Genetic analysis of foot-and-mouth disease virus serotype A of Indian origin and detection of positive selection and recombination in leader protease- and capsid-coding regions

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The leader protease (L^{pro}) and capsid-coding sequences (P1) constitute approximately 3 kb of the foot-and-mouth disease virus (FMDV). We studied the phylogenetic relationship of 46 FMDV serotype A isolates of Indian origin collected during the period 1968–2005 and also eight vaccine strains using the neighbour-joining tree and Bayesian tree methods. The viruses were categorized under three major groups – Asian, Euro-South American and European. The Indian isolates formed a distinct genetic group among the Asian isolates. The Indian isolates were further classified into different genetic subgroups (<5% divergence). Post-1995 isolates were divided into two subgroups while a few isolates which originated in the year 2005 from Andhra Pradesh formed a separate group. These isolates were closely related to the isolates of the 1970s. The FMDV isolates seem to undergo reverse mutation or convergent evolution wherein sequences identical to the ancestors are present in the isolates in circulation. The eight vaccine strains included in the study were not related to each other and belonged to different genetic groups. Recombination was detected in the L^{pro} region in one isolate (A IND 20/82) and in the VP1 coding 1D region in another isolate (A RAJ 21/96). Positive selection was identified at aa positions 23 in the L^{pro} (*P*<0.05; 0.046*) and at aa 171 in the capsid protein VP1 (*P*<0.01; 0.003**).

1. Introduction

Foot-and-mouth disease (FMD) is an acute, contagious viral disease of cloven-hoofed livestock such as cattle, pigs, buffalo, sheep and goats. FMD causes severe economic loss in developing countries. Seven serotypes (A, O, C, Asia-1, and South African Territories 1, 2, and 3) have been identified serologically, and multiple subtypes occur within each serotype. The foot-and-mouth disease virus (FMDV) consists of a single-stranded, positive-sense RNA genome of approximately 8500

bases surrounded by four structural proteins (VP1 [1D], VP2 [1B], VP3 [1C], and VP4 [1A]) to form an icosahedral capsid. The genome also codes for eight non-structural proteins (L^{pro}, 2A, 2B, 2C, 3A, 3B, 3C^{pro} and 3D-RNA dependant RNA polymerase) (Grubman and Baxt 2004).

FMD is endemic in India and serotypes A, O and Asia-1 are prevalent. The predominant serotype reported in India is type O, while serotype A has been identified in 10% of outbreaks. The uncontrolled movement of animals, low vaccination coverage and antigenic variation within

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Abbreviations used: BHK-21, baby hamster kidney cell line; BTy, bovine thyroid; CTE, C-terminal extension; FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; GARD, genetic analysis for recombination detection; L^{pro}, leader protease; MCMC, Markov Chain Monte Carlo; NJ, neighbour-joining; ORF, open reading frame; P1, capsid-coding sequence; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; SLAC, single likelihood ancestor counting; UTR, untranslated region; UV, ultra-violet; VMMS, virus maintenance medium with (1%) serum

and between serotypes are factors that cause problems in controlling FMD outbreaks (Azad *et al* 1995). Extensive studies on the genetic and antigenic variation of FMDV serotypes O and Asia-1 have been carried out worldwide. Unlike serotype O and Asia-1 virus, serotype A isolates have shown extensive antigenic variation (Arrowsmith 1975; Belwal *et al* 1986; Belwal *et al* 1987; Armstrong *et al* 1994; Azad *et al* 1995; Araujo *et al* 2002). Several workers (Tosh *et al* 2004; Mittal *et al* 2005; Jangra *et al* 2005; Mohapatra *et al* 2008) have studied genetic and antigenic variation in Indian FMDV isolates. Continuous monitoring of the disease is essential to understand the epidemiology of the virus and the mutations that occur over a period of time. Recently, Nagendrakumar *et al* (2006) reported the need for continuous monitoring of the isolates in order to update the primers and probes used for the detection of Indian FMDV isolates. The present study reports the analysis of the leader protease (L^{pro}) and P1 coding sequences of FMDV type A virus isolated from various parts of the country between 1968 and 2005.

2. Materials and methods

2.1 *Viruses*

Forty-six FMDV serotype A isolates from different regions of India collected during the period 1968–2005 available with the virus repository of the FMDV Laboratory, Indian Immunologicals Limited, Hyderabad were used in this study (table 1). Sequences of Indian and exotic serotype A virus available in the GenBank (Jangra *et al* 2005; Carrillo *et al* 2005) and sequences of two type A Indian isolates (Nick Knowles, EF 208771 and EF 208772) including eight vaccine strains (A IND 17/77, A IND 17/82, A IND 7/82, A IVRI, A_5 FRANCE 60, A_{10} HOLLAND 42, A_{22} IRAQ 64 and A_{24} BRAZIL 55) were included for comparison.

2.2 *Primary bovine thyroid cell cultures*

Primary bovine thyroid (BTy) cell cultures were prepared using thyroid glands harvested from day-old male calves as per the procedure described in Freshney (2004). The thyroid glands were harvested, dissected and trypsinized using 0.5% trypsin solution in 0.04 M phosphate buffer. The cells were seeded at a rate of $1x10^6$ cells/ml in 5 cm² Nunclon™ polystyrene tubes with a flat side (Nunc®, Denmark) with cell growth medium containing 10% adult bovine serum. Once the monolayers were formed, the culture was used for virus isolation.

2.3 *BHK-21 cells*

Baby hamster kidney cell line (BHK-21) maintained as monolayers by the tissue culture laboratory, Indian Immunologicals Limited, Hyderabad was used for isolation of the virus as well as preparation of virus master banks.

2.4 *HELY cell growth medium*

HELY cell growth medium (pH 7.4–7.6) was used for cultivating primary BTy cells. Growth medium was prepared by mixing 800 ml of Hanks BSS medium containing 0.2% yeast extract with 200 ml of 10% Eagle medium. Tissue culture-grade calf serum (10%, Selborne, Australia) and 6 ml of 4.4 M sodium bicarbonate were added. Sterility testing was carried out and the medium was stored at 4–8ºC until use.

2.5 *Virus maintenance medium with (1%) serum (VMMS)*

Virus maintenance medium was prepared by adding 10 ml of adult bovine serum, 50 ml of 4.4 M sodium bicarbonate solution and 40 ml of sterile distilled water to 900 ml of Glasgow Eagle medium containing Earle salts. The sterility of the medium was confirmed by inoculation into soyabean casein digest medium and tryptose broth for 7 days. Media that passed the sterility test were stored at 4–8ºC until use.

2.6 *Reagents for genome amplification and sequencing*

The viral RNA was isolated using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. The FMDV genome targets L^{pro} and capsid-coding P1 regions were reverse transcribed using the Thermoscript® Reverse Transcription System (Invitrogen, USA). The target in the genome was amplified by polymerase chain reaction (PCR) using a Platinum Taq PCR system for Long Templates (Invitrogen, USA). The target was sequenced using an ABI Prism BigDye Cycle Sequencing Ready Reaction kit v 3.1 as per the manufacturer's protocol on an ABI Prism Automatic Cycle Sequencer.

2.7 *Isolation and adaptation of the virus*

Epithelial samples from the tongue and feet were processed in 0.04 M phosphate buffer and made up as a 10% suspension. The suspension was inoculated on the primary thyroid cell monolayer cultures. The cultures were observed for 24–48 h for the appearance of a cytopathic effect. Virus typing was done (Hamblin *et al* 1984) and type A viruses adapted to BHK-21 cells were used in this study.

