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Partial sequence analysis of VP1 of Indian isolates of foot-and-mouth disease virus type Asia-1

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Abstract Nucleotide sequence of 3′ end of VP1 (1D region) was determined using RT-PCR amplified DNA of 31 foot and mouth disease virus (FMDV) type Asia-1 field isolates originating from 11 different geographically distinct states of India during the period 1987–2000. These field strains exhibited an average of 7.5% divergence among them and were found to be divergent from the Indian vaccine strains Asia-1 WBN 117/85, IND 8/79, and IND 63/72, by an average 5.9, 14.8, and 7.4% divergence, respectively. Phylogenetic analysis of these 31 field isolates including 3 of the vaccine strains of India and sequences of 22 Indian field isolates obtained from the GenBank revealed that all the Indian FMDV type Asia-1 isolates belonged to a single genotype comprising of two distinct lineages (Lineages A and B). All the field isolates under study belonged to the Lineage-B comprising 8 different clusters, which also includes the vaccine strains WBN-117/85 and IND 8/79. Surprisingly, another vaccine strain IND 63/72 formed Lineage-A. Phylogenetic analysis of sequences of another 23 exotic type Asia-1 isolates from 15 different countries obtained from the GenBank along with the 56 Indian isolates revealed the existence of three distinct genotypes. The prototype strain Asia-1 PAK 1/54 belongs to a separate genotype. Two strains from India along with one strain each from China and Russia belongs to another genotype. The third genotype is formed by the remaining isolates including all the 31 isolates from the present study and exotic viruses from 14 other different countries. Comparison of deduced amino acid (aa) sequence indicated that majority of the mutations were found within two distinct regions corresponding to amino acid positions 130–160 and 193–211. The motif at aa positions 138–141 in vaccine strains WBN 117/85, IND 8/79 and in all the field isolates was ETTS/P; however, the same motif in IND 63/72 was TQPT. The motif 153–156 in majority of Indian isolates including vaccine strains WBN 117/85 and IND 8/79 was LSGQ/R whereas the same motif seen in IND 63/72 was VSNR. The study revealed that the FMDV type Asia-1 isolates circulating in the country are not highly heterogeneous, but showed considerable genetic variations. Certain mutations were also observed in the residues, which have been proved to be contributing to the formation of neutralizing epitopes. In neutralization studies employing polyclonal antisera, type Asia-1 WBN 117/85 revealed broader serological spectrum than other vaccine strains of India used in this study.

Keywords FMDV · Serotype Asia-1 · 1D region

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Introduction

Foot and mouth disease (FMD) is a highly contagious and acute viral infection of cloven-hooved animals caused by foot and mouth disease virus (FMDV). FMDV belongs to genus *aphthovirus* of family Picornaviridae which exists as seven antigenically distinct serotypes viz. O, A, C, Asia-1, South African Territory-1 (SAT-1), SAT-2, and SAT-3.

Incidences of types O, A, and C have been recorded in different parts of the world. However, incidences of types Asia-1 and SAT-1–3 are mainly restricted to Asian continent and South African Territory, respectively. In India outbreaks due to serotypes viz. O, A, and Asia-1 are widespread but outbreak due to type C has not been recorded since 1995.

The FMDV has icosahedral symmetry and it consists of 60 copies of four structural polypeptides VP1 to VP4. The virus contains a single-stranded positive sense RNA genome of about 8.5 kb which undergoes a very high level of mutation resulting in the emergence of genetic and antigenic variants.

Among the four viral polypeptides, VP1 is the most immunogenic and most exposed (54% of residues) on the virion surface [1]. It plays a major role in conferring immunity against the disease. Immunological properties of the virus are also dependent on VP1 (1D region), especially amino acid 137–157 region and to a lesser extent aa residues 200–213 of C terminus. The region formed by aa residues 141–160 is a part of the G-H loop protruding from the surface of the virion [2] harboring critical antigenic determinants of the virus which are involved in the attachment of the virus to the cell [3]. The phylogenetic analysis based on nucleotide sequencing of a region formed by aa residues 130–213 of VP1 allows direct evaluation of the relatedness among virus strains [4].

The article describes the phylogenetic analysis of Indian FMDV type Asia-1 isolates and their serological spectrum in comparison to vaccine strains.

Materials and methods

FMDV type Asia-1 isolates

Thirty one FMDV type Asia-1 field isolates obtained from clinical cases of FMD-infected cattle or buffaloes were taken up for the study. These samples originated from 11 geographically distinct states of India during the period 1987–2000 (Table 1). All the isolates were adapted to BHK-21 cell line. The virus-infected culture fluids were stored at −70°C until further use. FMDV type Asia-1 vaccine strains were obtained from Indian Immunologicals Ltd. Hyderabad. Strains WBN 117/85, IND 8/79, and IND 63/72 originated from Indian states of West Bengal in 1985, Gujarat in 1979, and Maharashtra in 1972, respectively.

