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Genetic analysis of foot-and-mouth disease virus type O isolates responsible for field outbreaks in India between 1993 and 1999

D. HEMADRI[1], C. TOSH[1], R. VENKATARAMANAN[1]*, A. SANYAL[1], A. R. SAMUEL[2], N. J. KNOWLES[2] AND R. P. KITCHING[2]

[1] *Central Laboratory, All India Coordinated Research Project on Foot-and-Mouth Disease, Indian Veterinary Research Institute, Mukteswar-Kumaon, Nainital 263 138, India*
[2] *World Reference Laboratory for Foot-and-Mouth Disease, Institute for Animal Health, Pirbright Laboratory, Pirbright, Surrey, GU 24 ONF, UK*

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SUMMARY

Partial nucleotide sequence at the 3′ end of 1D (VP1-encoding) gene of 90 foot-and-mouth disease virus type O isolates recovered from field outbreaks in India between 1993–9 were determined. The sequences were compared with each other and reference viruses. The published sequences of 15 type O isolates recovered from different parts of Asia and one isolate (O1BFS) from Europe and one from Egypt (O1/Sharquia/Egypt/72) were also included in the analysis for comparison. On the basis of phylogenetic analysis the viruses could be grouped into four distinct genotypes (genotypes I–IV). All 90 isolates from India were genotype-I, as were the reference isolates from Bangladesh, China, Egypt, Iran, Saudi Arabia, Syria and Turkey. Genotype-I isolates were further subdivided into 16 sub-genotypes. The Indian isolates were found to be extremely heterogeneous in nature and clustered into 12 different genetic groups. In genotype-I, the nucleotide sequence difference seen between the isolates was 0–11·6%, while among the Indian isolates it is 0–8·8%. Viruses of similar genetic groups are circulating in India, Bangladesh and countries of the Middle East. Genotype-II and -III are represented by isolates from Lebanon (O1/South Lebanon) and Europe (O1-BFS), respectively. Genotype-IV is formed by isolates from China, Hong Kong and Taiwan. The present study reveals the occurrence of viruses belonging to multiple genetic groups over a short period of time and persistence of single genetic group in the same geographical area over several years. This is consistent with the endemic nature of the disease in the country.

INTRODUCTION

Foot-and-mouth disease causes serious economic losses to the livestock sector due to its effects on productivity. The causative agent, foot-and-mouth disease virus (FMDV), belongs to the genus aphthovirus of the family Picornaviridae, and exists as seven distinct serotypes (O, A, C, Asia1, SAT1, SAT2 and SAT3) and each serotype has, historically been divided in to a number of subtypes [1]. In India the disease is endemic and outbreaks occur due to types O, Asia1, A and C. The majority (> 85%) of the outbreaks in the country are due to type O followed by types Asia1 and A; the last reported type C outbreak was during 1995 [2]. Though vaccination is an important step in the control of the disease, antigenic diversity of the virus in endemic areas where regular vaccination is followed reduces its effectiveness. Serological tests with virus neutralization (VN) and an enzyme-linked immuno-

* Author for correspondence.

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Table 1. *Virus isolates used in the study*

