MOLECULAR EPIDEMIOLOGY OF FOOT-AND-MOUTH DISEASE VIRUS TYPE ASIA 1

Thesis

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Dated : 20/08/ *1997*

CERTIFICATE

Certified that the research work embodied in the Thesis entitled **"MOLECULAR EPIDEMIOLOGY OF FOOT-AND-MOUTH DISEASE VIRUS TYPE ASIA 1"** submitted by **Shri Gurumurthy, C.B., Roll No. 3602,** for the award of **Master's Degree** of the Deemed University, **Indian Veterinary Research Institute**, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that **Shri Gurumurthy, C.B.** has worked for more than 24 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the **Master's Degree** of this University, as required under the relevant ordinance.

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CERTIFICATE

Certified that the thesis entitled **"MOLECULAR EPIDEMIOLOGY OF FOOT-AND-MOUTH DISEASE VIRUS TYPE ASIA1"**,submitted by **Shri Gurumurthy, C.B., Roll No. 3602,** in partial fulfilment of **Master's Degree** of the Deemed University of Indian Veterinary Research Institute, embodies the original work done by the candidate. The candidate has carried out his work sincerely and methodically.

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of **Master's Degree** of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master's Degree** of the Deemed University of the Indian Veterinary Research Institute.

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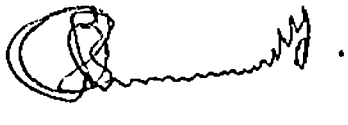

C.B. GURUMURTI

20/08/1997

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ABBREVIATIONS

AMV Reverse transcriptase	Avian Myeloblastosis virus reverse transcriptase
BHK-21	Baby Hamster Kidney-21
BVS	Bovine vaccinate serum
bp	Base pair
cDNA	Complementary DNA
CFT	Complement fixation test
Cm	Centrimeter
CO_2	Carbon dioxide
CPE	Cytopathic effect
ddATP	Dideoxy adenosine triphosphate
ddCTP	Dideoxy cytosine triphosphate
ddGTP	Dideoxy guanosine triphosphate
ddTTP	Dideoxy thymine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleoside triphosphate
EF	Electrofocussing
ELISA	Enzyme linked immunosorbent assay
FMD	Foot-and-mouth disease
fmol	Femtomole
FMDV	Foot-and-mouth disease virus
GPS	Guinea-pig serum
h	Hour
Hcl	Hydrochloric acid
H_2SO_4	Sulphuric acid
HRPO	Horse radish peroxidase
Ig	Immunoglobulin
IND	India
I.U.	International Unit
IVRI	Indian Veterinary Research Institute
Kcl	Potassium chloride
Km	Kilometer
M	Molar
Mab	Monoclonal antibody
MAR-mutant	Monoclonal antibody resistant mutant
MgCl2	Magnesium chloride
mM	Millimole
MNT	Micro-neutralization test

Continued.....

µg	Microgram
ml	Millilitre
µl	Microlitre
NaOH	Sodium hydroxide
ng	Nanogram
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
pmol	Picomole
PR	Percent reference
r	Serological relationship
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reversetranscription-Polymerasechainreaction
S	Svedberg unit
SAT	South African Territory
SNT	Serum neutralization test
NI	Neutralization index
Taq	Thermus aquaticus
$TCID_{50}$	Tissue culture infective $dose_{50}$
Tfl	Thermus flavus
2D-MNT	Two dimensional micro-neutralization test
Vpg	Viral protein attached to genome
v/v	Volume/Volume
w/v	Weight/Volume

CONTENTS

INTRODUCTION

INTRODUCTION

Foot-and-Mouth Disease (FMD) is an acute and highly contagious disease of farm livestock affecting cloven-hooved animals viz. cattle, pig, sheep and goats. The disease still remains a major scourge of animal production industry in many parts of the world. Although rarely fatal with less than 5% mortality in adult animals, FMD is econonically devastating due to the loss of productivity of animals following the disease and the trade embargo on export of animals and animal products. Many factors combine to make it one of the most damaging disease of animals. These include its high contagiousness, wide geographical distribution and host spectrum, protracted convalescence and carrier status, plurality of antigenic forms and relatively short duration of immunity to a given serotype. The virus occurs in the form of seven immunologically distinct serotypes viz. O, A, C, Asia 1, SAT 1, SAT 2, SAT 3 and more than 65 subtypes (Pereira, 1977). Animals recovered from infection with one serotype remain fully susceptible to infection with any other serotype.

The causal agent of the disease, the foot-and-mouth disease virus (FMDV) belongs to genus aphthovirus, family Picornaviridae (Cooper et al., 1978). The virus is icosahedral, nonenveloped with a diameter of 30 nm and 8.3 to 8.9 x 10^6 daltons in molecular weight and sedimentation co-efficient of 146S. The genome consists of a single stranded, positive sense, linear RNA molecule of about 8,500 nucleotides, which is polyadenylated at the 3' terminus and which carries a small protein, VPg, covalently attached to its 5' end. The 5' untranslated region of the RNA is extra-ordinarily (nearly 1,200 bases) long and includes a poly(C) tract whose function is not yet fully known. The genome is translated into a single polypeptide chain, which is then cleaved into structural and non-structural proteins (Fig.1). The capsid consists of 60 copies each of the four structural proteins VP1, VP2, VP3 and VP4 coded by 1D, 1B, 1C and 1A genes respectively. X-ray diffraction (Acharya et al., 1989) and immunological studies have shown that VP1, VP2 and VP3 have surface components, while

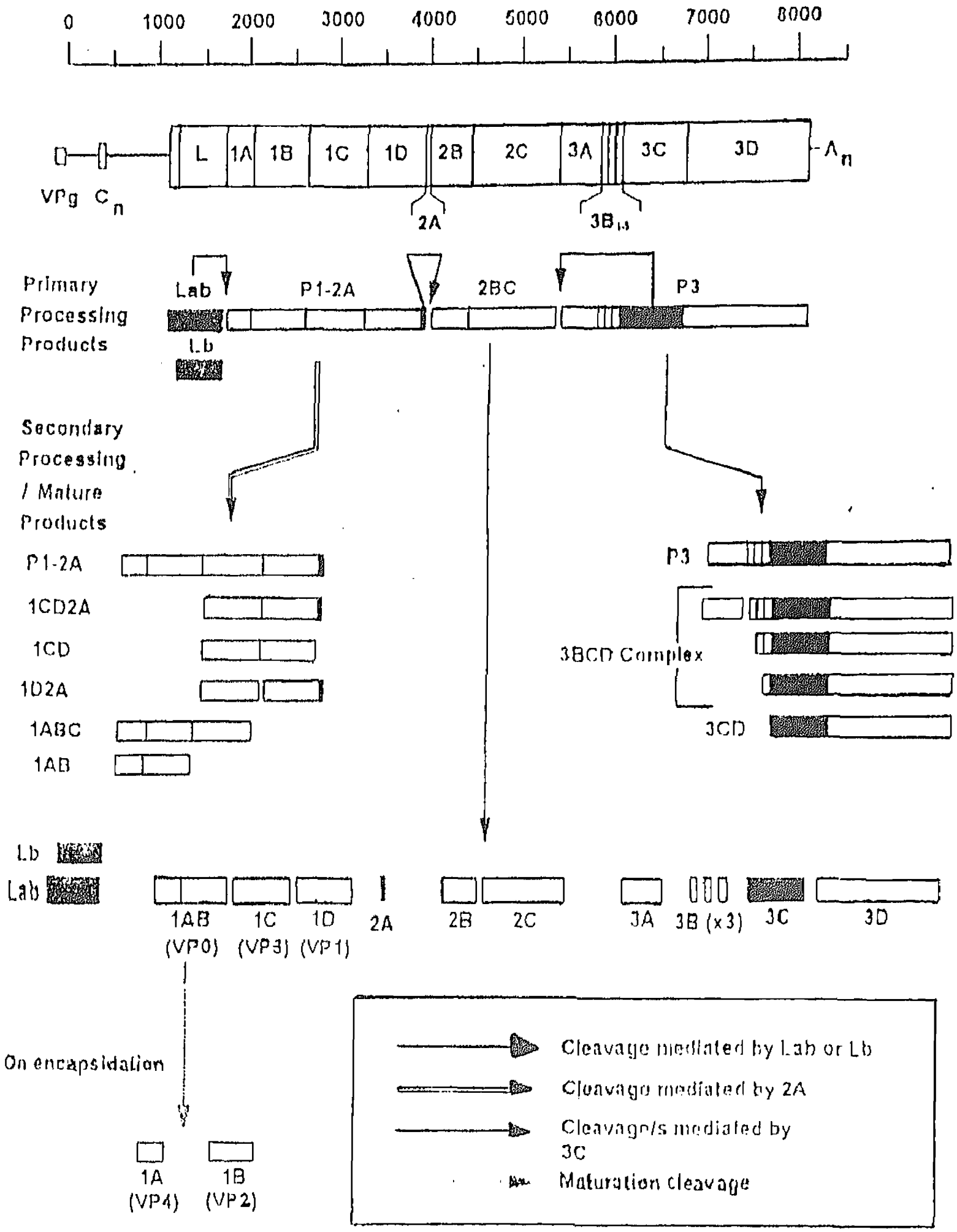


Fig. 1 *The FMDV Genome, and Protein processing cascade.*

VP4 is entirely internal. VP1 has been shown to be the most antigenically important of the four strucutral proteins. In particular, the region from amino acids 138 to 156, the major antigenic site of FMDV, has been shown to be hypervariable, and to contain several overlapping epitopes involved in neutralization of viral infectivity (Mateu et al., 1990).

Inspite of extensive vaccination [nearly 2 thousand million doses of vaccine are used annually (Domingo et al., 1990)] for control of FMD, the disease is still enzootic in South America, Africa, South Europe and several parts of Asia including India. At present, North and Central America, Australia, New Zealand and Japan are free of the disease (Barteling and Vreeswijk, 1991).

The most effective means of controlling the spread of FMD remains the slaughter of affected and in-contact susceptible animals, movement control and effective tracing of previous outbreaks. In countries where the disease is endemic and slaughter is not practical, vaccination and movement control provide an acceptable although less effective alternative (Kitching , 1992). Because of high contagiousness of FMD and quasispecies nature of the virus, success of control programmes depends initially on the efficiency of epidemiological surveillance in detecting the foci of infection, collecting samples and blocking the spread of the disease. This aspect is of vital importance in countries having extensive animal movement which enormously facilitates propagation of the virus (Alonso et al., 1987). It is probable that more rapid progress will be made in eliminating the disease, particularly from the developing countries if more information on its immunology and epidemiology were available (Kitching, 1992).

In countries where vaccination is the chief method of controlling the disease, epidemiological studies are mainly aimed at (i) evaluation and selection of the best strain for use in vaccine production and (ii) ensuring that the chosen vaccine strain provides effective protection against the strains circulating in the region. For these purposes, subtype characterization of field isolates is done using serological tests like complement fixation test

(CFT), serum neutralization test (SNT) and Enzyme-linked immunosorbent assay (ELISA). However, serological tests do not provide complete information on the ability of current and new vaccines to protect against circulating viruses and epizootiological tracing of new outbreaks (Beck and Strohmaier, 1987; Kitching et al, 1989). In fact, FMDV isolates belonging to an epizootic and indistinguishable by SNT may show incomplete cross-protection (Martinez et al., 1988) and have amino acid changes on the 140-160 VP1 region that strongly decrease the affinity of some of these viruses for a neutralizing monoclonal antibody (Mab) (Mateu et al., 1987b, 1989). Since monoclonal antibodies are specific for an epitope/antigenic determinant, they are excellent tools for demonstration of antigenic relatedness of the isolates with vaccine virus. Mabs have been used to understand antigenic features (Xie et al., 1987; Stave et al., 1988; Pfaff et al., 1988; Thomas et al, 1988a, b; Baxt et al., 1989; Bolwel et al., 1989; Parry et al., 1989; Mateu et al., 1990) and for profiling of field isolates (Samuel et al, 1991; Butchaiah et al., 1992; Sanyal, 1995), a standard method for establishing antigenic differences between strains (Samuel et al., 1991).

A valuable tool now available for epidemiological studies on FMD virus is the nucleotide sequencing of viral genome which allows precise characterization of virus isolates (Kitching,1992).Beck and Strohmaier(1987) observed that important epidemiological information could be gained from nucleotide sequence data. They concluded that nucleotide sequence analysis should be used as standard method of diagnosis because when compared to other techniques, it more clearly reveals the origin and course of epizootics and offers the possibility of preventing further outbreaks. For epidemiological investigations, field viruses are examined by nucleotide sequencing and by reactivity with a Mab panel whereever available (Kitching et al., 1989).The analysis of sequences for epidemiological purposes involves comparing the degree of nucleotide homology/divergence between virus strains and expressing this on a dendrogram (Kitching et al., 1989). As more nucleotide sequence data is accumulated, it is anticipated that it may be possible to place strains into closely related groups.By designating each of these groups a name or number, a field virus could be genetically classified. By combining this genomic classification

(G number) with its antigenic classification (R number) an almost complete description of the strain in this manner should satisfy the requirements of epidemiological investigative purposes as well as requirements of vaccine selection for its antigenic suitability (Kitching et al., 1989).

VP1, the main polypeptide determining the antigenic identity of the virus, includes important determinants of virus neutralization (Domingo, 1990). It is subjected to frequent and viable mutations (Acharya et al., 1989; Dopazo et al., 1988; Logan et al., 1993); this high mutation rate is the basis for using the 1D(VP1) gene sequence to evaluate the relationships between virus isolates (Stram et al., 1995).

In India, outbreaks of FMD are attributed to serotypes O, A, C and Asia 1. The disease occurs round the year and in all parts of the country. During recent years, the serotype Asia 1 type has emerged as one of the causes of FMD outbreaks next only to the serotype O. Though regular vaccination is practiced in some pockets of the country, failure of successful control of the disease in India is mainly because of large population of susceptible animals, absence of restriction on animal movement, limited availability of vaccines and other socio-economic conditions. By examining the distribution of strains of FMD virus and tracing their movement within India, it will be possible to understand the natural history of FMD in the country. This undoubtedly will highlight the importance of movement control in any program to eradicate the disease, but in addition may explain how it persists and key areas in which control efforts can be concentrated (Kitching and Knowles, 1993). A prerequisite to undertake any control programme is the thorough understanding of epidemiology of the disease. Several approaches using CFT, mouse cross protection test, ..NT and ELISA to study epidemiology of Asia 1 FMDV in India have been made (Rai and Goel, 1983; Antony ,1987;Belwal et al., 1989; Shridhara,1990; Butchaiah et al., 1992; Mishra et.al., 1995; Prabhudas et al., 1993). Recently Mabs have also been used for understanding the antigenic relatedness of field isolates with vaccine strain (Butchaiah et al., 1992; Prabhudas et al., 1993; Sanyal, 1995) but their molecular characterization at

genome level has not been done. The nucleotide sequence data for field isolates in combination with the data generated for their antigenic relatedness can reveal molecular characteristics of the strains prevailing in our country, their behaviour and detailed epidemiology of the disease. Keeping these points in view, the present study titled " Molecular Epidemiology of Foot-and-Mouth Disease virus type Asia 1 " was undertaken with the following objectives.

1.To study antigenic relatedness of Asia 1 field isolates with the vaccine strain (IND 63/72) by indirect sandwich ELISA, micro-neutralization test and Mab profiling.

2.To study genetic relatedness of Asia 1 field isolates with the vaccine strain (IND 63/72) by sequencing the 3' end of 1D genomic (VP1 coding) region.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

FMD is an acute and highly contagious viral disease of domestic and wild cloven-hooved animals. It adversly affects livestock productivity and trade and so is considered the most important disease of farm animals. The control of the disease by vaccination is complicated by antigenic diversity of the virus. There are seven immunologically distinct types and over 65 subtypes distributed unevenly in different parts of the world (Pereira, 1977). Such antigenic variation, particularly within the serotypes, necessiates careful monitoring of the virus strains occuring in a particular area.

VIRION ARCHITECTURE: ITS IMMUNOGENICITY AND ANTIGENICITY

The causative agent, foot-and-mouth disease virus belongs to the genus aphthovirus of the family Picornaviridae. The FMDV genome consists of a single stranded, positive sense RNA of about 8,500 nucleotides with a small protein (VPg) linked covalently to its 5' end. It also has a poly (C) tract internal to the 5' untranslated region and a poly (A) tail at its 3' end (Domingo et al., 1992). The genome is surrounded by an icosahedral capsid, composed of 60 copies each of the four structural proteins VP1 to VP4 (Cooper et al., 1978) which are produced by post-translational cleavage of a common precursor (Boothroyd et al., 1981). Of the four structural proteins, VP1 is the most exposed one on the capsid of the virus compared to VP2 and VP3, while VP4 is entirely internal (Morrel et al., 1987; Acharya et al., 1989).

The intact virus particle (146S virion) induces formation of type specific, complement fixing and neutralizing antibodies in cattle and guinea pigs (Cartwright et al., 1980). The 12S structural subunits produced from the 146S particles by mild acid treatment or by heating at 50°C contains 5 copies each of VP1, VP2 and VP3 (Morrel et al, 1983) and induce low levels of neutralizing antibodies (Cartwright et al., 1980). FMD virus also

produce RNA free particles which have a sedimentation coefficient of 75S (Planterose and Ryan, 1965) which are composed of VP0, VP1 and VP3 in contrast to 146S particles (Rowlands et al., 1975; Doel and Chang, 1982). Like 146S particles, they also elicit production of virus neutralizing antibodies (Cowan, 1973; Rweyemamu et al., 1979; Doel and Chang, 1982). In addition, FMDV harvests also contain a viral-infection- associated (VIA) antigen which is not serotype-specific (Brown, 1985a). This antigen has been shown to be the viral RNA polymerase (Newman et al., 1979).

The importance of VP1 in stimulating immunity against FMD has been proved from the fact that of the four structural proteins, only isolated VP1, or fragments of it, has been shown to elicit neutralizing antibodies in animals (Bolwel et al., 1989) and, secondly the immunogenicity of the virus is impaired by treatment with trypsin which cleaves only the VP1 (Meloen et al., 1979; Haresnape et al., 1981). Two sites in VP1, one located between amino acid residues 138- 154 and the other between 200-213 were found to have antigenic activity (Strohmaier et al., 1981). According to Rowlands et al (1983) and Brown (1985b) the 141-160 residues of VP1 contain the major immunogenic site of the FMD virus, which is immunogenically dominant and elicits much higher levels of neutralizing antibody than other regions of this protein. This region also contains the highly conserved RGD(arginine-glycine-aspartic acid) sequence involved in cell attachment (Fox et al., 1989).

ANTIGENIC VARIATION IN FMD VIRUS

Valee and Carre in 1922 recognised antigenic differences between strains of FMDV when they found that cattle in France which had recovered from the disease became re-infected almost immediately when they came in contact with sick animals from Germany. This wass the first instance of demonstration of antigenic differences between strains of any animal virus. The two serotypes were labelled 'O' (Oise) and 'A' (Allemagne). In 1926, Waldmann and Trautwein reported serotype 'C'. In 1948, at Animal Virus Research Institute,

Pirbright. SAT 1, SAT 2 and SAT 3 were identified. A seventh antigenically different type, Asia 1, was first identified in 1954 from samples submitted to Pirbright from Pakistan (Brooksby and Rogers, 1957). Retrospective studies of some atypical isolates from Izatnagar, India during 1951-52 were also found to belong to the Asia 1 serotype and are consequently the earliest documented Asia 1 virus isolates (Dhanda et al., 1957). Infection as well as vaccination with virus of one serotype do not confer protection against virus of another serotype. By CFT and cross-neutralization tests carried out with guinea pig sera raised against reference and field strains, more than 65 subtypes have been identified (Pereira, 1977).

Antigenic variation leading to emergence of variants in FMD is of great importance from the epidemiological point of view and for formulating suitable vaccination programmes. Pringle (1964) reported that the subtype variants probably arose in the field through genetic change followed by selection in the host population and might exhibit a range of susceptibility to the hosts as a result of earlier infection or immunization. The antigenic variation even within a serotype can be so great that immunity against the homologous strain of virus need not necessarily ensure protection against infection by other viruses within that serotype (Rowlands et al., 1983). Subseqently, Domingo et al. (1985) reported that cloned or uncloned populations of most RNA viruses do not consist of a single genome species of defined sequence, but rather a heterogeneous mixture of related genomes (quasispecies) and mutations at only one or a few sites may alter the phenotype of an RNA virus. FMDV also consists of distributions of genomic sequences (Domingo et al., 1985) and this ensure rapid evolution since many variants are present in any population and there is frequent generation of new mutant genomes. It has been proposed that both immune ·ection and size of infecting inocula are important factors in the rate of evolution of FMD vi.as (McCahon et al., 1985). Each FMDV population, such as a field isolate, is not only genetically heterogeneous, but probably also antigenically heterogeneous (Rowlands et al., 1983; Mateu et al., 1989) consisting of an indeterminate spectrum of variants, supporting the quasispecies model of Domingo et al. (1985).

ANTIGENIC COMPARISON STUDIES

The study of antigenic variation in foot-and-mouth disease virus is important from the point of view of epidemiology of the disease and virus classification, and for the selection of suitable vaccine viruses (Ouldridge et al., 1984). As attempts to control FMD by vaccination evolved, it was realised that antigenic differences existed between strains within each serotype. There was, therefore, a requirement to further classify the strains into antigenically similar groups or subtypes (Brooksby, 1968). A consequence of placing strains of FMD virus into groups or subtypes was the requirement to define the boundaries between subtypes. However, while defining these limits, Brooksby recognised that a dilemma would constantly undermine the concept of subtyping:namely that strains known to be epidemiologically linked could have low cross-fixation values. These two sometimes conflicting requirements remained to confuse and finally discredit subtyping. Later Rweyemamu et.al.(1977c) extended the concept that field strains should be related to a list of reference vaccine strains and they emphasized the importance of using antisera against vaccine strains rather than against original field isolates.

The types of study involved in the examination of variation can be divided into serological and biochemical (Samuel et al., 1991). Traditionally, the serological studies were undertaken to measure the antigenicity of viruses with the help of reference antisera either raised in guinea pigs or bovines, in assays like CFT, virus neutralization and enzyme-linked immunosorbent assays (ELISAs). These assays involved the interaction of virus with polyclonal antisera raised against both reference strains and each field isolate examined, to obtain two-way relationships between the field strains and the reference strains. Biochemical techniques used for differentiation of FMD virus isolates include polyacrylamide gel electrophoresis (PAGE) and isoelectric focussing (IEF) of viral proteins, T_1 oligonucleotide mapping and sequencing of viral genome. In the context of the present work, serological methodologies for subtype analysis using polyclonal and monoclonal antibodies and biochemical techniques involving nucleotide sequencing have been reviewed.