Micro-serum neutralization test

One-way serological relationship (‘*r*’ value) was determined using the bovine vaccinate serum (BVS) raised against the vaccine strains of type Asia-1 WBN 117/85, IND 63/72, and IND 8/79, by two-dimensional micro-serum neutralization test (MNT) [5]. For the raising of BVS against vaccine strains, mono-valent vaccines were prepared from inactivated and purified virus preparations of FMDV type Asia-1 vaccine strains. Seronegative calves above 4 months of age, maintained in FMD-free farm were vaccinated with these vaccines (3 ml dose). Serum antibody titer was estimated by MNT against homologous virus and the serum dilutions having serum neutralization titer (SN_{50}) of 2.0–2.5 were used in two-dimensional MNT for estimation of ‘*r*’ values.

The mean serum neutralization titer (SN_{50}) of three replicate tests was used for calculation of ‘*r*’ values, expressed as the ratio of SN_{50} of heterologous to homologous viruses. An ‘*r*’ value closer to 1.00 indicates that the field virus has a close antigenic relationship with the vaccine virus. The critical ‘*r*’ value is the highest value of ‘*r*’ which is distinguishable from 1.00 at a set probability for a given number of test replicates [6]. The strains were differentiated at a 99% significance level, which requires a critical ‘*r*’ value > 0.24 for a strain to be declared homologous.

RT-PCR and nucleotide sequencing

Total RNA was extracted from the cell culture-derived FMDV by guanidine isothiocyanate method using ‘RNeasy Total RNA extraction kit (Qiagen, Germany). 1D region of FMDV genome was amplified in one tube one buffer RT-PCR system (Qiagen, Germany) with the universal primer (NK61, 5′-GACATGTCCTCCTGCATCTG-3′) and type-specific primer (pAs-IC505, 5′-TACACTGCTTCTGAC GTGGC-3′) combinations [7]. The amplified product of size ∼911 bp was visualized by ethidium bromide staining in 1% agarose gel after electrophoresis. The PCR product was purified by using the Wizard PCR Prep DNA Purification system (Promega, USA) for removing the residual oligonucleotide primers, dNTPs, and enzyme.

The purified PCR products were subjected to cycle sequencing using Fmol cycle sequencing kit (Promega, USA) with the virus-specific antisense primer pNK72 (5′-GAAGGGCCCAGGGTTGGACTC-3′) [7]. Before subjecting to cycle sequencing the primer was endlabeled with $^{32}P\gamma ATP$ using T4 Polynucleotide Kinase. The product was electrophoresed in 6% polyacrylamide gel in the presence of SDS in a sequencing apparatus and nucleotide sequence of about 246 bp of 3′ 1D region was determined by autoradiography.

Sequence analysis

All the nucleotide sequences taken up for this study were initially aligned using Genedoc software [8]. Multiple

Table 1 GenBank accession numbers along with details of FMD virus Asia-1 field isolates for which nucleotide sequence data for 1D region have been submitted to the GenBank