Sl. no.	Isolates*	Date of isolation	Place of isolation (state)	Species
1	INDR2/75†	00/00/75	Tamilnadu	Bovine
2	IND53/79†	00/00/79	Tamilnadu	Bovine
3	IND27/97	00/01/97	Uttar Pradesh	Bovine
4	IND28/97	00/01/97	Uttar Pradesh	Caprine
5	IND31/97	30/12/96	Tamilnadu	Bovine
6	IND33/97	04/01/97	Tamilnadu	Bovine
7	IND37/97	09/01/97	Tamilnadu	Bovine
8	IND38/97	06/01/97	Tamilnadu	Bovine
9	IND39/97	00/01/97	Tamilnadu	Bovine
10	IND49/97	29/01/97	Tamilnadu	Bovine
11	IND61/97	00/00/96	Haryana	Bovine
12	IND63/97	00/01/97	Haryana	Bovine
13	IND64/97	00/00/97	Haryana	Bovine
14	IND70/97	00/00/97	Haryana	Bovine
15	IND74/97	00/00/97	Haryana	Buffalo
16	IND75/97	28/11/97	Himachal Pradesh	Bovine
17	IND78/97	25/01/97	Jammu & Kashmir	Bovine
18	IND79/97	17/02/97	Haryana	Bovine
19	IND82/97	10/02/97	Andhra Pradesh	Bovine
20	IND160/97	13/01/97	Maharashtra	Bovine
21	IND162/97	00/00/97	Maharashtra	Bovine
22	IND170/97	00/01/97	Haryana	Bovine
23	IND275/97	00/03/97	Haryana	Buffalo
24	IND278/97	11/02/97	Assam	Bovine
25	IND279/97	20/03/97	Assam	Bovine
26	IND281/97	21/03/97	Meghalaya	Bovine
27	IND289/97	19/04/97	West Bengal	Bovine
28	IND296/97	00/04/97	Haryana	Bovine
29	IND313/97	00/00/97	Haryana	Bovine
30	IND352/97	06/03/97	Karnataka	Bovine
31	IND380/97	24/02/94	Karnataka	Bovine
32	IND384/97	29/04/94	Karnataka	Bovine
33	IND391/97	06/08/94	Karnataka	Bovine
34	IND399/97	00/00/94	Karnataka	Bovine
35	IND407/97	21/12/95	Karnataka	Bovine
36	IND409/97	06/07/96	Karnataka	Bovine
37	IND410/97	04/09/96	Karnataka	Bovine
38	IND411/97	18/09/96	Karnataka	Bovine
39	IND414/97	22/11/96	Karnataka	Bovine
40	IND416/97	28/11/96	Karnataka	Bovine
41	IND417/97	04/12/96	Karnataka	Bovine
42	IND420/97	27/01/97	Karnataka	Bovine
43	IND423/97	00/00/96	Karnataka	Bovine
44	IND424/97	00/00/96	Karnataka	Bovine
45	IND461/97	00/10/96	Haryana	Buffalo
46	IND463/97	00/11/96	Haryana	Buffalo
47	IND464/97	00/12/96	Haryana	Ovine
48	IND465/97	00/12/96	Haryana	Bovine
49	IND485/97	00/00/93	Tamilnadu	Bovine
50	IND489/97	NA	Tamilnadu	Bovine
51	IND47/98	31/12/97	Punjab	Bovine

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Table 1 (*cont.*)