Studies using polyclonal sera

Different test systems have been used for studying antigenic variation.The differentiation of isolates into types and subtypes is based on complete or partial lack of cross-protection between given FMD viruses (Pereira, 1977). Following demonstration of complement fixing antibodies in FMD immunized cattle (Lourens, 1909),CFT has been used extensively for distinguishing different strains of FMD virus (Traub and Mohlmann, 1946; Brooksby, 1952; Davie, 1964; Forman, 1974a, b, 1975a, b; Arrowsmith, 1975; Rweyemamu et al., 1978; Rai, 1980; Ferris et al., 1984; Ivanov and Tekerlekov, 1989). However,CFT is of limited use for the selection of serologically appropriate vaccine strains,because it detects a wide spectrum of antigens not revelent to protection. This test has also been criticised for its lack of sensitivity and specificity (Rweyemamu et al.,1978;Pay et al.,1985).

Forman (1975a) employed neutralization test in microtitre plates using two fixed doses of virus and two-fold dilutions of sera. The test appeared to provide a satisfactory means of differentiation between strains. Rweyemamu et al. (1977c) found MNT to be more specific compared to CFT. Subsequently, serum neutralization test was recommended as the in vitro test for assessment of antigenic variation in field strains, as it correlated well with cattle protection test (Rweyemamu, 1984). Pay (1985) reported that SNT carried out with bovine antisera prepared against vaccine strains has been used as the test of relevance for analysis of new field strains. Different variations of neutralization test viz. metabolic inhibition test and two-dimentional micro-neutralization test (2D-MNT) have been used for strain differentiation studies (Forman 1975a; Rweyemamu, 1977a, b,c). Although both FMD virus guinea pig sera and bovine vaccinate sera have been used in neutralization tests (Rweyemamu et al., 1977c; Rweyemamu, 1984), bovine vaccinate sera has been advised for use in neutralization studies (Ahl, 1985; Pay, 1985).

Abu Elzein and Crowther (1978) introduced the technique of enzyme-linked immunosorbent assay (ELISA) for FMDV serological studies. Subsequently the same group

(1979a, b) demonstrated the sensitivity of three ELISA techniques over CFT and discussed its application for detection, typing and subtype differentiation of FMDV isolates. It has been reported that for strain differentiation, indirect sandwich ELISA was 5 -10 times more sensitive than CFT (Ouldridge and Rweyemamu, 1983) and the results were comparable to that obtained with neutralization tests (Ouldridge et al., 1984). Roeder et al. (1987) reported that the indirect sandwich ELISA achieved a detection sensitivity approximately 125 times that of CFT. ELISA has an advantage over neutralization tests in that the former can be used with killed virus preparations (Crowther, 1986), moreover it measures major immunogenic sites of FMDV (Ouldridge et al., 1981). ELISA results are much more reproducible than those obtained with virus neutralization test and are not influenced by variations in tissue culture susceptibility.

Since their introduction, indirect and sandwich ELISAs have been used by several workers for detection of antigen and strain differentiation analysis (Rai and Lahiri, 1981; Have et al., 1983; Hamblin et al., 1984; Pattnaik and Venkataramanan, 1989a, b).

The sandwich ELISA was used for characterization of Indian isolates of type O (Pattnaik et al., 1990; Tosh, 1991) and Asia 1 (Mishra et.al., 1995) FMD virus. Pattnaik et al. (1991) used a liquid phase ELISA developed by McCullough et al. (1985a, b) for characterization of Indian field isolates. Later the liquid phase ELISA technique was modified by Hamblin et al. (1986) and named, liquid phase blocking sandwich ELISA. This blocking ELISA was performed using bovine convelascent sera for characterization of type A FMD virus isolates and the results tallied with conventional virus neutralization test (Samuel and Kitching, 1987).

Studies using monoclonal antibodies(Mabs)

Antigenic characterization using polyclonal sera have several disadvantages . Such sera contain antibodies against different parts of each antigen as well as against many

different antigens present in the immunogen. So if a particular determinant is either not present or lost, it is likely to go undetected as the majority of antibodies will still bind to the antigen. A monoclonal antibody (Mab) is secreted by the clonal progeny of a single B lymphocyte sensitized with a single antigenic determinant or part of it and so contain antibodies of single specificity. It has been observed that the unique properties of Mabs can be exploited to link chemical, antigenic and immunological properties of FMDV (Crowther and Samuel, 1987). Mabs against FMD viruses are ideal reagents for the measurement and better understanding of antigenic differences in epidemiological studies (Crowther et al., 1990).

Hamblin et al. (1985) observed that use of Mabs in ELISA can provide more information on the identity, specificity and possible origin of viruses than methods like CFT and VNT. They used FMD virus type O_1 Suisse (Lausanne) Mabs identifying three different neutralizing antigenic sites to characterize heterologous O_1 virus isolates by indirect sandwich ELISA and results were expressed as a percentage of activity in relation to the results with homologous virus. Differences were observed in the epitopes expressed by the type O isolates examined. In many isolates absence of expression of some epitopes was observed and the number of shared epitopes àlso varied. They concluded that as the viruses were compared using Mabs defining neutralizable epitopes, the comparision was pertinent to protective antibodies induced in animals against these epitopes.

In order to study the epidemiology of outbreaks and the relationships of the isolates with respect to vaccine strains, Brocchi et al. (1986) characterised FMD virus isolates from Italy of serotypes O, A and C by ELISA using Mabs. This study revealed variation in trypsin-sensitive antigenic site of some type A isolates. In case of type C two groups were observed: one, homologous to the vaccine virus and two, not reactive with the Mabs identifying VP1, 140-160 epitope of vaccine virus. Isolates of type O reacted well with main neutralizing Mabs..

Barteling et al. (1986) described a 'trapping' ELISA for screening the interaction of different European field and vaccine strains with a panel of strongly neutralizing anti-A_{10}-Holland Mabs which had been shown to be directed against four different antigenic domains located on VP1, VP3 and probably VP2. The results showed that the A_{10} Holland virus was clearly different from the European A_5 strains. Only three Mabs were reactive with all A strains tested in the study.

Using a panel of 10 Mabs, against A Parma/1962, Brocchi et al. (1987) characterized forty FMD virus isolates from the Italian epizootic of 1986-87. Only six of the forty isolates were found to be different by Mabs 3H2 and 5G2. Both these Mabs, one neutralizing (3H2) and another non-neutralizing (5G2), were against trypsin-sensitive region of VP1. But these six isolates did not show any difference in CFT and MNT performed with bovine vaccinate sera. This showed that although changes in the 3H2 and 5G2 antigenic areas can occur in the field, such changes do not confer any selection advantage. The results also showed that the antigenic areas identified by Mabs 3H2 and 5G2, commonly considered as the main antigenic component of the virus, are frequently subjected to variation and other immunogenic epitopes are also important for the immune protection mechanism. Results of this study emphasized the better suitability of Mabs for investigating variations occuring in the field isolates. Mateu et al. (1987a) used a panel of 12 Mabs raised against serotype C_1 to characterise 14 isolates of type C virus by immunoelectrotransferblot, immunodot and neutralization test. Although none of the isolates could be distinguished by their reactivities in immunoelectrotransferblot and immunodot, the isolates could be classified into two groups by a 10^2 fold difference in their reactivity with 6 neutralizing Mabs. They observed that epidemiologically related strains differed in at least one epitope critical for virus neutralization with synthetic peptide antigen study. In another study, Mateu et al. (1987b) investigated 13 epidemiologically related FMDV isolates of serotype C_1 from Spain using Mabs. They observed that single amino acid substitutions in the epitopes greately affect the neutralization of virus infectivity by Mabs.

A panel of Mabs against O1K and O1 Suisse was used to characterize different subtypes and strains of type O FMD virus (Haas et al., 1988). The antigens either in the form of cattle tongue epithelium or BHK-21 cell culture supernatant, when tested with Mab panels, similar results were obtained as in plaque reduction test and cDNA sequencing. They concluded that Mab profiling by ELISA is a valuable tool for subtyping and characterization of strains and isolates.Samuel et al. (1991) evaluated a trapping ELISA for strain differentiation of FMD virus by Mab profiling. They defined the criteria for establishing antigenic differences between the strains with the help of Mabs.

Pattnaik (1993) characterized 29 type O field isolates of Indian origin from 1987 to 1992 by Mab profiling in sandwich ELISA using a panel of 26 neutralizing mabs raised against type O vaccine virus. The majority of the isolates showed reaction of homology with most of the Mabs. The Mabs raised against trypsin-sensitive site showed differences in antigenicity whereas Mabs against trypsin-resistant sites did not reveal much difference between the field isolates and vaccine virus.

Alonso et al (1993) selected a panel of Mabs raised against FMD virus of serotype O1 Campos, A24 Cruzeiro and C3 Indiail on the basis of their neutralizing titre, protective titre, sensitivity to trypsin and specificity for virus structural proteins. The Mabs were utilized in an ELISA test format to compare European and South American representative field isolates with the results obtained in CFT and SNT with polyclonal antibodies. The reactivity of Mabs with different strains showed varied amount of reactivity indicating antigenic differences between strains.

WORK DONE ON FMDV SEROTYPE ASIA 1

The earliest report of antigenic variation in FMDV type Asia 1 was that of Arrowsmith (1982) who found that type Asia 1 virus which caused disease in several Gulf countries and Asia between 1979 and 1980 was different from the old Asia 1 vaccine strain

(Asia 1/1, Pak 1/54). She concluded that the results indicated a movement away from earlier established subtypes. This change was found to be not dramatic at that time but rather a gradual shift from Pak 1/54 and it was recommended that in future, to control outbreaks, appropriate strains of the virus needs to be incorporated in vaccine production.

From 1979 onwards, in India, a large number of outbreaks were recorded in herds vaccinated with subtype Asia 1/1 (Pak 1/54). Rai and Goel (1983) showed 'r' values 0.25 and 0.13 with Asia 1/1 antiserum and reverse direction testing of few selected isolates revealed 'r' values of less than 0.25, indicating that these strains were different from subtype Asia 1/1 . They also subjected these isolates to subtyping with Asia 1/2 (Israel 3/63) antiserum which confirmed the homology of these isolates to Asia 1/2 . Based on these studies they concluded that the increased incidence of FMD outbreaks among vaccinated herds may be due to the emergence of Asia 1/2 . Using micro-neutralization, guinea pig protection and challenge tests in cattle , Goel and Rai (1983) confirmed their earlier report of emergence of Asia 1/2 in the country.

Belwal et al. (1989) while studying strain differentiation of FMDV type Asia1 isolates of Indian origin by using a two-dimensional micro-neutralization test (2D-MNT) reported considerable antigenic variation among 24 Asia 1 strains. However, most strains could be related to the vaccine strain, Asia 1 IND 63/72 but not to narrow spectrum vaccine strain Asia 1 IND 5/79. The broadest antigenic spectrum was observed from strain Asia 1 WBN 117/85 and recommended for incorporation in the quadrivalent vaccine.

Mishra et.al. (1995) studied the antigenic relationship of 20 FMDV type Asia1 field isolates from different parts of India isolated during the year 1987-92 with the IVRI vaccine strain IND 63/72 using micro-CFT, indirect sandwich ELISA , 2D-MNT, and plaque reduction neutralization test. A close antigenic relationship of the field isolates with the vaccine virus was observed.

Butchaiah et al.(1992) characterized seven isolates of Indian origin using a panel of 26 Mabs elicited against three isolates of Asia 1 FMD serotypes . Five of the seven isolates exhibited extensive variation particularly in conformation dependent neutralization epitopes.. They concluded that extensive antigenic heterogenicity observed among Indian Asia 1 virus field isolates could be the result of partially immune (vaccinated) host animals providing a strong selection force for the generation of antigenic variants.

Prabhudas et al.(1993) used two Mabs against the major antigenic viral polypeptide VP1 of Asia 1 vaccine strain and they were used to characterize 17 Asia 1 isolates from different regions of India by neutralization assay. Six of these field isolates were further characterized with polyclonal antibodies as well as with Mabs in neutralization assays and passive mouse protection assay. They observed the occurance of isolates with low, medium and high reactivity with Mabs, there were also isolates that resisted neutralization. This showed that there was variation in the major antigen of Asia 1 field isolates.

Sanyal (1995) used a panel of 10 neutralizable Mabs raised against vaccine strain (IVRI strain: IND 63/72), shown to be directed against four different antigenic sites with seven epitopes, to analyze 47 Asia 1 field isolates of Indian origin. Based on reactivity pattern the isolates were classified into 11 groups. He concluded that although in Mab profiling minor difference was observed at some Mab-binding sites (mabs B3, 1A, 24, 89 and 72) identified as trypsin sensitive sites on the virus, all the isolates were found to be related ($r = 0.45$ to 1.00) to the vaccine virus strain, when assayed in sandwich ELISA using polyclonal serum. Study of antigenic features of FMDV type Asia 1 using monoclonal antibody resistant mutants (MAR-mutants), has revealed the presence of four different antigenic sites containing seven distinct neutralization epitopes. All the sites are shown to be trypsin sensitive and conformation dependent.

GENETIC COMPARISON STUDIES

Over the last 10 years, subtyping , which was a method for antigenic classification of virus strains, has been replaced by characterization and comparision of viral proteins by polyacrylamide gel electrophoresis (PAGE) and electrofocussing (EF) and then by comparision at the genome level. The present stage in this evolution of techniques to identify and compare strains of FMDV is nucleotide sequencing. A portion of the genome, approximately 160 nucleotides at the 3' end of 1D gene (coding for the VP1 surface protein), is sequenced by primer extension (Sanger et al., 1977), and then compared with that of the same region from another strain. As more sequences are accumulated, family trees or dendrograms are generated, showing the genetic relationship between strains (Kitching and Knowles, 1993). The analysis of sequences for epidemiological purposes involves comparing the degree of nucleotide homology/divergence between virus strains and expressing this on a dendrogram (Ricco-Hesse et al, 1987).

The first of this kind of study pertaining to FMDV was carried out by Beck and Strohmaier (1987). They compared the primary genetic structure of 18 recent European outbreaks and of 9 strains isolated more than 20 years ago and used in part as vaccines, by cDNA sequencing of the VP1 coding regions. Comparision of the sequences revealed that most of the isolated outbreak viruses were closely related to the vaccine strains used. Only two minor outbreaks in Federal Republic of Germany, A' Aachen in 1976 and O' Wuppertal in 1982, did not correspond to the classical European strains but were obviously introduced from outside. They suggested that comparision of the primary genetic structures allows a much more precise evaluation of the degree of relationship among viral strains than do the serological and physico-chemical methods.

Dopazo et al. (1988) constrructed a phylogenetic tree and calculated the rate of nucleotide substitutions per site to define phylogenetic relationships among the VP1 coding regions of 15 isolates of FMDV serotypes A, C and O. They have statistically

documented high mutability, rapid generation of distributions of related, nonidentical genomes, and absence of correlation between time intervals between isolations and genetic distances in phylogenetic analysis. Their results clearly show that evolution of FMD viruses concerns complex, indeterminate mixtures of genomes rather than a single, determinate species and the VP1 based phylogenetic grouping correlated wtih the classical serological classification.

Samuel et al. (1990a) performed antigenic studies using polyclonal and monoclonal antibodies and nucleotide sequence analysis of strains of FMDV serotype O isolates from Saudi Arabia in 1988 and 1989. Their study revealed that virus isolates from Saudi Arabia formed a related genetic group distinct from both the vaccine virus strains used in the study, although the vaccine strain O1/Manisa/Turkey/69 was more closely related to vaccine strain O1/BFS 1860. Comparision of the deduced amino acid sequences showed that most differences occured within the known antigenic region (amino acids 140-160) which was excpected to give rise to serological differences between some of the virus isolates.

For epidemiological characterization of type O FMDV in Europe, Saiz et al (1993) constructed a phylogenetic tree based on the VP1 sequences of field isolates. Their analysis supports a close relationship between European O1 field isolates and vaccine strains, with the exception of two isolates which were probably of non-European origin. Analysis indicated that synonymous mutations play a major role in the evolution of FMDV. They concluded that the combination of phylogenetic approaches and the acquisition of sequences from PCR products provides a rapid, accurate and powerful tool for the improvement of epidemiological surveillance.

Stram et al. (1995) employed the reverse transcription- polymerase chain reaction (RT-PCR) and direct sequencing in the diagnosis and typing of foot and mouth disease virus (FMDV) in samples isolated during the 1994 disease outbreak in Israel. Using PCR, virus isolation and serological methods, it was shown that the 1994 disease outbreak

in Israel and other Middle-Eastern countries was caused by type O1 virus. Homology analysis of the VP1 gene sequences revealed that there were two distinct outbreaks in Israel. The first originated in Jordan, moved to the West Bank territory and then to the Lower Galilee. The second outbreak, caused by another virus, was responsible for disease outbreaks in South Lebanan, Upper Galilee and the Golan Heights. When viral sequences of isolates from the 1993 outbreaks in Egypt and Lebanan were included in the analysis, they showed a high degree of VP1 sequence homology between themselves, suggesting a common origin.

In addition to above cited studies, many other molecular epizootiological studies have been done for serotypes O, A, C, SAT 1 and SAT2 (Weddel et al., 1985; Knowles et al., 1988; Marquardt and Adam, 1988; Martinez et al., 1988; Piccone et al., 1988; Samuel et al., 1988; Marquardt and Adam, 1989; Sorbino et al., 1989; Carillo et al., 1990; Knowles and Samuel, 1990; Kerbs et al., 1991a, b; Armstrong et al., 1992; Marquardt and kerbs, 1992; Martinez et al., 1992; Samuel et al., 1993; Armstrong et al., 1994; Dave et al., 1994).

Ansell et al. (1994) studied the genetic relationship between foot-and-mouth disease type Asia 1 viruses by sequence analysis of 165 nucleotides at the 3' end of 1D (VP1) gene of 44 strains isolated throughout Asia between 1954 - 1992 and their analysis of the relationships between the virus genomes showed epidemiological links not previously evident. They used a difference of < 5% to indicate a close relationship and based on this criterion isolates were classified into 18 different related groups and none of the sequences showed a greater divergence than 14%. Their conclusion was that variation in the region sequenced was not as great as that seen in the other FMDV serotypes and all viruses shared > 85% nucleotide identity and thus all the virus isolates examined were considered to belong to a single genotype. Five Indian isolates which were included in this study were represented in four of the eighteen different groups they made based on dendrogram, indicating the wide diversity of virus isolates of Indian origin

MATERIALS AND METHODS

MATERIALS AND METHODS

Reference Virus

FMD virus type Asia 1 vaccine strain (IVRI Vaccine strain IND 63/72) adapted in BHK-21 clone 13 cell line and maintained at the respository of the Central Laboratory, All India Co-ordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar was used as the reference virus in the present study. The purity and specificity of the virus was tested in Enzyme-Linked Immuno Sorbent Assay (ELISA) using type specific guinea pig polyclonal serum.

Field virus isolates

A total of 40 isolates of type Asia 1 FMD virus recovered from outbreaks in different parts of the country and available at the Central Laboratory, All India Co-ordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar in the form of cell culture antigen were used in this study. The detailed history of the field isolates is given in Table 1.

Anti-146S sera

Anti-146S sera raised in guinea pig and rabbit against the reference type Asia 1 virus strain were available at the Central Laboratory, All India Co-ordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar.

Bovine vaccinate Serum

Bovine vaccinate serum used was available at the Central Laboratory,AICRP for Epidemiological Studies on FMD,IVRI, Mukteswar. This was produced by vaccinating hill bulls with 2.5 ml of type Asia 1 monovalent vaccine. On 21 day of vaccination, blood was collected, serum was separated and pooled. The serum was inactivated at 56°C for 30 minutes and tested for the presence of neutralizing antibodies and was stored at -20°C.

Table-1: Detatils of FMD Asia 1 field isolates used in the study.

Sl. No.	Isolate No	Year of Isolation	Place of outbreak	Species	Vaccination Status
1.	IND75/86	1985	Parbharagaon (Assam)	Cattle	N.A.
2.	IND46/87	1986	Jaipur (Rajasthan)	Cattle	N.A.
3.	IND22/88	1987	Gauhati (Assam)	Cattle	Vac.
4.	IND120/88	1988	Nasik Dist. (Maharashtra)	Cattle	Unvac.
5.	IND155/88	1988	Ahmed Nagar (Maharashtra)	Cattle	Unvac.
6.	IND267/88	1988	Baroda (Gujarat)	Cattle	Vac.
7.	IND19/89	1989	Raya,Mathura (Uttar Pradesh)	Buffalo	Unvac
8.	IND21/89	1989	Baldro, Mathura (Uttar Pradesh)	Buffalo	Unvac.
9.	IND45/89	1989	Izatnagar (Uttar Pradesh)	Buffalo	N.A.
10.	IND132/90	1990	Mevalika, Nagla,Mathura (Uttar Pradesh)	Cattle	Unvac.
11.	IND10/91	1990	Nanded (West Bengal)	Cattle	Unvac.
12.	IND13/91	1990	Ahmednagar (Maharashtra)	Sheep	Unvac.
13.	IND17/91	1990	NDA, Pune (Maharashtra)	Pig	Unvac.
14.	IND17/93	1993	Hissar (Haryana)	Cattle	Unvac.
15.	IND53/93	1993	Bangalore (Karnataka)	Cow	Unvac.
16.	IND293/94	1994	Calcutta (West Bengal)	N.A.	N.A.
17.	IND316/94	1994	Thane (Maharashtra)	Cattle	Unvac.
18.	IND1/95	1994	UAS, Bangalore (Karnataka)	Cattle	Vac.
19.	IND4/95	1994	Bangalore (Karnataka)	Cow	Vac.
20.	IND6/95	1994	Holalu, Mundya (Karnataka)	Cattle	N.A.
21.	IND14/95	1995	Nellore (Andra Pradesh)	Bullock	N.A.
22.	IND15/95	1995	Kurnul, Hyderabad (Andra Pradesh)	Cattle	N.A.
23.	IND26/95	1995	IVRI, Izatnagar (Uttar Pradesh)	Cattle	Vac.
24.	IND29/95	1994	Dharmapuri, Hosur (Tamil Nadu)	Cattle	N.A.
25.	IND40/95	1995	Nilgiri (Tamil Nadu)	Cattle	N.A.
26.	IND50/95	1995	Gorakhpur (Uttar Pradesh)	Buffalo	Vac.
27.	IND57/95	1995	Kurnul (Andra Pradesh)	Cattle	N.A.