S. No.	Laboratory reference No. of virus sample	Outbreak date	Origin of the specimen			Species	V/UV	Specimen collected	Date of submission	GenBank Accession No.
			Village	District	State					
1	Asia-1, GUKh-1/87	Feb-87	Ravipura	Kheda	Gujarat	C	V	TE	25.01.06	DQ319903
2	Asia-1, APKh-2/88	Aug-88	Nachapalli	Khammam	Andhra Pradesh	B	UV	TE	25.01.06	DQ376235
3	Asia-1, KEC-3/88	Aug-89	Taliparamba	Cannanore	Kerala	C	UV	TE	25.01.06	DQ376236
4	Asia-1, KAKo-4/90	Jan-90	Hosahudya	Kolar	Karnataka	NA	V	NA	25.01.06	DQ376237
5	Asia-1, MAP-5/90	Dec-90	Wagalwadil	Pune	Maharashtra	C	UV	TE	25.01.06	DQ376238
6	Asia-1, KEC-6/91	Sep-91	NA	Cannanore	Kerala	C	V	TE	25.01.06	DQ376239
7	Asia-1, GUM-7/92	Jan-92	Patan	Mehsana	Gujarat	C	UV	FE	25.01.06	DQ376240
8	Asia-1, MPK-8/92	Jan-92	Khidikya	Khandwa	Madhya Pradesh	C	UV	TE	25.01.06	DQ376241
9	Asia-1, MPKr-9/92	Feb-92	Bhulgonw	Khargove	Madhya Pradesh	C	UV	TE	25.01.06	DQ376142
10	Asia-1, APR-10/92	May-92	Nandipalli	Rangareddy	Andhra Pradesh	NA	NA	NA	26.01.06	DQ376243
11	Asia-1, MAC-11/92	Aug-92	Madheli	Chandrapur	Maharashtra	C	UV	TE	28.01.06	DQ381805
12	Asia-1, GUA-12/92	Sep-92	Amnagar	Ahmedabad	Gujarat	NA	NA	TE	28.01.06	DQ381806
13	Asia-1, KAN-13/93	Sep-93	Pandavapur	Mandya	Karnataka	NA	V	TE	15.02.06	DQ404399
14	Asia-1, TNN-14/93	Sep-93	Bokka Farm	Nilgiris	Tamil Nadu	NA	UV	TE	15.02.06	DQ404400
15	Asia-1, GUSk-15/94	Feb-94	Lakshipura	Sabarkantha	Gujarat	C	UV	TE	13.02.06	DQ400358
16	Asia-1, WBP-16/94	Aug-94	Telidih	Purulia	West Bengal	NA	UV	TE	15.02.06	DQ404398
17	Asia-1, MAB-17/94	Sep-94	Goregaon East	Mumbai	Maharashtra	B	V	FE	15.02.06	DQ404397
18	Asia-1, APN-18/94	Nov-94	AHC (D.LAB)	Nizamabad	Andhra Pradesh	NA	NA	TE	15.02.06	DQ404401
19	Asia-1, TPA-19/94	Nov-94	NA	Agartala	Tripura	C	UV	TE	15.02.06	DQ404402
20	Asia-1, WBN-20/94	Nov-94	SLF, Mohanpur	Nadia	West Bengal	C	UV	TE	15.02.06	DQ404403
21	Asia-1, MAA-21/94	Dec-94	Koliwadi	Ahmedanagar	Maharashtra	C	V	TE	15.02.06	DQ404404
22	Asia-1, MAN-22/94	Dec-94	Shingave	Nasik	Maharashtra	C	V	TE	16.02.06	DQ404405
23	Asia-1, KAB-23/94	Dec-94	Damlun	Bangalore	Karnataka	C	V	TE	16.02.06	DQ404406
24	Asia-1,GUKt-24/94	Dec-94	Anjar	Kutch	Gujarat	C	V	TE	16.02.06	DQ404407
25	Asia-1, MAJ-25/95	Feb-95	Faizpur	Jalgaon	Maharashtra	C	V	TE	16.02.06	DQ404408
26	Asia-1, TNDg-26/95	Mar-95	Vilanpatti	Dindigul	Tamil Nadu	C	V	TE	16.02.06	DQ404409
27	Asia-1, GOA-27/95	Jul-95	Goa Vehla	North Goa	Goa	C	V	TE	18.02.06	DQ406753
28	Asia-1, GUR-28/98	May-98	Peddock	Rajkot	Gujarat	C	V	TE	18.02.06	DQ406754
29	Asia-1, DED-29/98	Mar-99	Gazipur	Delhi	Delhi	NA	V	TE	18.02.06	DQ406755
30	Asia-1, GUBv-30/99	Jun-99	Parvadi	Bhavnagar	Gujarat	C	UV	TE	18.02.06	DQ406756
31	Asia-1, GUSk-31/2000	Apr-00	Bayad	Sabarkantha	Gujarat	B	UV	TE	18.02.06	DQ406757

NA = Not available; V = Vaccinated; UV = Unvaccinated; C = Cattle; B = Buffalo; TE = Tongue epithelium; FE = Foot epithelium

alignments of the sequence data and phylogenetic analysis were carried out with ClustalX 1.81m (PC/Gene software). The phylogenetic tree was constructed as per Felsenstein [9] using NJplot of the ClustalX software. The nucleotide sequences were piled up and multiple data sets were generated using bootstrap resampling and values shown in the model were bootstrap values for 1000 replicates. Phylogeny was estimated by algorithms from distance matrix data under additive model, according to which the distances are expected to be equal to the sum of the length of the lines between the isolates. Rooted phylogenetic trees were drawn.

The nucleotide sequences of all the strains were subjected to similarity plots analysis using SimPlot software [10] with window size of 20 bp (step: 10 bp) with gap-stripping and Kimura (2-parameter) correction so as to visualize the relatedness of one sequence to a panel of other sequences.

Selection pressure analysis was undertaken on the deduced amino acid sequences (130–211) of VP1 for determining the positively selected sites using single Likelihood Ancestor Counting (SLAC) analysis [11].

