried out in automatic cycle sequencer (ABI Prism, Applied Biosystems, Grand Island, NY, USA). These sequences were aligned with Clustal X 1.8 programs (Thompson *et al.* 1994) with a few reference sequences like O/IND/R2/75, O_1/Manisa/Turkey/69, O_5/India/1/62 (GenBank Accession numbers AF207523, AF283435 and AF283435, respectively). The Mega 5.05 program (Tamura *et al.* 2011) was used for construction of the neighbour-joining (NJ) tree with Kimura 2-parameter model with uniform rates and bootstrap of 1000 replicates. The L^{pro} sequences of this study were compared with a few serotype A viruses available in GenBank (Nagendrakumar *et al.* 2009). Furthermore, VP1 sequences of the corresponding serotype A viruses were also used to construct a phylogenetic tree for comparison.

Results and discussion

Twenty-one isolates of FMDV serotype O collected from various outbreaks in India were used in this study for the phylogenetic analysis of L^{pro} region

Table I. *Details of the field viruses analysed in the study.*

S. No	Virus	Source						
		Village	District	State	Animal	Tissue	Vaccination	L^{pro} Accession Number
1	O/MER/57/2001	Eleven Miles	Rihboi	Meghalaya	Cattle	TE[a]	UV[b]	KF297682
2	O/MAA/28/2002	Hungaewadi	Ahmednagar	Maharashtra	Cross Bred	TE	V[c]	KF297683
3	O/GOA/120/2002	Neteavali	Sanguemsouth	Goa	Cattle	TE	V	KF297684
4	O/GUK/142/2002	Bidaj	Bidaj	Gujarat	Cross Bred	TE	V	KF297685
5	O/POP/144/2002	Thevankudy	Karikal	Pondicherry	Cross Bred	TE	V	KF297686
6	O/RAJ/47/2003	Bassi	Jaipur	Rajasthan	Cattle	TE	V	KF297687
7	O/APMb/78/2004	Macharam	Mahaboobnagar	Andhra Pradesh	Cattle	TE	UV	KF297688
8	O/APV/86/2004	Rellivalasa	Vijiyanagaram	Andhra Pradesh	Cross Bred	FE[d]	NA[e]	KF297689
9	O/APV/93/2004	Kondakarakam	Vijiyanagaram	Andhra Pradesh	Goat	FE	UV	KF297690
10	O/HAS/34/2005	Daulpriya	Sirsa	Haryana	Buffalo	TE	UV	KF297691
11	O/PUL/88/2005	Ludhiana	Ludhiana	Punjab	Cross Bred	TE	V	NA(e)
12	O/TNS/03/2006	Manivilundhy colony	Salem	Tamil Nadu	Crossbred	TE	UV	KF297692
13	O/APR/20/2006	NA	Rangareddy	Andhra Pradesh	Crossbred	TE	V	KF297693
14	O/TNKr/57/2007	Jumpukuttapaty	Krishnakiri	Tamil Nadu	Crossbred	TE	UV	KF297694
15	O/KETr/24/2008	NA	Thiruvananthapuram	Kerala	NA	NA	NA	KF297695
16	O/CHRn/05/2009	Godmara	Rajnandgaon	Chattisgarh	Crossbred	TE	V	KF297696
17	O/APR/19/2009	Kokapet	Ranga Reddy	Andhra Pradesh	Crossbred	TE	UV	KF297697
18	O/ORB/21/2009	Sambalpur	Bardhwan	Odisha	Crossbred	TE	V	KF297698
19	O/GUK/29/2009	Kheda	Kheda	Gujarat	NA	NA	NA	KF297699
20	O/KEKm/06/2010	Kurichy	Kottayam	Kerala	Cross Bred	TE	V	KF297700
21	O/KEPt/07/2010	Peringara	Patahnamthitta	Kerala	Cross Bred	TE	V	KF297701

(a) = Tongue epithelium; (b) = unvaccinated; (c) = vaccinated; (d) = foot epithelium; (e) = not available.