28.	IND33/96	1995	Siliguri Dist. (West Bengal)	Cattle	Unvac.
29.	IND43/96	1996	Military Farm, Kirki, Pune (Maharashtra)	Cattle	N.A.
30.	IND70/96	1996	Nadia (West Bengal)	N.A.	N.A.
31.	IND71/96	1996	Guntur (Andra Pradesh)	N.A.	N.A.
32.	IND72/96	1996	Nasik (Maharashtra)	N.A.	N.A.
33.	IND73/96	1996	Pondicherry	N.A.	N.A.
34.	IND80/96	1996	Teliyur, Bangalore (Karnataka)	Cow	Unvac.
35.	IND81/96	1996	Teliyur, Bangalore (Karnataka)	Cow	Unvac.
36.	IND82/96	1996	Chirgaon, Shimla (Himachal Pradesh)	Cattle	N.A.
37.	IND89/96	1996	Modhopur, Ropar (Punjab)	Buffalo	Unvac.
38.	IND172/96	1996	Rohtak (Haryana)	Buffalo	N.A.
39.	IND173/96	1996	Rohtak (Haryana)	Buffalo	N.A.
40.	IND26/97	1997	Hyderabad (Andra Pradesh)	Cattle	Vac.

Vac-Vaccinated, Unvac-Unvaccinated, NA-Not Available.

Monoclonal antibodies

Mouse monoclonal antibodies (Table 2) developed against complete virus particles of type Asia 1 vaccine virus strain at the Central Laboratory, All India Co-ordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar were used in the present study (Venkataramanan et al., 1990-92). The Monoclonal antibodies (Mabs) either in the form of cell culture supernatant and/or mouse ascites fluid were used in the study. The Mabs were characterized earlier (Sanyal et al., 1997).

Cell culture

BHK-21 clone 13 cell line maintained at the Central FMD Typing Laboratory, IVRI, Mukteswar was used for propagation of virus isolates.

Oligonucleotide primers

Details of the oligonucleotide primers used in the study are given in table 3, Fig. 2

Table -2: Details of Type Asia 1 monoclonal antibodies (Mabs) used in the study*.

Sl. No.	Mab designation	Isotype	Virus neutralizing activity (Log_{10} NI)	Trypsin sensitivity of the binding site
1	B3	IgG2b	3.5	Pts
2	1A	IgG2b	4.0	Ts
3	24	IgG2b	3.5	Pts
4	2A	IgG2b	4.0	Ts
5	40	IgG2b	2.5	Ts
6	63**	IgG2b	3.5	Pts
7	34	IgG2b	3.5	Ts
8	81**	IgG2b	1.0	Ts
9	72	IgG2a	4.5	Pts
10	82	IgG2a	3.0	Pts

*, The Mabs were characterized earlier (Sanyal et al., 1997).

**, Used as mouse ascites fluids, rest all were used in the form of supernatants.

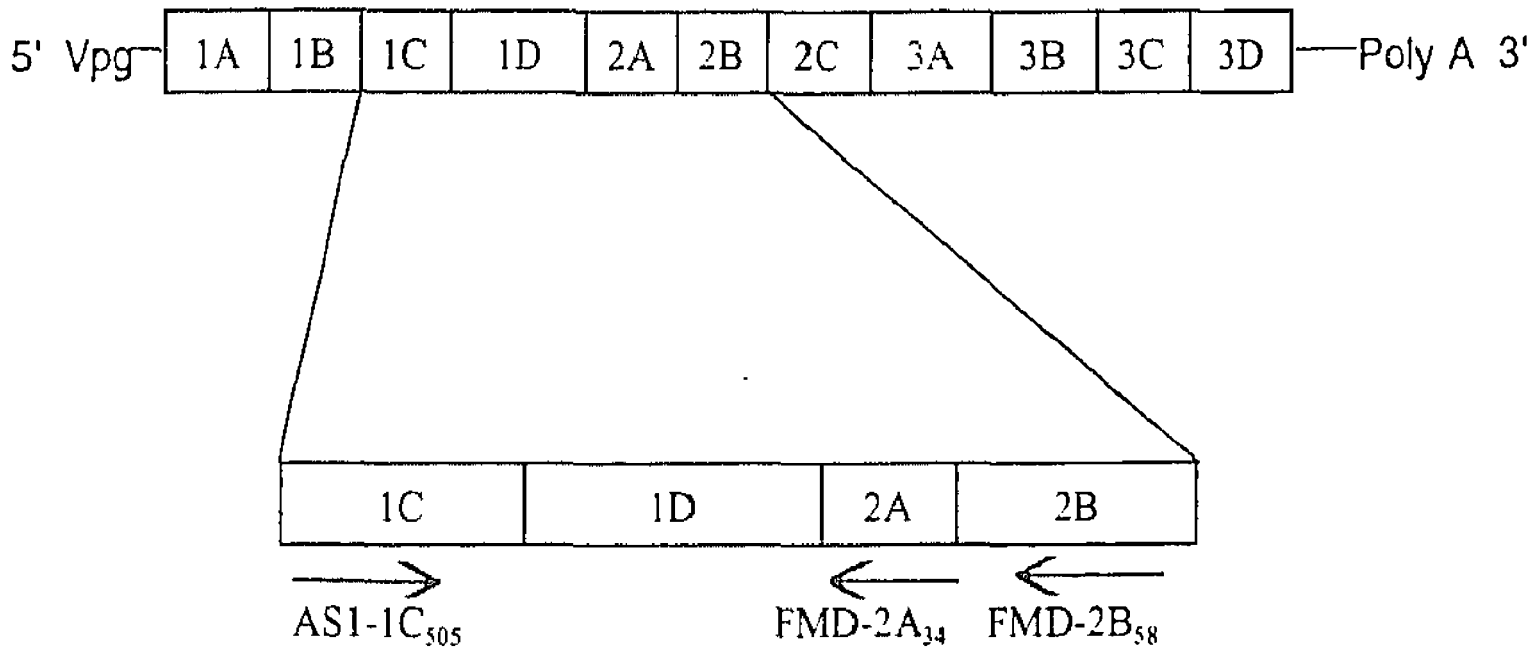
Ts, Antigenic site sensitive to trypsin.

Pts, Antigenic site partially sensitive to trypsin.

Table.3: Oligonucleotide primers used in the study

Sl. No	Primer designation	Primer sequence	Polarity and position	Remarks
1	As1-1C505	TACACTGCTTCTGACGTGGC (20-mer)	Positive, 1C region	Primer for RT and PCR amplification
2	FMD-2B58 (NK61)	GACATGTCCTCCTGCATCTG (20-mer)	Negative, 2B region	Primer for PCR amplification
3	FMD-2A 34 (NK72)	GAAGGGCCCAGGGTTGGACTC (21-mer)	Negative, 2A region	Sequencing primer

Fig. 2: Sequences of oligonucleotide primers and their location on the FMDV genome.



AS1-$1C_{505}$ 5' TACACTGCTTCTGACGTGGC 3'

FMD-$2A_{34}$ 5' GAAGGGCCCAGGGTTGGACTC 3'

FMD-$2B_{58}$ 5' GACATGTCCTCCTGCATCTG 3'

Nucleic acid manipulation kits

1.RNeasy total RNA kit (Qiagen, Cat. No. 74104):	for RNA extraction
2.Access RT-PCR system (Promega, Cat. No.A1250):	for RT-PCR
3.Wizard™ PCR preps DNA purification system (Promega, Cat. No. A7170):	for purification of RT-PCR product
4.fmol® DNA cycle sequencing system (Promega, Cat. No.Q4100):	for cycle sequencing
5.Silver sequencing™ DNA staining reagents (Promega, Cat.No.Q4132):	for silver staining of sequencing gels.

ı ropagation and type confirmation of virus isolates

The field isolates of FMDV serotype Asia 1 preserved at the Central Laboratory,AICRP for Epidemiological Studies on FMD, IVRI, Mukteswar, in the form of BHK-21 cell culture infected fluid were used for propagation. Each culture tube was inoculated with 0.2 ml of inoculum after washing the monolayer with maintenance medium, and virus was allowed to adsorb at 37°C for 45 minutes. The unadsorbed virus was discarded, cell-sheet was washed twice with maintenance medium, 2 ml of maintenance medium was added to each tube and incubated at 37°C. The tubes were observed daily for cytopathic effect (CPE) as evidenced by rounding of cells followed by detachment from the glass surface. Serial passage(2-3) in cell culture tubes was given to obtain complete CPE within 24 hours. The working stock of all the field isolates was obtained by infecting them in 25 cm^2 cell culture flasks (Nunc, Cat. No. 163371). When complete cytopathic effect was observed, the infected culture fluid was collected and centrifuged at 2500 rpm for 15 minutes to sediment the cell debris. The clear supernatants were collected separately, confirmed for type specificity by a sandwich ELISA (Battacharya et al., 1996), and stored at -70°C in aliquotes for subsequent use in the study.

ANTIGENIC ANALYSIS OF FIELD ISOLATES

Strain differentiation of isolates by sandwich ELISA

Indirect sandwich ELISA described by Ouldridge et al., (1982) with some modifications was employed in the study using 96-well flat bottomed polyvinyl immunoassay plates (Greiner 655061, Germany). The steps followed in the test were as follows:

(i) Wells of the plates were coated with rabbit anti-146S serum diluted to1:5000 in coating buffer in 50µl volumes. Coating was effected at 37°C for 1 hour or at° 4 C overnight.

(ii) The wells of the plate were washed 3 times at 5 minutes intervals using PBS-Tween 20 washing buffer.

(iii) Type Asia 1 vaccine strain virus in the form of infected BHK-21 cell culture fluid was added to all the wells of row A of the plate and accdordingly 6 field isolates were added to the wells of row B to G (one isolate/row) in 50 µl volumes. Row H was kept as background control and to these wells 50 ul of blocking buffer in the place of antigen was dispensed. The plate was incubated at 37°C for 1 hour and then washed as in step (ii).

(iv) Serial dilutions(1:500, 1:1000, 1:1500, 1:2000, 1:2500 and 1:3000) of type Asia 1 anti-146S guinea pig serum in ELISA blocking buffer was added to the wells(two wells/dilution) in 50 µl volumes. The serum was allowed to react at 37°C for 1 hour.

(v) After washing the plates as in step (ii), 50 µl of 1:2500 dilution of rabbbit-anti-guineapig HRPO conjugate (P-141,Dakopatts, Denmark) in ELISA blocking buffer was added to all the wells. The plates were incubated at 37°C for 1 hour and the unbound conjugate was washed off as in step (ii).

(vi) 50 µl of OPD substrate solution was added to all the wells and incubated at 37°C for about 10 minutes to facilitate enzyme-substrate reaction.

(vii) 50 µl of stopper solution (1M H_2SO_4) was added to all the wells.

(viii) The optical density (OD) was recorded at 490 nm in an ELISA reader (Dynatech Minireader II).

Corrected OD values for each antigen and serum dilution was calculated by subtracting OD values in background control wells from that of the test wells. The average OD values of the different antigen and serum combinations were converted as percentage of the average OD of reference virus reaction, known as percent reference (PR) to avoid variation between tests and plates.

From the PR value, the relationship 'r' (Ouldridge et al., 1982) was calculated as follows:

$$r = \frac{\text{PR maximum Heterologous}}{\text{PR maximum Homologous}}$$

Test was repeated on three separate days and average 'r' value was calculated.

Strain differentiation of isolates by Micro-neutralization test

Two dimentional micro-neutralization test (2D-MNT) was carried out in 96-well flat bottomed tissue culture plates (Nunc, Cat. No. 167008) to asses the serological relationship with vaccine strain using bovine vaccinate serum. The procedure described by Rweyemamu et al., 1978 with some modifications was followed:

(i) Two fold dilutions (1:16 to 1:256) of type Asia 1 bovine vaccinate serum was prepare in BHK-21 maintenance medium (Glasgow modification) and inactivated at 56°C for 30 minutes in a waterbath. Each dilution of the serum was added to two columns of each in 50 µl volumes.

(ii) Serial $\log_{10}$ dilutions of BHK-21 adapted virus was prepare in BHK-21 maintenance medium and dispensed to wells of rwos A to G from 2 $\log_{10}$ dilution to 8 $\log_{10}$ dilution in 50 µl quantities.

(iii) The plates were properly shaken for thorough mixing of the serum and virus and kept for incubation at 37°C for 1 hour.

(iv) BHK-21 Clone 13 cell suspension at a concentration of 1.5×10^6 cells/ml in maintenance medium containing 4% serum was dispensed to the wells in 50 µl quantities.

(v) The plates were shaken thoroughly, sealed with adhesive tape and incubated at 37°C for 48 hours under 5% CO_2 tension.

Appropriate controls viz. antigen control (no serum) and cell control (no antigen and no serum) were kept in all the plates.

The plates were read after 48 hours of incubation for the presence or absence of CPE. The highest dilution of serum neutralizing 100$TCID_{50}$ (calculated according to Reed and Muench, 1938) of the virus was determined graphically. This value was taken as the serum titre. Then the serological relationship expressed as 'r' value was obtained as follows:

$$\text{'r'} = \frac{\text{Serum titre with heterologous virus}}{\text{Serum titre with homologous virus}}$$

Antigenic characterization of field isolates by Mab profiling

The sandwich ELISA procedure described by Samuel et al.,(1991) was followed for Mab profiling of Asia 1 field isolates. The plate layout is shown in Fig. -3. The procedure broadly includes:

(I) Wells of immunoassay plates were coated with rabbit anti-146S serum diluted to 1:4000 in coating buffer in 50 µl volume and incubated at 4°C for overnight.

(ii) The wells were washed 3 times each at 5 minutes intervals using PBS-Tween washing buffer.

(iii) Type Asia1 vaccine strain virus in the form of infected BHK-21 cell culture fluid was added to all the wells of row A of each plate. Three field isolates in the form of infected BHK-21 cell culture fluid were dispensed to the wells of row B to G (two rows/isolate) in 50 µl volumes. Row H was kept as background control and to these wells 50 µl of blocking buffer (1% skimmed milk powder in washing buffer) was dispensed. The virus was allowed to react at 37°C for 1 hour and then washed as in step (ii).

(iv) Each antigen trapped plate was vertically divided and each column from 1 to 10 received 50 µl of ten different Mabs at a single pretitrated dilutions (this dilution was obtained from titration of Mabs against vaccine virus in sandwich ELISA) in blocking

Fig. 3: Plate layout for Mab profiling of field isolates by sandwich ELISA.

	Monoclonal Antibodies										Polyclonal Antibodies	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Homologous virus											
B	Virus A											
C												
D	Virus B											
E												
F	Virus C											
G												
H	Background (No virus)											

buffer(1% skimmed milk powder in washing buffer). To column 11 & 12, 50 µl type Asia 1 anti-146S guinea pig serum in ELISA blocking buffer at the pretitrated dilution of 1:1000 was added. Antibodies were allowed to react at 37°C for 1 hour.

(v) *After washing the plates as in step (ii), anti-mouse HRPO conjugate* (Sigma, Cat. No. A-4416) diluted to 1:5000 in blocking buffer (1% SMP in washing buffer) and anti-guinea pig HRPO conjugate (Dakopatts, Denmark, Cat. No. P-141) diluted to 1:2500 in ELISA blocking buffer were dispensed to the appropriate wells in 50µl volumes. The plates were incubated at 37°C for 1 hour and washed as in step (ii).

(vi) After washing, 50 µl of OPD substrate solution was added and kept at 37°C for enzyme substrate reaction for 10 minutes.

(vii) 50 µl of stopper solution (1M H_2SO_4) was added to all the wells.

(viii) The optical density was taken at 490 nm in a Dynatech Minireader *II*(ELISA reader).

Calculations:

Corrected OD values of each Mab with each antigen was obtained by subtracting background OD values from those of test proper. The OD values of each Mab with each antigen was expressed as percentage of OD of the polyclonal antibody with respective antigens and finally relationship between vaccine virus and field isolates was expressed as percentage of the homologous (vaccine virus) value of each Mab. The percent relationship was expressed with minor modification of Samuel et al.(1991) and was qualified by 4 ranges of reaction, viz. 70% and above reflects equal reaction to the homologous virus, 46-69% and 20-45% reflects reduced affinity and below 20% reflects no reaction.

GENOMIC ANALYSIS OF FIELD ISOLATES

Extraction of FMDV RNA

Genomic RNA of the field isolates and vaccine strain of FMDV serotype Asia 1 by Guanidine thiocyanate method. " RNeasy total RNA kit "(Qiagen, Cat. No. 74104) was used for this purpose with the following protocol:

(i) To the 460 µl of cell culture supernatant (free of any cell debris) in 1.5 ml tube, equal volume of Lysis buffer RLT (containing 1% 2-mercaptoethanol) was added and mixed by vortexing.

(ii) 460 µl of 70% ethanol in 1% DEPC treated water was added and mixed by vortexing.

(iii) The mixture was added to RNeasy spin columns and spun in a microfuge for 15 seconds at 10,000 rpm, flow-through was discarded and procedure was repeated.

(iv) After discarding the flowthrough, column was washed with 700 µl of wash buffer RW1 by centrifugation at 10,000 rpm for 15 seconds.

(v) The column was washed twice with 500 ul wash buffer RPE.

(vi) The column was centrifuged at 12,000 rpm for 2 minutes to dry the membrane completely.

(vii) The column was transferred to a new 1.5 ml collection tube and 50 µl of 1% DEPC treated water was pipetted directly onto the column membrane and centrifuged at 10,000 rpm for 60 seconds to elute RNA. RNA obtained was labelled and stored at -70°C till it was used for further steps.

REVERSE TRANSCRIPTION&POLYMERASE CHAIN REACTION

Reverse transcription and PCR amplification of viral RNA was done using the "Access RT-PCR system" (Promega, Cat. No. A1250).

The reaction mix in 50 µl volume contained, 10 µl AMV/Tfl 5x Reaction buffer, 1µl of 10 mM dNTP mix, 2µl FMD-$2B_{58}$ (NK61) universal primer (22 pmol/µl), 1µl As1-$1C_{505}$ primer (22pmol/µl), 2 µl 25 mM $MgSO_4$ 1µl AMV Reverse transcriptase (5U/µl), 0.5 ul Tfl DNA polymerase (5U/µl), 4µl RNA sample and 28.5µl nuclease free water. The reaction mixture was overlaid with 25 µl of PCR grade mineral oil and mixed for 2 minutes by centrifugation. The sterile PCR tubes (Axygen Scientific, Cat. No. PCR-05-C, thin walled) containing the reaction mixture were loaded onto a thermal cycler block (Hybaid, Omnigene) and RT-PCR amplification was done as given in table below:

Temperature	Time	Cycle	Remarks
48°C	45 minutes	1	RT
94°C	4 minutes	1	Denaturation
94°C 60°C 68°C	30 seconds 1 minute 2 minutes	40	Amplification
68°C	7 minutes	1	Final elongation

Confirmation of RT-PCR product

The RT-PCR products were tested by Agarose gel electrophoresis using 2% Agarose gel in TBE.25 ml of 2% Agarose (Boehringer Manheim) was prepared in 1X TBE and placed in boiling water until melted. Molten agarose was allowed to cool to about 45°C and ethidium bromide was added to give a final concentration of 0.5 µg/ml. The gel was poured into electrophoresis trough and well former (comb) was inserted. The gel was allowed to set on a flat surface for about 15 minutes. Electrophoresis trough was placed in electrophoresis tank filled with 1X TBE containing 0.5 µg/ml of ethidium bromide and the comb was removed. Samples were prepared on a parafilm by mixing 1µl loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cynole FF and 40% w/v sucrose in water) and 5µl of PCR product. Samples were loaded in parallel with molecular weight marker VI. Marker was prepared by mixing 0.5 µl of molecular weight marker VI (Boehringer Manheim), 1 µl loading buffer and 4.5 µl of water. Electrophoresis was done at 100 volts for 20 minutes. Gel was viewed under a UV-transilluminator for presence of bands and photographed. Length of the amplified DNA fragment was estimated in relation to the migration of DNA sizing ladder.

Purification of RT- PCR product

After confirmation of the RT-PCR products, these were purified to remove primer dimers and free nucleotides etc. using " Wizard ™ PCR preps DNA Purification System" (Promega, Cat. No. A7170).

(i) Aqueous phase of RT-PCR product was carefully transferred into a clean 1.5 ml eppendorf tube. 100 µl of direct purification buffer (50 mM Kcl, 10 mM Tris Hcl pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100) was pipetted into the tube and vortexed briefly to mix.

(ii) 1 ml of DNA purification Resin (6 M Guanidine thiocyanate) was added and vortexed briefly 3 times over a one minute period.

(iii) 2 ml sterile disposable syringe barrel was attached to the Luer-Lok^R extension of minicolumn and tip of the minicolumn/ syringe barrel assembly was inserted into the vaccum manifold.

(iv) Resin/DNA mix from step (ii) was pipetted into the syringe barrel and the contents of the barrel was pushed into vaccum manifold. Syringe alongwith piston was ached from the minicolumn and syringe was refixed to minicolumn after removing the sion from it.

(v) 2 ml of 80% isoproponol (Sigma) in 1% DEPC treated water was added to the barrel to wash the column by pushing into vaccum manifold as explained in step (iv). Syringe was discarded and minicolumn was transferred to a new 1.5 ml eppendorf tube.

(vi) The column was centrifuged at 10,000 rpm for 2 minutes to remove any residual isopropanol.

(vii) The column was transferred to a new clean 1.5 ml eppendorf tube and 50 µl of nuclease free water was applied. After 15 minutes the column was centrifuged at 10,000rpm for 20 seconds to elute the DNA into the tube.

Purified RT- PCR products were stored at -80°C until used.

Cycle Sequencing

The RT-PCR products were sequenced using a negative sense primer FMD-$2A_{34}$ (NK-72) (5'-GAAGGGCCCAGGGTTGGACTC-3') internal to FMD-$2B_{58}$ (NK-61). The "fmol^R DNA cycle sequencing system" (Promega, Cat. No. Q4100) was used for the purpose.

Sets of 4 PCR tubes were labelled and 2 µl of the appropriate dd/dNTPs were dispensed to tubes, centrifuged briefly and kept on ice till needed.

A sequencing reaction mixture contained 7 µl template DNA (RT-PCR product), 4 µl 5X sequencing buffer (250 mM Tris Hcl pH 9.0, 10 mM $MgCl_2$), 1.5 µl pNK72 (25 pmol/µl), 1 µl of sequencing grade Taq DNA polymerase (5U/µl)(in 50% glycerol, 100 mM Kcl, 20 mM Tris Hcl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20 and 0.5% NP40) and 6.5 µl DEPC treated water. After mixing, 4 µl reaction mixture was transferred to one set of the dd/d NTP tubes (T, C, G, A). Then a drop (about 15 µl) of PCR grade mineral oil was despensed into the tubes and were briefly centrifuged to collect the contents at the bottom of the tubes with oil layer on top of it. The sequencing tubes were loaded on to a heating block (Omnigene, Hybaid) and it was run as follows:

Temperature	Time	Cycle	Remarks
95°C	2 minutes	1	Denaturation
95°C 42°C 72°C	30 seconds 30 seconds 1 minute	60	Sequencing reaction

The sequencing reaction was stopped by adding 3µl of sequencing stop solution and the tubes were stored at -20°C till loading onto a sequencing gel.