2.8 *Preparation of cDNA by reverse transcription*

The total RNA extracted (5 μl) was subjected to reverse transcription (final reaction volume of 20 μl) using the

Table 1. List of viruses used in the study and their place of origin

S. No.	Virus identification	Place of origin		Year of isolation	GenBank accession no:	
		District and State	Country		1D region	L^{pro} region
1	A5 IVRI	-	HOLLAND	1942	EF120378	-
2	A IND 2/68	Pune, Maharashtra	INDIA	1968	EF120380	EF108183
3	A IND 5/68	Calcutta, West Bengal	INDIA	1968	-	EF108185
4	A IND 1/70	Lucknow, Uttar Pradesh	INDIA	1970	EF120381	-
5	A IND 3/73	Roorkee, West Bengal	INDIA	1973	EF120382	-
6	A IND 8/76	North Arcot, Tamil Nadu	INDIA	1976	EF120383	-
7	A IND 19/76	Dehradun, Uttar Pradesh	INDIA	1976	-	EF108190
8	A IND 3/77	Haringhatta, West Bengal	INDIA	1977	-	EF108184
9	A IND 17/77	West Bengal	INDIA	1977	-	-
10	A IND 54/79	Tirunelveli, Tamil Nadu	INDIA	1979	-	EF108195
11	A IND 57/79	Dharmapuri, Tamil Nadu	INDIA	1979	EF120385	EF108196
12	A IND 73/79	Mehboob Nagar, Andhra Pradesh	INDIA	1979	-	EF108197
13	A IND 85/79	Bombay, Maharashtra	INDIA	1979	-	EF108198
14	A IND 86/79	Ahmedabad, Gujarat	INDIA	1979	-	EF108199
15	A IND 25/81	Ahmedabad, Gujarat	INDIA	1981	-	EF108193
16	A IND 7/82	Kheda, Gujarat	INDIA	1982	-	EF108186
17	A IND 16/82	Jind, Haryana	INDIA	1982	-	EF108187
18	A IND 17/82	Bilaspur, Himachal Pradesh	INDIA	1982	-	EF108189
19	A IND 20/82	Jammu, Jammu & Kashmir	INDIA	1982	-	EF108191
20	A IND 22/82	Not known	INDIA	1982	-	EF108192
21	A IND 26/82	Not known	INDIA	1982	-	EF108194
22	A APA 25/84	Anantapur, Andhra Pradesh	INDIA	1984	-	EF108200
23	A GUM 33/84	Mehsana, Gujarat	INDIA	1984	EF120386	EF108208
24	A APN 41/84	Nellore, Andhra Pradesh	INDIA	1984	-	EF108201
25	A ORS 66/84	Sambalpur, Orissa	INDIA	1984	EF120379	EF108209
26	A ORS 75/88	Sambalpur, Orissa	INDIA	1988	EF120387	EF108210
27	A GUA 24/91	Ahmedabad, Gujarat	INDIA	1991	EF120389	-
28	A GUA 21/91	Ahmedabad, Gujarat	INDIA	1991	EF120390	-
29	A GUA 27/91	Ahmedabad, Gujarat	INDIA	1991	EF120388	-
30	A MAT 18/93	Thane, Maharashtra	INDIA	1993	EF120391	-
31	A MAN 47/94	Nagpur, Gujarat	INDIA	1994	-	-
32	A TNAn 60/94	Arakkonam, Tamil Nadu	INDIA	1994	EF120393	EF108211
33	A TNC 71/94	Chennai, Tamil Nadu	INDIA	1994	EF120394	EF108182
34	A GUK 14/95	Kheda, Gujarat	INDIA	1995	EF120395	-
35	A BIM 46/95	Mirzapur, Bihar	INDIA	1995	EF120396	EF108207
36	A HAH 51/95	Hissar, Haryana	INDIA	1995	EF120397	-
37	A RAJ 21/96	Jaipur, Haryana	INDIA	1996	EF120398	-
38	A APG 35/96	Guntur, Andhra Pradesh	INDIA	1996	-	-
39	A HAH 14/00	Hissar, Haryana	INDIA	2000	EF120399	-
40	A APR 51/01	Ranga Reddy, Andhra Pradesh	INDIA	2001	EF120400	-
41	A MEU 19/03	Umroi, Meghalaya	INDIA	2003	EF120401	-
42	A WBJ 30/04	Jalpaiguri, West Bengal	INDIA	2004	-	-

Table 1 *(Continued)*

S. No.	Virus identification	Place of origin		Year of isolation	GenBank accession No:	
		District and State	Country		1D region	L^pro region
43	A APS 44/05	Srikkakulam, Andhra Pradesh	INDIA	2005	EF120402	EF108202
44	A APS 50/05	Srikkakulam, Andhra Pradesh	INDIA	2005	-	EF108203
45	A APS 55/05	Srikkakulam, Andhra Pradesh	INDIA	2005	-	EF108204
46	A APS 66/05	Srikkakulam, Andhra Pradesh	INDIA	2005	-	EF108205
47	A APS 68/05	Srikkakulam, Andhra Pradesh	INDIA	2005	EF120403	EF108206

Thermoscript® Reverse Transcription System (Invitrogen, USA). For each sample, reverse transcription was carried out in duplicate using negative sense primer P33 (CGG TTT GGG ACC ATG TTC GA, 2B region of the genome; Knowles and Samuel 1995). The two sets of cDNA were used for PCR amplification as duplicate reactions.

2.9 *Amplification of L^pro-coding region 'L' and capsid-coding region 'P1' by PCR*

The cDNA (5 μl) was subjected to PCR reaction using the Platinum Taq PCR System for Long Templates (Invitrogen, USA) with primer pairs LO1F (GTC CCC CAG TTT AAA AAG CTT, positive sense primer, 5' untranslated region [UTR]; Roberts and Belsham 1995) and NK72 (GAA GGG CCC AGG GTT GGA CTC, negative sense primer, 2B region; Knowles and Samuel 1995) for amplifying the LP1 region. Primer pairs A-1C612 (TAG CGC CGG CAA AGA CTT TAG, positive sense primer; Vangrysperre and de Clercq 1996) and NK61 (GAC ATG TCC TCC TGC ATC TG, negative sense primer; Knowles and Samuel 1995) were used to amplify the complete 1D region, while primer pairs DH6 (TTG TTC TGA GTG TTG GTT GTG TG, positive sense primer; Sabarinath 2001) and NK61 were used to amplify the complete capsid-coding P1 region.

2.10 *Analysis of reverse transcriptase-polymerase chain reaction product*

The reverse transcriptase-polymerase chain reaction (RT-PCR) products obtained were analysed on a 0.8% agarose gel with 0.01% ethidium bromide under ultraviolet (UV) illumination and documented using a gel documentation system (BioRad, USA). Positive amplification was the appearance of a ~3 kbp PCR product for the LP1 region, ~2.1 kbp PCR product for the complete P1 region and ~900 bp PCR product for the 1D region.

2.11 *Purification of the RT-PCR product*

The RT-PCR products were run on a 1.5% low-melting agarose gel and the respective bands were cut and removed.

The PCR product from the excised portion of the gel was eluted using Qiaquick® Gel Extraction Kit (Qiagen, USA) and reconstituted in 30 μl of elution buffer.

2.12 *Sequencing of RT-PCR products*

The eluted RT-PCR products were subjected to direct cycle sequencing with different sets of forward and reverse sequencing oligonucleotide primers using the BigDye Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems, USA) as per the manufacturer's protocol on an automatic cycle sequencer (ABI Prism, Applied Biosystems, USA).

2.13 *Genomic comparison by phylogenetic analysis*

The L^pro and VP1 coding sequences (complete 1D region) were aligned using the ClustalW 1.8x program (Thompson *et al* 1994). Manual editing at codon positions 153 and 155 was done to accommodate for the reported deletion of amino acids in some of the exotic virus sequences referred to. A phylogenetic neighbour-joining (NJ) tree was constructed (bootstrap replicates = 1000) using Mega 3.1 (Kumar *et al* 2004). The genetic heterogeneity of the viruses has been defined as genetic groups if the genetic relationship has <15% nucleotide divergence (Vosloo *et al* 1992; Samuel and Knowles 2001; Tosh *et al* 2002a) and as lineage if the genetic relationship has <7.5% nucleotide divergence (Mohapatra *et al* 2002).

Phylogenetic analysis using a Bayesian algorithm (MrBayes) was also drawn using the software described by Huelsenbeck and Ronquist (2001). The maximum likelihood model employed 2 substitution types ("nst = 2"), with base frequencies set to fixed values ("statefreqpr = fixed [equal]"). Rate variation across sites was modelled using a gamma distribution ("rates = gama"). The Markov Chain Monte Carlo (MCMC) search was run with 4 chains for 5 000 000 generations, with trees being sampled every 1000 generations (the first 10 000 trees were discarded as "burnin"). The topology of the consensus tree was viewed using Treeview (Page 1996).

2.14 *Similarity analysis and detection of recombination events*

Similarity plots for L^{pro} sequences, complete P1 sequence and complete 1D sequence were performed using the Simplot software (Lole *et al* 1999) with windows 200 bp in size (step: 20 bp) with gap stripping and Kimura 2-parameter correction. Recombination events were detected by a bootscan of sequences under different genetic groups. Recombination events were also detected using the Datamonkey website (Pond and Frost 2005) by genetic analysis for recombination detection (GARD) analysis and by breakpoint placement support in the GARD analysis.

2.15 *Selection pressure analysis*

Positively selected sites (codon-specific analyses of dN/dS) were identified using single likelihood ancestor counting (SLAC) analysis (Pond and Frost 2005), a modification of the Suzuki–Gojobori method (Suzuki and Gojobori 1999) available at the Datamonkey website (Pond and Frost 2005). The analysis was done with the HKY85 substitution model on phylogenetic trees inferred using the NJ method with a cut-off *P* value of 0.1 as described by Klein *et al* (2006). Only those sites that showed dN–dS>0 with *P*<0.05 were considered as positively selected.

