SEQUENCE ANALYSIS OF RECENT INDIAN ISOLATES OF FOOT-AND-MOUTH DISEASE VIRUS SEROTYPES O, A AND ASIA 1 FROM CLINICAL MATERIALS

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Summary. – Partial nucleotide sequences of 1D gene of 38 isolates of foot-and-mouth disease virus (FMDV) of serotypes O, A and Asia 1 originating from various parts of India were determined. Field materials were subjected straight to RNA extraction, reverse transcription – PCR (RT-PCR) and sequencing. Also 3 FMDV vaccine strains, IND R2/75 (serotype O), IND 63/72 (serotype Asia 1) and IND 17/77 (serotype A) were included in the analysis. The seqences were compared mutually as well as with available corresponding sequences of other FMDV isolates, and their phylogenetic relationships were calculated. The deduced amino acid sequences showed that the serotype O isolates were relatively conserved as compared to serotype Asia 1 or A isolates from India. In phylogenetic analysis, the serotype O viruses clustered in two genotypes, one including the European vaccine strain (O1/K) and the other represented by the isolates from Bangladesh, India, Nepal and Turkey. The serotype Asia 1 viruses clustered in two groups of single genotype where the prototype strain from Pakistan (PAK 1/54) formed one group and the other was formed by the isolates from Bangladesh, Bhutan, India, Israel and Nepal. In serotype A viruses three well-differentiated genotypes were observed. The isolates from Azerbaijan, Bangladesh, Malaysia and India formed the first genotype. The second genotype was formed by isolates from Iran, Saudi Arabia and Turkey, while two recent Iranian isolates represented the third genotype. In India, the prevalence of at least one genotype could be identified in each serotype. This evolutionary clustering of isolates from the neighbor countries is not surprising, since these countries share border with India The genetic relatedness between sequences of isolates from India and those from distant places is indicative of spread of the virus between the countries. Of importance is the fact that clinical materials proved useful for rapid generation of sequences and subsequent studying of molecular epidemiology of the disease.

Key words: FMDV; Indian isolates; 1D gene; VP1; phylogeny

Introduction

FMDV, a small RNA virus (genus *Aphthovirus*, family *Picornaviridae*), is responsible for one of major economically important diseases of farm animals. Globally, there are seven immunologically distinct serotypes (O, A, C, Asia 1 and SAT 1-3) and multiple subtypes within each serotype (Pereira, 1977). The disease scenario in most of the Asian countries is grave. In India, the disease is caused by the serotypes O, Asia 1, A and C in that order. The incidence of the disease caused by the serotype C in the country has not been reported since 1995 (Anon, 1997). FMDV, like other RNA viruses, has high mutation rate due to the absence of the 3′→ 5′ exonuclease activity in viral RNA polymerase and exists as non-identical but closely related population termed viral quasispecies (Domingo *et al.*, 1992). Antigenic variants are selected from these highly

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Abbreviations: FMD = foot-and mouth disease, FMDV = FMD virus; nt = nucleotide; PBS = phosphate-buffered saline pH 7.4; RT-PCR = reverse transcription – PCR