Sl. no.	Isolates*	Date of isolation	Place of isolation (state)	Species
52	IND48/98	31/12/97	Punjab	Bovine
53	IND54/98	00/01/98	Haryana	Bovine
54	IND55/98	16/01/98	Punjab	Bovine
55	IND56/98	29/01/98	Punjab	Bovine
56	IND57/98	01/02/98	Punjab	Buffalo
57	IND64/98	11/02/98	Punjab	Bovine
58	IND65/98	12/02/98	Punjab	Bovine
59	IND66/98	12/02/98	Punjab	Buffalo
60	IND81/98	25/03/98	Uttar Pradesh	Bovine
61	IND304/98	18/05/98	Meghalaya	Porcine
62	IND307/98	05/06/98	Assam	Bovine
63	IND309/98	03/07/98	Mizoram	Bovine
64	IND427/98	03/11/98	Andhra Pradesh	Bovine
65	IND469/98	00/12/98	Uttar Pradesh	Ovine
66	IND23/99	13/12/98	Kerala	Bovine
67	IND81/99	21/12/98	Karnataka	Bovine
68	IND141/99	24/01/99	Tamilnadu	Bovine
69	IND143/99	00/00/99	Uttar Pradesh	Buffalo
70	IND146/99	04/02/99	Andhra Pradesh	Buffalo
71	IND148/99	04/02/99	Andhra Pradesh	Buffalo
72	IND153/99	00/02/99	Tamilnadu	Bovine
73	IND160/99	11/02/99	Karnataka	Bovine
74	IND164/99	29/12/98	Assam	Bovine
75	IND174/99	18/01/99	Mizoram	Bovine
76	IND175/99	18/01/99	Assam	Porcine
77	IND178/99	09/09/98	West Bengal	Bovine
78	IND185/99	21/12/98	West Bengal	Bovine
79	IND195/99	15/10/98	Orissa	Bovine
80	IND205/99	01/03/99	Tamilnadu	Bovine
81	IND207/99	09/03/99	Tamilnadu	Bovine
82	IND208/99	00/09/98	Maharashtra	Bovine
83	IND209/99	00/09/98	Maharashtra	Bovine
84	IND210/99	00/11/98	Maharashtra	Bovine
85	IND246/99	00/00/99	Gujarat	Bovine
86	IND249/99	24/01/99	Gujarat	Bovine
87	IND256/99	07/02/99	Gujarat	Bovine
88	IND282/99	10/06/99	Kerala	Bovine
89	IND285/99	00/00/99	Haryana	Bovine
90	IND287/99	00/00/99	Haryana	Porcine

* Laboratory accession number; † Vaccine virus; NA, not available.

sorbent assay (ELISA) using defined serum/monoclonal antibodies are useful in showing antigenic diversity, but they are unable to individually characterize strains and cannot be used to trace the origin and course of epizootics. Nucleotide sequence analysis has now replaced serology as the method of choice for FMDV strain identification and epidemiological investigation [3].New molecular techniques such as reverse transcription-polymerase chain reaction and cycle sequencing has improved the speed and accuracy of these studies. The phylogenetic grouping correlates with geographical origin of isolates and provides a powerful tool for epidemiological surveillance [4]. Recently several authors [5–9] including us [10] have used the nucleotide sequence within the 1D gene to make phylogenetic grouping of FMDV isolates [11].

In the present communication we have determined and compared the nucleotide sequence at the 3′ end of the 1D (VP1-encoding) gene of 90 FMDV type O isolates recovered from different outbreaks in India during the last 7 years (1993–9) including two of the vaccine viruses (INDR2/75 and IND53/79) used in

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the country. The published sequences of 17 type O isolates from Asia and Europe were also included in the analysis.

METHODS

Viruses

The type O FMDV isolates studied are listed in Table 1. They were initially isolated on primary calf kidney cells and subsequently grown in BHK-21 cells. The infected cell culture supernatant was used for RNA extraction.

Nucleotide sequencing

Total RNA was extracted from infected cell culture supernatants using TriReagent (Sigma) according to the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously [12] using two oligonucleotide primers (NK61, Sense, 5′-GACATGTCCTCCTG-CATCTG and ARS4, anti-sense, 5′-ACCAACCT-CCTTGATGTGGCT) [13]. The PCR products were-purified using the Wizard PCR Preps (Promega).Cycle sequencing of the PCR products was performed using Silver Sequencing kit (Promega) with an anti-sense primer (NK72, 5′-GAAGGGCCCAGGGTTGGAT-CTC) [13]. The reaction was carried out on a thermo-cycler (Hybaid) using the following thermal conditions: 94 °C for 2 min, 1 cycle; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, 60 cycles. The sequencing products were resolved in 6% polyacrylamide gel containing 7.5 M urea. The sequencing gel was stained with silver nitrate using silver staining kit (Promega).