Results and discussion

Comparison of 31 FMDV type Asia-1 isolates with the vaccine strain Asia-1 WBN 117/85, revealed that 26 out of 31 (83.87%) isolates were homologous as evident from the '*r*' values. However, '*r*' values of remaining 5 isolates were found to be significantly different ($P = 0.01$) from this vaccine strain (Table 2). However, comparisons of these field isolates with the then vaccine strain, IND 8/79 showed that 22 out of 31 (70.97%) isolates were homologous and remaining 9 were heterologous. Surprisingly, comparison of these isolates with a vaccine strain IND 63/72 showed only 22.58% isolates to be homologous.

In the present study, nucleotide sequencing of 3′ end of 1D (VP1 encoding) region encompassing 130–211 amino acids residues of VP1 (total 246 nucleotides) of 31 field isolates of type Asia-1 originating from 11 states of India collected during a 14-year period from 1987 to 2000 was carried out. Two type Asia-1 vaccine strains viz. WBN-117/85 and IND 8/79 were also taken up for comparison. The sequence data of these field isolates have been submitted to the GenBank and the accession numbers were obtained.

However, for drawing a more interesting epidemiological conclusion and hypothesis, nucleotide sequences of additional 23 isolates from India including another type Asia-1 vaccine strain of India, IND 63/72 (Accession # Y09949), and 23 other exotic strains of type Asia-1 from 15 different countries including the prototype strain Asia-1 PAK 1/54 (Accession # AJ251478), were also included for comparison. The sequences of 22 field isolates of India isolated during the period 1981–2004 were obtained from the GenBank. The sequences of 16 exotic strains originating from 13 different countries viz. Afghanistan, Bhutan, China, Greece, Hong Kong, Iran, Lebanon, Myanmar, Pakistan, Russia, Tajikistan, Thailand, and Turkey, isolated during the period 1983–2005 were also obtained from the GenBank. The sequences of 7 more exotic strains of type Asia-1 viz. Shamir 1989 from Israel, Asia-1 48/97 from Russia, Asia-1 GR00 from Greece, IRN 0600 from Iran, and few strains from the neighboring Bangladesh Asia-1 BAN 10/01, BAN 17/01, and BAN 18/

Table 2 '*r*' values obtained for FMD virus type Asia-1 field isolates in MNT against BVS of WBN 117/85, IND 8/79, and IND 63/72

S. No.	Asia-1 field isolates	WBN-117/85	IND-8/79	IND-63/72
1	Asia-1, GUKh-1/87	*1.00*	*0.65*	**0.06**
2	Asia-1, APKh-2/88	*0.86*	*0.35*	**0.04**
3	Asia-1, KEC-3/88	*0.28*	*1.00*	*1.00*
4	Asia-1, KAKo-4/90	*1.00*	*0.60*	**0.03**
5	Asia-1, MAP-5/90	*0.48*	*0.34*	**0.17**
6	Asia-1, KEC-6/91	*0.91*	*0.59*	*1.00*
7	Asia-1, GUM-7/92	*0.38*	*0.36*	**0.06**
8	Asia-1, MPK-8/92	*0.81*	**0.15**	*0.25*
9	Asia-1, MPKr-9/92	**0.16**	**0.16**	*0.27*
10	Asia-1, APR-10/92	*0.49*	*0.68*	**0.03**
11	Asia-1, MAC-11/92	*0.59*	**0.17**	**0.04**
12	Asia-1, GUA-12/92	**0.21**	*0.81*	*0.26*
13	Asia-1, KAN-13/93	**0.22**	*1.00*	**0.13**
14	Asia-1, TNN-14/93	**0.22**	*1.00*	**0.02**
15	Asia-1, GUSk-15/94	**0.12**	**0.05**	**0.02**
16	Asia-1, WBP-16/94	*0.50*	*0.50*	**0.05**
17	Asia-1, MAB-17/94	*0.29*	**0.17**	**0.12**
18	Asia-1, APN-18/94	*0.95*	*1.00*	*0.74*
19	Asia-1, TPA-19/94	*0.32*	**0.11**	**0.13**
20	Asia-1, WBN-20/94	*0.71*	*0.63*	**0.12**
21	Asia-1, MAA-21/94	*0.34*	**0.04**	**0.03**
22	Asia-1, MAN-22/94	*0.39*	*0.45*	**0.09**
23	Asia-1, KAB-23/94	*0.60*	*0.43*	*0.31*
24	Asia-1, GUKt-24/94	*0.56*	*0.41*	**0.10**
25	Asia-1, MAJ-25/95	*0.98*	*0.25*	**0.06**
26	Asia-1, TNDg-26/95	*0.74*	*0.60*	**0.12**
27	Asia-1, GOA-27/95	*0.50*	**0.16**	**0.08**
28	Asia-1, GUR-28/98	*1.00*	*0.54*	**0.10**
29	Asia-1, DED-29/98	*0.71*	*0.43*	**0.10**
30	Asia-1, GUBv-30/99	*0.49*	*1.00*	**0.05**
31	Asia-1, GUSk-31/2000	*0.56*	**0.15**	**0.05**

Values in italics are homologous; values in bold are heterologous

01, kindly provided by Dr. Otfried Marquardt, Tubingen, Germany, were also included for comparison.