Table 1. FMDV isolates originating from India in 1994–1998

Isolate No	Isolation date	Place of isolation (outbreak)	Host
O/ R2/75*	00/00/1975	Tamilnadu	Bovine
O/ IND 319/97	31/01/1997	Latur, Maharashtra	Bovine
O/ IND 333/97	09/05/1997	Nagaon, Assam	Bovine
O/ IND 18/98	12/11/1997	Cooch Behar, West Bengal	Bovine
O/ IND 20/98	13/11/1997	Cooch Behar, West Bengal	Bovine
O/ IND 31/98	09/11/1997	Jalpaigudi, West Bengal	Bovine
O/ IND 37/98	09/02/1998	Moga, Punjab	Bovine
O/ IND 45/98	00/00/1998	Pune, Maharashtra	Bovine
O/ IND 51/98	00/01/1998	Sirsa, Haryana	Bovine
O/ IND 53/98	00/01/1998	Sirsa, Haryana	Bovine
O/ IND 67/98	17/12/1998	Moga, Punjab	Bubaline
O IND 75/98	26/12/1997	Ribhoi, Meghalaya	Bovine
O/ IND 77/98	20/01/1998	Kamrup, Assam	Bovine
O/ IND 131/98	00/00/1998	Muzaffarnagar, Uttar Pradesh	Bovine
O/IND 304/98	18/05/1998	Upper Shillong, Meghalaya	Porcine
O/IND 307/98	05/06/1998	Kamrup, Assam	Bovine
O/IND 309/98	03/07/1998	Aizawl, Mizoram	Bovine
Asia 1/ IND 63/72*	00/00/1972	Tamilandu	Bovine
Asia 1/ IND 11/95	23/11/1994	Mandya, Karnataka	Bovine
Asia 1/ IND 14/95	00/00/1994	Nellore, Andhar Pradesh	Bovine
Asia 1/ IND 17/95	00/00/1994	Chittur, Andhra Pradesh	Bovine
Asia 1/ IND 49/95	09/02/1995	Kottayam, Kerala	Bovine
Asia 1/ IND 88/95	27/02/1995	Sangli, Maharashtra	Bovine
Asia 1/ IND 101/95	28/01/1995	Sangli, Maharashtra	Bubaline
Asia 1/ IND 8/96	00/00/1995	Arakonam, Tamilnadu	Bovine
Asia 1/ IND 11/96	00/00/1995	Ayampatcheri, Tamilnadu	Bovine
Asia 1/ IND 198/96	12/04/1996	Jalna, Maharashtra	Bovine
Asia 1/ IND 202/96	17/04/1996	Nasik, Maharashtra	Bovine
Asia 1/ IND 252/97	09/12/1994	Dindigul, Tamilnadu	Bovine
Asia 1/ IND 447/97	17/03/1997	Meshana, Gujarat	Bubaline
Asia 1/ IND 130/98	23/02/1998	Bhubaneswar, Orissa	Bovine
Asia 1/IND 305/98	23/05/1998	Khanapara, Assam	Bovine
Asia 1/IND 323/98	00/08/1998	Kaithal, Haryana	Bovine
Asia 1/IND 325/98	00/08/1998	Kaithal, Haryana	Bovine
Asia 1/IND 333/98	18/09/1998	Kaithal, Haryana	Bovine
A/ IND 17/77*	00//00/1977	West Bengal	Bovine
A/ IND 77/96	03/02/1996	Bangalore, Karnataka	Bovine
A/ IND 93/ 96	22/11/1995	Hisar, Haryana	Bovine
A/ IND 252/ 96	00/08/1996	Karnal, Haryana	Bovine
A/ IND 289/96	00/00/1996	Hisar, Haryana	Bovine
A/ IND 432/97	05/10/1997	Meerut, Uttar Pradesh	Bovine

*Vaccine strains

heterogeneous viral quasispecies populations in the presence (Carrillo *et al.*, 1989) or absence of immune pressure (Domingo *et al.*, 1993). By the advent of RT-PCR and cycle sequencing techniques, monitoring of circulation of outbreak strains has become easier and faster. VP1, the product of 1D gene and one of the structural proteins, is the target of this study as it is the most exposed protein which contains major antigenic determinants on the surface of the virion. To date, a large number of molecular epidemiological studies of foot-and mouth disease (FMD) based on comparison of partial or full 1D gene region are available (Beck and Strohmaier, 1987; Saiz *et al.*, 1993; Ansell *et al.*, 1994; Stram *et al.*, 1995; Vosloo *et al.*, 1995; Samuel *et al.*, 1987, 1999; Knowles *et al.*, 1998; Pattnaik *et al.*, 1998; Hemadri *et al.*, 2000; Nayak *et al.*, 2001). However, in all the abovementioned reports, the molecular studies have been performed on viruses isolated and propagated in cell cultures. The propagation of viruses in cell cultures for these purposes is a time consuming process as it involves a number of passages until a sufficient amount of viral RNA is obtained. The heterogeneous nature of a field isolate means that this procedure may artificially

select a particular phenotype. Furthermore, the emergence of antigenic variants during propagation in cell cultures has been observed (Sobrino *et al.*, 1983; Meyer *et al.*, 1994) which may hide the true genetic picture of outbreak strains as there is chance of accumulation of mutations during replication.