Sequence analysis

Nucleotide sequences of two Bangladesh strains (BAN/3/96, BAN/5/96) [14] and one European strain (O1-BFS) [15] were used for comparisons. In addition to these strains, the following 14 sequences of type O strains from outside of India were obtained from the GenBank and included in the analysis (GenBank accession numbers are given in parenthesis): China/GD/86, China/A/58, HK/93 (AJ-131468, AJ131469, AJ131470), Golan/Israel/81 (Z-21860), O1/South Lebanon (S77354), SAU/1/88, SAU/100/94, SAU/2/95, SAU/5/95 (AJ004659, AJ-004660, AJ004661, AJ004662), O1/Sharquia/72, Iran/3/87, Syria/1/87, O1/Manisa/Turkey/69 (AJ-004655, AJ004656, AJ004657, AJ004658), and TL/Taiwan/97 (AF030259). The nucleotide sequences of individual isolates were analysed on a personal computer using the programme 'Seqprog' written by one of the authors (N.J.K). All pairwise comparisons were performed by giving each base substitution equal statistical weight (ambiguities were ignored). A phylogenetic tree was constructed using the UPGMA method as implemented in the computer programme Neighbor and dendrogram plotted using the programme DRAWGRAM both from the PHYLIP 3.5c phylogeny package [16]. The UPGMA method constructs a tree by successive (agglomerative) clustering using an average-linkage method of clustering. The scale below the tree was drawn from the nucleotide sequence distance matrix.

RESULTS

On the basis of nucleotide sequence analysis at the 3′ end of 1D (VP1-encoding) gene, we have classified the type O FMDV in to four distinct genotypes. A divergence of $> 15\%$ in nucleotide sequence distinguishes genotypes [17] and strains which differ by $< 5\%$ are considered to be closely related [8].

We determined the nucleotide sequence within the 1D region (165 nucleotides, positions 475–639) of 90 FMDV type O isolates including two vaccine strains (Table 1) recovered from different parts of India (Fig. 1*a*). In addition to these strains recovered from India, we have also included 17 more type O exotic sequences in our analysis. The result is shown in the form of a dendrogram (Fig. 1*b*). On the basis of nucleotide sequence divergence, the FMDV isolates included in the study were clustered into four different genotypes. All the Indian isolates along with isolates from Bangladesh, Egypt, Israel, Saudi Arabia, Syria, Turkey and one isolate (China/A/58) from China were grouped in genotype-I. The genotype-I was further subdivided into 16 different sub-genotypes/groups (groups 1–16, groups are numbered randomly). The criteria used for defining sub-genotypes/groups was 5% nucleotide sequence divergence. The Indian isolates were distributed in 12 different groups, reflecting the heterogeneous nature of the viruses causing the outbreaks in the field. Sequences of viruses isolated from different regions/states, on different occasions are identical. This situation was observed between IND48/98, IND56/98, IND57/98 and IND65/98 isolated from the state of Punjab