Per cent nucleotide homology/divergence among these type Asia-1 isolates was calculated and the Indian isolates under study (except IND 63/72) exhibited 0–12.6% divergence among them. The divergence of the field isolates with the vaccine strain WBN 117/85 ranged from 0% to 7.7% (average 5.9%) whereas the divergence of the field isolates with another vaccine strain of India IND 63/72, ranged from 12.7% to 17.6% (average 14.8%). The divergence of these field strains with IND 8/79 was found to range from 0.8% to 11% (average 7.4%). Two vaccine strains WBN 117/85 and IND 63/72 were found to be divergent from each other by 15.5%. Similar divergence (average 15.2%) was also reported [12] between these two strains when complete VP1 sequences were compared.

In the phylogenetic analysis, based upon the poliovirus genotyping studies [13], a divergence of more than 15% in nucleotide sequence could distinguish genotypes. However, FMDV strains differing by less than 5% are considered to be closely related and placed in the same cluster.

Phylogenetic analysis including 31 Indian field isolates collected during this study and 3 vaccine strains of India WBN-117/85, IND-8/79, and IND 63/72, and 22 other Indian field isolates from the GenBank revealed that all the Indian type Asia-1 strains belonged to a single genotype comprising of two distinct lineages (Lineages A and B). All the field isolates under study belonged to the Lineage-B, which also includes the vaccine strains of India viz. WBN-117/85 and IND 8/79, respectively. Surprisingly, another vaccine strain of India (IND 63/72) formed a separate lineage (Lineage-A) and none of the Indian isolates under study was a part of Lineage-A. All the Indian field isolates of Lineage-B were found to be distributed into 8 different clusters (Fig. 1).

It has already been stated that the Indian isolates under study (except IND 63/72) exhibited 0–12.6% divergence among them. Hence, the FMDV type Asia-1 isolates circulating and causing FMD in India are relatively heterologous on the basis of the partial nucleotide sequencing of 1D (VP1) region. Existence of such heterologous population of virus isolates may be due to the quasispecies nature of the virus which may be attributed to several epidemiological factors. In India the FMD outbreaks due to type Asia-1 is around 15–20% of total FMD outbreaks. Such outbreaks are generally recorded nearly throughout the year. Very low vaccination coverage, presence of large number of FMD susceptible livestock population of more than 470 millions animals including several species viz. cattle, buffalo, sheep, goat, and pigs, and unrestricted movement of susceptible animals might have resulted in such a heterologous population of virus in India.

In many of the clusters the relationship among the virus isolates are very close, especially when the virus isolate originated from the same region of Central, Western, or Southern Indian states. On the other hand, some isolates are very closely related to the virus isolated from far off places than an isolate from the same area (district/state). Absence of any specific distribution pattern of the sequences among Indian isolates may be due to the unrestricted movement of clinically or sub-clinically infected livestock with in the country [14, 15].

Phylogenetic analysis of 23 exotic type Asia-1 isolates along with 56 Indian isolates revealed the existence of three different genotypes (Fig. 2). The prototype strain Asia-1 PAK 1/54 formed a separate genotype. Two strains from India isolated during the years 1980 and 1981 along with one strain each from China and Russia originating from an outbreak in the year 2005 formed a separate genotype. The third genotype is formed by the remaining strains. However, as discussed earlier, the vaccine strains IND 63/72 and WBN 117/85 formed two different lineages in the third genotype. None of the Indian isolates, examined were closely related to PAK 1/54. In fact, two Indian vaccine strains IND 63/72 and WBN 117/85 were found to be divergent from the prototype strain PAK 1/54 by 15.92% and 20.00%, respectively. All the 31 isolates of the present study belong to a single genotype (genotype 3) which also includes majority of the other isolates from India as well as isolates from 14 different countries. Many of the isolates in the present study isolated during the period 1994–1998 were found to form the same cluster along with more recent isolates from Pakistan, Bangladesh, Greece, Hong Kong, Iran, Myanmar, Tajikistan, and Turkey. It is interesting to note that the recent isolates of India were found to be closely related to an isolate from Bhutan and isolates from Iran, Greece, and Turkey originated during 1999–2000. Thus these close relationships among the virus isolates originating from different countries rather than circulation of similar virus strains in different countries does not exclude the probable inter-regional spread of the virus from one country to another [16].