3. Results

3.1 *Viruses*

Forty-six FMD virus samples originating from various parts of India including vaccine strains were phylogenetically compared for their genetic relatedness. The field samples were collected from the states of Andhra Pradesh (10); Gujarat (8); Tamil Nadu (5); Haryana and West Bengal (4), Maharashtra (3), Orissa and Uttar Pradesh (2 each) and Bihar, Jammu and Kashmir, and Meghalaya (1 each). The identity of two field samples was not known. Vaccine strains of Indian origin, viz. A IND 17/82 (Himachal Pradesh), A IND 7/82 (Gujarat), A IND 17/77 (West Bengal), A IVRI and exotic strains A IRAQ 24/64, A_{10} HOLLAND 42, A_5 FRANCE 60 and A_{24} BRAZIL 55 (Carrillo *et al* 2005) originating from Iraq, Holland, France and Brazil, respectively, were included for genetic comparison.

3.2 *Genetic comparison of FMDV serotype A isolates*

All the 46 viruses were isolated using BTy cell culture or directly using BHK-21 cell culture. Out of the 46 isolates, an LP12A region of ~3.0 kb size could be amplified from 25 isolates, P1 region of ~2.2 kb from 8 isolates and 1D region

of ~700 bp from another 13 isolates used in the study. The RT-PCR products could be purified and visualized using 0.8% agarose gel with ethidium bromide staining.

3.3 *L^{pro} coding region*

The NJ tree and Bayesian tree, constructed using the L^{pro} coding nucleotide sequences (figure 1a and 1b) revealed the existence of isolates that were related to the A_{22} subtype and not the A_5 or A_{10} subtypes, as thought earlier. The various FMDV serotype A isolates could be broadly classified as being of South American, European, Euro-South American and Asian lineages. This finding indicates that the Indian isolates were derived from a single major lineage related to A_{22} represented by A_{22} IRAQ 24/64, and later diverged into different sublineages. Five major sublineages (L1, L2, L3, L4 and L5) could be recognized. The predominantly circulating sublineage L5 consisted of Indian isolates that emerged after 1994. Post-1995, the virus diverged into many groups and the latest of them were isolates from the state of Andhra Pradesh, A APS 44/05, A APS 50/05, A APS 55/05 and A APS 66/05. None of the Indian isolates except ORS 64/84 was related to the vaccine strain A IVRI. Incidentally, this vaccine strain was closely related to A_{10} HOLLAND 42. A significant finding is that the L^{pro} region of A IND 57/79 closely matched that of A IRAN 98, while A IND 19/76 and A IND 5/68 closely matched that of A IRAN 96.

3.4 *Partial 1D region*

Serotype A isolates of the world were grouped as European, Euro-South American, South American, African and Asian (tree not shown). The Asian group of viruses constitutes those predominantly of Middle Eastern origin and those from the Indian subcontinent. The virus strains from the Indian subcontinent have evolved into different genetic groups during the past 37 years and post-1995, serotype A viruses established themselves as the major genetic groups. Most of the circulating viruses had evolved from this lineage. The recent isolates of 2005 were categorized into a separate subgroup and were closely related to A IND 57/79. Based on the NJ tree analysis, the FMDV serotype A virus was divided into 13 distinct genetic groups (G-I to G-XIII) and the Indian isolates could be classified under four genetic groups (G-IV, G-VI, G-VII and G-XII). The vaccine strain A IND 17/82 represents G-VI and A IND 17/77 was placed in G-IV. A IVRI was grouped with European isolates in G-I, which included A_{10} HOLLAND 42.