In this study, we have sequenced approximately 168 nucleotides of the 3′-terminus of the 1D gene encoding the VP1 polypeptide of FMDV serotypes O, Asia 1 and A from a large number of field materials. These sequences were compared with those of other FMDV isolates.

Materials and Methods

Field materials. Fifty-seven field samples consisting of tongue epithelium of different host animals from outbreaks in different parts of India during 1994–1998 and three vaccine strains of the serotypes O, Asia1, and A from the virus repository of our laboratory were subjected to analysis. Tongue epithelium samples were preserved in a glycerol-phosphate (1:1) buffer pH 7 4 at 4°C, while the vaccine strains also in the form of tongue epithelium were stored in the same medium at -80°C. A 10% suspension of each sample was made in a phosphate-buffered saline pH 7.4 (PBS) and, after two extractions in equal volume of chloroform and centrifugation, the aqueous phase was collected for further processing. Those field materials that, after RNA extraction and RT-PCR, yielded 38 FMDV isolates, are listed in Table 1.

Sandwich ELISA was employed for serotyping of field samples (Bhattacharya *et al.*, 1996).

RNA extraction, RT-PCR, gel electrophoresis and nucleotide sequencing. Total RNA was extracted from 460 µl of 10% suspension of each sample using the RNeasy Total RNA Isolation kit (Qiagen) according to the manufacturer. The viral VP1-coding (1D) region was amplified from the extracted total RNA by RT-PCR described earlier (Tosh *et al* , 1997) The list of primers used in RT-PCR and sequencing is given in Table 2. The PCR products were resolved on a 1.5% agarose gel, stained with ethidium bromide and the bands were visualized under UV light. The PCR products (cDNA) were purified with Wizard PCR Prep (Promega) before sequencing. The purified cDNA was then subjected to cycle sequencing using fmol DNA Cycle Sequencing System (Promega) under the following conditions: 1 cycle of denaturation at 95°C for 2 mins, followed by 40 cycles, each consisting of 30 secs of denaturation at 95°C, 30 secs of annealing at 55°C, and 1 min extension at 72°C. The sequencing reaction products were run in the 6% Cast Away Precast Sequencing polyacrylamide gel with 7 mol/l urea in the Cast Away Sequencing device (Stratagen). The sequencing gels were silver stained (Promega) and read manually after drying.

Phylogenetic analysis. Multiple nucleotide sequence alignments were performed by the Clustal W algorithm (Thomson *et al.*, 1994), implemented in the OMIGA 2.0 program (Oxford Molecular Ltd., UK). The sequence alignments were made independently for serotypes O, Asia 1 and A. The phylogenetic analyses were performed using the Phylip package, version 3.5c (Felsenstein, 1993). The trees were generated by the neighbor-joining algorithm (Saito and Nei, 1987). The tree diagram was displayed in the TreeView program (Page, 1996). The tree topology was statistically evaluated by the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs using 1000 replicas of the original data. The dendrograms based on the UPGMA (unweighted pair group method using arithmetic average) method for the same region of the 1D genes were constructed for each serotype using the EpiSeq programs (written by N.J. Knowles). Pairwise sequence identities were calculated using the DNASTAR program package (DNASTAR Inc, Madison, USA).

The following sequences from GenBank and published literature were included in the study. GenBank Accession Number and published references wherever applicable are given in the parenthesis. Type O sequences: Bangladesh, BAN 3/96 and BAN 5/96 (Marquardt, 1998); Nepal, NEP 46/95 (AJ004652); Turkey, Manisa/Turkey/69 (AJ004658); Europe, O1/Kaufbeuren (Forss *et al.*, 1984). Type Asia 1 sequences: from Bangladesh, BAN 57/80 and BAN 1/87 (Ansell *et al* , 1994); Bhutan, BHU 1/86 (Ansell *et al.*, 1994); Pakistan, PAK 1/54 (AJ251478); Israel, Shamir/89 (Stram *et al* , 1994) Type A sequences· from Bangladesh, BAN 2/87 (Freiberg *et al.*, 1999); Iran, IRN/87 (Marquardt and Adam, 1988); IRN 2/97 and IRN 17/97 (Freiberg *et al.*, 1999); Malaysia, MAL 10/97 (Freiberg *et al.*, 1999); Turkey, TUR/92 and TUR/96 (Freiberg *et al.*, 1999), Saudi Arabia, SAU/92 (Marquardt and Haas, 1998); Iraq, A22 Iraq 24/64 (Bolwell *et al.*, 1989); Azerbaijan, A22/ 55 Azerbaijan/65 (X74812).