Similarity plots were also drawn using SimPlot software [10] with window size of 20 bp (step: 10 bp) with gap-stripping and Kimura (2-parameter) correction so as to visualize the relatedness of one sequence to a panel of other sequences. SimPlot analysis incorporating 31 type Asia-1 field isolates (Asia-1) of the present study along with 3 vaccine strains revealed that these isolates were highly similar and especially at the hyper variable region (aa 133–160), similarity among the strains were more than 70%. However, one of the vaccine strains IND 63/72 exhibited lower similarity with respect to the hyper variable region with the rest of the isolates. When SimPlot analysis was done incorporating an additional 22 Indian type Asia-1

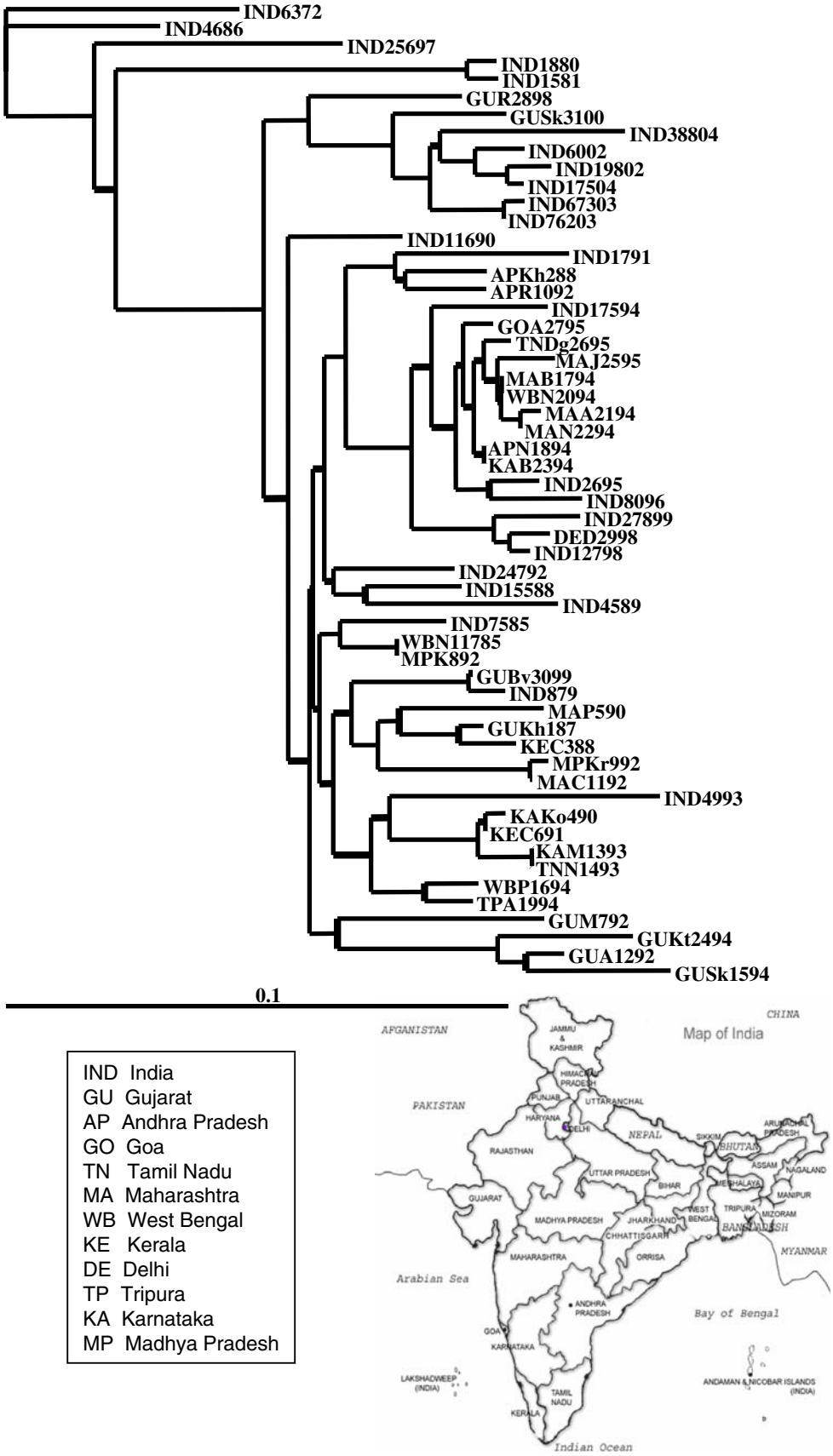

Fig. 1 Neighbor-joining tree showing genetic relationship among Indian isolates of FMDV type Asia-1 based on partial nucleotide sequence of VP1 (130–211 amino acid). Last two digits in identification number of each isolate indicate year of isolation and first two letters indicate the state of origin, where as IND denotes India