Vaccine strains A_{24} BRAZIL 55 represented the South American isolates (G-V). Further, the isolates of South American origin were categorized in three different genetic groups (G-I, G-II and G-V). A_{22} IRAQ 24/64 represented
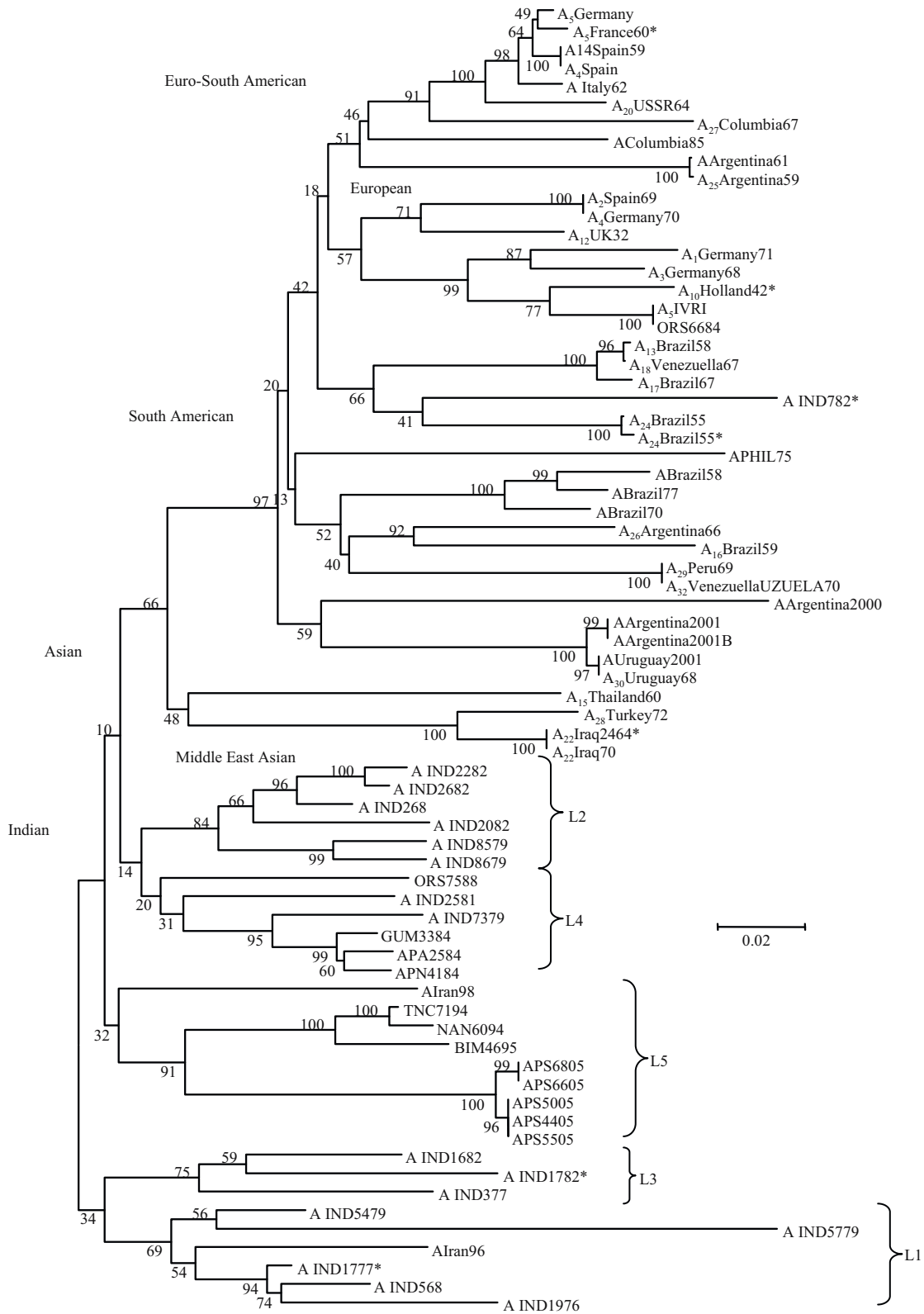
Figure 1a. Dendrogram showing the relationship between type A Indian isolates based on nucleotides constituting the leader protease (L^{pro}) region – neighbour-joining (NJ) tree.
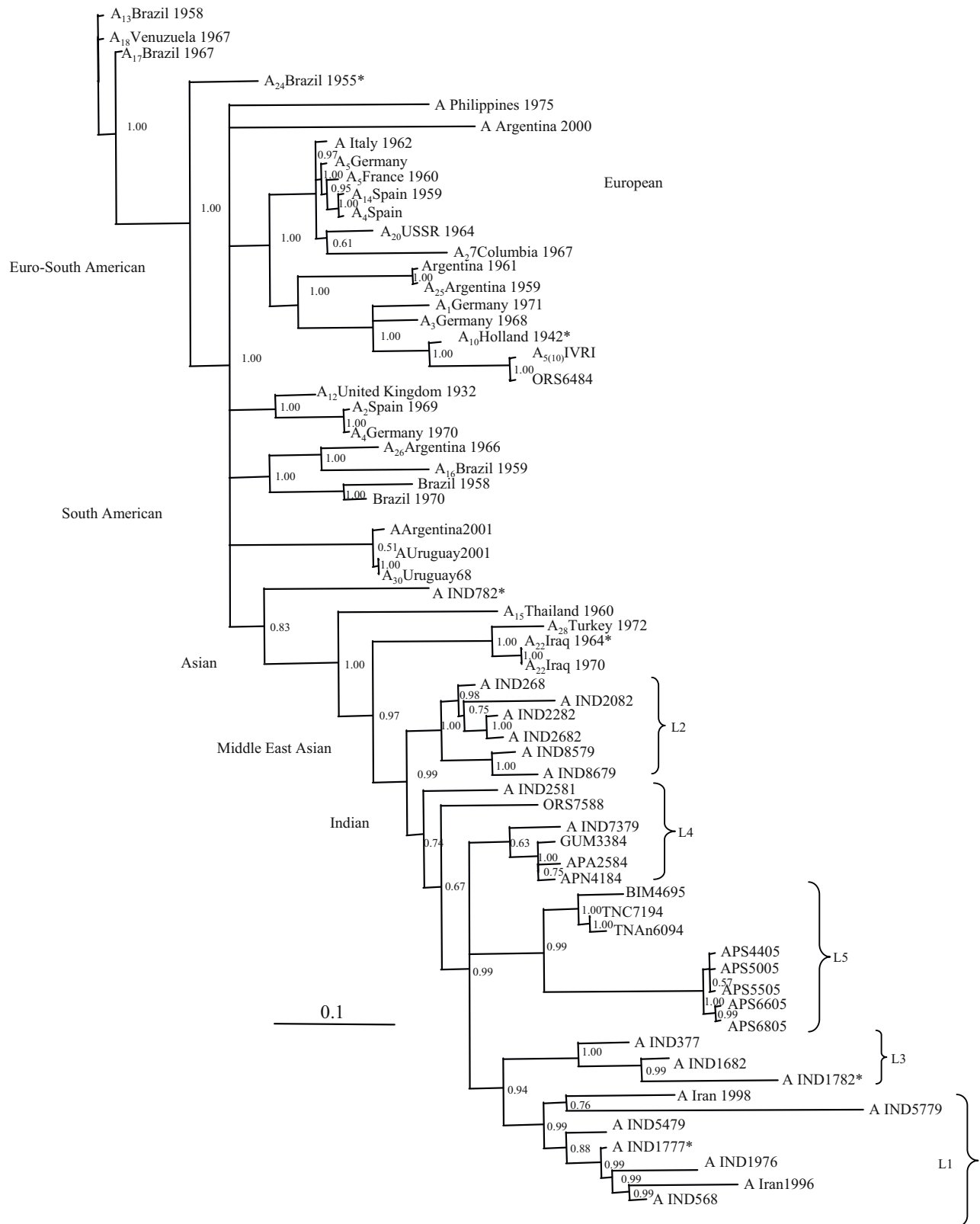
Figure 1b. Dendrogram showing the relationship between type A Indian isolates based on Bayesian analysis of nucleotides constituting the leader protease-coding sequence.
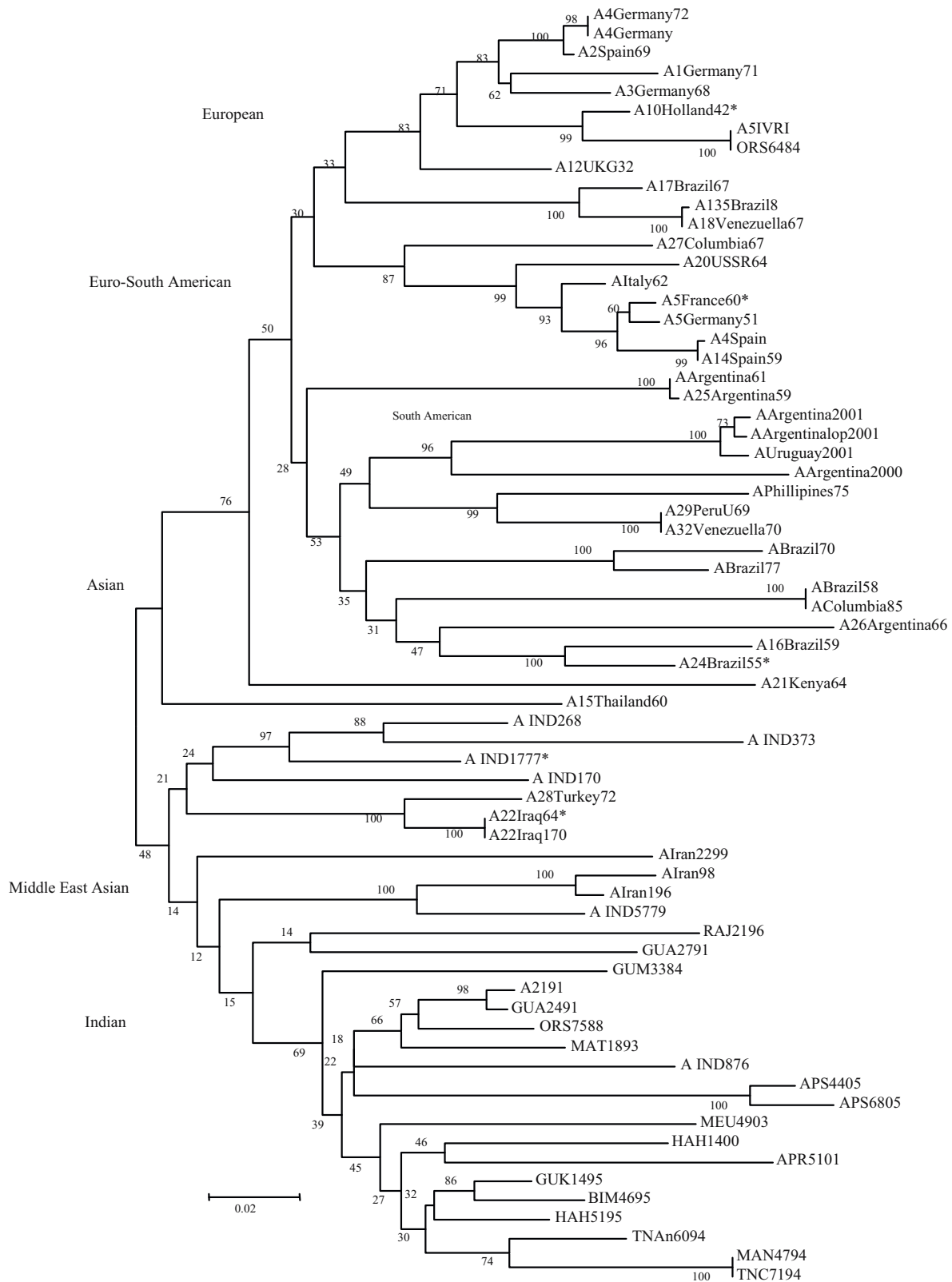
Figure 2a. Dendrogram showing the relationship between type A Indian isolates based on neighbour-joining (NJ) analysis of nucleotides constituting the complete 1D sequence.
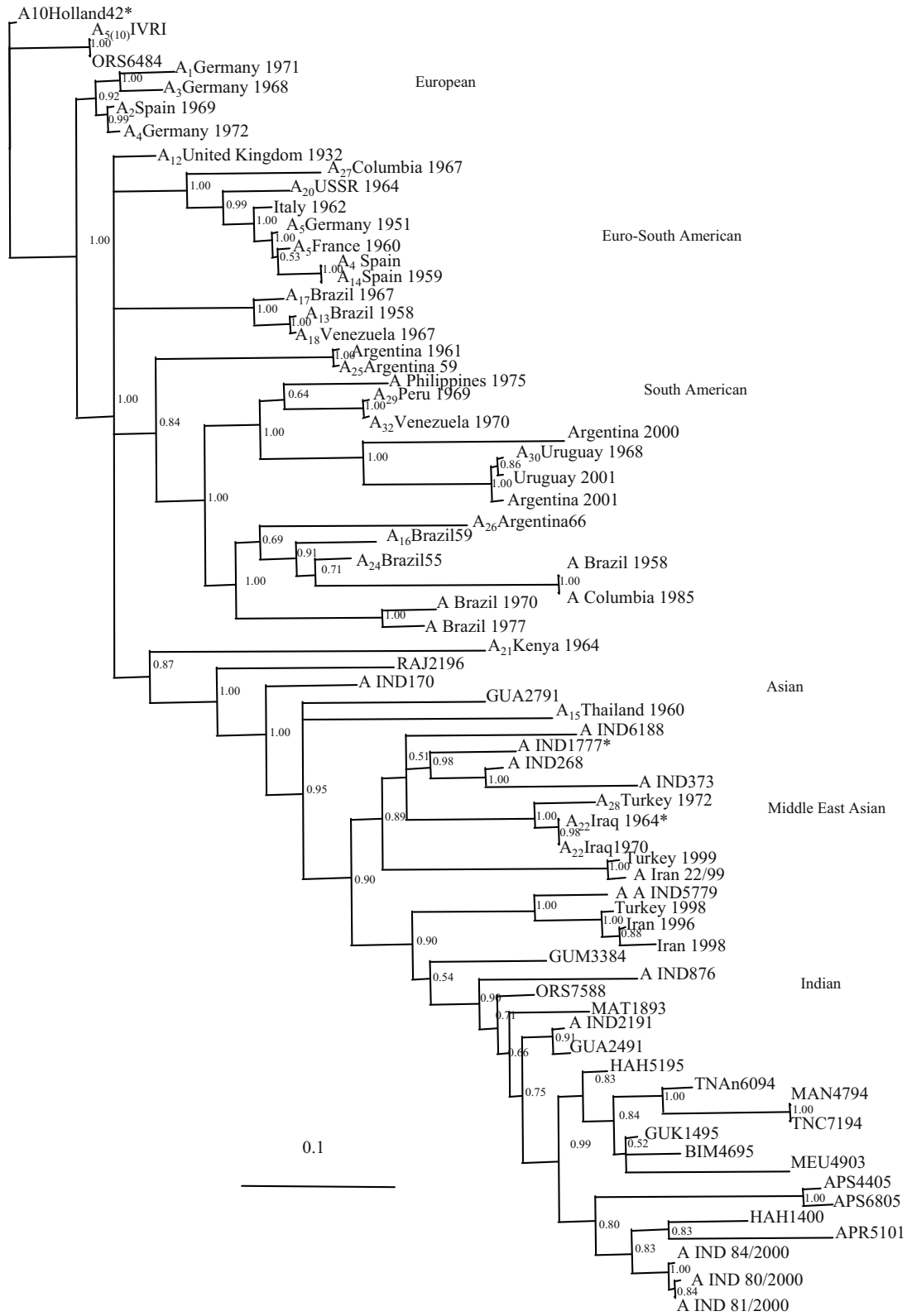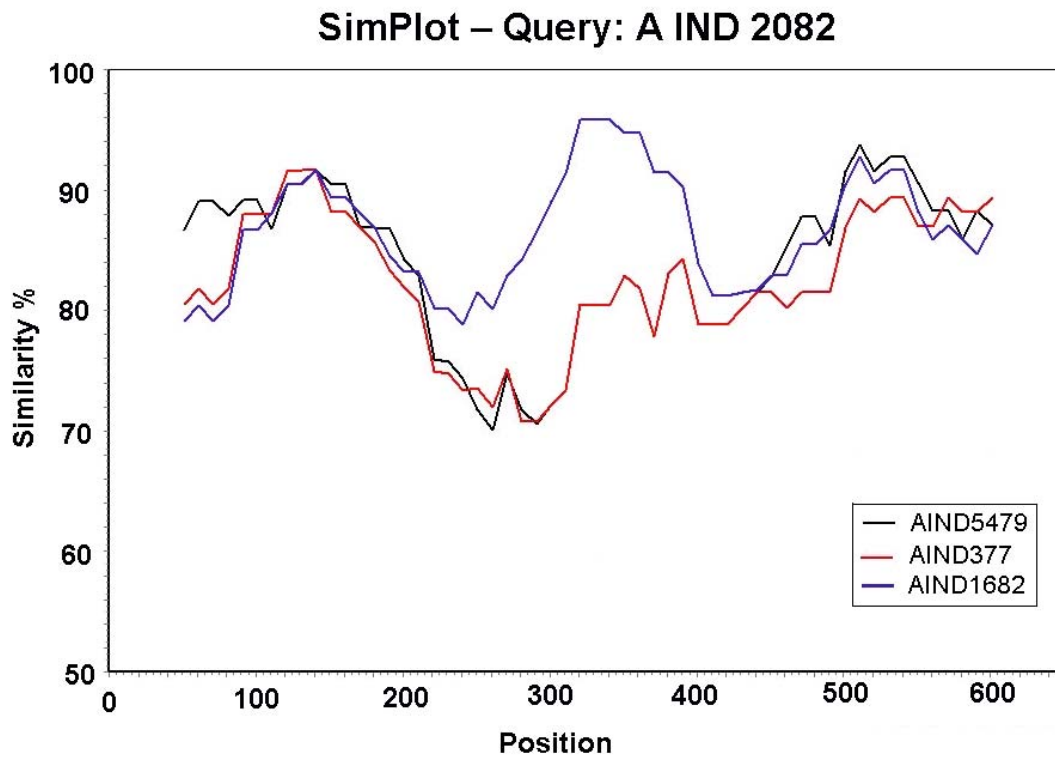
Figure 2b. Dendrogram showing the relationship between type A Indian isolates based on Bayesian analysis of nucleotides constituting the complete 1D sequence.
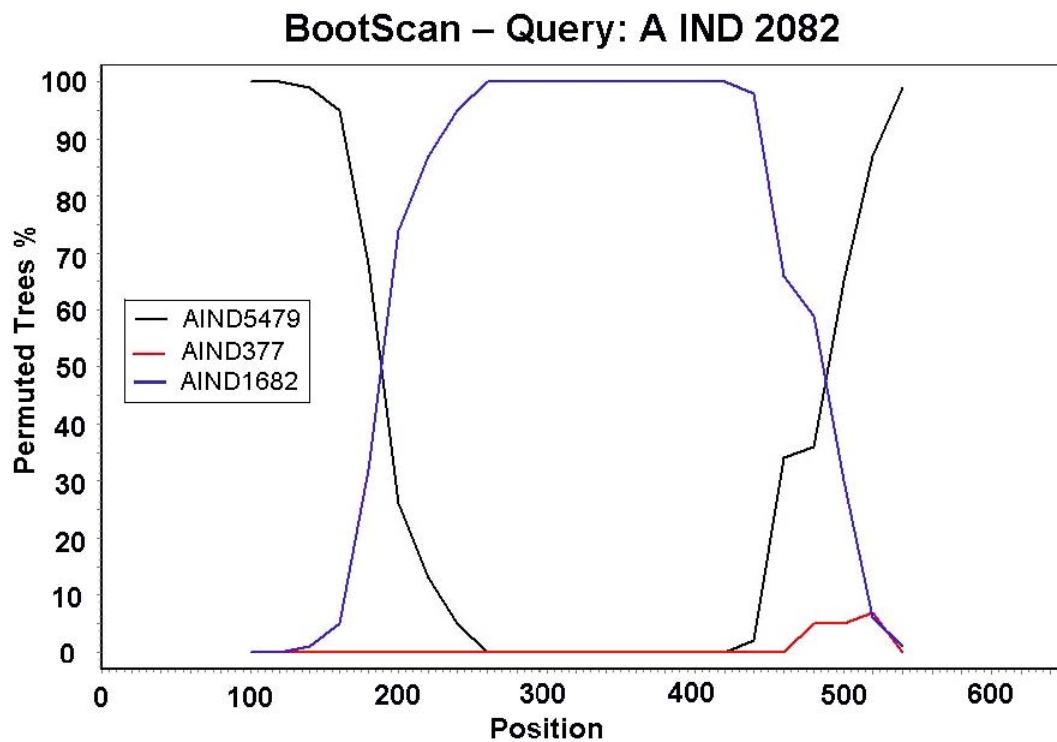
(A)