Polyacrylamide Gel Electrophoresis of Cycle Sequencing product

Electrophoresis of cycle sequencing products was done using Cast Away Precast Sequencing Gels (Stratagene, Cat. No. 401090) and Cast Away Sequencing Device (Stratagene Cat. No. 401070).

Cast Away Precast Sequencing Gel was assembled into the sequencing device following manufacturer's instructions. Pre-electrophoresis was done for 30 minutes at a set wattage of 80 to warm the gel to more than 30°C. Power was turned off and the gel top was flushed with 1X TBE (running buffer) to ensure that the gel top is free of urea and a shark-tooth comb was inserted into the gel.

Cycle sequencing samples were heated upto 85°C for 2 minutes in a thermalcycler and were loaded (2µl/well) onto the gel following the order T,C,G,A. The gel was run at a set wattage of 80 for appropriate time (2.5h or 6h as required).

Fixing: After the electrophoresis was over the sequencing device was disassembled and gel alongwith plates was taken out, spacers and comb were removed and the glass plates were pried apart. The shorter glass plate alongwith the gel sticking to it was immersed (gel side up) in a tray containing 10% glacial acetic acid (Sigma, Cat. No. A-0808) in ultrapure water and agitated gently for 20 minutes. The gel was stored in the fixing solution for additional 3 hours without shaking.

Staining: Gel was stained using "Silver Sequencing ™ DNA staining reagents" (Promega, Cat. No. Q4132).Staining solution was prepared by dissolving 2g of Silver nitrate in 2 liter of ultrapure water and adding 3ml of 37% formaldehyde to the solution.The gel was lifted from the fixing tray and rinsed in ultrapure water for 3 times (2 minutes each) with gentle agitation. After transferring into the staining tray containing 2 liters of freshly prepared stain, the gel was agitated well for 30 minutes and left overnight in the stain. The fixing solution was saved to use as stop solution after developing the gel.

Developing: Developing solution was prepared by dissolving 60g of Sodium Carbonate (Na_2CO_3) in 2 liter of ultrapure water and chilled to 10°C. Immediately before use 3 ml of 37% formaldehyde and 400 µl of Sodium Thiosulphate (10mg/ml) was added. The developing solution was filled into 2 developer trays (1 liter each).

The gel was removed from the staining tray, rinsed in ultrapure water for about 5 seconds and immediately transferred to developing solution 1.The gel was gently agitated and when the tracks started appearing(2-3 min), the gel was transferred to the developer tray 2 and agitated till all bands became visible(2-3 min). The developing reaction was then stopped by adding 1 liter of fix/stop solution (10% acetic acid, saved after fixing) directly to the developer tray and agitating for 2 to 3 minutes. After rinsing the gel twice in ultrapure water for 2 minutes each, it was dried at 37°C and viewed on a light box to read the sequences.

RESULTS

RESULTS

All the 40 field isolates showed characteristic CPE in BHK21 clone 13 cell line within 18 to 24 hours post infection. The infectivity titres are given in Table 4. All the virus isolates were of FMDV serotype Asia 1 in sandwich ELISA done using anti-146S guinea pig serum.

ANTIGENIC ANALYSIS OF FIELD ISOLATES

Reactivity of isolates with polyclonal sera

Most of the field isolates reacted well with the Asia 1 anti-146S guinea pig serum in indirect sandwich ELISA (Table 4).Of the 40 isolates tested, only 4 isolates (IND /91, IND 17/91, IND 17/93 and IND 53/93) showed 'r' values in the range of 0.20 to 0.39 and rest of the isolates had 'r' values above 0.40. The relatively low 'r' values (0.22 to 0.36) of these 4 isolates tallied with their 'r' values (0.43 to 0.50) in 2D-MNT.

Two dimentional micro-neutralization test (2D-MNT) was performed using bovine vaccinate serum. The infectivity titre (Log_{10} $TCID_{50}$/ml) of the isolates varied from 5.5 to 7.5 (Table 4). The results of neutralization test are given in Table 4. All the field isolates gave 'r' value of > 0.40, indicating their close relationship with the vaccine virus..

Reactivity of Asia 1 field isolates with monoclonal antibodies (Mab profiling)

All the field isolates used in the study were subjected to antigenic profiling using a panel of 10 Mabs to type Asia 1 vaccive virus.Table 5. shows the level of relationship between field isolates and vaccine strain virus expressed as percent homologous reaction. Percentage reactivity of each Mab against the field isolates in relation to their reactivity with the vaccine virus strain (homologous) is given in Table 6. Based on reactivity pattern the isolates could be clustured into 9 groups (Fig.4).

Eight isolates viz. IND 22/88, IND 120/88, IND 267/88, IND 132/90, IND 10/91, IND 293/94, IND 33/96 and IND 43/96 showed reaction of homology (73-109%) with all the Mabs (group 1).

Table -4: Antigenic relationship (r value) of the field isolates in sandwich ELISA and Micro neutralization test.

Sl.No.	Isolate No.	Virus titre*	Polyclonal 'r' value	
			Sandwich ELISA	Micro neutralization
1.	IND 75/86	6.5	0.53	1.00
2.	IND 46/87	6.5	0.93	1.00
3.	IND 22/88	6.0	0.97	1.00
4.	IND 120/88	6.0	0.72	1.00
	IND 155/88	6.5	0.64	1.00
	IND 267/88	7.0	0.72	1.00
7.	IND 19/89	7.0	0.57	1.00
8.	IND 21/89	7.0	0.65	1.00
9.	IND 45/89	6.5	0.59	0.75
10.	IND 132/90	7.0	0.69	1.00
11.	IND 10/91	7.0	0.84	0.50
12.	IND 13/91	7.0	0.35	0.50
13.	IND 17/91	6.0	0.32	0.90
14.	IND 17/93	6.5	0.22	0.43
15.	IND 53/93	6.0	0.36	0.46
16.	IND 293/94	6.5	0.54	1.00
17.	IND 316/94	5.5	0.63	1.00
18.	IND 1/95	6.0	0.54	1.00
19.	IND 4/95	6.5	0.54	0.50
20.	IND 6/95	7.0	0.66	0.83
21.	IND 14/95	7.5	0.54	1.00
22.	IND 15/95	6.0	0.52	1.00

23.	IND 26/95	6.0	0.48	1.00
24.	IND 29/95	5.5	0.47	1.00
25.	IND 40/95	6.0	0.65	1.00
26.	IND 50/95	6.0	0.52	0.75
27.	IND 57/95	6.5	0.71	1.00
28.	IND 33/96	7.0	0.94	0.50
29.	IND 43/96	7.0	0.86	1.00
30.	IND 70/96	7.0	0.66	1.00
31.	IND 71/96	7.0	0.69	1.00
32.	IND 72/96	6.5	0.78	1.00
33.	IND 73/96	6.5	0.72	1.00
34.	IND 80/96	6.0	0.64	0.83
35.	IND 81/96	7.0	0.63	1.00
36.	IND 82/96	6.5	0.65	0.83
37.	IND 89/96	7.0	0.64	0.75
38.	IND 172/96	5.5	0.51	1.00
39.	IND 173/96	5.5	0.53	1.00
40.	IND 26/97	6.5	0.48	1.00

*Virus titre expressed as log 10 $TCID_{50}$ /ml

The isolate IND 155/88 showed reaction of homology (72-111%) with eight Mabs and no affinity (0%) for the Mabs 72 and 82 (group 2).

The isolate IND 46/87 showed reaction of homology (79-91%) with seven Mabs and reduced reactivity (67-69%) for Mabs 63, 72 and 82 (group 3).

The isolate IND 45/89 showed reaction of homology (71-92%) with Mabs B_3, 2A, 40, 34 and 81, reduced reactivity (22-61%) for Mabs 1A , 24 and 63 and no reaction (8%) with Mabs 72 and 82 (group 4).

Seven of the isolates viz. IND 29/95, IND 57/95, IND 6/95, IND 50./95, IND 71/96 ,IND26/95 and IND 316/94 showed reaction of homology with any three Mabs of the panel. Five of the isolates viz. IND 6/95, IND 50/95, IND 71/96, IND 26/95 and IND 316/94 showed reaction of homology (71-94%) with Mabs 81, 72 and 82. Among these five isolates, IND 6/95 showed reduced reaction (31-61%) with seven of the Mabs and isolates IND 50/95, IND 71/96 and IND 26/95 showed reduced reaction (21-65%) for rest seven of the Mabs except for Mab 63 with shich no reaction (0%) is observed. The isolate IND 316/94 showed reduced reaction (33-47%) with Mabs B_3, 24, 2A, 40 and 34 and no affinity (11-15%) for Mabs 1A and 63. Isolates IND 29/95 and IND 57/95 of this group showed reaction of homology (78-92%) with Mabs B_3, 81 and 82, reduced reaction(35-68%) with rest of the Mabs except for Mab 63 with which no reaction(0%) is noticed (group 5).

Six of the isolates showed reaction of homology with any two of the Mabs. The isolate IND 4/95 showed reaction of homology (75-76%) with Mabs 81 and 72 and reduced reaction (24-62%) with rest of the mabs. The isolates IND 15/95 and IND 70/96 showed reaction of homology (73-991%) with Mabs 81 and 82, and reduced reaction (22-69%) with all other Mabs except for Mab 63 with which they showed no reaction (0%). The isolates IND 73/96 and IND 82/96 showed reaction of homology (70-93%) with Mabs B_3 and 81 and reduced reaction (24-68%) with rest of the Mabs except for Mab 63 with which no reaction (0%) is observed. The isolate IND 89/96 showed reaction of homology (92-116%) with Mabs 63 and 81 and reduced reactivity (25-63%) with all other Mabs except Mab 1A with which no reaction (19%) is observed (group 6).

Seven of the isolates viz. IND 14/95, IND 75/86, IND 1/95, IND 21/89, IND

80/96, IND 81/96 and IND 19/89 showed reaction of homology (71-99%) with Mab 81 except isolate IND 14/95 which showed reaction of homology (92%) with Mab 82 and reduced reaction (25-69%) with all other Mabs. The isolate IND 1/95 showed reduced reaction (20-67%) with rest of the mabs except for Mabs 1A and 63 with which no reaction (0-17%) is observed. The isolates IND 75/86 and IND 21/89 showed reduced reaction (20-67%) with Mabs B_3, 1A, 24, 2A, 40 and 34 and no reaction (0%) with Mabs 63, 72 and 82. The isolates IND 80/96 and IND 81/96 showed reduced reaction (23-47%) with Mabs B_3, 2A, 40, 34, 72 and 82 and no reaction (7-15%) with Mabs 1A, 24 and 63. The isolate IND 19/89 showed reduced reaction (20-27%) with Mabs B_3, 24, 2A, 40 and 34 and no reaction (3-15%) with Mabs 1A, 63, 72 and 82 (group 7).

Seven of the isolates viz. IND 72/96, IND 40/95, IND 13/91, IND 17/91, IND 17/93, IND 53/93 and IND 26/97 did not show reaction of homology with any of the Mabs. Isolate IND 72/96 showed reduced reaction (30-65%) with all the Mabs except for Mabs 1A and 63 with which it showed no reaction (0-19%). The isolate IND 40/95 showed reduced reaction (26-59%) with Mabs B3, 81, 72 and 82 and no reaction (0-17%) with rest of the Mabs. The isolates IND 13/91 and IND 17/91 showed reduced reaction (50-57%) for Mab 63 and no reaction (0-17%) with all other Mabs. The isolates IND 17/93 and IND 53/93 showed reduced reaction (24-66%) for Mab 81 and no reaction (0-7%) with all other Mabs. Isolate IND 26/97 showed reduced reaction (40-45%) with Mabs 72 and 82 and no reaction (0%) with rest of the eight Mabs (group 8).

The remaining two isolates viz. IND 172/96 and IND 173/96 did not show either reaction of homology or reduced reaction with any of the Mabs (group 9).

Table -5: Percentage reactivity values of the Asia 1 virus isolates with the Mab panel and the 'r' values of the isolates obtained in sandwich ELISA using anti-146S guinea / pig serum.

Sl. No.	Isolate number	Monoclonal antibodies										'r'* value
		B3	1A	24	2A	40	63	34	81	72	82	
1.	IND 75/86	67	31	39	53	67	0	60	95	0	0	0.53
2.	IND 46/87	86	79	86	85	91	68	84	98	69	67	0.93
3.	IND 22/88	99	95	93	98	96	73	96	97	90	91	0.97
4.	IND 120/88	100	92	91	93	96	86	93	100	83	88	0.72
5.	IND 155/88	104	72	78	84	75	109	79	111	0	0	0.64
6.	IND 267/88	108	109	109	107	105	94	108	95	102	102	0.72
7.	IND 19/89	27	13	20	21	21	11	21	92	03	15	0.57
8.	IND 21/89	56	20	23	34	49	0	43	93	0	0	0.65
9.	IND 45/89	90	59	61	72	73	22	71	92	8	8	0.59
10	IND 132/90	101	93	96	94	95	75	104	98	82	85	0.69
11	IND 10/91	83	73	77	77	77	100	77	91	74	75	0.84
12	IND 13/91	0	12	12	10	13	50	0	0	15	9	0.35
13	IND17/91	5	15	12	10	17	57	15	0	12	5	0.32
14	IND 17/93	7	0	0	0	0	0	0	66	0	0	0.22
15	IND 53/93	0	0	0	0	0	0	0	24	0	0	0.36
16	IND 293/94	85	74	73	75	82	79	84	80	75	76	0.54
17	IND 316/94	47	15	33	34	35	11	35	84	71	88	0.63
18	IND 1/95	57	17	20	32	31	0	38	74	62	67	0.54
19	IND 4/95	62	24	30	43	54	40	52	76	58	75	0.54
20	IND 6/95	61	24	31	40	51	52	59	87	73	90	0.66
21	IND 14/95	61	25	32	41	54	44	56	47	69	92	0.54

22	IND 15/95	64	26	40	46	69	0	55	91	57	73	0.52
23	IND 26/95	62	21	25	37	35	0	42	80	70	80	0.48
24	IND 29/95	92	39	51	65	68	0	67	79	66	89	0.47
25	IND 40/95	26	6	10	16	17	0	17	59	48	54	0.65
26	IND 50/95	48	22	25	37	57	0	36	84	70	89	0.52
27	IND 57/95	78	35	46	66	63	0	63	85	67	92	0.71
28	IND 33/96	94	90	86	90	88	103	91	90	96	98	0.94
29	IND 43/96	100	93	92	93	92	82	90	88	92	92	0.86
30	IND 70/96	67	22	27	42	42	0	52	90	67	75	0.66
31	IND 71/96	65	21	30	40	38	0	54	94	76	88	0.69
32	IND 72/96	58	19	30	35	40	0	39	62	59	65	0.79
33	IND 73/96	70	24	33	43	44	0	53	93	61	68	0.72
34	IND 80/96	40	14	15	24	30	7	34	71	38	46	0.64
35	IND 81/96	32	11	12	23	24	12	29	72	40	47	0.63
36	IND 82/96	72	29	39	48	60	0	63	86	42	54	0.65
37	IND 89/96	63	19	25	36	51	116	51	92	46	56	0.64
38	IND 172/96	0	0	0	0	0	0	0	0	9	12	0.51
39	IND 173/96	0	0	0	0	0	0	0	0	10	12	0.53
40	IND 26/97	0	0	0	0	0	0	0	0	40	45	0.48

* Obtained with anti-146S guinea pig serum.

Fig.4: Grouping of Asia1 field isolates using Mabs (Mab Profiling)

Sl. No.	Isolate number	Monoclonal antibodies										Group
		B3	1A	24	2A	40	63	34	81	72	82	
1.	IND22/88	●	●	●	●	●	●	●	●	●	●	1
2.	IND 120/88	●	●	●	●	●	●	●	●	●	●	
3.	IND 267/88	●	●	●	●	●	●	●	●	●	●	
4.	IND 132/90	●	●	●	●	●	●	●	●	●	●	
5.	IND 10/91	●	●	●	●	●	●	●	●	●	●	
6.	IND 293/94	●	●	●	●	●	●	●	●	●	●	
7.	IND 33/96	●	●	●	●	●	●	●	●	●	●	
8.	IND 43/96	●	●	●	●	●	●	●	●	●	●	
9.	IND 155/88	●	●	●	●	●	●	●	●	○	○	2
10	IND 46/87	●	●	●	●	●	◆	●	●	◆	◆	3
11	IND 45/89	●	◆	◆	●	●	▼	●	●	○	○	4
12	IND 29/95	●	▼	◆	◆	◆	○	◆	●	◆	●	5
13	IND57/95	●	▼	◆	◆	◆	○	◆	●	◆	●	
14	IND 6/95	◆	▼	▼	▼	◆	◆	◆	●	●	●	
15	IND 50/95	◆	▼	▼	▼	◆	○	▼	●	●	●	
16	IND 71/96	◆	▼	▼	▼	▼	○	◆	●	●	●	
17	IND 26/95	◆	▼	▼	▼	▼	○	▼	●	●	●	
18	IND 316/94	◆	○	▼	▼	▼	○	▼	●	●	●	
19	IND 4/95	◆	▼	▼	▼	◆	▼	◆	●	◆	●	6
20	IND 15/95	◆	▼	▼	◆	◆	○	◆	●	◆	●	
21	IND 70/96	◆	▼	▼	▼	▼	○	◆	●	◆	●	
22	IND 73/96	●	▼	▼	▼	▼	○	◆	●	◆	◆	
23	IND 82/96	●	▼	▼	◆	◆	○	◆	●	▼	◆	

24	IND 89/96	◆	○	▼	▼	◆	●	◆	●	◆	◆	6
25	IND 14/95	◆	▼	▼	▼	◆	▼	◆	◆	◆	●	7
26	IND 1/95	◆	○	▼	▼	▼	○	▼	●	◆	◆	
27	IND 75/86	◆	▼	▼	◆	◆	○	◆	●	○	○	
28	IND 21/89	◆	▼	▼	▼	◆	○	▼	●	○	○	
29	IND 80/96	▼	○	○	▼	▼	○	▼	●	▼	◆	
30	IND 81/96	▼	○	○	▼	▼	○	▼	●	▼	◆	
31	IND 19/89	▼	○	▼	▼	▼	○	▼	●	○	○	
32	IND 72/96	◆	○	▼	▼	▼	○	▼	◆	◆	◆	8
33	IND 40/95	▼	○	○	○	○	○	○	◆	◆	◆	
34	IND 13/91	○	○	○	○	○	◆	○	○	○	○	
35	IND 17/91	○	○	○	○	○	◆	○	○	○	○	
36	IND 17/93	○	○	○	○	○	○	○	◆	○	○	
37	IND 53/93	○	○	○	○	○	○	○	▼	○	○	
38	IND 26/97	○	○	○	○	○	○	○	○	▼	▼	
39	IND 172/96	○	○	○	○	○	○	○	○	○	○	9
40	IND 173/96	○	○	○	○	○	○	○	○	○	○	

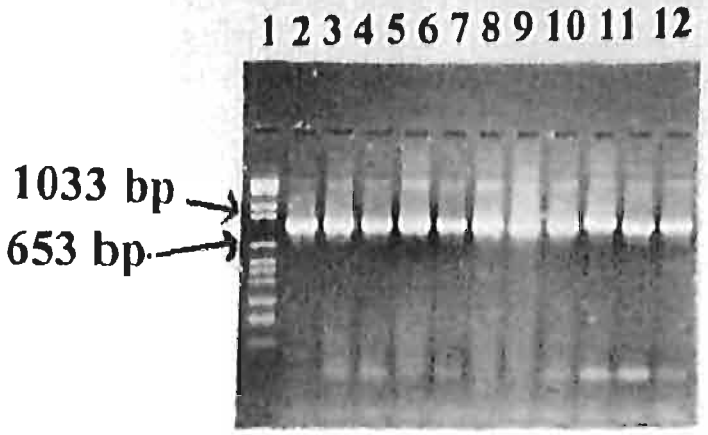
● 70% and above

◆ 46-69%

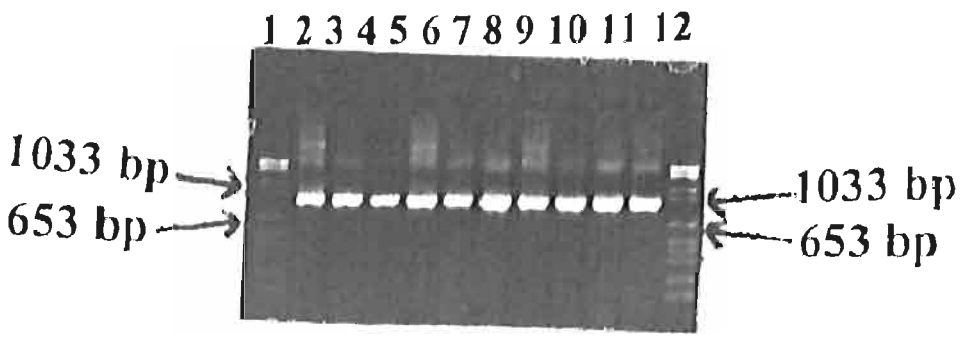
▼ 20-45%

○ 19% and below

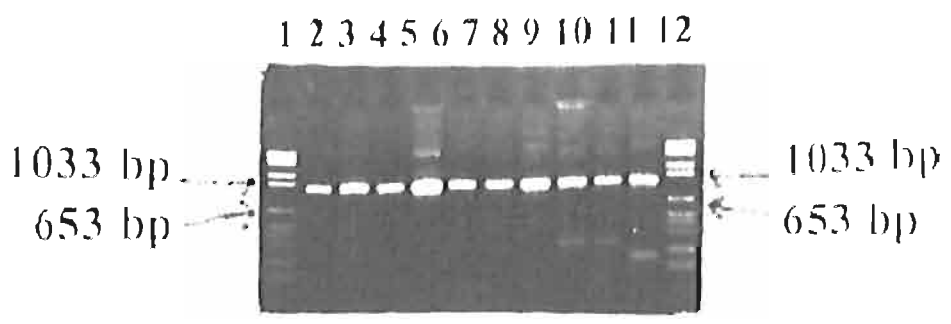
Fig. 5: Photographs showing Agarose gel electrophoresis of RT-PCR products. RT-PCR products of Asia 1 isolates amplified using primers NK_{61} and AS1-$1C_{505}$, are of 908-914 bp length and lie between 1033 and 653 bp of Marker VI.