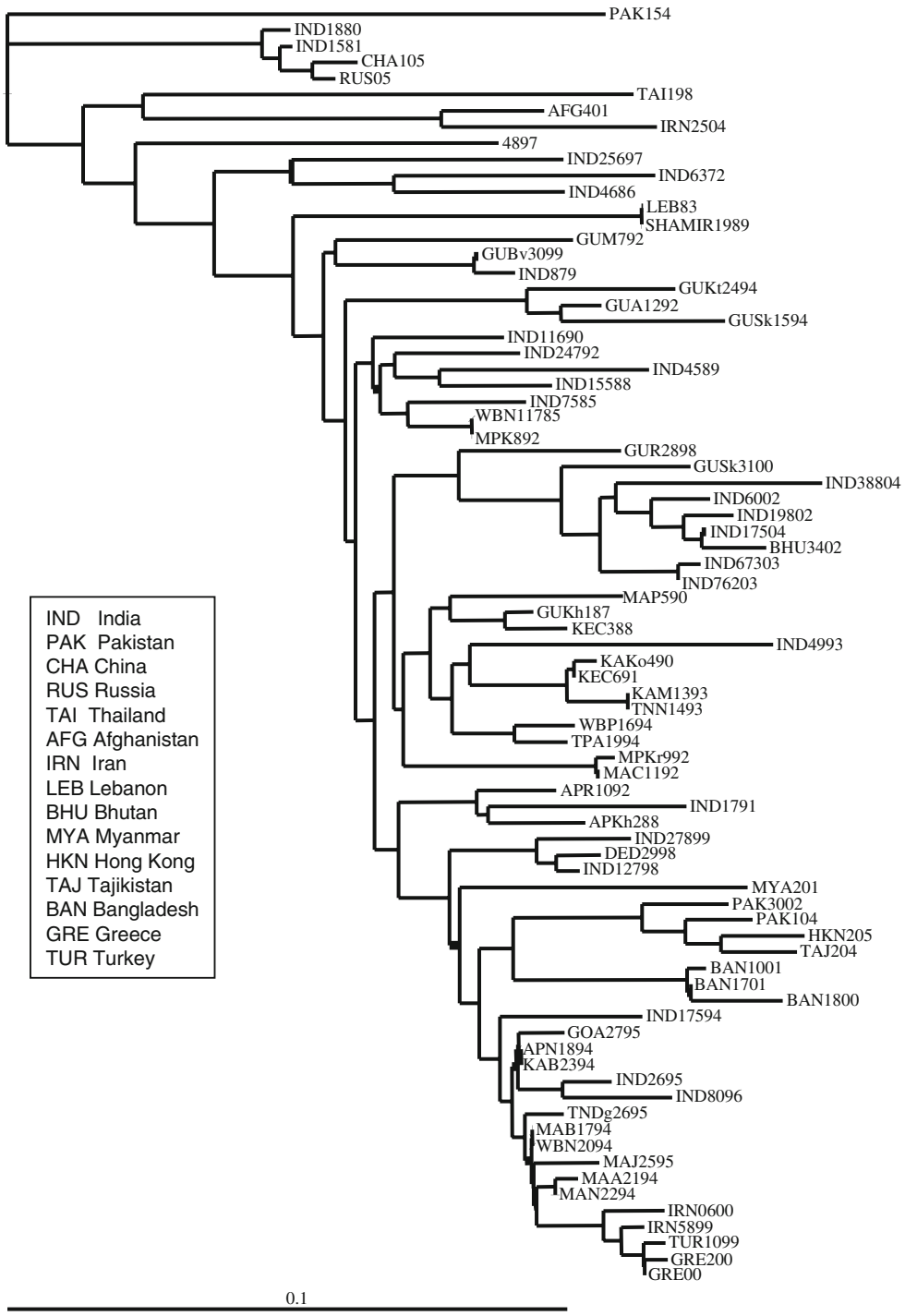

Fig. 2 Neighbor-joining tree showing genetic relationship among 56 Indian isolates along with 23 isolates from 15 other countries of FMDV type Asia-1 based on partial nucleotide sequence of VP1 (130–211 amino acid). Last two digits in identification number of each isolate indicate year of isolation and first three letters indicate the country of origin

field isolates, majority of the isolates were found to be similar (more than 70%) at the hyper variable region, but around 10% of the isolates were found to be divergent in this region showing much lower per cent of similarity. A very low similarity was seen in the hyper variable region when SimPlot analyses were conducted incorporating Indian type Asia-1 isolates along with exotic isolates from 15 other countries.