(B)

Figure 3. **(A)** Similarity plot for a type A Indian isolates showing a recombination event in the L^{pro}-coding region. **(B)** Recombination event in L^{pro}-coding region in a serotype A virus of Indian origin.

the A_{22} strains (G-IV). The Middle-East Asian isolates were divided into six genetic groups – G-IV, G-VIII, G-IX, G-X, G-XI and G-XII. Isolates from Thailand were grouped in G-VIII while the Chinese isolates represented G-III. G-IX consisted of isolates from Malaysia and Thailand identified between 1993 and 1997, while G-X consisted of isolates from Iran identified in 1997 and 1998, which were different from G-XI. A IRAN 96, A IRAN 98 and A TURKEY 2000 were included in G-XII while A IRAN 99 was included in G-XI. The recent variant of serotype A from the Middle East, A IRAN 2005, was included in G-VIII along with an earlier Iranian isolate, A IRAN 87 and isolates from Thailand. The recent Indian isolates (Andhra Pradesh) were distantly related to A IND 57/79 and A IND 17/77, forming a distinct subgroup. The isolates of 2000, 2001 and 2003 have a common ancestor that diverged in the year 1994 from the isolates of G-VI. Incidentally, the vaccine strain from Saudi Arabia, A SAU 25/87, was grouped with G-VI, which showed a close relationship with the Indian type A isolates of the 1980s and early 1990s. The African serotype A isolates formed a distinct genetic group not previously described (G-XIII), which included two distantly related isolates A_{21} KENYA 64 and A EGYPT 06.