Table 2. Primers used in RT-PCR and nucleotide sequencing

Primer*	Sequence	Sense	Gene	Methods	Remarks
pNK61	GACATGTCCTCCTGCCATCTG	–	2B	RT-PCR	Universal
pNK72	GAAGGGCCCAGGGTTGGACTC	–	2A	Sequencing	Universal
ARS4	ACCAACCTCCTTGATGTGGCT	+	1C	PCR	Serotype O-specific
As1-1C505	TAGCACTGCTTCTGACGTGGC	–	1C	PCR	Serotype Asia 1-specific
A-1C562	TACCAAATTACACACACGGGA	+	1C	PCR	Serotype A-specific

*All primers are from Knowles and Samuel (1994)
(+) = positive, (–) = negative

Results and Discussion

All the 57 field samples, which were found positive for FMDV in sandwich ELISA, and 3 vaccine virus samples were subjected to RNA extraction and RT-PCR amplification. Forty-five of 57 field samples were found to be RT-PCR-positive with varying band intensities in agarose-gel electrophoresis. Seven of the 45 PCR-positive field samples yielded too weak PCR products to be used for sequencing studies and hence, only the remaining 38 field samples and 3 vaccine virus samples were subjected to sequencing.

The fact that though all 57 field samples were FMDV-positive in sandwich ELISA but we could generate PCR products only from 45 of them, could be due to any of the following reasons:(i) putrefaction in a poor quality sample can result in degradation of RNA, (ii) incorrect pH of the buffer during transport of the sample, (iii) high environmental temperature during the transport of the field sample, (iv) degradation of viral RNA during storage (Reid *et al.*, 1998), and (v) lower sensitivity of the primers used in RT-PCR for some samples. However, it was interesting that we could successfully extract RNA and subsequently amplify cDNA from all the three vaccine virus samples, which were cattle passaged, collected and stored at -80°C since 1981. This indicates that if the samples are timely collected and properly stored, the success rate of generation of good PCR product could be as high as 100%.

Thirty eight FMDV field samples comprising 16 of serotype O, 17 of serotype Asia1 and 5 of serotype A, collected from different outbreaks in India during 1994–1998 and three vaccine virus samples of serotype O, Asia1 and A were subjected to RNA extraction, RT-PCR, gel electrophoresis and sequencing. The nucleotide sequences determined for 1D gene corresponded to the codon positions 159 to 213 for serotype O, 156 to 210 for serotype Asia 1, and 158 to 213 for serotype A. Sequences of additional FMDV strains/isolates of serotype O (6), Asia 1 (7) and A (11), available in GenBank and published literature were included in the comparative analysis. The sequences were aligned separately for the serotypes O, Asia 1 and A.

Nucleotide and amino acid sequences comparison

In FMDV serotype O, the nucleotide identity among the isolates from India, Bangladesh, Nepal and Turkey varied from 85% to100%, while these isolates in comparison with the European vaccine strain O1/Kaufbeuren exhibited the nucleotide identity from 77.6% to 84%.

In contrast, these relationships are not maintained at amino acid level. The amino acid identity between the European vaccine strain O1/Kaufbeuren and the other isolates varied from 92.7% to 98.2% as among the isolates from India, Bangladesh, Nepal and Turkey (90.9–100%). The higher identity at amino acid level is due to mostly synonymous substitutions of nucleotides which do not alter the amino acid sequence. It has already been observed that synonymous nucleotide mutations play a major role in the evolution of FMDV and non-synonymous nucleotide mutations are not accumulated in the viral sequence (Martinez *et al.*, 1992).

In FMDV serotype Asia 1, pairwise alignment of the 165 nucleotides at the 3'-terminus of 1D gene have indicated that the nucleotide identity among the isolates from India, Israel and one from Bangladesh (BAN 1/86) was 87.3–100%. In contrast, the strain from Pakistan (PAK 1/54) exhibited nucleotide identity of 81.2–86.5% with the abovementioned isolates except for the Indian vaccine strain IND 63/72 (88.5%). In no case the identity at amino acid level was below 85%. Within the Indian isolates the amino acid identity of 90.6–100% was observed.