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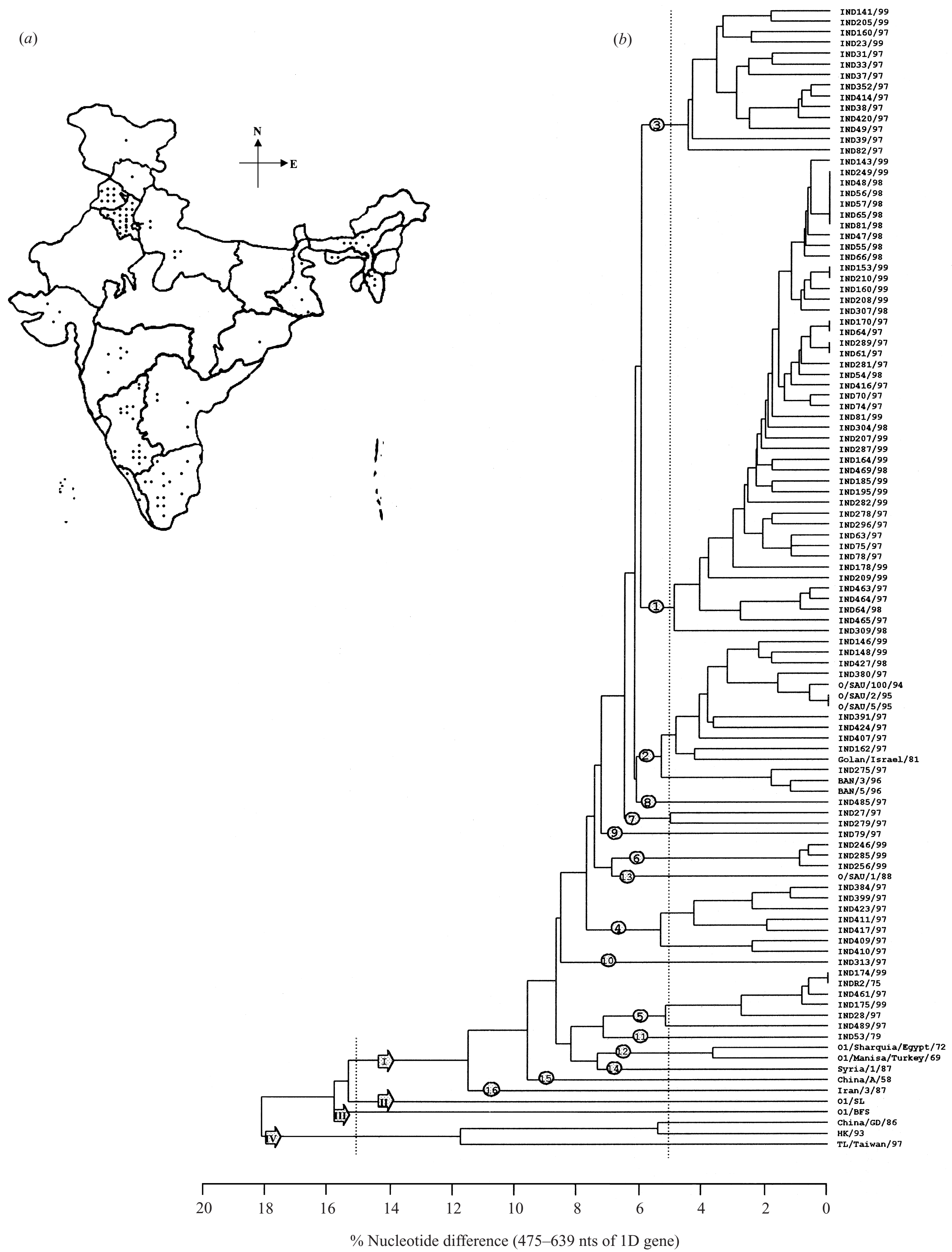

Fig. 1. (*a*) Distribution within India of FMDV type O field isolates used in the study. (*b*) Dendogram showing genetic relationship between FMDV type O strains.

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during late 1997 and early 1998 and IND81/98, isolated from Bhagpat (Uttar Pradesh) during March 1998. These isolates along with a Gujarat isolate (IND249/99) share 100% nucleotide similarity. A similar result was observed with IND61/97, isolated from Haryana during December 1996 and IND289/97, isolated from West Bengal during April 1997 which had a 100% nucleotide sequence identity. Yet another, similar observation was made with the isolates IND210/99, recovered from Maharashtra in November 1998 and IND153/99, recovered from Tamilnadu in February 1999.

Group 2 is the second largest genetic group formed by 9 (of 90) Indian isolates, along with 3 (SAU/100/94, SAU/2/95, SAU/5/95), 2 (BAN/3/96, BAN/5/96) and 1 (Golan/81) isolates from Saudi Arabia, Bangladesh and Israel, respectively. The remaining one isolate from Saudi Arabia (SAU/1/88) formed a distinct group (group 13), with approximately 7·5% (average) nucleotide sequence difference from the former three Saudi Arabian isolates. The Saudi Arabian isolates are closely related to an isolate from Karnataka (IND380/97) recovered during February 1994.