3.5 *Complete 1D region*

Based on the NJ and Bayesian trees (figure 2a, b), the serotype A isolates from different parts of the world can be grouped as being of European, Euro-South American, South American, African and Asian origin as in the case of the partial 1D region phylogeny. The Asian group consisted predominantly of Middle-East Asian, Indian and a few South-East Asian type A isolates.

Close examination revealed that there were only two major lineages and the viruses from the Indian subcontinent evolved over the years into different genetic groups. The virus post-1995 constituted the major or dominant genetic group and most of the circulating virus strains have evolved from this lineage. The genetic drift from the different vaccine strains used in the subcontinent was very high. The recent isolates of Iran (1996 and 1998) and Turkey (1998) were distantly related to A IND 57/79. Here again, only one Indian isolate A ORS 64/84 was related to A IVRI.

3.6 *Similarity plot analysis and detection of recombination events*

The similarity plot analysis of the L^{pro} coding region revealed a recombination event in at least one Indian isolate, A IND 20/82 by Simplot® analysis and boost scan analysis for recombination (figure 3a, b). This isolate was a chimera of two other isolates, A IND 16/82 and A IND 54/79. The recombination was confirmed by GARD analysis and by breakpoint placement support in the GARD analysis. The

isolates shared sequence similarity with A IND 16/82 at nucleotide positions 1–265 and 450–651, while at positions 266–449 they shared sequence similarity with A IND 54/79.

Similarly, the isolate A RAJ 21/96 appeared to be a recombinant of A IVRI and A TNAn 60/94 in the 1D region of the P1 sequence. The Simplot® analysis and boost scan analysis for recombination (figure 4a, b) revealed that the recombination was in the VP1 coding 1D region. The GARD analysis and breakpoint placement support in the GARD analysis also confirmed this recombination. The recombination had occurred between nucleotide positions 1572 and 1956 of the P1 region. The isolates shared sequence similarity with A TNAn 60/94 at positions 1–1572 and 1957–2211, while at positions 1573–1756 they shared sequence similarity with A IVRI.

3.7 *Selection pressure analysis*

3.7.1 L^{pro} *coding region:* Examination of the deduced amino acid sequences of the L^{pro} region for selective pressures revealed that 2 sites (residues 23 and 34) were positively selected (figure 5a). The positive selection at site 23 was significant ($P<0.05$; 0.046) while at site 34 it was not significant ($P>0.05$; 0.061). These residues were located close to the second initiation codon of the L^{pro} and are therefore exposed to factors influencing virus protein synthesis. The histories of these two residues show that at site 23, the codons AAA, TCA, ACA or CTA (Lys, Ser, Thr, Leu or Ser, respectively) were selected in place of ACA, CCA or TCA (Thr, Pro or Ser) and at site 34, the codon TAC (Tyr) was selected for CAC (His).

3.7.2 *VP1 coding region:* Examination of the deduced amino acid sequences of the VP1 coding region for selective pressures also revealed 2 sites (residues 154 and 171) that were positively selected (figure 5b). Residue 171 displayed significant evidence of positive selection ($P<0.01$; 0.0031), while positive selection at residue 154 was not statistically significant ($P>0.05$; 0.066). These two residues were located within the immunodominant GH-loop of the VP1 protein, which indicates the vulnerability of this region for mutation due to constant immune pressure. The history of these two residues shows that at site 171, the codons AAC, GAC, GCC, TCC, GGC or GTC (Asn, Asp, Ala, Ser, Gly or Val, respectively) were selected for ACC (Thr) and GCC (Ala), while at site 154, the codons GCG and ATC (Ala and Ile, respectively) were selected for GTG and GTC (Val).

4. **Discussion**

4.1 *Phylogenetic analysis of sequences*

FMDV is a positive-sense RNA virus and hence has error-prone replication (Sobrino *et al* 2001). It is therefore
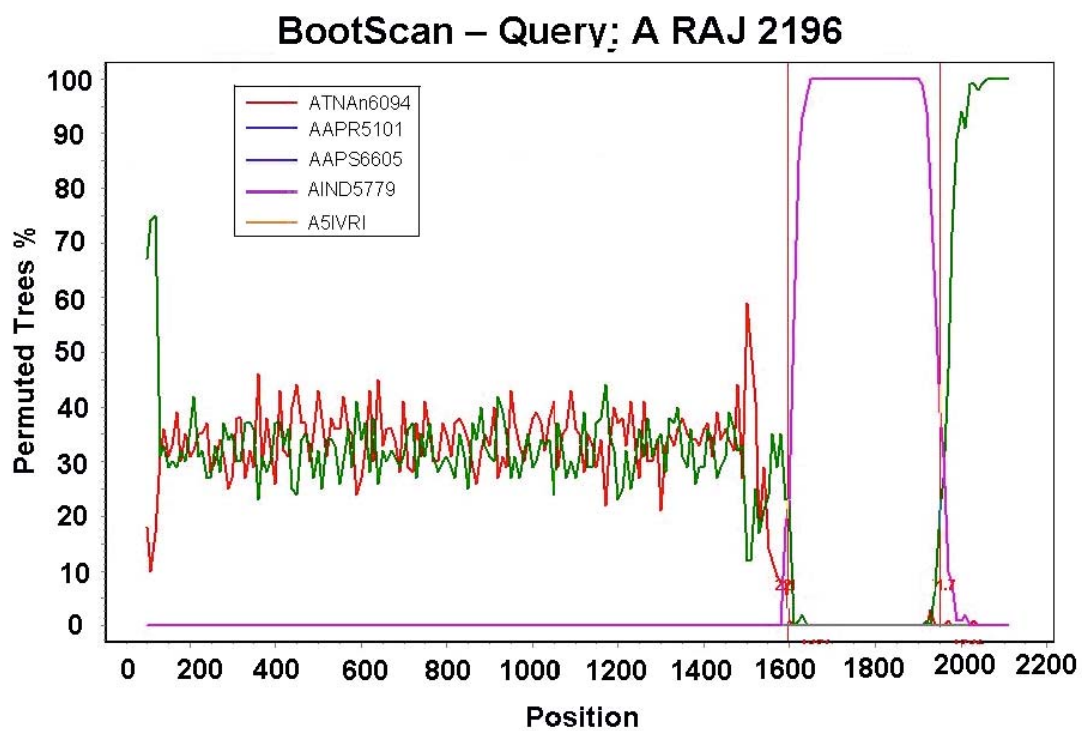
(A)
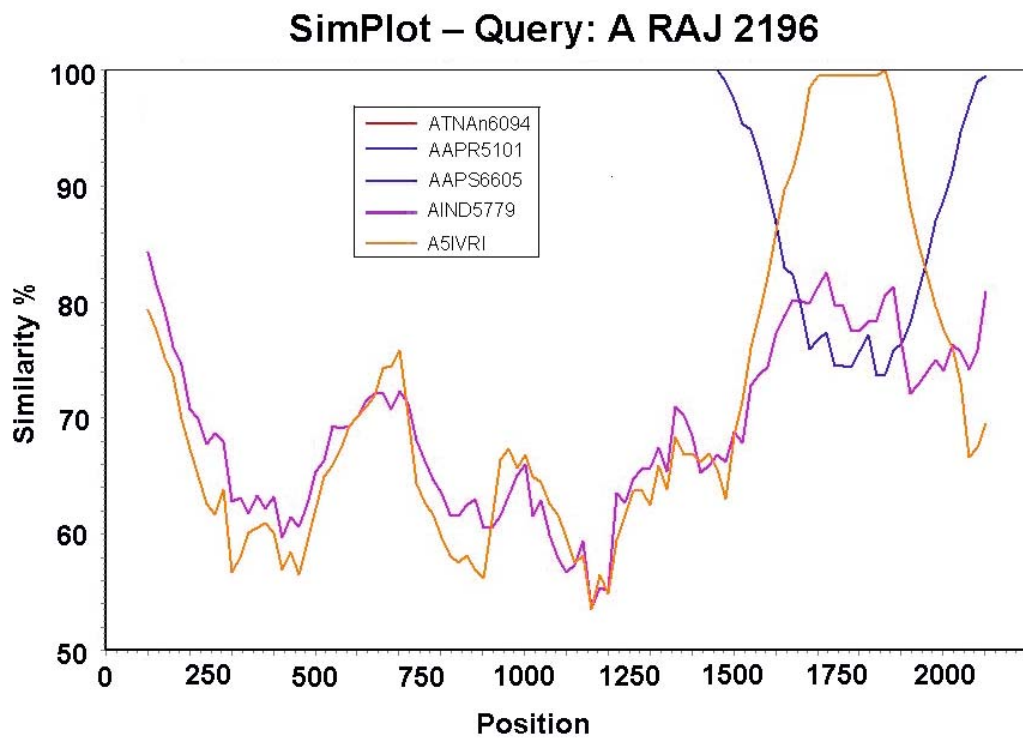
(B)