Nucleotide identity among the Indian serotype A isolates varied from 91.1% to 100%, while these isolates are more divergent (86.9–89.9%) from the vaccine strain IND 17/77. The serotype A isolates from India, Iraq (A22 Iraq 24/64), Azerbaijan (A22/ Azerbaijan/ 65) and Bangladesh (BAN 2/87) show a similar nucleotide identity (87.5–93.5%).

In contrast, nucleotide identity among serotype A isolates from India, Turkey, Iran, Malaysia and Saudi Arabia is of 76.2–85%. However, the amino acid identity was of 91.1–100%.

The deduced amino acid sequences (approximately 56 amino acids) of VP1 region of serotypes O, Asia 1 and A were aligned (Fig. 1). The alignment indicated that the sequences of serotype O isolates are relatively conserved (substitution at 8 of 55 positions) compared to serotype Asia 1 (substitution at 14 of 55 positions) or A (substitution at 14 of 56 positions). The CPRP (cystein-proline-arginine-proline) tetrapeptide is fully conserved across the serotypes compared supporting the previous observation (Acharya *et al.*, 1989). In serotype O isolates, three different substitutions (S→T/N/A) were seen at position 197. Genotype II (O1/Kaufbeuren) had a substitution at position 212 (L→T), while in genotype I this position was invariant.

In serotype Asia 1 frequent substitutions were seen at positions 209 and 210. Three different substitutions were seen at position 209 (M→T/V/L), while two different substitutions were observed at position 210 (L→M/I). Another substitution at position 168 (D→E) was found in 20% of the isolates compared. In PAK 1/54, two positions, 172 and 175 were invariant, while there were substitutions at positions 172 (L→M) and 175 (M→I).

Substitutions in serotype A isolates were more pronounced than in those of serotypes O or Asia 1. Four different substitutions were seen at position 171 and three at 173 (Fig. 1C). Amino acid at position 169 near the region of

(A) Serotype O

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                160       170       180       190       200       210
Majority        LPTSFNYGAIKATRVTELLYRMKRAETYCPRPLLAIHPSEARHKQKIVAPVKQLL
IND 304/98      ---------------A-----------------------------------------
BAN 5/96        ----------------------------------------------------------
IND 131/98      ----------------------------------------------------------
IND 18/98       ---------V----------------------------------------------------
IND 20/98       ----------------------------------------------------------
BAN 3/96        -------------------------M--------------------------------
IND 37/98       -------A-------------------------------T----------------
IND 309/98      ----------------------------------------------------------
IND 31/98       ----------------------------------------------------------
IND 319/97      ------------------------------------N----------------
IND 333/97      ------------------------------------N----------------
IND 307/98      ----------------------------------------------------------
IND 77/98       ----------------------------------S---------------------  I
IND 51/98       ----------------------------------------------------------
IND 53/98       ----------------------------------------------------------
IND 67/98       ----------------------------------------------------------
IND 75/98       ----------------------------------------------------------
IND 45/98       ----------------------------------------------------------
O1 MANISA       -H--------------------------------------AQ----------------
IND R2/75       ---------------------------------------N----------------
NEP 46/95       ---------------------------------------T----------------
O1K             ---------------------------------------T-------------T-  II
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(B) Serotype Asia 1

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                160       170       180       190       200       210
Majority        LPTSFNYGAVKADTITELLIRMKRAETYCPRPLLALDTTQDRRKQEIIAPEKQML
IND 11/96       ----------------------------------------------------------M
BAN 57/80       ----------------------------------------------------------T-
BHU 1/86        ------------E-------------------------------------------V-
IND 101/95      --------------------H----------------------------------M
IND 11/95       --------------------H----------------------------------M
BAN 1/87        ------------EN-----------------------------------------V-
IND 252/97      ----------------------------------P----A------------L-
IND 14/95       --------------------H----------------------------------M
IND 17/95       ----------------------------------------------------------M
IND 198/96      ----------------------G-----------P------------------M
IND 202/96      ----------------------------------------------------------L-
IND 130/98      ----------------------------------------------------------M
IND 63/72       -----T-----------------------------------------------V-   I
IND 323/98      ----------------------------------------------------------LM
IND 325/98      -------------N---------N-----------------------------LI
IND 447/97      ------------------------------------------R---------V-
IND 49/95       ----------------------------------------------------------M
IND 305/98      ------------EN-------------------------------------
IND 88/95       ----------------------------------------------------------M
IND 8/96        ----------------------------------P------------------M
IND 333/98      -------R----------------G-------------------------
IND 28/90         ----------E--A-------------------------------------V-
IND 63/72       -----T-------------------------------H-------------V-
SHAMIR          ----------------------------------------------------------V-
PAK 1/54        ------------EN---M---I-----------------------------L-
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(C) Serotype A