Group 3 is the third largest genetic group comprising field strains isolated between 1994–9. Members of this group are mainly confined to four southern states of Andhra Pradesh, Karnataka, Tamilnadu and Kerala. This genetic group is still responsible for FMD outbreaks in this region. In this group IND160/97 is the only isolate from Maharashtra state which neighbours the southern states of Andhra Pradesh and Karnataka. IND57/96 isolated during 1994 from the state of Andhra Pradesh is the earliest outbreak strain in this genetic group. The nucleotide sequence difference seen in this group varies from 0·6–4·5%.

Group 4 comprises seven isolates from the state of Karnataka (Fig. 1*b*), all isolated from bovines between 1994–6. Two isolates, IND410/97 and IND411/97, from the same district, but isolated within 15 days intervals, showed nucleotide divergence as high as 5·4% between them. Group 5 comprises of six isolates including one vaccine strain (INDR2/75). These isolates are related to each other by 0–5·2%. Group 6 consists of three isolates from Gujarat and Haryana, which share more than 99% sequence homology over the region sequenced. Group 7 consists of isolates IND27/97 and IND279/97 from Uttar Pradesh and Assam, respectively, and show approximately 95% nucleotide sequence homology. Groups 8, 9, 10, and 11 are represented by single isolates. Group 8 is formed by IND485/97, which was isolated from Tamilnadu in 1993. Isolates representing groups 9 (IND79/97) and 10 (IND313/97) are from the state of Haryana, with a nucleotide difference of $> 8\%$. Another vaccine virus IND53/79 used in the country forms group 11.

DISCUSSION

In India FMD is endemic and occurs throughout the year. Of the four serotypes O, Asia1, A and C prevalent in India, type O accounts for nearly 85–90% of the outbreaks, followed by Asia1 and A. Disease due to type C has not been recorded since 1995. The country has a very large number of susceptible animals ($>$ 470 million) comprising cattle, buffaloes, sheep, goats and pigs besides a large number of wild ungulates. Very few animals (less than 2%) are regularly vaccinated, mostly on the larger farms. The disease situation is further complicated by the unrestricted movement of animals. FMD samples included in this study were collected over a large geographical area of the country (Fig. 1*a*) and provide a clearer picture of the epidemiology of FMD in India. There is no restriction on movement of animals across the state boundaries which may explain the lack of correlation between geographical origin and genetic groups. Group 1, which is the largest genetic group in the genotype-I, is formed by 45 (of 90) Indian isolates from 15 different states of the country. This group is formed by isolates recovered in the past 3 years (between 1996–9). A nucleotide difference of 4·95% was the highest observed among the isolates in this group. These results, which show the same strain in locations over 100 km apart, suggest the long distance movement of infected animals. A similar situation was observed in SAT-type FMDV in Kruger National Park and other regions of Southern Africa [7], where 100% homology was found between isolates collected from different areas; in this instance it was thought due to fragmentation of animal herds, after a culling programme. The isolate IND81/98, was recovered from Bhagpat in Uttar Pradesh and there was a history of animals being brought from Punjab in the midst of a FMD outbreak to villages near to Bhagpat.

The isolates IND47/98, IND48/98, IND55/98 to IND57/98, IND64/98 to IND66/98 were all recovered from different districts of Punjab during an FMD epidemic. It started in December 1997 and

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continued during the following 2 months, affecting a large number of animals. All the isolates from this epidemic are clustered at one place within group 1, except IND64/98, which is placed separately in the phylogenetic tree, indicating that it probably had a different origin from the other isolates. This isolate shares a minor branching with the strains IND463/97 and IND464/97, which were isolated in November 1996 from the neighbouring Haryana state. All of them share > 98·5% sequence identity. This suggests that there were at least two different but closely related populations of virus circulating and causing the disease during the Punjab epidemic. This is further substantiated by the fact that the isolates IND64/98, IND463/97 and IND464/97 have methionine instead of proline at amino acid position 160 of VP1.