Figure 4.	**(A)** Similarity plot for a type A Indian isolates showing recombination event in the VP1 coding 1D region. **(B)** Recombination event in VP1 coding region in a serotype A virus of Indian origin.
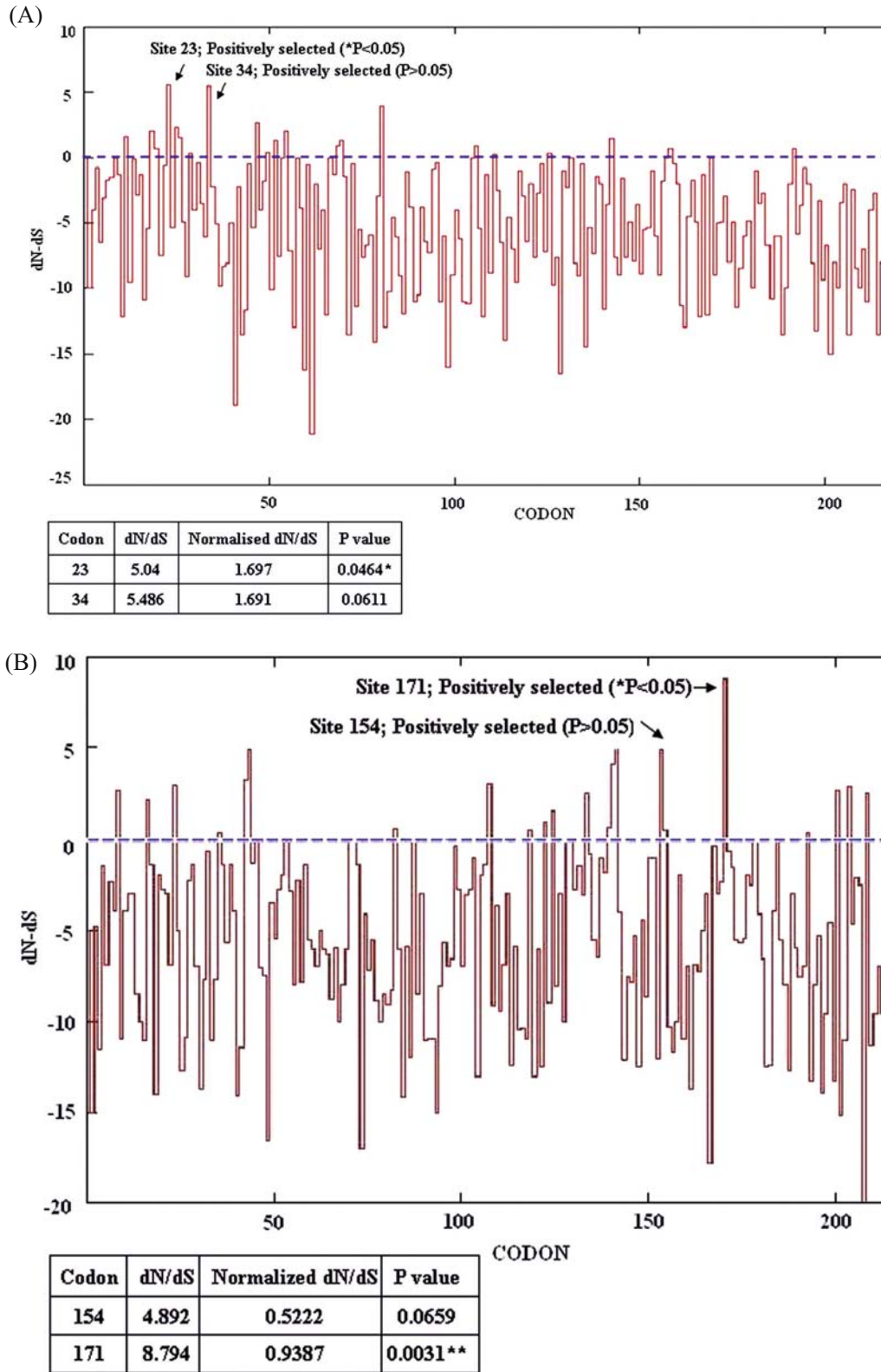
(A)

Codon	dN/dS	Normalised dN/dS	P value
23	5.04	1.697	0.0464*
34	5.486	1.691	0.0611

(B)

Codon	dN/dS	Normalized dN/dS	P value
154	4.892	0.5222	0.0659
171	8.794	0.9387	0.0031**

Figure 5. **(A)** Sliding window representing the rate of synonymous and non-synonymous nucleotide substitutions per site from serotype A L^{pro}-coding sequences. Zero value indicates dN = dS. **(B)** Sliding window representing the rate of synonymous and non-synonymous nucleotide substitutions per site from serotype A VP1 coding sequences. Zero value indicates dN = dS.

not uncommon to observe virus isolates with different sequences present in a pool of viruses isolated from a single animal. However, all the virus isolates with different sequences do not survive and establish themselves in the environment due to selection pressure. Those that establish themselves result in newer strains that are genetically and antigenically different from the parent strain. As a result, single-type variants with varying antigenic make-up emerge in the field from time to time. These isolates can cause outbreaks, despite the fact that a good inactivated vaccine against FMDV is available.

The NJ and Bayesian trees, constructed using the L^{pro} coding nucleotide sequences revealed many sublineages that circulated in India between 1968 and 2005. All the isolates were related to the A_{22} subtype and none of the Indian strains showed a relationship to the A_5 or A_{10} subtypes. The various FMDV serotype A isolates could be broadly classified as belonging to the South American, European, Euro-South American and Asian lineages. The Indian isolates were derived from a single major lineage that diverged from the A_{22} strains represented by A_{22} IRAQ 24/64. Five sublineages (L1, L2, L3, L4 and L5) could be recognized. The sublineage L1 consisted of A IND 7/82; L2 consisted of A IND 85/79, A IND 86/79, A IND 2/68, A IND 20/82, A IND 22/82, A IND 26/82, A IND 25/81 and ORS 75/88; L3 consisted of A IND 73/79, A GUM 33/84, A APA 25/84 AND AA AP 41/84; L4 consisted of Indian isolates that emerged post-1994, namely, A TNAn 60/94, A TNC 71/94, A BIM 46/95, A APS 44/05, A APS 50/05, A APS 55/05, A APS 66/05 and A APS 68/05; while L5 consisted of A IND 3/77, A IND 16/82, A IND 17/82. Sublineage L4 is the predominant circulating one. The virus post-1995 diverged into many groups and the latest are the isolates from the state of Andhra Pradesh. The results confirmed the high variability of type A FMDV isolates in India.

Globally, FMDV type A is classified into 10 major genotypes (genotypes I–X) based on the 1D (VP1) gene sequence with >15% nucleotide divergence among the genotypes (Tosh *et al* 2002a). Since then, more variant strains have emerged in different parts of the world and the present study identified three more genotypes belonging to the A IRAN 96 and A IRAN 99 lineages from the Middle East, and A_{21} KENYA 64 and A EGYPT 06 from Africa. Earlier studies have shown that the Indian isolates have four distinct genotypes (Tosh *et al* 2002a). Examination of the complete 1D region in the present study showed that most of the Indian isolates belong to only two major genetic lineages, though newer virus strains have appeared over time and appear to have evolved from existing Indian serotype A virus strains. Only one Indian isolate (A ORS 64/84) could be grouped with the vaccine strain A IVRI which, in turn, could be grouped with A_{10} HOLLAND 42. Similar findings were reported by Tosh *et al* (2004) wherein the phylogenetic relationships between 35 Indian type A FMDV isolates were reconstructed by Lb gene

sequences using quartet-puzzling steps. The possibility of the virus originating from outbreaks in animals that had been administered a vaccine containing partially or incompletely inactivated virus could not be ruled out. Outbreaks due to escape of live virus in vaccine preparations have been documented earlier (King *et al* 1981). In P1 phylogeny, the genotype distinctions were supported by bootstrap values ≥98 of the 1000 bootstrap replicates. However, as reported by Tosh *et al* (2002a), IND 17/77 (genotype IV) was supported by low bootstrap values that could be due to limited phylogenetic information on related viruses. The probable extinction of genotype IV was the explanation provided, since none of the isolates were grouped with genotype IV in both the phylogenies. Further, the isolates were clustered into distinct genetic subgroups (<5% divergence) within genotypes VI and VII. Examination of the P1 phylogeny (phylogenetic trees not shown) showed temporal grouping patterns of the isolates rather than geographical clustering within subgenotypes, indicating their widespread distribution. This geographical intermixing of isolates could be due to unrestricted animal movement in the country. Partial vaccine coverage, indiscriminate movement of cattle within India and involvement of small ruminants in the epidemiology might have contributed to the distinct evolutionary pattern in India. Similar findings have been reported by Araujo *et al* (2002), wherein a high degree of genetic and antigenic variability was seen among the strains of serotype A FMDV isolated from foci of FMD in Brazil in 1994 and 1995. Such variability was particularly evident when these viruses were compared with the A_{24} BRAZIL 55 strain.