sequences. All L^{pro} sequences generated in this study are available at GenBank under accession number KF297682 - KF297701 (Table I). The NJ tree of these L^{pro} sequences showed that the viruses did not group strictly according to the serotypes. There was a dissimilarity in clustering isolates (Figure 1b) based on the VP1 sequences (Nagendrakumar *et al.* 2009, Yuvaraj *et al.* 2013). Five isolates that were grouped under the Ind 2001 lineage in VP1 sequence analysis clustered together along with few other serotype A L^{pro} sequences. However, all the 6 PanAsia-2 lineage isolates clustered into a distinct branch as seen in VP1 sequence analysis. Three PanAsia isolates clustered together along with the O/PUL/88/2005 isolate. O/GOA/120/2002 isolate clustered along with the O_1/Manisa/Turkey/69 and O_5/India/1/62 as noticed in VP1 analysis. Conversely, the O/IND/R2/75 L^{pro} sequence could be grouped with a serotype A virus sequence. Thus, phylogenetic analysis of the L^{pro} sequence showed a difference in clustering isolates from that of the structural protein as observed by few other authors (Chitray *et al.* 2013, Mohapatra *et al.* 2009, van Rensburg *et al.* 2002).

Two initiation codons

All the sequences compared in this study showed 2 initiation codons at 1st and 29th amino acid sites, which is a characteristic feature found in all the 7 serotypes of FMDV (Sangar *et al.* 1987). Hinton and Crabb (Hinton and Crabb 2000) reported about an existence of hairpin like structure between the 2 initiation codons that plays an important role in internal ribosome entry site (IRES) activity. A high amount of amino acid sequence variability was noticed (Figure 2) in the N terminus end between the 2 initiation codons with more than 70% of residues showing changes (George *et al.* 2001, Mohapatra *et al.* 2009). In this study, it was observed that more than 75% of the amino acid residues between the 2 initiation codons showed variations among themselves. Zhu and colleagues (Zhu *et al.* 2010) reported that both the forms of L^{pro} showed amino acid variability with high mutation rates. However, the L^{b} form was relatively less variable than the L^{ab} form of L^{pro} (George *et al.* 2001, Sangar *et al.* 1987).