Grouping of Saudi Arabian isolates with the Indian isolates is not surprising as a close relationship has been reported earlier. Importation of a large number of sheep and goats into the Middle East from India during 1994 has been attributed to the spread of FMD in that region [8]. The Israeli isolate has been placed along with an isolate from Maharashtra (IND162/97) in group 2. They share more than 95% sequence homology indicating a close genetic relationship. An isolate from Haryana (IND275/97) and two strains from Bangladesh (BAN3/96 and BAN5/96) are about 5·5% divergent in nucleotide sequence from the former group of isolates and are placed in group 2. Strains of Bangladesh show more than 98% sequence identity with the isolate from Haryana indicating circulation of viruses of similar genetic groups in India and Bangladesh. The prevalence of genetically related type O viruses between the two countries has been attributed to the intensive animal trade [18]. The northern states of Punjab, Haryana, Uttar Pradesh and Rajasthan have a large population of buffaloes and these buffaloes are transported to West Bengal, Kerala and Andhra Pradesh for purposes of slaughter. Thus the genetic relatedness of isolates from geographically distant states like Haryana could also be due to such unrestricted animal movement. Genetic relatedness of isolates from India and Nepal were reported by us earlier [10].

Within genotype-I, isolates from Syria, Iran and China formed distinct groups. An isolate from Turkey (O1/Manisa/Turkey/69) along with the Egyptian isolate O1/Sharquia/72 formed another group, and are divergent by 3·7% from each other.

In genotype-I, nucleotide sequence difference seen among the Indian isolates varied from 0 to 8·8%. Though the field isolates vary from 0–8·8% in nucleotide sequence from the vaccine strains, they showed close antigenic relationship in enzyme-linked immunosorbent assay (unpublished observation) with the vaccine strain INDR2/75.

The results of this study show that in India during the last 6-year period there were at least 12 genetic groups active. Group 1 seems to be the dominant genetic group as it is responsible for the majority of the outbreaks. But this genetic group did not remain confined to any particular region of the country and was widespread. This is not unexpected considering the fact that no restriction is imposed on animal movement at the state boundaries. Furthermore, this genetic group was not restricted to any particular species of animal, instead, it caused disease outbreaks in large ruminants, small ruminants and pigs. Members of this genetic group showed 92% sequence homology with the vaccine viruses used in the country.

The appearance of different genetic groups in the same state/region within a period of one year is evident in the state of Karnataka, during 1994 and 1996 when outbreaks were caused by viruses belonging to three different genetic groups and, in the state of Haryana, where outbreaks due to four different genetic groups (groups 1, 3, 9 and 10) were evident during 1997. In contrast to these observations, there are examples of outbreaks due to the same genetic groups over a long period of time. Group 3 isolates from the state of Karnataka and Andhra Pradesh, were prevalent for a period of 4 (1994–7) and 6 (1994–9) years respectively. The conservation of genetic groups for such a long period of time may be due to persistence of virus in the cattle [19].

All the Indian type O isolates are not related to the European type O virus (O1 BFS) as reported earlier by us [10]. The genetic relatedness of Indian field isolates with those from Nepal and Saudi Arabia are reported earlier [8, 10]. The results of the present study also revealed a similar situation. O1 Manisa which is widely used in Asia as a vaccine strain and originated in Turkey is related to the Indian isolates in genotype I by 8·8%. Although there is considerable variation of strains within India, they all fall in to one genotype (genotype I) and are serologically related to the vaccine strain. Occurrence of epidemiologically unconnected outbreaks over a short period is consistent with the endemic nature of the disease and uncontrolled animal movement. The present study shows a clear picture of the epidemiological situation of the disease due to type O, which accounts for more

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than 85% of the outbreaks in India. Such continuous monitoring of the field strains help in selection of vaccine strains and evolving a proper control strategy for the disease.

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