Recently, in a study related to the Turkish type A isolates, Klein *et al* (2006) reported the presence of two independent lineages among the currently circulating Turkish isolates. These isolates are closely related to the two earlier identified lineages of Iranian origin, A IRAN 96 and A IRAN 99 along with the traditional A_{22} subtype viruses based on phylogenetic analysis. These two new lineages have also been reported from Iran and other Middle-East countries. A fourth lineage was reported in the year 2005, which diverged from the A_{22} virus subtype and is antigenically related to the A_{22} IRAQ 24/64 and A_{22} Mahmattli strains.

Comparison of deduced amino acid consensus sequences for the P1 region of the different type A viruses showed genotype-specific amino acid substitutions, which confirm the earlier findings of Tosh *et al* (2002a). For instance, the variations at 8 amino acid positions (VP2 134, 191, 195, VP3 197, VP1 43, 83, 141 and 149) in the P1 region shown earlier were confirmed as characteristics of the Indian isolates belonging to genotypes IV, VI and VII. Of the 8 positions, 6 were mapped to/near antigenically critical residues. The highest variability was found not only at the amino acid positions corresponding to the main immunogenic region of the FMDV capsid, the G-H loop (amino acid residues

130–160), but also in other amino acid residues of the VP1 molecule, especially in sequences 40–47 and 164–171. Thomas *et al* (1988) described in VP1 another antigenic site constituted by the residues around the amino acid 170 (H-I loop). Apart from this, relevant modifications in the amino acid sequences 43–47 and in the residue 174 of VP1 were reported in the A ARG 76 and A ARG 79 strains (Cheung *et al* 1984; Weddel *et al* 1985).

4.2 *Reverse mutation or convergent evolution?*

The current study throws some light on the reverse mutation in FMDV or convergent evolution of viruses that are separated by geography and time. Genetic comparison of the L^{pro} and VP1 coding sequences of A IRAN 96 and A IRAN 98 reveal that these two isolates are related to one of the earliest vaccine strains A IND 57/79. The similarity of the sequences may be due to the extensive mutations that occurred in these two isolates, which reverted to those isolates that were in circulation earlier rather than being introduced from outside sources. However, the VP1 and L^{pro} trees show non-congruous results and it is only in the VP1 tree that the Middle Eastern and A IND 57/79 viruses look similar. This suggests a common origin for these Middle East Asian and Indian viruses, perhaps due to a recombination event or due to back mutations in VP1. The current WRL reports reveal that the outbreaks of 2005 in Iran and Turkey were caused by isolates that were different from both A IRAN 96 and A IRAN 99 but similar to A$_{22}$ IRAQ 64 (WRL-FMD Network Report 2006), and this could be an evidence for reverse mutations or, more likely, convergent evolution. This may be due to vaccine pressure and re-emergence of old virus strains. However, it must be noted here that A IRAN 99 does not relate to A IND 57/79 or any of the Indian isolates, at least on comparison of the VP1 coding sequences, though it belongs to the same geographical area as the other Middle East Asian isolates.

4.3 *Recombination events in L^{pro} and P1 regions*

Recombination events have been documented in the 3' end of the genomes encoding the non-structural proteins of picornavirus (Cammack *et al* 1988; Wilson *et al* 1988; Furione *et al* 1993; Santti *et al* 1999). Until recently, there was only one report available on recombination within the FMDV coat proteins involving isogenic parents (King *et al* 1982). The report of Tosh *et al* (2002b) provided direct evidence of recombination in the structural protein-coding region in type A FMDV involving two heterogenic parents. One Indian serotype A isolate A IND 170/88 was discovered as a recombinant of genotypes VI and VII that circulated in India. Recombination, particularly in the structural protein-coding region, may provide selective advantage to

the virus, as the pre-existing immunity towards either of the parental viruses may not afford complete protection. This is of particular concern in countries where multiple FMDV genotypes co-circulate (Tosh *et al* 2002b). In our study, we found that recombination can also occur at the 5' end of the virus, which includes the L^{pro} coding region. However, the progenies of the isolate A IND 20/82 failed to establish themselves into separate genetic groups over a period of time. Also, the recombination event in the VP1 region of isolate A RAJ 21/96 showed that recombination can occur at any place in the genome. However, it was not necessary that such recombinant virus strains could become ancestors to form a distinct genetic group. Moreover, this isolate was not neutralized by any of the reference vaccine sera except rabbit convalescent sera raised using A TNAn 90/94. This could be due to a sharing of the immunodominant sites that are present in the VP1, VP2 and VP4 regions but not in the VP3 region, which matched with A IVRI and A ORS 64/84.

4.4 *Positive selection*

Studies based on monoclonal antibody-resistant mutants have identified 36 amino acid sites that are critical in the antigenicity of FMDV type A (Thomas *et al* 1988; Baxt *et al* 1989; Bolwell *et al* 1989; Saiz *et al* 1991). Mittal *et al* (2005) identified six additional sites. In this paper, we report another site (171 in VP1) that is under positive selection. Incidentally, this site is present within the major immunodominant site or the antigenic epitope within the G-H loop and is a conformation-dependent site. However, positive selection of site 154 described by earlier workers (Tosh *et al* 2003) was not significant (*P*>0.05) in our study. This could be due to the epidemiological nature of the disease and the distinct evolutionary pattern that different virus strains in India underwent. Aggarwal and Barnet (2002) found that the antigenic sites reported are far from complete, and yet-unidentified sites might also be important in the induction of a protective immune response. Importantly, some of these sites may be present in and around the loop region of structural capsid proteins such as 171, which was significant in the Indian isolates compared. It was previously proposed, through amino acid sequence alignments, that L^{pro} of FMDV belongs to the family of cysteine proteinases (Gorbalenya *et al* 1991). Recently, the crystal structure of the FMDV L^{pro} from O1Kaufbeuren was determined (Guarné *et al* 1998, 2000), confirming Cys51 and His148 to be the catalytic residues as well as the papain-like fold. As expected, the catalytic triad of the proteinase (Cys52–His149–Asp164) was conserved. In this study, no difference was found in the length of L^{pro}. However, it has been reported that in type A isolates from Kenya, namely, KEN/37/84 and KEN/1/76, the leader proteinase L^{pro} was found to be 202 and 201 amino acids in length, respectively. All the European type A, O and

C isolates had 201 amino acids. The corresponding region of the SAT serotypes, and the type A and O isolates from West Africa had 199 residues. The most variable regions in L^{pro} were found to be a-helices 2 and 3, b4, the loop to b5 as well as the C-terminal extension (CTE). The evidence for positive selection in the L^{pro} region indicates that the immune mechanisms are active even at the L^{pro} region where the viral protein initiation sites are present. The viruses circulating in these regions are under immune pressure in some part of their evolutionary path due to a low level of antibodies. Therefore, antibody-combining sites are more likely to be under positive selection due to immune pressure (Tosh *et al* 2003); however, positive selection in the L^{pro} region identified in this study shows that some intracellular selection pressure is in operation for the initiation of viral replication in the host. Such a selection may be responsible for selective binding of the ribosome for initiation of virus protein synthesis resulting in the generation of virus that can cause clinical signs even in vaccinated animals. Such a hypothesis needs to be confirmed using *in vitro* studies using modified L^{pro} sequences encoding these changes in the amino acid sequences. The significance of positive selection on residue 23 is important as it is close to the second initiation codon (AUG) in the open reading frame (ORF) of the viral genome, as most of the viral proteins are said to be initiated from the second initiation codon in FMDV to produce L^{b} predominantly rather than using the first initiation codon in the ORF to synthesize L^{pro}.

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