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            160       170       180       190       200       210
Majority    LPASFNFGAIRATTIHELLVRMKRAELYCPRPLLAVEVSSQDRHKQKIIAPAKQLL
IND 17/77   ---------VR----------------------M----------------------
A22 IRAQ    ----------Q----------------------------------------------
A22 USSR    --T-------Q----------------------------------------------
IND 77/96   ----------------------------------------------------------
IND 93/96   ------------D-------------------------F----------------
IND 252/96  ------------------------------------F----------------  I
IND 289/96  ----------------------------------------------------------
IND 432/97  ------------NA--------------------------------------------
BAN 2/87    -------------N-R----------------------T-----------------
MAL 10/97   -------------G-Q----------------------L-------R---------
IRAN/87     --S-------------------R-----------M---AEG---------------
SAU/92      --S-------------------------------VES---------------
TURKEY/92   --S------------R-------------------L--TAG---------------  II
TURKEY/96   --S------------R-------------------L--TAG--------G------
IRAN 2/97   -------------D-S----------------------TA----------------
IRAN 17/97  -------------D-S----------------------TA----------------  III
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Fig 1

Alignment of deduced amino acid sequences of serotype O (A), Asia 1 (B) and A (C) isolates

Genotypes defined by the UPGMA method are indicated on the right. The conserved CPRP tetrapeptide is shaded.

concern has been reported to contain a minor antigenic site in FMDV A10 (Thomas *et al.*, 1988).

Amino acid substitutions at the region of concern suggest that there is exerted a positive Darwinian selection to produce antigenically variant viruses.

Genotype assignment of isolates

Isolates with nucleotide divergence below 15% are grouped in single genotype in the similar way that has been defined for polioviruses (Rico-Hasse *et al.*, 1987). In the UPGMA tree (not shown), isolates are clustered in different genotypes. In serotype O, all the isolates compared could be assigned to two genotypes; the isolates from India (inclusive of the vaccine strain IND R2/75), Bangladesh, Nepal, and Turkey were grouped in genotype I, while the European vaccine strain O1/Kaufbeuren was assigned to genotype II. Earlier studies of serotype O isolates from India (Pattnaik *et al.*, 1998, Hemadri *et al.*, 2000) have shown prevalence of a single genotype (genotype I).

All the isolates of serotype Asia 1 studied here were grouped in single genotype. PAK 1/54 showed the highest divergence (14.7%) from the average of the group. Comparison of serotype Asia1 isolates mostly from Asian countries also showed the prevalence of a single genotype (Ansell *et al.*, 1994).

The isolates of serotype A compared here were more divergent and could be grouped in 3 genotypes. All the Indian isolates, the Indian vaccine strain IND 17/77 and isolates from Iraq, Azerbaijan, Bangladesh and Malaysia were grouped in genotype I. In a recent report (Nayak *et al.*, 2001), there was prevalence of a single genotype in serotype A isolates from India. Genotype II comprised isolates from Turkey (TUR/92 and TUR/96), Saudi Arabia (SAU/92) and one earlier isolate from Iran (IRN/87), while other two isolates from Iran (IRN/2/97and IRN/17/97) were grouped in genotype III. Reports from other countries (Knowles *et al.*, 1998, Freiberg *et al.*, 1999) have shown prevalence of multiple genotypes in serotype A isolates.

Phylogenetic analysis

To explore the evolutionary relationship between FMDV isolates neighbor-joining trees (Fig. 2) were constructed using the partial nucleotide sequences (168 nt for serotype A and 165 nt each for serotypes O and Asia1) at the 3′-terminus of the VP1-encoding gene. The evolutionary distances between isolates are proportional to the branch lengths in the tree. The phylogenetic clustering of the isolates in the neighbor-joining tree is similar to the designated genotypes in UPGMA tree.