1 2 3 4 5 6 7 8 9 10 11 12
1033 bp
653 bp

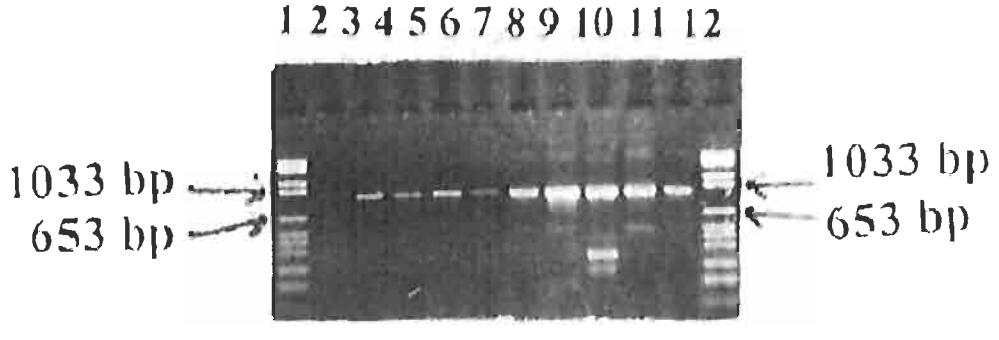
A


1 2 3 4 5 6 7 8 9 10 11 12
1033 bp
653 bp
1033 bp
653 bp

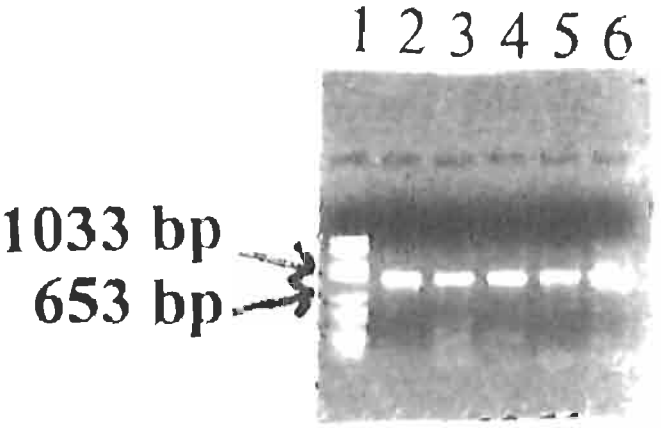
B


1 2 3 4 5 6 7 8 9 10 11 12
1033 bp
653 bp
1033 bp
653 bp

C


1 2 3 4 5 6 7 8 9 10 11 12
1033 bp
653 bp
1033 bp
653 bp

D


1 2 3 4 5 6
1033 bp
653 bp

E

(Fig.2). Seqeuncing reaction samples were electrophoresed on 6% polyacrylamide gels and stained by silver staining method. The gels were visualised on a light box and sequences read and aligned. Aligned nucleotide sequences at the 3' end of 1D genomic region of all field isolates and vaccine virus are given in Fig 6. When the sequences of isolates were compared with the corresponding sequences of vaccine virus, substantial divergence among isolates was found. All observed mutations were base substitutions. Total nucleotide substitutions per isolate (Table 7), when compared with vaccine virus, ranged from 1 to 22 (0.63 to 13.84%) which were scattered throughout the length (at 42 different positions) sequenced, however, certain regions were highly conserved. Most of the substitutions (29 out of 42) noticed were in third piositions of the triplet codon, than first (7 out of 42) and 2nd (6 out of 42) positions.

Of the total nucleotide substitutions per isolate, non-synonymous mutations (which lead to change in amino acid) were relatively low, when compared to synonymous mutations, which ranged from 14.29% to 45.45%, but for certain isolates like IND 22/88, IND 120/88, IND 267/88 IND 19/89, IND 132/90, IND 33/96 and IND 43/96 they ranged from 50 to 100% since there were only few substitutions (1 to 4) and many of them were non-synonymous .

Deduced amino acid sequences of the isolates and vaccine virus are given in Fig. 7. A length of 53 amino acid (156-208) residues in VP1 protein was deduced for all the viruses sequenced. The sequences were aligned and a consensus sequence was derived. When compared with the vaccine virus sequence, isolates showed substitutions at 13 different positions. All the isolates showed substitution from threonine to asparagine at residue 161, and except four, all the other isolates showed change from histidine to glutamine at residue 195. Ten isolates showed substitutions at the amino acid residue 168 from aspartic acid to glutamaic acid. Nine of the isolates showed amino acid substitution from glutamic acid to leucine at the residue 201. Only two isolates (IND 21/89 and IND 45/89) showed substitution from alanine to glutamine at the residue 204. Other substitutions observed were ; IND 155/88 at residue 160-from phenyl alanine to threonine, IND 70/96 at 173- from lysine to proline, IND 50/95 at 175- from isoleucine to valine, IND 72/96 at 177-

Fig. 6: Nucleotide sequences of part of 1D (VP1 coding) gene of FMDV Asia 1 isolates and vaccine virus. Nucleotides identical to the consensus (majority) sequence are shown by periods. Pak 1/54-previously published sequence (Ansell et al., 1994). Nucleotide numbered 1 in the figure corresponds to 466th nucleotide of the gene.

```
C T G C C C A C C T C T T T C A A C T A C G G C G C T G T A A A G G C T G A C A  Majority
          10        20        30        40
1  . . . . . C . . . . . . . . . . . . . . . . . . G . . . . . . . . A .  10-91-1.SEQ
1  . . . . . C . . . . . . . . . . T . . . . . G . . . . . . . . . . .  120-88-1.SEQ
1  . . . . . C . . . . . . . . . . T . . . . . G . . . . . . . . . . .  132-90-1.SEQ
1  . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . A .  13-91-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  14-95-1.SEQ
1  . . . . . C . A . . . . . . . . . T . . . . . G . . . . . . . . . A .  155-88-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  15-95-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  172-96-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  173-96-1.SEQ
1  . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . A .  17-91-1.SEQ
1  . . . . . C . . . . C . . . . . . . . . . . . . . . . . . . . . . . . A .  17-93-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1-95-1.SEQ
1  . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A .  19-89-1.SEQ
1  . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A .  21-89-1.SEQ
1  . . . . . C . . . . . . . . . . T . . . . . G . . . . . . . . . . .  22-88-1.SEQ
1  . . . . . C . . . . . . . . . . T . . . . . G . . . . . . . . . . .  267-88-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  26-95-1.SEQ
1  . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  26-97-1.SEQ
1  . . . . . C . . . . . . . . . . T . . . . . G . . . . . . . . . . .  293-94-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  29-95-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  316-94-1.SEQ
1  . . . . . C . . . . . . . . . . T . . . . . G . . . . . . . . . . .  33-96-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  40-95-1.SEQ
1  . . . . . C . . . . . . . . . . T . . . . . G . . . . . . . . . . .  43-96-1.SEQ
1  . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . A .  45-89-1.SEQ
1  . . . . . C . . . . . . . . . . T . . . . . . . . . . . . . . . . .  46-87-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  4-95-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  50-95-1.SEQ
1  . . . . . C . . . . . T . . . . . . . . . . . . . . . . . . . . . T .  53-93-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  57-95-1.SEQ
1  . . . . . C . . . . C . . . . . T . . . . G . . . . . . . . . . .  63-72-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  6-95-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . G .  71-96-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  72-96-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  73-96-1.SEQ
1  . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . A .  75-86-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  80-96-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  81-96-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  82-96-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  89-96-1.SEQ
1  . . . . . C . . T . . . . . . . T . . A . . G . . . . . . . . G .  PAK1-5-1.SEQ
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  70-96-1.SEQ
```

```
                C C A T C A C T G A G C T T T T G A T C C G C A T G A A A C G C G C G G A G A C  Majority
                                  50                  60                  70                  80
41  . . . . T . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  10-91-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . G . . T . . . . . . . . . . . . . .  120-88-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . G . . T . . . . . . . . . . . . . .  132-90-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  13-91-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  14-95-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  155-88-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . .  15-95-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  172-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  173-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  17-91-1.SEQ
41  . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  17-93-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1-95-1.SEQ
41  . . . . T . . . . . . . . C . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . .  19-89-1.SEQ
41  . . . . T . . . . . . . . C . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . .  21-89-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . G . . T . . . . . . . . . . . . . .  22-88-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . G . . T . . . . . . . . . . . . . .  267-88-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  26-95-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  26-97-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . C . . T . . . . . A . .  293-94-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . C G . . . . . . . . . . . . . . . . . . . . . . .  29-95-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  316-94-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . C G . . T . . . . . . . . . . . . .  33-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  40-95-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . G . . T . . . . . . . . . . . . . .  43-96-1.SEQ
41  . . . . T . . . . . . . . C . . . . . A . . . . . . . . . . . . . . . . . . . . . . . . . .  45-89-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  46-87-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  4-95-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . . . . . . .  50-95-1.SEQ
41  . . . . . . . . . . . . . . C C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  53-93-1.SEQ
41  . . . . . . . G . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . .  57-95-1.SEQ
41  . . . . . . . G . . . . . G . . . . . . . . . . . . . . G . . T . . . . . . . . . . . . . .  63-72-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  6-95-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  71-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . .  72-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  73-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  75-86-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . .  80-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . .  81-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . .  82-96-1.SEQ
41  . . . . . . . . . . . . . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . .  89-96-1.SEQ
41  A . . . . . G . . . A . G . . A . . . . . . . A . . G . . T . . . . . A . .  PAK1-5-1.SEQ
41  . . . . . . . . . . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  70-96-1.SEQ
```

```
                A T A C T G C C C C A G G C C T T T G C T A G C T C T T G A C A C C A C T C A G Majority
                                   |                   |                   |                   |
                                  90                  100                 110                 120
81  . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 10-91-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . C 120-88-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . C 132-90-1.SEQ
81  . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 13-91-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 14-95-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 155-88-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 15-95-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 172-96-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 173-96-1.SEQ
81  . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 17-91-1.SEQ
81  . . . . . . . . . T . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . . . . 17-93-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1-95-1.SEQ
81  . . . T . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 19-89-1.SEQ
81  . . . T . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 21-89-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . C 22-88-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . C 267-88-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 26-95-1.SEQ
81  . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . 26-97-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . C 293-94-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 29-95-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . . 316-94-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . C 33-96-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 40-95-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . 43-96-1.SEQ
81  . . . T . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 45-89-1.SEQ
81  . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . 46-87-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 4-95-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 50-95-1.SEQ
81  G . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 53-93-1.SEQ
81  . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 57-95-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . C 63-72-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 6-95-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 71-96-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 72-96-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 73-96-1.SEQ
81  . . . . . . . . . T . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . . 75-86-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 80-96-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 81-96-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 82-96-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 89-96-1.SEQ
81  . . . . . . . . . . . . . . . . . C C . . . . G . . . . . . . . . . . . . . . . . . A PAK1-5-1.SEQ
81  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 70-96-1.SEQ
```

```
                GACCGCCGTAAACAGGAGATCATTGCACCTGAGAAGCAG   Majority
                        130       140       150     159
121 ...............................................   10-91-1.SEQ
121 ..........G.....................................A   120-88-1.SEQ
121 ..........G.....................................A   132-90-1.SEQ
121 ................A....T..............A...........   13-91-1.SEQ
121 ........C.......................A..............   14-95-1.SEQ
121 ................A.....................A.....A   155-88-1.SEQ
121 ...............................................A   15-95-1.SEQ
121 ...........................................A....   172-96-1.SEQ
121 ...............................................   173-96-1.SEQ
121 ................A....T..............A...........   17-91-1.SEQ
121 ..........G..A..A..T..............................   17-93-1.SEQ
121 ...............................................A   1-95-1.SEQ
121 ...............................................A   19-89-1.SEQ
121 ................A....T...CAG.......A...........   21-89-1.SEQ
121 ..........G.................A.....................   22-88-1.SEQ
121 ..........G.....................................A   267-88-1.SEQ
121 ...............................................   26-95-1.SEQ
121 ...............................................   26-97-1.SEQ
121 ..........G.....................................A   293-94-1.SEQ
121 ...............................................   29-95-1.SEQ
121 ................A.....................A.....   316-94-1.SEQ
121 ..........G.....................................A   33-96-1.SEQ
121 ...........................................A....   40-95-1.SEQ
121 ..........G....................................   43-96-1.SEQ
121 ................A....T...CAG.......A...........   45-89-1.SEQ
121 ........C..G....................................   46-87-1.SEQ
121 ...............................................A   4-95-1.SEQ
121 ..........G.....................................A   50-95-1.SEQ
121 ...............................................A...   53-93-1.SEQ
121 ...........................................A....   57-95-1.SEQ
121 ..........G.....................................A   63-72-1.SEQ
121 ................A.....................A.....   6-95-1.SEQ
121 ..............A.....................A.....   71-96-1.SEQ
121 ...............................................A...   72-96-1.SEQ
121 ...............................................   73-96-1.SEQ
121 ................A....................   75-86-1.SEQ
121 ...........................................A....   80-96-1.SEQ
121 ...............................................   81-96-1.SEQ
121 ................A.....................A.....   82-96-1.SEQ
121 ...............................................   89-96-1.SEQ
121 ........C..G....................................   PAK1-5-1.SEQ
121 ...............................................   70-96-1.SEQ
```

Fig. 7: Deduced amino acid sequences of 3' end of 1D gene of FMDV Asia 1 isolates. - indicates same as vaccine virus.

	156				160									170										180									190									200								208
	L	P	T	S	F	T	Y	G	A	V	K	A	D	T	I	T	E	L	L	I	R	M	K	P	A	E	T	Y	C	P	R	P	L	L	A	L	D	T	T	H	D	R	R	K	Q	E	I	I	A	P	E	K	Q
IND 63/72																																									Q						L							
IND 75/86						N							E																												Q													
IND 46/87						N																																													T			
IND 22/88						N																																															
IND 120/88						N																																															
IND 155/88					T	N							E																												Q						L							
IND 267/88						N																																															
IND 19/89						N							E																												Q						L			Q				
IND 21/89						N							E																												Q						L			Q				
IND 45/89						N							E																																									
IND 132/90						N																																			Q													
IND 10/91						N							E																												Q						L							
IND 13/91						N							E																												Q						L							
IND 17/91						N							E																												Q													
IND 17/93						N							E																								I				Q													
IND 53/93						N							E																												Q													
IND 293/94						N																																															
IND 316/94						N																																			Q						L							
IND 1/95						N																																			Q													
IND 4/95						N																																			Q						L							
IND 6/95						N																																			Q													
IND 14/95						N																																			Q													
IND 15/95						N																																			Q													
IND 26/95						N																																			Q													
IND 29/95						N																																			Q													
IND 40/95						N																																			Q													
IND 50/95						N															V																				Q													
IND 57/95						N																																			Q													
IND 33/96						N																			T																	Q													
IND 43/96						N																																			Q													
IND 70/96						N																		P																	Q													
IND 71/96						N																																			Q													
IND 72/96						N																									I										Q													
IND 73/96						N																																			Q													
IND 80/96						N																																			Q													
IND 81/96						N																																			Q													
IND 82/96						N																													S						Q								L					
IND 89/96						N																																			Q													
IND 172/96						N																																			Q													
IND 173/96						N																																			Q													
IND 26/97						N																																			Q													

from methionine to isoleucine, IND 33/96 at 178-from lysine to threonine , IND 82/96 at 185-from proline to serine, IND 17/93 at residue 191-from leucine to isoleucine and IND 22/88 at residue 205-from proline to threonine. Total number of amino acid substitutions per isolate varied from 1 to 5 (1.89 to 9.43%).

To establish relationships between isolates and vaccine virus a phylogenetic tree (Fig.8) was constructed using 159 nucleotides (coding from amino acid 156 to 208) at the 3'end of 1D gene, which is a standard method followed for studying genetic relatedness between FMDV isolates (Kitching and Knowles, 1993). For comparison, sequence of Asia 1 virus Pak 1/54 strain, widely used as vaccine virus in many countries (Ansell et al., 1994) was also included.

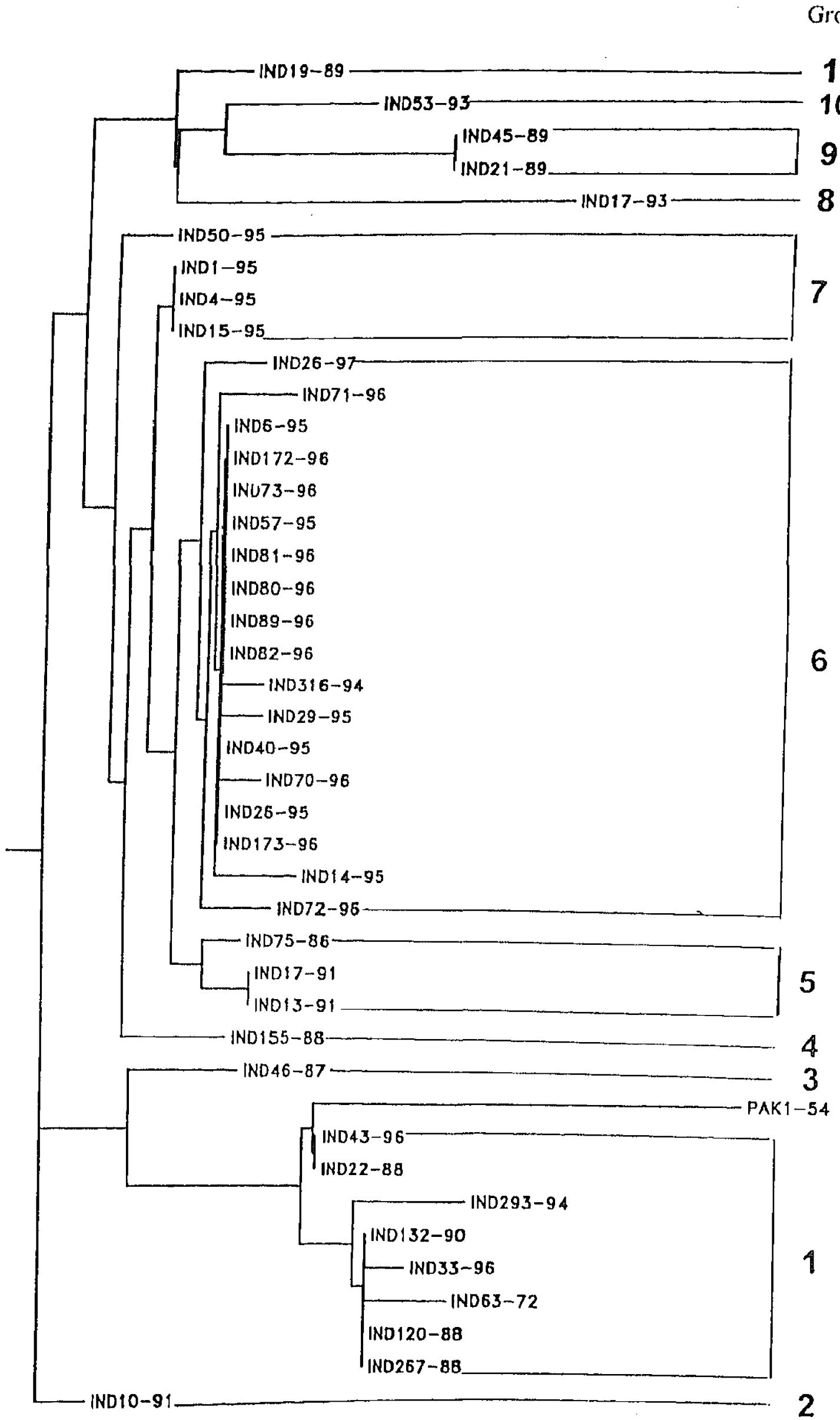
Nucleotide sequences were aligned with CLUSTAL using PC/Gene software. Phylogenetic analysis were carried out with the SEQBOOT, DNADIST, and FITCH algorithms in the PHYLIP version 3.41 package. The phylogenetic tree is constructed as per Felsenstein, J. (1993). The sequences were piled up using DNASTAR software and multiple data sets were generated using bootstrap resampling. The resampled samples were used in DNADIST to generate distances using kimura's 2-parameter method. Phylogenies were estimated by FITCH algorithm from distance matrix data under an "additive tree model" according to which the distances are expected to equal the sums of branch lengths between the isolates. Finally, a phylogentic tree was constructed (Fig .8).

Based on the divergence in the relationship between any two sequences, all the viruses could be clustered into 11 different groups (Fig.8). Within each group, divergence levels between the isolates were below 5% (Appendix -Fig.9).

Group 1: Comprised of 7 isolates collected between 1988-1996 from different regions of India (North eastern- Assam; IND 22/88, Eastern- West Bengal; IND293/94 and IND 33/96, Northern- Uttar Pradesh ; IND 132/90, Western- Maharastra; IND 120/88 and IND 43/96,Gujarat; IND267/88) and the vaccine virus. The divergence in the nucleotide sequence between each pair varied from 0 to 2.5%, with 1 to 3 nucleotide substitutions from vaccine virus.

Group 2: Consisted of only one isolate (IND 10/91) collected from West

Fig. 8: Phylogenetic tree constructed from partial 1D gene sequences of FMDV Asia 1 isolates (see Table 1 for details of the isolates).


Group
IND19-89
IND53-93
IND45-89
IND21-89
IND17-93
IND50-95
IND1-95
IND4-95
IND15-95
IND26-97
IND71-96
IND6-95
IND172-96
IND73-96
IND57-95
IND81-96
IND80-96
IND89-96
IND82-96
IND316-94
IND29-95
IND40-95
IND70-96
IND25-95
IND173-96
IND14-95
IND72-96
IND75-86
IND17-91
IND13-91
IND155-88
IND46-87
PAK1-54
IND43-96
IND22-88
IND293-94
IND132-90
IND33-96
IND63-72
IND120-88
IND267-88
IND10-91
11
10
9
8
7
6
5
4
3
1
2

Bengal in the year 1990, with 7.5% divergence and nucleotide substitutions at 13 positions when compared to vaccine virus.

Group 3 :The isolate IND 46/87 from Rajasthan, collected in the year 1986, formed the single isolate in group 3 with 5% divergence from vaccine virus and nucleotide changes at 7 positions.

Group 4: Similarly, a 1988 isolate (IND 155/88) from Maharashtra showed 7.5% divergence and 11 point mutations when compared to vaccine virus. This isolate showed 4.4 to 8.5% divergence from isolates of other groups.

Group 5: Two isolates (IND 13/91 and IND 17/91) from Maharashtra, isolated in 1990, clustered with an isolate (IND 75/86) from Assam (year of isolation-1986) with 3.6% divergence between them. These isolates showed 10.3 to 12.7% divergence from vaccine virus with point mutations at 15 to 18 positions.