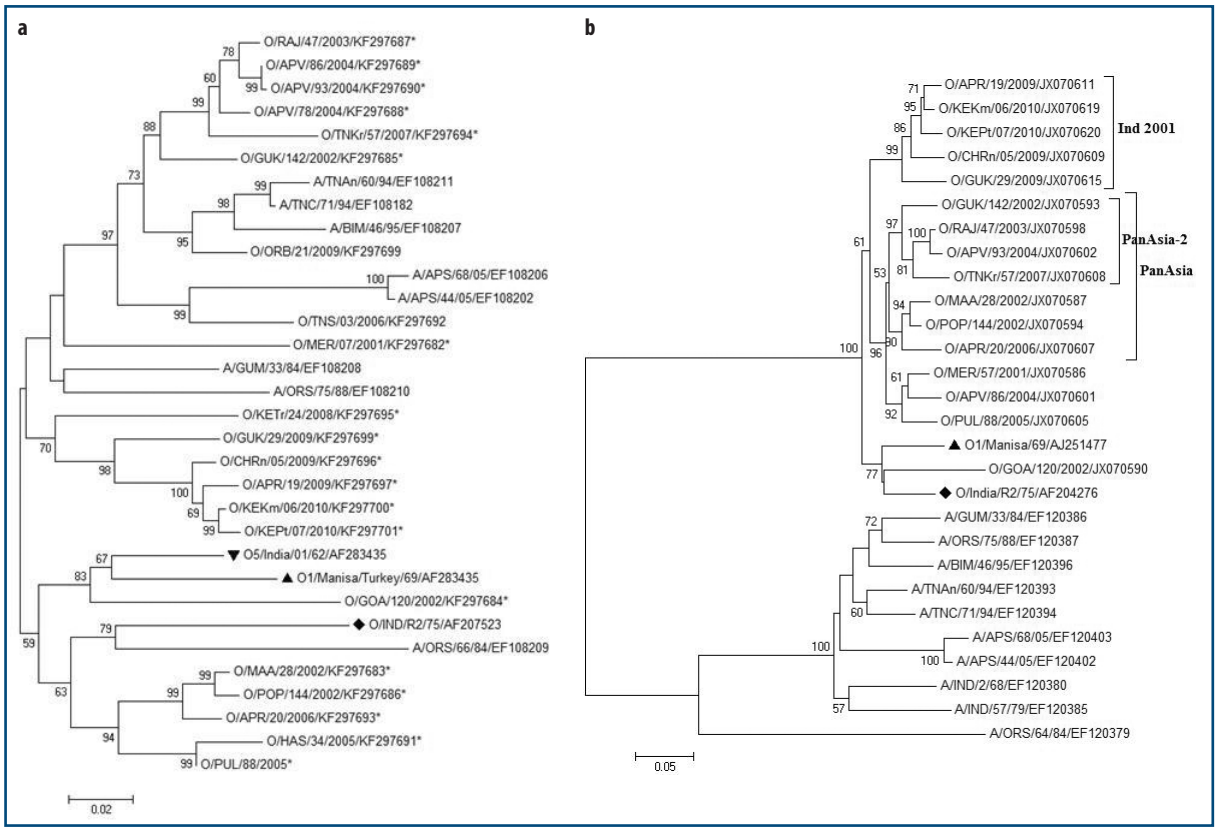

Figure 1. a) *Neighbour joining (NJ) tree of complete L^{pro} sequences (603 nt) of 21 Indian serotype O FMDV isolates along with other reference sequences.* Kimura 2-parameter model with uniform rates and bootstrap of 1000 replicates was used for construction of NJ tree with MEGA 5.05. Only bootstrap values P50% are shown. **b)** *Neighbour joining (NJ) tree of complete VP1 sequences (639 nt) of Indian serotype O and A FMDV isolates with GenBank Accession Number.* Only bootstrap values P50% are shown.
Scale bar indicates nucleotide substitutions per site. ♦ = current Indian vaccine strain; ▼ = older Indian strain; ▲ = exotic vaccine strain; * = sequences generated in this study.

Cleavage site amino acid residues

The L^{pro}/VP4 cleavage site at C terminus end of L^{pro} showed amino acid sequences of KRL(K/R)↓GAG in all the isolates used in this study. An identical amino acid sequence at this site had been observed previously by Seipelt and colleagues (Seipelt *et al.* 1999) among serotype O isolates. Mayer and colleagues (Mayer *et al.* 2008), using the molecular modelling and energy optimization studies, had found that the 143rd amino acid residue was critical for the restricted specificity of L^{pro} at L^{pro}/VP4 cleavage site. They also noticed the existence of exclusively either lysine or methionine amino acids at this site in all the FMDV serotypes. All the isolates sequenced in this study had either Lysine or methionine as the 143rd amino acid residue.

Active site cleft

The catalytic dyad formed by cysteine and histidine residues at the 51st (top of the central α helix region) and at the 148th amino acid sites (found in the turn between β sheets), respectively, were reported to be critical for the L^{pro} activity (Piccone *et al.* 1995, Seipelt *et al.* 1999). Any mutation of amino acid at these residues was shown to result in the loss of L^{pro} activity (Roberts and Belsham 1995). Both these amino acid residues were conserved in all the isolates analysed in this study. Crystallographic structural studies by Guarne and colleagues (Guarne *et al.* 1998) had showed that the orientation of H148 with respect to C51 was maintained by the hydrogen bonds of amino acid aspartate at the 163rd site. Furthermore, they had reported that the presence of asparagine (at the 46th site, 5 amino acids upstream to C51) along with D49, N54 and D164 were also essential for the structural stability and activity of the L^{pro} enzyme. All these amino acid residues (N46, D49, N54, D163 and D164) were found to be conserved in the L^{pro} sequences compared in this study.

Cysteine and histidine residues at sites C6, C125, C133 & C153 and H109, H138 & H148, respectively, were conserved among all the isolates in this study as noticed in O1Kaufbeuren isolate (Roberts and Belsham 1995). Few other amino acid residues at T55 and E147 that were reported to be related to cleavage and activity (George at al. 2001, Mohapatra *et al.* 2002) of the L^{pro} enzyme were also conserved in all the sequences except one isolate (T55A in O/