In phylogenetic analysis, the serotype O isolates were clustered in two groups as in the UPGMA tree. One group was formed by the European vaccine strain O1/Kaufbeuren and the other was formed by isolates from India, Bangladesh, Nepal and Turkey. Though the grouping was supported by a lower bootstrap value (48% of 1000 replicas), the genetic distances between the strain O1/Kaufbeuren and all other

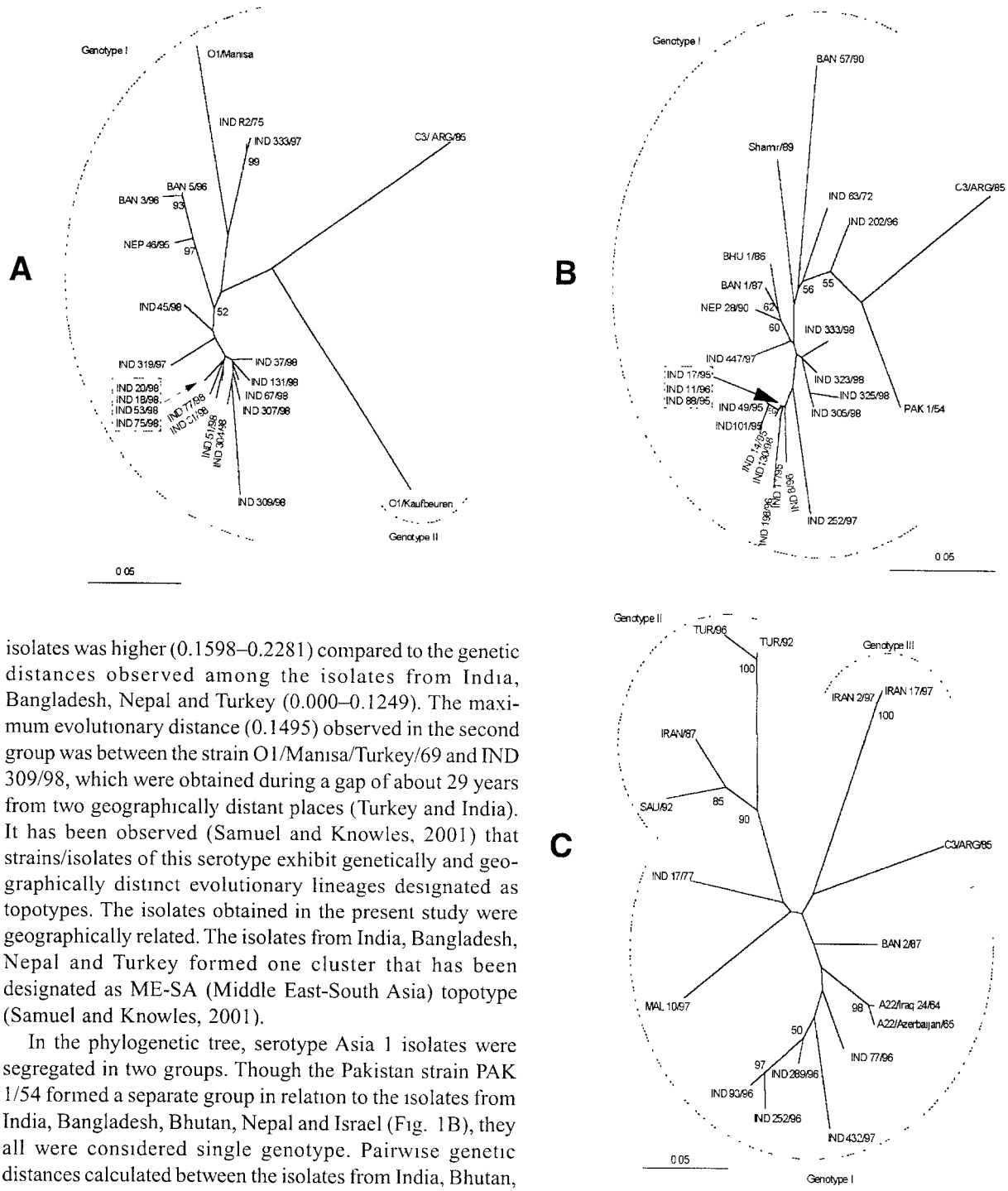

Fig 2

Neighbor-joining tree, based on partial nucleotide sequence (approx. 168 nucleotides) of VP1-encoding gene showing evolutionary relationships within FMDV serotype O (A), Asia 1 (B) and A (C) The bootstrap values above 50% (out of 1000 bootstrap replicates) are shown at nodes of the tree Genotypes (indicated in roman numerals) are defined by the UPGMA method. The scale bar indicates genetic distance Branch length of the outgroup, C3/ARG/85 (Piccone *et al*., 1988), was reduced by 75% to save space.

isolates was higher (0.1598–0.2281) compared to the genetic distances observed among the isolates from India, Bangladesh, Nepal and Turkey (0.000–0.1249). The maximum evolutionary distance (0.1495) observed in the second group was between the strain O1/Manisa/Turkey/69 and IND 309/98, which were obtained during a gap of about 29 years from two geographically distant places (Turkey and India). It has been observed (Samuel and Knowles, 2001) that strains/isolates of this serotype exhibit genetically and geographically distinct evolutionary lineages designated as topotypes. The isolates obtained in the present study were geographically related. The isolates from India, Bangladesh, Nepal and Turkey formed one cluster that has been designated as ME-SA (Middle East-South Asia) topotype (Samuel and Knowles, 2001).

In the phylogenetic tree, serotype Asia 1 isolates were segregated in two groups. Though the Pakistan strain PAK 1/54 formed a separate group in relation to the isolates from India, Bangladesh, Bhutan, Nepal and Israel (Fig. 1B), they all were considered single genotype. Pairwise genetic distances calculated between the isolates from India, Bhutan, Nepal, Israel and one isolate (BAN 1/87) from Bangladesh showed a close relationship (0.0000–0.1314) among themselves, while the strain PAK 1/54 was more divergent (0.1509) from most of the isolates except the Indian vaccine strain (IND 63/72) and the isolate IND 305/98 where it was relatively less distant (0.1119–0.1374).