Group 6: This group consisted of eighteen isolates comprising of ten isolates from Southern part of India (Karnataka, Andhra Pradesh, Tamil Nadu and Pondichery) viz. IND6/95, IND 80/96. IND 81/96, IND 14/95, IND 29/95,IND 57/95, IND 26/97, IND 71/96, IND 40 95 and IND 73/96, five isolates from Northern part (Uttar Pradesh, Hissar, Punjab and Himachal Pradesh) viz. IND 26/95, IND 82/96, IND 172/96, IND 173/96 and IND 89/96), two isolates from Western part (IND 316/94 and IND 72/96) and one isolate from Eastern part of India (IND 70/96) recovered between 1994 and 1997. These isolates showed varied level of inter-relationships among themselves ranging from 0 to 4.2% and showed 12 to 16 nucleotide changes, with >5% divergence from vaccine virus.

Group 7: The remaining three isolates from Southern part of India (IND 1/95 and IND 4/95 from Karnataka and IND 15/95 from Andhra Pradesh) , clustered with an isolate from Northern India (IND 50/95 from Uttar Pradesh) to form a group with 0 to 2.5% divergence between them. These isolates recovered in 1994 and 1995, showed 6.7 to 10.3% divergence level from vaccine virus with 11 to 12 nucleotide substitutions.

Group 8: This group consisted of only one isolate (IND 17/93) recovered from Hissar in the year 1993 which showed 5.7 to 10.7% divergence from all other isolates and 9.4% from vaccine virus. This isolate had 15 nuclotide substitutions compared to vaccine

virus.

Group 9: Two isolates (IND 21/89 and IND 45/89) recovered from Uttar Pradesh in the year 1989 showed similar changes at 22 positions when compared to vaccine virus.These isolates, did not differ (0%) among themselves, but showed a maximum divergence of 14.5% from vaccine virus and 6.3 to 14.5% divergence from all other isolates.

Group 10: The isolate IND 53/93 from Karnataka, collected in the year 1993, formed a group with 5 to 11.5% divergence from other isolates. This isolate showed nucleotide substitutions at 17 positions with 11.5% divergence from vaccine virus.

Group 11: A 1989 isolate (IND 19/89) from Uttar Pradesh, with 9.1% divergence from vaccine virus formed a separate group. This isolate showed nucleotide substitutions at 14 positions when compared to vaccine virus and showed varied level of divergence (3.1 to 9.9%) from other isolates.

The virus strain Pak 1/54 was very much divergent (8.8 to 19.4%) from all Indian isolates and also from vaccine virus (10.9%). It showed nucleotide substitutions at 15 and 22 positions when compared with vaccine virus sequence and consensus sequence respectively. No separate group number is designated for this virus strain.

Table 7: Nucleotide and amino acid changes of FMDV Asia 1 field isolates.

Sl. No.	Isolate number	Nucleotides sequenced		Nucleotide substitutions		Non-synonymous mutations		Amino acid substitutions	
		From -To	No.	No.	%	No.	%	No.	%
1.	IND 75/86	466-624	159	14	8.81	4	28.87	4	7.55
2.	IND 46/87	466-624	159	7	4.40	2	28.57	2	3.77
3.	IND 22/88	466-624	159	3	1.89	2	66.67	2	3.77
4.	IND 120/88	466-624	159	1	0.63	1	100.0	1	1.89
5.	IND155/88	466-624	159	11	6.92	5	45.45	5	9.43
6.	IND267/88	466-624	159	2	1.26	1	50.0	1	1.89
7.	IND19/89	466-624	159	14	8.81	3	21.43	3	5.66
8.	IND21/89	466-624	159	22	13.84	6	27.27	5	9.43
9.	IND45/89	466-624	159	22	13.84	6	27.27	5	9.43
10	IND132/90	466-624	159	1	0.63	1	100.0	1	1.89
11	IND10/91	466-624	159	14	8.81	3	21.43	3	5.66
12	IND13/91	466-624	159	18	11.32	4	22.22	4	7.55
13	IND17/91	466-624	159	18	11.32	4	22.22	4	7.55
	IND17/93	466-624	159	15	9.43	3	20.0	4	7.55
15	IND53/93	466-624	159	17	10.69	3	17.65	3	5.66
16	IND293/94	466-624	159	3	1.89	1	33.33	1	1.89
17	IND316/94	466-624	159	10	6.29	3	30.00	3	5.66
18	IND1/95	466-624	159	11	6.92	2	18.18	2	3.77
19	IND4/95	466-624	159	11	6.92	2	18.18	2	3.77
20	IND6/95	466-624	159	18	11.32	3	16.66	3	5.66
21	IND14/95	466-624	159	14	8.81	2	14.29	2	3.77
22	IND15/95	466-624	159	12	7.55	2	16.66	2	3.77

23	IND26/95	466-624	159	12	7.55	2	16.66	2	3.77
24	IND29/95	466-624	159	12	7.55	2	16.66	2	3.77
25	IND40/95	466-624	159	13	8.18	2	15.38	2	3.77
26	IND50/95	466-624	159	12	7.55	3	25.00	3	5.66
27	IND57/95	466-624	159	13	8.18	2	15.38	2	3.77
28	IND33/96	466-624	159	2	1.26	2	100.00	3	5.66
29	IND43/96	466-624	159	3	1.89	2	66.00	2	3.77
30	IND70/96	466-624	159	13	8.18	3	23.08	2	3.77
31	IND71/96	466-624	159	14	8.81	2	14.29	2	3.77
32	IND72/96	466-624	159	14	8.81	3	21.43	3	5.66
33	IND73/96	466-624	159	12	7.55	2	16.66	2	3.77
34	IND80/96	466-624	159	14	8.81	2	14.29	2	3.77
35	IND81/96	466-624	159	13	8.18	2	15.38	2	3.77
36	IND82/96	466-624	159	16	10.06	4	25.00	4	7.55
37	IND89/96	466-624	159	13	8.18	2	15.38	2	3.77
38	IND172/96	466-624	159	13	8.18	2	15.38	2	3.77
39	IND173/96	466-624	159	12	7.55	2	16.66	2	3.77
40	IND26/97	466-624	159	14	8.81	3	21.43	2	3.77

DISCUSSION

DISCUSSION

Foot-and- mouth disease is one of the economically most important diseases of cloven-hoofed animals such as cattle, buffaloes, sheep, goats and pigs. In India, FMD is endemic, and occurs throughout the country during all seasons. The country is a habitat for four of the seven serologically distinct serotypes i.e. O, A, C and Asia 1. Out of the four prevalent types, type Asia 1 comes next only to type O in the number of outbreaks per year. The plurality of FMDV serotypes and emergence of variant strains in the field poses a major problem in disease control despite the fact that good quality vaccines are availabe. A central need for defining a strategy and an action plan to control FMD is the thorough understanding of epidemiology of the disease. By examining the distribution of strains and tracing their movement within India, it will be possible to understand the natural history of FMD in the country which will facilitate formulation of better control programmes. As a small step in this direction, 40 FMDV Asia 1 field isolates recovered from different parts of the country between 1985 and 1997, were subjected to antigenic and genetic comparison studies.

Antigenic analysis of FMDV Asia 1 field isolates using polyclonal sera

All the isolates were revived and propagated in BHK 21 clone 13 cell line in which they produced characteristic CPE. The type specificity of the isolates were confirmed by sandwich ELISA to be Asia 1 using anti-146S guinea pig serum.

The prieiminary serological (antigenic) analysis of field isolates was carried out by sandwich ELISA and 2-D MNT using polyclonal sera to determine their relationship with the vaccine strain (IVRI vaccine strain IND 63/72). These tests are used as an alternative to cross protection trials in cattle (Ouldridge, 1987).

The set of criteria proposed by Samuel et al. (1990b) for interpretation of 'r' values obtained in seroslogical tests using polyclonal sera has been followed. The 'r' values under this system are proposed as guidelines rather than as absolute values and are as follows:

r = 0.00-0.19, indicates highly significant serological variation from the reference strain. r = 0.20-0.39. These values represent an area of concern in that they show a difference from the reference strain; but protection may be satisfactory if a sufficiently potent vaccine is used. r = 0.40-1.00. These values show that the strains are more closely related to the reference vaccine strain as measured by the particular test system used.

Unidirectional testing of all the field isolates by sandwich ELISA using anti-146S guinea-pig serum revealed that 36 out of 40 isolates had 'r' values of above 0.40 and only 4 isolates showed 'r' values in the range of 0.20-0.39. From the 'r' values it was evident that all the field isolates were related and there was not much antigenic variation from the vaccine virus. These results are in concurrence with the findings reported by Antony (1987);Shridhara (1990) and Mishra et al.(1995).

As neutralizing antibody titres correlate well with protection in the animal, the virus neutralization test is more widely used as the reference test system for vaccine virus selection and has been adopted since 1977 (Pereira, 1977). This test is not influenced by antigen-antibody reactions involving non-immunogenic antigen (Pay, 1985). In the present investigation, the field isolates were compared with the corresponding vaccine virus by 2-D MNT using bovine vaccinate serum and the results of this test revealed that all the 40 isolates had 'r' values of above 0.40 indicating their close relationship with vaccine virus. Although results of both tests point to the same conclusion, some differences between 'r' values of 2D-MNT and sandwich ELISA were observed. These may reflect differences of the antibody populations measured by the two assays.

Antigenic analysis of FMDV Asia 1 field isolates using monoclonal antibodies

Use of polyclonal sera, in different serological tests, for antigenic profiling of field isolates, has a major disadvantage. Such sera contain mixture of antibodies against different parts of the antigen and if a particular determinant in the testing antigen is either not present or lost, it is likely to go undetected as the majority of antibodies will still bind to the antigen (Pollock et al., 1984). Since monoclonal antibodies (Mabs) are produced by the clonal progeny of a single B lymphocyte, they contain identical population of antibodies with

single specificity and hence they can differentiate between isolates differing even slightly. This property of Mabs makes them useful as ideal reagents for the measurement and understanding of finer antigenic differences between FMD viruses in epidemiological studies (Crowther et al., 1990). It has also been observed that Mab-profiling by ELISA is a valuable tool for subtyping and characterization of FMDV isolates (Haas et al., 1988) and it also provides more information on the identity, specificity and possible origin of viruses (Hamblin et al., 1985).

Samuel et al (1991) evaluated a sandwich ELISA using Mabs for the differentiation of strains of FMD viruses and proposed a simplified approach for comparing the field strains. They designated four ranges of percentage reactions based on the reactivities of Mabs with different viruses. They are as follows:

(i) below 20 reflects no reaction

(ii) 20-45 and 46-75 reflect reduced affinity

(iii) 76-100 reflects equal reactions to the homologous.

These ranges of values were followed with little modifications to cluster the isolates in the study (Table 5, Fig. 4).

In the present study a panel of 10 neutralizing Mabs were used for antigenic profiling of FMDV Asia 1 field isolates. Mabs were characterised earlier (Sanyal et al., 1997). Each of the Mab recognizes an area on the virus surface which is either partially or completely sensitive to trypsin digestion and all the Mabs also recognize conformation-dependent antigenic sites on the virus surface. The reactivity pattern of the Mab panel with the field isolates was found to vary between the field isolates and enabled them to be clustered into 9 different groups.

Eight isolates of group-1 viz. IND 22/88, IND 120/88, IND 267/88, IND 132/90, IND 10/91, IND 293/94, IND 33/96 and IND 43/96 showed reaction of homology (70% and above) for all the Mabs.

The isolate IND 155/88 fall into group-2, which retained homologous affinity with eight Mabs of the panel and showed no reaction with Mabs 72 and 82.

Reactivity pattern of group-3 isolate IND 46/87 was almost identical for seven Mabs of the panel with which it showed homologous reaction and showed reduced reaction with Mabs 63, 72 and 82.

The isolate IND 45/89 of group-4 retained homologous affinity for Mabs B_3, 2A, 40, 34 and 81, reduced affinity for Mabs 1A, 24 and 63 and showed no reaction with Mabs 72 and 82.

Seven isolates of group-5 viz IND 29/95, IND 57/45, IND 6/95, IND 50/95, IND 71/96, IND 26/95 and IND 316/94 retained homologous affinity for any three Mabs of the panel while their reactivity with other Mabs varied. Isolates IND 29/95 and IND 57/95 showed reaction of homology with Mabs B3, 81 and 82, reduced reaction with Mabs 24, 2A, 40, 34 and 72 and no affinity for Mab 63. The isolates IND 6/95, IND 50/95, IND 71/96, IND 26/95 and IND 316/94 showed reaction of homology with Mabs 81, 72 and 82 and except IND 6/95 all showed reduced reaction with Mabs B_3, 1A, 24, 2A, 40 and 34 and no affinity for Mab 63, while IND 6/95 showed reduced reaction with remaining seven Mabs.

Six isolates viz IND 4/95, IND 15/95, IND 70/96, IND 73/96, IND 82/96 and IND89/96 formed group-6 in which they retained homologous affinity with any two Mabs of th panel. The isolates IND 4/95, IND 15/95 and IND 70/96 showed reaction of homology with mabs 81 and 82, reduced reaction for Mabs B_3, 1A, 24, 2A, 40 and 34 and with Mab 63, isolate IND 4/95 showed reduced reaction whereas isolates IND 15/95 and IND 70/96 showed no reaction. Isolates IND 73/96 and IND 82/96 showed homologous reaction with Mabs B_3 and 81, reduced reaction with Mabs 1A, 24, 2A, 40, 34, 72 and 82 and no reaction with Mab 63. The isolate IND 89/86 showed reaction of homology (92-116%) with Mabs 63 and 81, reduced reaction (25- 63%) with all other Mabs except Mab 1A with which no reaction (19%) was observed.

Except the isolate IND 14/95, all the seven isolates of group-7 showed homologous reaction with Mab 81 whereas isolate IND 14/95 showed homologous reaction with Mab 82. Isolate IND 14/95 showed reduced reaction with rest of the mabs while other isolates showed either reduced or no reaction with rest of the mabs. The isolate IND 1/95 showed reduced reaction with all other Mabs except for Mabs 1A and 63. The isolates IND

75/86 and IND 21/89 showed no affinity for Mabs 63, 72 and 82 and reduced reaction with Mabs B_3, 1A, 24, 2A, 40 and 34. The isolates IND 80/96 and IND 81/96 showed reduced reaction with Mabs B_3, 2A, 40, 34, 72 and 82 and no reaction with Mabs 1A, 24 and 63. The isolate IND 19/89 showed reduced reaction with Mabs B_3, 24, 2A, 40 and 34 and no affinity for other remaining four Mabs.

A total of seven isolates viz. IND 72/96, IND 40/95, IND 13/91, IND 17/91, IND 17/93, IND 53/93 and IND 26/97 of group-8 did not show reaction of homology with any of the Mabs and showed either reduced or no reaction for all the Mabs. Except for Mabs 1A and 63, the isolate 72/96 showed reduced reaction with all the mabs and isolate IND 40/95 showed reduced reaction with Mabs B_3, 81,72 and 82 and no reaction with other mabs. The isolates IND 13/91 and IND 17/91 showed no reaction with all the Mabs except for 63 with which reduced reaction was observed. The isolates IND 53/93 and IND 17/93 showed reduced reaction with Mab 81 and no reaction with all other Mabs. The isolate IND 26/97 showed reduced reaction with Mabs 72 and 82 and no reaction with other 8 Mabs of the panel.

Two isolates viz. IND 172/96 and IND 173/96 of group-9 showed neither reaction of homology nor reduced reaction with any Mabs of the panel.

Results of Mab-profiling showed that majority of the FMDV Asia 1 field isolates of India were homologous to the vaccine virus, though some of them showed either reduced or no reactivity at some, or, all Mab binding sites. Reduction in reactivity to Mabs may result from amino acid substitutions at Mab binding sites and in some cases, single amino acid substitutions have been found to greately reduce the Mab reactivity (Mateu et al., 1987a). It has been shown that the fixation of amino acid substitutions at epitopes involved in neutralization of FMDV is the basis for the extensive antigenic variation of the virus (Domingo et al., 1990). Mateu et al. (1994) have showed that within a serotype of FMDV, antigenically highly divergent viruses can arise in the field by very limited sequence variation at exposed key residues of each of several antigenic sites. Similar findings of extensive antigenic heterogeneity among Indian FMDV Asia 1 isolates have been reported: Butchaiah et al. (1992) have characterized seven Asia 1 isolates using a panel of 26 Mabs

and shown that viruses isolated even a few months apart, from different regions of India, exhibited extensive variation particularly in conformation independent neutralization epitopes. Prabhudas et al. (1993) have characterized 17 Indian isolates using two Mabs against vaccine virus, by neutralization assay, demonstrating the occurance of isolates with low, medium and high reactivity with Mabs and some isolates even resisted neutralization. Sanyal (1995) noticed the high variantion among 47 Indian FMD Asia 1 isolates, in relation to each Mab-binding site, using the same panel of Mabs used in this study. Mateu et al. (1987a) have observed that epidemiologically related FMD virus isolates differ in at least one epitope critical for neutralization of the virus. It may be noted that in the Mab profiling results, no correlation could be found between vaccination status of the animal, geographical area from which the virus was isolated or time of virus isolation, indicating that there is no specific distribution pattern of viruses with particular antigenic profiles in the country. This is possible because of unrestricted movement of animals within the country and irregular vaccination of large population of susceptible animals. The extensive antigenic variation observed among Indian FMDV Asia 1 field isolates could result from partially immune (vaccinated) host animals providing a strong selection force for the generation of variants (Butchaiah et al., 1992).

Genetic relationships between Asia 1 FMDV field isolates and vaccine virus

Prior to the initiation of this study, the laboratory has been analysing in detail, over the years, the antigenic relationship of Asia 1 outbreak strains with the vaccine strain (IVR1 vaccine strain, IND 63/72) to study epidemiology of the disease using both polyclonal and monoclonal antibodies (Antony, 1987; Mishra et al., 1995 ; Sanyal, 1995). In the present study, in addition to antigenic relationship analysis, genetic relationship analysis was undertaken by comparing nucleotide sequences of the 3' end of 1D (VP1 coding) gene of 40 Indian FMDV Asia 1 isolates recovered between 1985-1997.

The genomic RNA of the viruses was extracted by guanidine thiocyanate method and the 1D (VP1 coding) gene was amplified by RT-PCR using primers flanking the 1D gene (Table3). The RT-PCR products were used for cycle sequencing of nucleotides

at the 3' end of the gene according to the dideoxy termination protocol of Sanger et al.(1977) using an internal negative sense primer. Sequencing reaction samples were electrophoresed on 6% polyacrylamide sequencing gels and stained by silver staining method. The gels were visualised on a light box and sequences read and aligned.

The sequence data analysed for epidemiological studies is derived from the 3' end of the 1D gene. This region, though not subject to the high rate of mutations as in neighbouring hypervariable region, is nevertheless also mutable, but to a lesser extent. Most of the substitutions are found at the third nucleotide position of codons and do not often result in amino acid changes (synonyomous mutations) or even when the amino acid is changed, it has no influeance on the antigenic characteristics of the virus. Studies show that such mutations play a major role in FMDV evolution (Dopazo et al., 1988; Saiz et al. 1993) and are also important in an epidemiological point of view in that they help to establish relationships between different isolates circulating in a region. Phylogenetic trees constructed using about 160 nucleotides at the 3' end (downstream to hypervariable region i.e. from 465th nucleotide onwards) of 1D gene (VP1 coding region) have been shown to be satisfactory for molecular epidemiological studies on FMDV (Knowles et al., 1988; Knowles and Samuel, 1990; Samuel et al., 1990; Kitching and Knowles, 1993).

The 159 nucleotides at the 3' end of all the isolates and vaccine virus and their deduced amino acid sequences are given in Fig. 6 and Fig. 7. The sequence alignment and phylogenetic analysis (Fig.8) indicates the wide diversity of Asia 1 FMDV isolates of Indian origin which clustured into 11 different groups almost irrespective of chronology or geographical origin. However, none of the sequences analysed showed a greater divergence than 14.5% from vaccine virus. By applying the criteria similar to those used by Vosloo et al (1992) for FMDV type SAT2 viruses to the Indian Asia 1 isolates analysed in the present study, they could be regarded as members of a single genotype. The high degree of similarity between geographically and chronologically diverse group of isolates included in the present study indicates that they are more conserved genetically than the other FMDV serotypes. The genetic relatedness of different isolates are described below.

Group 1:

This group comprised of two isolates from Eastern part of India (West Bengal, IND 293/94 and IND 33/96), one isolate from North Eastern (Assam, IND 22/88), three isolates from the Western part (Maharashtra, IND 120/88 and IND 43/96 and Gujarat IND267/88) and one from Northern India (Uttar Pradesh, IND132/90). All these isolates were very much similar to each other and the vaccine virus with < 3% divergence between them. In the serological tests also, these isolates showed close relationship with the vaccine virus. This shows the wide distribution and prevalance of genotypes similar to the vaccine virus in geographically distant areas of the country spanning a period of nearly a decade.Though sh these isolates were very similar to the vaccine virus,none were totally identical to it. Taking into consideration the quasispecies nature of the FMD virus it can be explained that each of the isolates in this group is an individual entity by itself.

Group 2, Group 3 and Group 4:

The sequence analysis results showed that three isolates recovered from Rajasthan (IND 46/87), Maharashtra (IND 155/88) and West Bengal (IND 10/91) in the years 1986, 1988 and 1990 respectively, are divergent (5% and above) from rest of the isolates including vaccine virus and were clustered into three different groups. Mab profiling also clustered these isolates into individual groups containing single isolates except IND 10/91 which was grouped along with seven other isolates to form group 1 in Mab profiling results. This indicates that these are distinct isolates quite different from others.

Group 5:

Two isolates viz. IND 13/91 and IND 17/91 recovered from sheep and pig respectively, from Maharashtra in the year 1990 showed a divergence of only 1.2%. These two isolates were grouped with a 1985 isolate of Assam (IND 75/86) recovered from cattle, with 3.6% divergence between them. This showed that viruses isolated from different species of animals, different places and during different periods of time, were closely related and no restriction of genetic lineages to specific species was observed. The variety

of susceptible animal species and the air-borne transmission of FMD virus often over long distances, play different roles in epizootiology of FMD (Terpestra, 1990).