Comparison of deduced amino acid sequence of the region 130–211 amino acid of 1D region (VP1 protein) of 31 Indian types Asia-1 field isolates in this study along with 3 vaccine strains showed majority of the mutations were clustered within two distinct regions corresponding to antigenic site, comprising the G-H loop (aa positions 130–160) and the C terminus region (aa positions 193–211). The isolates exhibited variation in the sequence

Table 3 Alignment of the deduced amino acid sequences of positions 130-211 of 1D genome region of FMD virus type Asia-1

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                  *        20        *        40        *        60        *        80
WBN11785 : YNGKTTYGETTSRRGDMADLAQRLSGRLPTSFNYGAVKAETITELLIRMKRAETYCPRPLLALDTTQDRRKQEIIAPEKQVL : 82
GUKh187  : ...........P......A.......Q.................................................... : 82
APKh288  : .................A....M..Q.................................................... : 82
KEC388   : ...........P......A.......Q.................................................... : 82
KAKo490  : .................A.G....RQ.............N.................................... : 82
MAP590   : .................A......KQ.............A.................................... : 82
KEC691   : .................A......RQ.............N.................................... : 82
GUM792   : .................A.....NR.....................................H.............S. : 82
MPK892   : ...................................................................... : 82
MPKr992  : ...........P....L.S...................D.....................................A. : 82
APR1092  : .................A....V..Q.................................................... : 82
MAC1192  : ...........P....L.S.....TR.............D.....................................A. : 82
GUA1292  : .................A.......Q.............N.....L.....................................A. : 82
KAM1393  : .................A......RQ.............N.................................... : 82
TNN1493  : .................A......RQ.............N.................................... : 82
GUSk1594 : ................T.A.......Q.............N.....L.....................................A. : 82
WBP1694  : .................A......E.............N.................................... : 82
MAB1794  : ...........P......A.......Q.............D.....................................MM : 82
APN1894  : ...........P......A.......Q.............D.....................................MM : 82
TPA1994  : .................A.....................N.................................... : 82
WBN2094  : ...........P......A.......Q.............D.....................................MM : 82
MAA2194  : ...........P......A.T.....Q.............D.........................R.............MM : 82
MAN2294  : ...........P......A.T.....Q.............D.....................................MM : 82
KAB2394  : ...........P......A.......Q.............D.....................................MM : 82
GUKt2494 : .................A.....................R..N.....L.....................................T. : 82
MAJ2595  : ...........PQ...V.A.......Q.............D.....................................MM : 82
TNDg2695 : ...........P......A.......Q.............D.....................................MM : 82
GOA2795  : ...........P......A.......Q.............D.....................................M. : 82
GUR2898  : .................A.T.................D.....................................Q.... : 82
DED2998  : .................A.......Q.............D.....................................MM : 82
GUBv3099 : .................A.....................................H.....................A. : 82
GUSk3100 : .................A......E.............D.....................................A. : 82
IND879   : .................A......R.....................................H.....................A. : 82
IND6372  : ........TQPT....L.V....V.N.............D.........................H.................. : 82
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(.) indicated in relation to the vaccine virus Asia-1 WBN 117/85

region, especially in amino acid positions 138–142, 146–150, 153–156, 169–170, 193, 196, and 207–211 of VP1. However, with in the hyper variable region the motifs NGK (131–133), TYG (135–137), RGD cell attachment site (143–145), LPTS (157–160), aa 147 (A), and aa 149 (L) remained constant in all the isolates under study. These sites were also reported to be conserved across the other strains from India and abroad which were taken from the GenBank for comparison in the present study, except the motif TYG which was present as AYG in certain isolates from Iran (IRN2504), Lebanon (LEB83), Thailand (TAI198), Israel (Shamir1989), and an isolate from India (IND38804). Two amino acid stretches, viz. 137–140 and 152–155 of majority of the isolates in the present study was seen as ETTS/P and LSGQ/P, respectively (Table 3). Surprisingly, in many of the Indian isolates, including in the vaccine strain IND 63/72, these motifs were TQPT and VSNR, respectively [17]. Similar mutational changes were also reported in the motif 152–155 in type Asia-1 isolates from other countries, which might be resulting from natural mutation of parent viruses.

Fifty six Indian isolates including 31 isolates from the present study and 23 exotic strains of type Asia-1 were subjected to analysis for positive selection by examining the partial nucleotide sequence of 1D region (130–211 amino acid). Statistically insignificant ($P > 0.5$) positive selection was recorded at amino acid position 170 of VP1.

Careful analysis of the data generated helps to better understand the molecular basis of antigenic variation among the type Asia-1 isolates. A considerable genetic variation among Asia-1 isolates could be recorded. Some mutation was also recorded in some of the residues which have been proved to contribute to the formation of neutralizing epitopes [18]. It is also clear that genetic variation among type Asia-1 virus isolates is not very high and is much lower than what is found with other serotypes. It has also become evident from neutralization studies using polyclonal antisera that vaccine strains WBN 117/85 and IND 8/79 have broad serological spectrum for the field isolates under study.

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