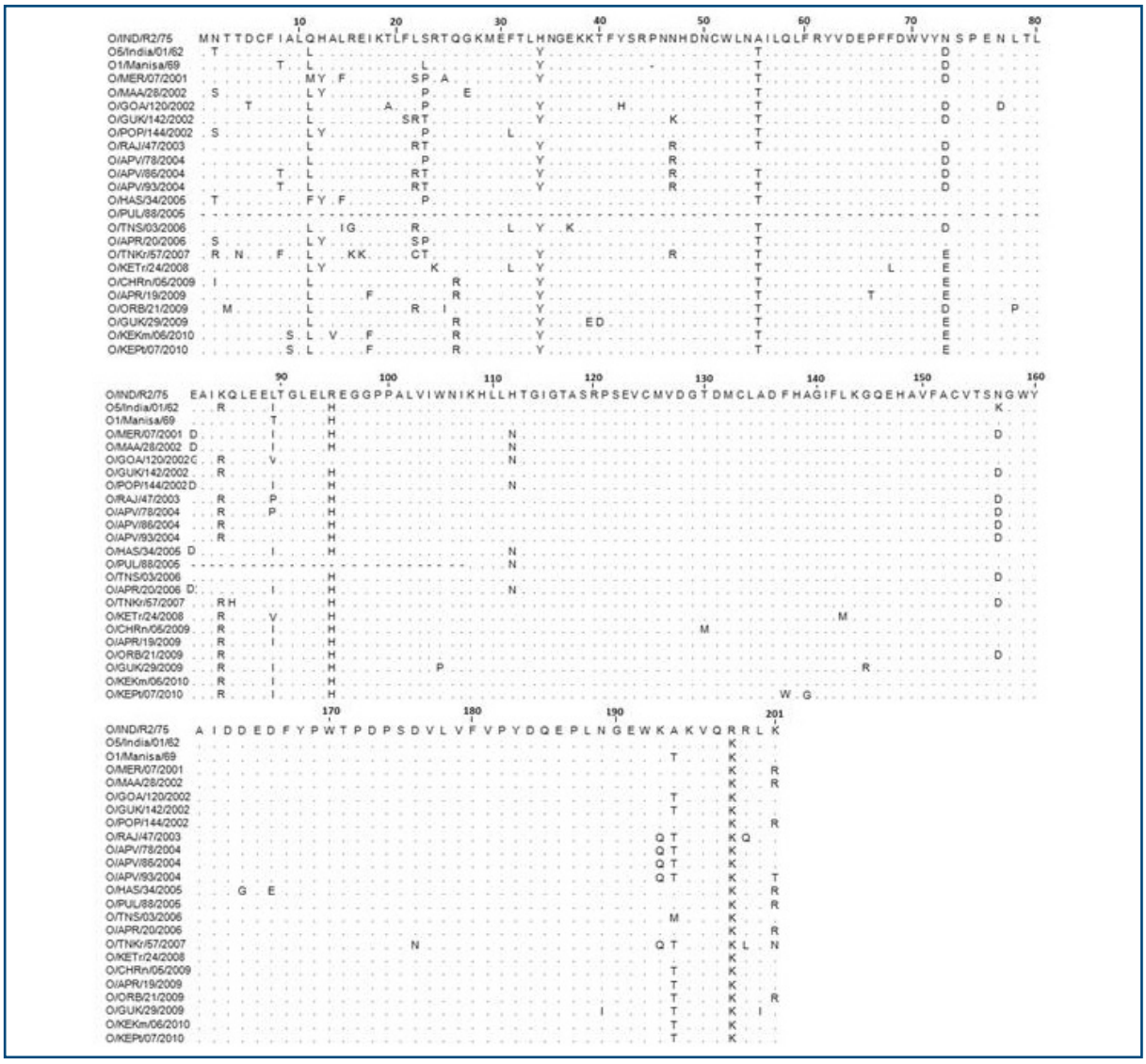

Figure 2. *Comparison of the amino acid sequences of the L^{pro} region of the FMDV Indian strains.*

APV/78/2004). However, a similar mutation (T55A) was observed in 1 of the reference sequence O/IND/R2/75, the Indian vaccine strain.

George and colleagues (George *et al.* 2001) reported that 80 amino acid residues out of total 201 were tolerant to amino acid replacements. At the nucleotide level these L^{pro} sequences showed >85 % identity with the vaccine strains O/IND/R2/75 and O_1/Manisa/Turkey/69. As this NSP was not under the influence of immune pressure of selection, the nucleotide variations are selected mainly considering the loss of L^{pro} activity. While, the random variations were fixed in the genome as they might not interfere with the viability of the virus particles (George *et al.* 2001). The study of this highly variable non-structural protein could be useful for the phylogenetic comparison of the FMDV isolates as the L^{pro} phylogeny resembles that of the capsid regions.

The phylogenetic analysis of the L^{pro} region sequences of 21 Indian serotype O isolates showed a difference in clustering of isolates as observed with the VP1 capsid coding region sequencing. The N terminus end of the L^{pro} sequences showed increased amino acid variations with 2 conserved start codons at 1st and 29th site. Among the sequences of 2 forms of leader proteinases (L^{pro}), the L^{ab} showed a high variability than the L^{b} form. All the critical amino acid residues of the active cleft site were conserved. Thus, the L^{pro} region phylogeny could be used for the comparison of the FMDV isolates.

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