Group 6:

Most of the isolates from Southern India (Karnataka, Andhra Pradesh, Tamil nadu and Pondichery) viz. IND 6/95, IND 80/96, IND 81/96, IND 14/95, IND 57/95, IND 26/97, IND 71/96, IND 29/95, IND 40/95 and IND 73/96 recovered between 1994 and 1997 were clustured together in this group with < 4.2% divergence between them. These isolates were grouped alongwith isolates from Uttar Pradesh (IND 26/95), Hissar (IND 172/96 and IND 173/96), Punjab (IND 89/96), Himachal Pradesh (IND 82/96), West Bengal (IND 70/96) and Maharashtra (IND 316/94 and IND 72/96) which were also isolated during the same period. This finding of serial outbreaks throughout the country within a period of four years emphasised that the viruses of related genotypes which formed foci of infection and mainly circulated in Southern India were also responsible for outbreaks outside this region. This was not surprising since there is absence of restriction of animal movement within the country which enormously facilitates spread of the virus. Moreover, it is shown that herds often fragment after the disturbance due to disease outbreaks, and the detached animals may join other herds or establish themselves in small separate groups leading to dissemination of the virus to the distant lands (Vosloo et al., 1995). Among the isolates studied, the sequence analysis results strongly suggested that more than 70% of the outbreaks during the recent four years (1994- 1997) were caused by viruses with high degree of sequence homology and could have originated from the same Asia 1 FMDV. The earliest isolate, viz. IND 316/94 in this group of viruses which originated from Maharashtra in the year 1994, could be suspected to be the common ancestor and it must have moved from there to Southern India establishing major foci of infection there to cause repeated outbreaks. However, because of the similarity between this group of viruses, the precise origin, based on the data presented, cannot be specified and the technique cannot identify the means by which the strains move from one place to another.

Group 7:

The remaining three isolates of Southern India (IND 1/95, IND 4/95 and IND 15/95) recovered during 1994 and 1995 were not closely related to the main group (group 6) of viruses of this region, instead they clustered along with an isolate from Uttar Pradesh (IND 50/95) collected in the year 1995. All the isolates of this group were from vaccinated herds except IND 50/95 (vaccination status not known) with 6.7 to 7.4% divergence from vaccine virus. Further, though these isolates showed close relationship with vaccine virus in polyclonal 'r' values, they showed either reduced or no reaction with 7 or more Mabs of the panel in Mab profiling ELISA. This result could be most likely due to failure of effective vaccination. The value of vaccination against FMD depends on the proper use of a potent inactivated vaccine containing strains of virus antigenically close to those likely to challenge the vaccinated animals. But the level of protective immunity achieved by vaccination is never complete throughout the herd and should those animals that have inadequate immunity become infected, they then become a potent source of infection which could overcome the immunity of others(Samual et al., 1990).

Group 8 and Group 10:

One of the isolate from Haryana (IND 17/93 of group 8), which was isolated from an unvaccinated cattle in the year 1993 was divergent (5.7 to 10.7%) from rest of the isolates including the vaccine strain. This result also correlated well with the antigenic analysis results. Of all the viruses studied, this isolate showed the least 'r' values in both sandwich ELISA (0.22) and 2D-MNT (0.43). In Mab profiling also, it showed no reaction with any of the Mabs of the panel except Mab 81, with which it showed reduced reaction (66%). Similarly, an isolate (IND 53/93 of group 10) from Karnataka, also isolated from unvaccinated cattle, was divergent from rest of the isolates and vaccine virus (4.8 to 11.5%) with comparatively low 'r' values in sandwich ELISA (0.36) and 2D-MNT (0.46). With the Mab panel, this isolate showed no reaction to any of the Mabs except Mab 81 with which it showed reduced reaction, again suggesting its distant relationship with vaccine virus. As these two isolates had similar history and were chronologically related, epidemiological

relationships between them if had been inferred based only on serological tests (employing polyclonal and monoclonal antibodies) it would not have been able to distinguish between them. In contrast to this, nucleotide sequencing studies revealed that they were deivergent not only from the vaccine virus but also from each other (6.9%). To come to such a conclusion conventional serological methods would have required two-way relationship tests using sera raised against both the isolates which is a very time consuming and tedious process and hence has become obsolete (Kitching et al., 1989). The value of nucleotide sequenc analysis in studying the relationship between each pair of large number of outbreak viruses has been emphasised by several workers. It is established by several studies that the nucleotide sequence analysis of the field isolates allows a much more precise evaluation of the degree of relationship among viral strains than do serological and other methods(Knowles et al., 1988; Samuel et al., 1988; Knowles and Samuel, 1990 and Armstrong et al., 1994).

Group 9 and group 11:

Two isolates viz. IND 21/89 and IND 45/89 of group 9,which were identical to each other, collected in the year 1989 from two places situated about 300 Km. away from each other, in Uttar Pradesh showed the highest divergence (14.5%) with vaccine virus and also with other isolates (5.5 to 14.5%). The isolate IND 19/89 of group 11, collected from the same place in the same year also showed higher divergence (5 to 9.9%) with other isolates , with vaccine virus the divergence was 9.1%.This isolate (IND 19/89) showed 5.5% divergence with isolates of group 9. A point worthy of mention is that these three isolates were collected from buffaloes which were unvaccinated. This showed the co-circulation of widely divergent viruses in the same geographic area during the same period. This might be due, at least in part, to factors like presence of multiple variants in any infected animal, the unknown nature of the selective constraints and random resampling events during virus multiplication and spread (Domingo et al., 1992).

The sequence of Pak 1/54 strain which is a widely used vaccine strain in other countries in Asia , included for the comparision in this study, revealed that it was very much divergent from any of the Indian isolates (8.8 to 19.4%) as well as vaccine virus (10.9%) and formed a separate clustur (no separate group was designated). Similar findings have also been reported by Ansell et al. (1994). This divergence of the Pak 1/54 strain with the Indian Asia 1 field strains was possibly due to the high rate of mutation of FMD virus in nature leading to accumulation of genomic changes (Carrillo et al., 1991). During replication of FMD virus, a high rate of fixation of mutations take place which leads to polymorphism in virus populations (Domingo et al., 1990).

Deduced amino acid sequences of the isolates (Fig. 7) showed that though there were substitutions ranging from 1 to 22 in the nucleotide sequences (Fig. 6), there were only 1 to 5 amino acid substitutions per isolate when compared with vaccine virus. Since the region sequenced for epidemiological investigation did not include the hypervariable region, most of the nucleotide substitutions observed were synonymous which did not lead to any amino acid substitutions. The nonsynonymous mutations which lead to amino acid substitutions varied from 1 to 5 per isolate when compared with vaccine virus and since the knowledge about the epitopes and their exact location in the VP1 protein of FMDV type Asia 1 is not available, antigenic features attributed to these substitutions could not be detected. Amino acid substitutions also did not correlate with phylogenetic grouping of isolates since synonymous mutations which play important role in FMDV evolution (Dopazo et al., 1988; Saiz et al., 1993) outnumbered the nonsynonymous mutations.

The present study, the first of its kind in India, where the analysis of the nucleotide sequence of 40 field isolates of type Asia 1 has been undertaken along with their antigenic analysis, has revealed valuable information on the prevalence and distribution of FMDV serotype Asia 1 in the country. They can be summarised as follows:

1.The Indian Asia 1 isolates are genetically heterogeneous occuring in 11 distinct groups (Fig. 8), having a sequence homology of > 85% and can be regardede as members of a single genotype .

2.The Indian isolates are very divergent from the Pak 1/54 strain of Asia 1 virus which is widely used as a vaccine virus in several countries in Asia. This is in agreement with the findings reported by Ansell et al., (1994).

3.The phylogenetic relationship between viruses isolated from different parts of the country clearly indicates that regular and unrestricted movement of animals from place to place is the cause for the wide spread of viruses of varied genetic nature over the country. Accordingly , no specific distribution pattern of Asia 1 virus sequences could be observed. Therefore, it was not possible to determine whether certain sequences occur only in certain areas/states/regions.

4.The changes in the nucleotide sequences between the Asia 1 vaccine strain (IND 63/72) and the field isolates is obvious. This is a normal phenomenon because FMD virus undergoes high rate of mutation in nature and this can lead to accumulation of genomic changes.

5.Three isolates recoved from buffaloes in Uttar Pradesh in the year 1989 showed the highest divergence (9.1 - 14.5%) with the vaccine virus and also showed a divergence of 5.0 to 14.5% with all other isolates included in the study. However, out of these three isolates, two of them were identical to each other (IND 21/89 and IND 45/89) though they were recovered from two places situated about 300 km from each other (Mathura and Izatnagar). This clearly showes that the virus have spread most probably due to movement of animals. Howe[illegible], the third isolate (IND 19/89) also recovered from Mathura showed a divergence of >5% with the other two isolates recovered during the same time. This reveals the circulation of viruses with widely divergent genetic lineagens in the same geographic area during the same period and genomic changes in FMD virus RNA increases when the disease is transmitted between related species of animals.

6.The results of the present study revealed that the unrestricted movement of animals is a majaor factor responsible for new outbreaks, This is clearly evident from group No. 6 in which the isolates (total 18) drawn from the states of (Karmataka, Pondicherry, Andhra Pradesh, Tamilnadu, Himachal Pradesh, Uttar Pradesh, Haryana, Punjab, West Bengal and Maharashtra) are grouped together, having a high level of sequence homology (0 to 4.2%)

between them. Some of the isolates are more closely related to the viruses isolated from far-off places than to an isolate from the same area/state [for ex. isolate No: IND 82/96 with IND 80/96 and IND 81/96; IND 70/96 with IND 72/96; IND 26/95 with IND 6/95 etc.]. These observations not only indicate that there are several distinct populations of FMD virus type Asia 1 in the country responsible for the outbreaks but also strongly suggest that as the movement of livestock in the country is unrestricted due to several factors like sale and purchase, shifting of animals from one herd to another, movement of animals to slaughter houses, seasonal migration of sheep and goat flocks, transport of agricultural products by bullock carts etc., the animals themselves carry the virus from place to place.

7.The detailed antigenic analysis of the field isolates carried out in the present study by using polyclonal and monoclonal antibodies also revealed the presence of distinct clusters of antigenically related viruses as is observed in the genetic analysis. The antigenic analysis also revealed that the field isolates are related to the current vaccine virus antigenically. However, minor antigenic differences could be observed in Mab profiling studies.

Thus, the present study reveals the importance of undertaking nucleotide sequence analysis studies in conjunction with detaiaied antigenic analysis studies of field isolates of type Asia 1 to trace the origin, spread and the prevalence of dominant/distinct viruses which will be very useful to undertake a planned strategy for the control of the disease .

SUMMARY

SUMMARY

There is probably no infectious disease of livestock that engenders more discussion than foot-and-mouth disease. It still remains a major scourge of livestock industry and the control of the disease by vaccination is complicated by the existence of the causative agent (foot-and-mouth disease virus) in several distinct immunological types and subtypes. Of the four FMDV serotypes viz. O, A, C, Asia 1 prevalent in India type Asia 1 causes the second largest number of outbreaks. Therefore, continuous surveillance of the antigenic and genetic nature of the field strains is a dire necessity wihich will help in planning and undertaking successful control measures.

Conventionally,antigenic analysis of FMDV strains was done using polyclonal sera and during the last decade Mabs against FMD viruses were also used for this purpose. In the last few years, in addition to antigenic analysis studies, genetic relationships between the field strains have been studied by nucleotide sequence analysis to understand the origin, source and movement (spreading) pattern of the virus strains .

The present work was undertaken by coupling both conventional antigenic analysis studies and genetic (nucleotide sequence) analysis studies to understand the antigenic and genetic nature of Indian FMDV Asia 1 outbreak strains and spread of the disease in the country.

A total of forty FMDV type Asia 1 isolates recovered from field outbreaks of the disease in vaccinated and unvaccinated herds from different parts of India during the period, 1985- 1997 were selected to study their antigenic and genetic relatedness. All the isolates were propagated in BHK 21 Clone 13 cell line and their type specificity was confirmed by sandwich ELISA using anti-146S guinea pig serum.

To study whether there is any antigenic divergence between the viruses, they

were subjected to antigenic analysis by sandwich ELISA using anti-146S guinea pig serum raised against vaccine virus. As neutralization is important in protection, and since virus neutralization test is found to be a more accurate serological test for detecting antigenic variation, a two dimentional microneutralization test (2D-MNT) was also undertaken using bovine vaccinate serum. In case of sandwich ELISA, 36 isolates gave an 'r' value in the range of 0.40- 1.00 and rest 4 isolates in the range of 0.20 to 0.39 indicating a high degree of relationship of all these isolates with the vaccine virus. The results of 2D-MNT revealed that all the field isolates gave 'r' values in the range of 0.43 to 1.00 indicating that they are closely related to the vaccine strain.

To study the minor antigenic differences between the isolates, which showed similar results in antigenic analysis studies using polyclonal sera, monoclonal antibodies were used for antigenic profiling of field isolates . A panel of 10 monoclonal antibodies known to bind at four different antigenic sites on the virus were used to profile the field isolates. Field isolates varied in their reactivity pattern with the Mab panel and this enabled them to be clustured in to 9 differnt groups. Isolates (a total of eight) of group 1 retained homologous affinity for all the Mab-binding sites, whereas isolates (two) of group 9 showed neither homologous reactivity nor reduced reactivity for all the Mabs. Remaining isolates of different groups showed varying levels of reactivity with th ' Mabs of the panel. The existence of heterogeneous population of viruses as observed in Mab-profiling revealed the high antigenic diversity of field isolates though they showed strong serological relationship with the vaccine virus in sandwich ELISA and 2D-MNT conducted using polyclonal sera.

Genetic analysis of the field isolates was undertaken by constructing a phylogenetic tree using 159 nucleotides at the 3' end of 1D (VP1 coding) gene of outbreak viruses and the vaccine virus. For comparision , the sequence of Pak 1/54 strain, widely used as vaccine strain in many countries, was also included in the analysis. Based on the divergence level between any two sequences, all the fourty isolates could be clustured in to 11 different groups. Divergence of less than 5% was taken as the criterion to classify isolates

into groups. Group 1 included seven isolates collected over a period of one decade from different regions of India and all isolates in this group showed high degree of similarity with the vaccine virus. Isolates of group 9 showed maximum divergence from the vaccine virus.The isolates in the remaining groups showed varying levels of divergence from the vaccine virus and with the isolates of other groups. Similar to the grouping based on antigenic differences, phylogenetic grouping also showed no correlation between vaccination status, geographical area and time of virus isolation.This may be due to the fact that the disease is highly endemic in India and there is unrestricted animal movement within the country. However, there was a high degree of correlation between antigenic and genetic analysis studies. Antigenic analysis studies revealed the close relationship of the outbreak strains with the vaccine strain indicating the wide antigenic coverage of the currently used vaccine strain (IVRI vaccine virus IND 63/72), whereas genetic analysis studies revealed the existence of genetically diverse groups of virus strains with complex spreading pattern in the country. Further molecular epidemiological studies involving antigenic and genetic analysis of more number of outbreak strains would help in understanding the natural history of the disease and the nature of virus strains prevailing in the country ; insights gained from such studies would contribute to the plan and implementation of suitable strategies for control of FMD in the country.

MINI ABSTRACT

Foot-and-mouth disease (FMD) is an economically important disease of cloven-hoofed animals and it is endemic in India. Of the four FMDV serotypes prevalent in India, Type Asia 1 causes the second largest number of outbreaks. The present work was undertaken to study the molecular epidemiology of type Asia 1 using 40 isolates collected from different parts of the country, between 1985 - 1997.

All the 40 isolates could be propogated in BHK 21 cell line. They were subjected to antigenic analysis studies using polyclonal and monoclonal antibodies (Mabs) raised against vaccine virus (IND 63/72). Sandwich ELISA using anti-guinea pig serum and micro-neautralization test using bovine vaccinate serum revealed that all the isolates are closely related to vaccine virus. Isolates were subjected to Mab profiling using a panel of, 10 Mabs and depending on reactivity pattern, they could be clustered into 9 different groups. Phylogenetic tree was constructed by determining sequence of 159 nucleotides at the 3' end of 1D gene for antigenic analysis, and in this, isolates could be clustered into 11different groups. The results of nucleotide sequence analysis , in addition, reinforced the view that genetic relatedness correlate well with antigenic relatedness, however, it was not possible to associate viruses of specific genetic or antigenic profiles to particular geographic areas over long periods of time. The intricate pattern of disease spreading revealed by this study could be mainly due to unrestricted animal movement within the country. This work is first of its kind in India and molecular epidemiological studies like this should contribute to the plan and implementation of suitable strategies for control of FMD in the country.

लघु सारांश

खुर व मुँह पका रोग पालतू पशुओं का आर्थिक रूप से एक महत्वपूर्ण रोग है । ये रोग हमारे देश में अति प्रचलित रूप में होता है । जो चार खुर-मुँह रोग सीरमप्ररूप ⟨सीरोटाइप⟩ भारत में सदैव दिखाई पड़ते हैं उनमें से टाइप ⟨प्ररूप⟩ एशिया 1 दूसरा सर्वाधिक रोग विस्फोट का कारण है । वर्तमान अध्ययन देश के विभिन्न भागों से प्राप्त ⟨उपलब्ध⟩ किये गये चालीस आइसोलेट्स ⟨पृथकृकर्तों⟩ को लेकर खुर व मुँह पका रोग सीरोटाइप एशिया 1 के मोलिक्युलार एपिडीमियोलोजी ⟨परमाणु सम्बन्धी - जानपदिक रोग विज्ञान ⟩ पर किया गया है ।

सबसे प्रथम समस्त आइसोलेट्स को बी0ऐच0के0 21 सेल लाइन में प्रचलित किया गया, तथा इन्ड 63/72 टीका ⟨वेक्सीन⟩ विषाणु के विरुद्ध उत्पन्न किया गया पोलिक्लोनल एवं मोनोक्लोनल प्रतिरोधियों ⟨मैब⟩ का प्रयोग करके समस्त आइसोलेटों का ऐन्टिजैनिक अनोलेटिक अध्ययन किया गया । ऐन्टी गिनियापिग सीरम लेकर किये गये सैन्डविच इलीसा तथा बोवाइन वैक्सीनेट सीरम लेकर किये गये माइक्रोन्यूट्रलाइजेशन ⟨सूक्ष्म-अप्रभावीकरण⟩ परीक्षण से यह ज्ञात हुआ कि समस्त आइसोलेट्स वैक्सीन विषाणु से निकटतम सम्बन्ध प्रस्तुत करते हैं । 10 मैब वाली एक पेनल ⟨श्रंखला⟩ में आइसोलेटों का मैब प्रोफाइलिंग किया गया तथा इस जाँच के परिणाम ⟨निष्कर्ष⟩ को लेकर आइसोलेटों को 9 समूहों में विभाजित किया गया । समस्त आइसोलेट के 1 डी जीन के 3' अंत का 159 न्यूक्लियोटाइड श्रेणी का निर्गम करके उसी से एक फेलोजैनेटिक ट्री की रचना की गयी । जिससे समस्त आइसोलेट्स11 समूहों में बँट गये । न्यूक्लियोटाइड श्रेणी का विघटन, परिणाम तथा ऐन्टीजैनिक विघटन परिणाम के मध्य सुन्दर व उत्तम पारस्परिक सम्बन्ध प्रकट है परन्तु प्रत्येक जनानिक अथवा ऐन्टिजैनिक कक्ष ⟨समूह⟩ के विषाणुओं का प्रत्येक प्रदेश या राज्य से अथवा प्रत्येक समय से कोई सम्बन्ध नही था । रोग फैलाव का गहन साँचा इस अध्ययन से प्रकट हुआ जिसका कारण ये हो सकता है कि हमारे देश में पशु गति पर कोई भी प्रतिरोध नहीं है । इस प्रकार का अध्ययन हमारे देश में प्रथम कार्य है और इसके द्वारा कार्य क्षम रोग नियंत्रण के प्रति बहुमूल्य जानकारी प्राप्त होती है ।

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APPENDIX

APPENDIX

Carbonate-bicarbonate buffer (ELISA coating buffer) pH 9.6:

Solution A

0.2 M sodium carbonate anhydrous	- 21.2 g
Distilled water to make	- 1000 ml

Solution B

0.2 M sodium bicarbonate	- 16.8 g
Distilled water to make	- 1000 ml

Working buffer

16 ml of solution A is mixed with 34 ml of solution B and volume adjusted to 200 ml with distilled water.

PBS-Tween 20 buffer (ELISA washing buffer):

$NaH_2PO_4.H_2O$	- 0.345 g (0.0025M)
$Na_2HPO_4.12H_2O$	- 2.680 g (0.0075M)
NaCl	- 29.224 g
Tween 20	- 1.0 ml
Distilled water to make	- 1000 ml

ELISA blocking buffer:

This was freshly prepared on the day of use.

Lactalbumin hydrolysate (LAH, Difco)	- 3.0 g
Healthy rabbit serum (neat)	- 5.0 ml
Healthy calf serum (neat)	- 5.0 ml
ELISA washing buffer to make	- 100 ml

Skimmed milk powder solution:

This solution was prepared at a concentration of 1% (w/v) in PBS-Tween 20 buffer and used as blocking reagent in Mab profiling sandwich ELISA.

0.1 m Citric acid-phosphate buffer, pH 5.0:

Citric acid	- 7.3 g (0.0347 M)
$Na_2HPO_4.12H_2O$	- 23.87 g (0.0667 M)
Distilled water to make	- 1000 ml
Stored at 4°C.	

Orthophenylene diamine (OPD) solution (Substrate solution):

OPD dihydrochloride(Sigma, P-1526)	- 10 mg
Citric acid-phosphate buffer, pH 5.0	- 15 ml
H_2O_2 (30%)	- 8 µl

This solution was prepared just before use.

1 M H_2SO_4 Stopper solution for ELISA

H_2SO_4	- 5.56 ml (96% H_2SO_4 of Special gravity 1.84)
Distilled water	- 94.44 ml

10mM dNTP mix

dATP	10mM
dCTP	10mM
dGTP	10mM
dTTP	10mM

Looding buffer (6X)

Bromophenol blue	0.25% w/v
Xylene cynole FF	0.25% w/v
Sucrose in water	40% w/v

Direct purification buffer

Kcl	50 mM
Tris Hcl	10 mM
$MgCl_2$	1.5 mM
Triton X-100	0.1% v/v

Purification resin

Guanidine thiocyanate	6 M

dd/dNTPs mixes

dd/dATP mix		dd/dCTP mix	
ddA	350 um	ddC	200 um
ddG	20 um	ddA	20 um
ddC	20 um	ddG	20 um
ddT	20ul	ddT	20 um

dd/dGTP mix		dd/dTTP mix	
ddG	30 um	ddT	600 um
ddA	20 um	ddA	20 um
ddC	20 um	ddG	20 um
ddT	20 um	ddC	20 um

includes 7-deaza dGTP1

5X sequencing buffer

Tris Hcl (pH 9.0 at 25oC)	250 nm
MgCl2	10 mM

Sequencing stop solution

NaOH	10 mM
Formamide	95%
Bromophenol blue	0.05%
Xylene cyanole	0.05%

Amino acid stock solution (For 2 ltrs)

Arginine	- 1 68 g
L-Cystine	- 1.137 g
Histidine	- 0.84 g
Isoleucine	- 2.096 g
Leucine	- 2.096 g
Lysine	- 2.924 g(monohydrochloride salt)
Phenylalanine	- 1.32 g
Threonine	- 1.904 g
Tryptophane	- 0.326 g
Tyrosine	- 1.801 g
Valine	- 1.872 g

Methionine	- 0.6 g
Inositol	- 0.14 g
Phenol red (0.5%)	- 0.16 ml

(L-cystine is dissolved separately in 5 ml in In HaOH solution)
Stored at -20°C

Vitamin stock solution (For 500 ml)

Choline chloride	- 250 mg
Folic acid	- 250 mg
Nicotinamide	- 250 mg
Pantothenic acid	- 250 mg
Pyridoxine HCl	- 250 mg
Thiamine HCl	- 250 mg
Riboflavine	- 25 mg

(Folic acid is dissolved separately in 5 ml of IN NaOH solution)
stored at -20°C.