The serotype A strains/isolates under comparison were clustered in three well-differentiated groups in the neighbor-

joining tree. The isolates from Iran (IRN/87), Saudi Arabia (SAU/92), Turkey (TUR/92 and TUR/96), Malaysia (MAL 10/97) and the Indian vaccine strain (IND 17/77) fell in the first group. The second group was formed by the isolates from India (IND 77/96, IND 432/97, IND 289/96, IND 252/96, and IND 93/96), Bangladesh (BAN 2/87), Iraq (A22 Iraq 24/64), and Azerbaijan (A22/Azerbaijan/65). Two recent isolates from Iran (IRN 2/97 and IRN 17/97) formed the third group. The genetic distance was greater (0.1669–0.2213) between the third group and all the other isolates except one isolate (BAN 2/87) from Bangladesh (0.1294–0.1364). Though the serotype A Indian vaccine strain IND 17/77 and an isolate from Malaysia (MAL 10/97) shared branching with the first group, its grouping was not statistically significant (26% bootstrap value).

In conclusion, the present study showed that (i) field samples in the form of infected tongue epithelium can be straightly used as starting material for FMDV sequencing, (ii) the success rate of generation of a PCR-amplified product suitable for further sequencing can be as high as 100%, (iii) sequence data for useful molecular epidemiology can be generated within 48 hrs, (iv) viruses, which could not be adapted to cell cultures, can also be characterized at molecular level. From the molecular epidemiological point of view this study provides evidence of exchange of genetic material between India and neighbor countries like Bangladesh, Nepal and Bhutan. The FMDV isolates causing the disease in India belong to single genotype in all the serotypes studied. At this stage, from these relatively limited data, it is difficult to predict the number of FMDV genotypes circulating in India. In recent studies (Hemadri *et al.*, 2000; Nayak *et al.*, 2001) on serotype O and A FMDV isolates recovered from different field outbreaks in India, prevalence of single genotype has been observed in each serotype. The isolates circulating in endemic countries, like India, are certainly very heterogeneous in nature. The endemic co-circulation of heterogeneous viruses could be attributed to the low vaccine coverage (less than 2% of susceptible populations) in the country.

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