Trypsin-versene solution

NaCl	- 5.0 g
Kcl	- 0.125 g
Na2HPO4.2H2O	- 0.950 g
KH2PO4	- 0.125 g
Trypsin	- 0.850 g
Versene (EDTA)	- 0.700 g
Phenol red (0.5%)	- 0.5 ml
Distilled water	- To make 500 ml
pH adjusted to 7.4	

Mixed by stirring on a magnetic stirrer and sterilized by positive pressure seitz filtration. Incubated overnight at 37°C before use. Stored at 4°C.

BHK-21 maintenance medium (Glasgow modification)

(Composition for making 2 ltr of medium)

NaCl	- 12.8 g
Kcl	- 0.800 g
$CaCl_2.2H_2O$	- 0.530 g
$MgSO_4.7H_2O$	- 0.400 g
$NaH_2PO_4.2H_2O$	- 0.280 g
Glucose	- 9.0 g
L-glutamine	- 1.170 g

(Part of distilled water is added to dissolve these salts)

Phenol red (Sodium salt)	- 0.034 g
$NaHCO_3$	- 5.5 g
Penicillin	- 2 Lakh I.U.
Streptomycin	- 0.2 g
Amino acid stock	- 100 ml
Vitamin stock	- 8 ml
Tryptose phosphate broth	- 6.0 g
Distilled water	- Upto 2000 ml
pH adjusted to 7.4	

Sterilized by positive pressure seitz filtration. Incubated for overnight at 37°C before use. Stored at 4°C.

BHK-21 growth medium (Glasgow modification)

BHK-21 maintenance medium	- 900 ml (pH 7.4)
Healthy calf serum	- 100 ml

Sterilized by positive pressure seitz filtration. Incubated for overnight at 37°C before use.
Stored at 4°C.

1X TBE buffer

Tris-HCl	- 89mM
Sodium borate	- 89mM
EDTA	- 2mM

The Genetic Code

TTT	TCT	TAT	TGT
TTC F	TCC S	TAC Y	TGC C
TTA	TCA	TAA*	TGA*
TTG L	TCG S	TAG*	TGG W
CTT	CCT	CAT	CGT
CTC L	CCC P	CAC H	CGC R
CTA	CCA	CAA	CGA
CTG L	CCG P	CAG Q	CGG R
ATT	ACT	AAT	AGT
ATC I	ACC T	AAC N	AGC S
ATA I	ACA	AAA	AGA
ATG M	ACG T	AAG K	AGG R
GTT	GCT	GAT	GGT
GTC V	GCC A	GAC D	GGC G
GTA	GCA	GAA	GGA
GTG V	GCG A	GAG E	GGG G

* Stop codons.

Amino acid abbreviations

Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Trptophan	Trap	W
Tyrosine	Tyr	Y

Fig. 9 Divergence percentages between sequences of Asia 1 FMD viruses used in the study.

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		89.7	89.7	91.5	91.5	92.1	91.5	92.1	92.7	91.5	90.9	92.1	93.3	90.3	89.7	89.7	92.7	92.1
2	6.9		100.0	89.1	89.7	92.7	93.3	91.5	89.7	87.3	86.7	93.9	91.5	86.1	97.0	100.0	89.7	87.9
3	6.9	0.0		89.1	89.7	92.7	93.3	91.5	89.7	87.3	86.7	93.9	91.5	86.1	97.0	100.0	89.7	87.9
4	5.0	10.9	10.9		92.7	92.7	94.5	93.3	92.7	98.2	89.7	95.2	93.3	93.3	87.3	89.1	92.7	92.1
5	5.0	9.7	9.7	6.7		92.7	95.2	97.6	95.2	91.5	89.1	95.8	91.5	89.7	88.5	89.7	97.6	93.3
6	4.4	7.3	7.3	6.7	6.7		93.9	92.1	91.5	92.7	87.9	94.5	93.3	91.5	90.3	92.7	92.7	89.7
7	5.0	6.7	6.7	5.5	4.2	5.5		97.0	95.2	92.7	89.1	99.4	95.8	90.3	90.3	93.3	95.2	93.3
8	4.4	8.5	8.5	6.1	1.8	6.7	3.0		95.8	92.1	89.7	97.6	93.3	89.1	90.3	91.5	98.2	93.9
9	3.8	6.9	6.9	3.8	1.3	4.4	1.3	0.6		92.7	90.3	95.8	91.5	88.5	89.7	89.7	96.4	94.5
10	5.0	12.1	12.1	1.2	7.3	6.7	6.7	6.7	3.8		89.7	93.3	91.5	93.3	87.3	87.3	92.7	92.1
11	5.7	10.1	10.1	6.3	7.5	8.2	7.5	6.9	6.3	6.3		89.7	90.3	87.8	86.7	86.7	90.3	89.1
12	4.4	6.1	6.1	4.8	3.6	4.8	0.6	2.4	0.6	6.1	6.9		95.8	90.3	90.9	93.9	95.8	93.9
13	3.1	8.5	8.5	6.7	7.9	6.7	4.2	6.7	5.0	7.9	6.3	4.2		94.5	88.5	91.5	91.5	90.9
14	6.3	13.9	13.9	6.7	10.3	8.5	9.7	10.3	8.2	6.7	8.2	9.7	5.5		84.8	86.1	89.7	87.9
15	6.9	1.2	1.2	11.1	9.3	8.0	8.0	8.0	6.9	11.1	10.1	7.4	9.9	13.6		97.0	89.7	87.9
16	6.9	0.0	0.0	10.9	9.7	7.3	6.7	8.5	6.9	12.1	10.1	6.1	8.5	13.9	1.2		89.7	87.9
17	3.8	9.1	9.1	6.1	2.4	6.1	3.6	1.8	0.0	6.1	6.3	3.0	7.3	9.7	8.0	9.1		94.5
18	4.4	8.8	8.8	4.4	3.1	6.3	3.1	2.5	1.9	4.4	6.9	2.5	5.7	8.8	8.8	8.8	1.9	
19	7.5	1.2	1.2	11.5	10.3	7.9	7.3	9.1	7.5	12.7	10.7	6.7	9.1	14.5	2.5	1.2	9.7	9.4
20	5.0	10.3	10.3	7.3	3.6	7.3	4.8	3.0	1.3	6.7	7.5	4.2	8.5	10.9	9.3	10.3	1.8	3.1
21	5.7	10.9	10.9	6.7	3.0	6.7	5.5	2.4	1.9	7.3	8.2	4.8	9.1	10.3	10.5	10.9	3.0	3.8
22	7.5	0.6	0.6	11.5	10.3	7.9	7.3	9.1	7.5	12.7	10.7	6.7	9.1	14.5	1.9	0.6	9.7	9.4
23	4.4	7.5	7.5	4.4	0.6	5.0	1.9	0.0	0.6	4.4	6.9	1.3	5.7	8.8	7.5	7.5	0.6	2.5
24	5.7	1.3	1.3	9.4	6.9	6.3	6.9	6.3	5.7	9.4	8.8	6.3	8.8	11.9	1.3	1.3	5.7	7.5
25	6.3	13.9	13.9	6.7	10.3	8.5	9.7	10.3	8.2	6.7	8.2	9.7	5.5	0.0	13.6	13.9	9.7	8.8
26	5.0	4.4	4.4	7.5	5.0	6.9	6.3	5.7	5.0	7.5	6.9	5.7	6.9	10.1	4.4	4.4	5.0	5.7
27	4.4	6.1	6.1	4.8	3.6	4.8	0.6	2.4	0.6	6.1	6.9	0.0	4.2	9.7	7.4	6.1	3.0	2.5
28	5.7	6.8	6.8	6.8	3.7	6.2	2.5	3.1	1.9	6.8	6.9	1.9	6.2	11.1	8.0	6.8	3.1	3.8
29	5.0	10.9	10.9	4.8	7.9	7.9	7.3	7.3	5.0	4.8	6.9	6.7	6.7	7.9	10.5	10.9	7.3	5.7
30	6.3	9.1	9.1	7.9	3.6	8.5	3.6	1.8	2.5	8.5	8.8	4.2	6.7	10.3	8.6	9.1	3.6	4.4
31	7.5	0.6	0.6	11.5	10.3	7.9	7.3	9.1	7.5	12.7	9.4	6.7	9.1	14.5	1.9	0.6	9.7	9.4
32	5.0	8.2	8.2	3.8	1.3	4.4	2.5	0.6	1.3	3.8	7.5	1.9	6.3	8.2	8.2	8.2	1.3	3.1
33	5.0	9.7	9.7	6.7	3.0	7.3	4.2	1.2	1.9	7.3	6.3	3.6	7.3	10.9	9.3	9.7	3.0	3.8
34	5.0	8.2	8.2	3.8	2.5	5.7	2.5	1.9	1.3	3.8	7.5	1.9	6.3	8.2	8.2	8.2	1.3	3.1
35	3.8	9.1	9.1	6.1	2.4	5.5	3.6	3.0	0.0	4.8	6.3	3.0	7.3	9.1	8.0	9.1	1.8	1.9
36	3.1	9.7	9.7	3.6	6.1	4.2	5.5	5.5	3.1	3.6	5.7	4.8	5.5	6.7	9.3	9.7	5.5	3.8
37	5.0	9.7	9.7	7.3	3.0	7.9	3.0	1.2	1.3	7.3	7.5	3.6	7.3	10.9	8.6	9.7	2.4	3.1
38	4.4	7.5	7.5	4.4	1.9	5.0	0.6	1.3	0.6	4.4	6.9	1.3	5.0	8.2	7.5	7.5	0.6	2.5
39	5.7	10.3	10.3	6.7	3.6	7.3	3.6	1.8	1.9	6.7	8.2	4.2	7.9	10.3	9.3	10.3	2.4	3.8
40	4.4	8.5	8.5	6.1	3.0	6.7	1.8	1.2	0.6	6.7	6.9	2.4	6.1	9.7	8.0	8.5	1.8	2.5
41	12.6	10.3	10.3	17.6	13.9	13.9	15.2	14.5	13.2	17.6	15.7	14.5	16.4	19.4	9.9	10.3	15.2	13.8
42	4.4	7.5	7.5	4.4	1.9	5.0	1.9	1.3	0.6	4.4	6.9	1.3	5.7	8.8	7.5	7.5	0.6	2.5
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

Percent Divergence

19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
89.1	91.5	90.9	89.1	92.1	90.9	90.3	91.5	92.1	90.9	91.5	90.3	89.1	91.5	91.5	91.5	92.7	93.3	91.5
98.8	88.5	89.1	99.4	89.1	95.2	86.1	92.1	93.9	91.5	89.1	90.9	99.4	88.5	90.3	88.5	89.7	90.3	89.7
98.8	88.5	89.1	99.4	89.1	95.2	86.1	92.1	93.9	91.5	89.1	90.9	99.4	88.5	90.3	88.5	89.7	90.3	89.7
88.5	91.5	93.3	88.5	92.1	87.3	93.3	89.1	95.2	90.9	95.2	91.5	88.5	92.7	92.7	92.7	92.7	96.4	91.5
89.1	96.4	97.0	89.1	95.8	89.7	89.7	91.5	95.8	94.5	91.5	95.8	89.1	95.2	96.4	93.9	97.0	93.3	96.4
92.1	91.5	92.1	92.1	90.9	90.3	91.5	89.7	94.5	90.9	92.1	90.3	92.1	91.5	91.5	90.3	93.9	95.8	90.3
92.7	93.3	94.5	92.7	94.5	89.7	90.3	90.3	99.4	95.2	92.7	96.4	92.7	93.9	95.8	93.9	95.2	94.5	96.4
90.9	97.0	97.6	90.9	96.4	90.3	89.1	90.9	97.6	95.2	92.1	98.2	90.9	95.8	98.8	94.5	95.8	93.9	98.8
89.1	95.2	94.5	89.1	95.8	90.9	88.5	91.5	95.8	94.5	91.5	93.9	89.1	95.2	94.5	95.2	96.4	93.3	95.2
86.7	92.7	92.1	86.7	92.1	87.3	93.3	89.1	93.3	90.9	95.2	90.3	86.7	92.7	91.5	92.7	94.5	96.4	91.5
86.1	89.1	87.9	86.1	89.7	87.9	87.9	89.7	89.7	89.7	89.7	87.9	87.3	88.5	90.3	89.1	90.3	89.7	89.1
93.3	94.5	95.2	93.3	95.2	90.3	90.3	90.9	100.0	96.4	93.3	95.8	93.3	94.5	96.4	94.5	95.8	95.2	95.8
90.9	89.7	90.9	90.9	90.9	87.9	94.5	89.7	95.8	91.5	93.3	93.3	90.9	90.3	92.7	90.3	91.5	94.5	92.1
85.5	87.9	89.1	85.5	87.9	84.8	100.0	86.7	90.3	86.1	92.1	89.1	85.5	88.5	88.5	88.5	90.9	93.3	87.9
95.8	88.5	87.9	96.4	89.1	95.2	84.8	92.1	90.9	90.3	87.9	89.7	96.4	88.5	89.1	88.5	89.7	89.1	89.7
98.8	88.5	89.1	99.4	89.1	95.2	86.1	92.1	93.9	91.5	89.1	90.9	99.4	88.5	90.3	88.5	89.7	90.3	89.7
89.1	98.2	97.0	89.1	95.8	90.9	89.7	91.5	95.8	94.5	91.5	96.4	89.1	95.2	97.0	95.2	97.6	93.3	97.6
87.3	93.3	92.7	87.3	93.9	89.1	87.9	90.9	93.9	92.7	90.9	92.1	86.7	93.3	92.7	93.3	94.5	92.1	93.3
[redacted]	87.9	88.5	98.2	88.5	93.9	85.5	91.5	93.3	90.9	88.5	90.3	98.2	87.9	89.7	87.9	89.1	89.7	89.1
10.9	[redacted]	95.8	87.9	94.5	89.7	87.9	90.3	94.5	93.3	90.3	94.5	87.9	93.9	95.8	93.9	97.6	92.1	95.8
11.5	4.2	[redacted]	88.5	95.2	89.1	89.1	89.7	95.2	92.7	90.9	95.8	88.5	95.8	96.4	93.3	94.5	93.9	96.4
1.8	10.9	11.5	[redacted]	88.5	94.5	85.5	91.5	93.3	90.9	88.5	90.3	98.8	87.9	89.7	87.3	89.1	89.7	89.1
8.2	1.9	1.3	8.2	[redacted]	90.3	87.9	90.9	95.2	93.9	90.9	94.5	88.5	95.8	95.2	94.5	95.8	92.7	95.8
2.5	6.9	7.5	1.9	6.3	[redacted]	84.8	93.3	90.3	90.3	87.9	89.7	94.5	89.7	89.1	89.7	90.9	89.1	89.7
14.5	10.9	10.3	14.5	8.8	11.9	[redacted]	86.7	90.3	86.1	92.1	89.1	85.5	88.5	88.5	88.5	90.9	93.3	87.9
5.0	6.3	6.9	5.0	5.7	3.1	10.1	[redacted]	90.9	90.9	89.7	90.3	91.5	90.3	89.7	90.3	91.5	90.9	90.3
6.7	4.2	4.8	6.7	1.3	6.3	9.7	5.7	[redacted]	96.4	93.3	95.8	93.3	94.5	96.4	94.5	95.8	95.2	95.8
7.4	4.3	5.6	7.4	2.5	6.3	11.1	5.7	1.9	[redacted]	89.7	92.7	90.9	93.3	93.9	93.3	95.8	91.5	93.9
11.5	8.5	9.1	11.5	5.7	8.8	7.9	6.9	6.7	8.0	[redacted]	90.3	88.5	90.3	91.5	91.5	92.7	95.2	90.3
9.7	4.8	4.2	9.7	1.9	6.9	10.3	6.3	4.2	4.9	9.1	[redacted]	90.3	93.9	97.0	92.7	93.9	92.1	98.2
1.8	10.9	11.5	1.2	8.2	1.9	14.5	5.0	6.7	7.4	11.5	9.7	[redacted]	87.9	89.7	87.9	89.1	89.7	89.1
8.8	2.5	0.6	8.8	0.6	6.9	8.2	6.3	1.9	3.1	6.3	2.5	8.8	[redacted]	94.5	93.9	95.2	93.3	95.2
10.3	4.2	3.6	10.3	1.3	7.5	10.9	6.9	3.6	4.3	7.9	3.0	10.3	1.9	[redacted]	93.3	94.5	93.3	97.6
8.8	2.5	3.1	8.8	1.9	6.9	8.2	6.3	1.9	3.1	5.0	3.8	8.8	2.5	3.1	[redacted]	95.2	92.1	93.9
9.7	2.4	4.2	9.7	0.6	5.7	9.1	5.0	3.0	2.5	6.7	4.8	9.7	1.3	4.2	1.3	[redacted]	94.5	95.2
10.3	6.7	6.1	10.3	3.8	7.5	6.7	5.7	4.8	6.2	4.8	7.3	10.3	3.1	6.1	4.4	4.8	[redacted]	92.1
10.3	3.6	3.6	10.3	0.6	6.9	10.9	6.3	3.6	3.7	8.5	1.8	10.3	1.3	2.4	2.5	3.6	6.7	[redacted]
8.2	1.9	2.5	8.2	1.3	6.3	8.2	5.7	1.3	2.5	5.7	1.9	8.2	1.9	2.5	1.9	0.6	3.8	0.6
10.9	4.2	3.0	10.9	1.3	7.5	10.3	6.9	4.2	4.3	9.1	2.4	10.9	0.6	3.0	3.1	4.2	6.1	1.2
9.1	3.0	3.6	9.1	1.3	6.3	9.7	5.7	2.4	3.1	7.3	1.8	9.1	1.9	2.4	1.9	3.0	5.5	1.2
10.3	16.4	15.8	10.9	13.8	8.8	19.4	10.7	14.5	13.6	16.4	15.2	10.9	14.5	14.5	13.8	13.9	15.2	15.8
8.2	1.9	2.5	8.2	1.3	6.3	8.8	5.7	1.3	2.5	5.7	3.1	8.2	1.9	2.5	1.9	0.6	3.8	1.9
19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37

Percent Divergence

38	39	40	41	42		
92.1	90.9	92.1	83.6	92.1	1	10-91-1.SEQ
89.1	89.1	91.5	89.1	89.1	2	120-88-1.SEQ
89.1	89.1	91.5	89.1	89.1	3	132-90-1.SEQ
92.1	92.1	93.3	80.6	92.1	4	13-91-1.SEQ
94.5	95.8	96.4	84.8	94.5	5	14-95-1.SEQ
90.9	90.9	92.1	84.2	90.9	6	155-88-1.SEQ
95.8	95.8	98.2	83.6	94.5	7	15-95-1.SEQ
95.2	98.2	98.8	84.2	95.2	8	172-96-1.SEQ
95.8	94.5	95.8	82.4	95.8	9	173-96-1.SEQ
92.1	92.1	92.1	80.6	92.1	10	17-91-1.SEQ
89.7	87.9	89.7	79.4	89.7	11	17-93-1.SEQ
95.2	95.2	97.6	84.2	95.2	12	1-95-1.SEQ
91.5	91.5	93.9	82.4	90.9	13	19-89-1.SEQ
88.5	88.5	89.7	78.8	87.9	14	21-89-1.SEQ
89.1	89.1	90.3	87.9	89.1	15	22-88-1.SEQ
89.1	89.1	91.5	89.1	89.1	16	267-88-1.SEQ
95.8	97.6	98.2	82.4	95.8	17	26-95-1.SEQ
93.9	92.7	93.9	81.8	93.9	18	26-97-1.SEQ
88.5	88.5	90.9	89.1	88.5	19	293-94-1.SEQ
93.9	95.2	96.4	81.2	94.5	20	29-95-1.SEQ
93.9	97.0	96.4	83.0	93.9	21	316-94-1.SEQ
88.5	88.5	90.9	87.9	88.5	22	33-96-1.SEQ
95.2	95.2	95.2	81.8	95.2	23	40-95-1.SEQ
90.3	89.1	90.3	87.3	90.3	24	43-96-1.SEQ
88.5	88.5	89.7	78.8	87.9	25	45-89-1.SEQ
90.9	89.7	90.9	85.5	90.9	26	46-87-1.SEQ
95.2	95.2	97.6	84.2	95.2	27	4-95-1.SEQ
93.3	93.3	94.5	83.6	93.9	28	50-95-1.SEQ
90.9	89.7	92.1	81.2	90.9	29	53-93-1.SEQ
94.5	97.6	98.2	83.6	93.3	30	57-95-1.SEQ
88.5	88.5	90.9	87.9	88.5	31	63-72-1.SEQ
94.5	95.8	94.5	81.2	94.5	32	6-95-1.SEQ
93.9	97.0	97.6	84.8	93.9	33	71-96-1.SEQ
94.5	93.3	94.5	81.8	94.5	34	72-96-1.SEQ
95.8	94.5	95.8	84.8	95.8	35	73-96-1.SEQ
92.7	92.7	93.9	83.0	92.7	36	75-86-1.SEQ
95.8	98.8	98.8	82.4	94.5	37	80-96-1.SEQ
[redacted]	95.2	96.4	81.8	95.2	38	81-96-1.SEQ
1.3	[redacted]	98.2	81.8	93.9	39	82-96-1.SEQ
0.0	1.8	[redacted]	84.2	95.2	40	89-96-1.SEQ
13.8	16.4	14.5	[redacted]	82.4	41	PAK1-5-1.SEQ
1.3	2.5	1.3	13.8	[redacted]	42	70-96-1.SEQ
38	39	40	41	42		

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