STUDIES ON THE ANTIGENIC SITES OF FOOT-AND-MOUTH DISEASE VIRUS SEROTYPE ASIA–1

Thesis

SUBMITTED IN PARTIAL FULFILMENT OF THE
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BY

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Roll No 502

TO

DEEMED UNIVERSITY

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2000

Dedicated

To My

Father And Mother

Central Laboratory
All India Coordinated Research Project On Foot-And-Mouth disease
Indian Veterinary Research Institute,
Mukteswar-Kumaon, Distt.Nainital 263138,(U.P)

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Dated: 12/09/ *2000*

CERTIFICATE

Certified that the research work embodied in the Thesis titled "STUDIES ON THE ANTIGENIC SITES OF FOOT-AND-MOUTH DISEASE VIRUS SEROTYPE ASIA-1" submitted by **Shri Gurumurthy, C.B., Roll No. 502,** for the award of **Doctor of Philosophy 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 28 months in the Institute and has put in more than 200 days attendance under me from the date of registration for the **Doctor of Philosophy Degree** of this University, as required under the relavent ordinance.

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CERTIFICATE

Certified that the Thesis titled ""**STUDIES ON THE ANTIGENIC SITES OF FOOT-AND-MOUTH DISEASE VIRUS SEROTYPE ASIA-1**", submitted by Shri **Gurumurthy,C.B., Roll No.502,** in partial fulfilment of **Doctor of Philosophy 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 **Doctor of Philosophy Degree** of this Institute.

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

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[Gurumurthy, C. B.]

ABBREVIATIONS

AMV	:	Avian Myeloblastosis Virus
BHK	:	Bovine Hamster Kidney
CPE	:	Cytopathic Effect
DMSO	:	Dimethyl Sulfoxide
DTT	:	Dithiothreitol
dNTP	:	Deoxynucleotide triphosphate
ddNTP	:	Dideoxynucleotide triphosphate
dUMP	:	DeoxyUracil Monophosphate
DNA	:	Deoxyribonucleic Acid
E.coli	:	*Escherichia coli*
ELISA	:	Enzyme Linked Immunosorbent Assay
FMDV	:	foot-and-mouth disease virus
GMM	:	Glasgow's Minimum Essential Medium
GMEM	:	Glasgow's Modification of Minimum Essential Medium
HRPO	:	Horse Radish Peroxidase
IPTG	:	Isopropyl Thio Galactopyranoside
kb	:	Kilobase
m	:	minute
Mabs	:	Monoclonal Antibodies
MAR	:	Monoclonal Antibody-Resistant
MNT	:	Microneutralization Test
µg	:	microgram
nm	:	Nanometer
ng	:	nanogram
OD	:	Optical Density
OPD	:	Orthophenylene Diamine
PBS	:	Phosphate Buffered Saline
RNA	:	Ribonucleic Acid
rpm	:	Revolutions per minute
RT-PCR Reaction	:	Reverse Transcription-Polymerase Chain
RT	:	Reverse Transcriptase
S	:	Swedberg Unit
s	:	second
TAE	:	Tris-Acetate Ethylene Diamine Tetra Acetic acid
TBE	:	Tris-Borate Ethylene Diamine Tetra Acetic acid
$TCID_{50}$	:	Tissue Culture Infective Dose 50
TE	:	Tris- Ethylene Diamine Tetra Acetic acid
UDG	:	Uracil DNA glycosylase
UV	:	Ultra-Violet

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INTRODUCTION

1. INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals characterized by the formation of vesicles on the tongue, lips, gums and in the inter-digital space. The disease affects domestic livestock like cattle, buffaloes, sheep, goats and pigs, it also affects many wild ungulates. Due to its remarkable speed of spread within susceptible populations, the almost 100% morbidity, total loss of production in affected and recovered animals and trade embargo associated with this disease, FMD remains one of the most feared animal diseases. While countries free of the disease impose strict import and trade regulations to ensure the safety of their livestock industry, endemic countries take all measures to reduce its incidence.

The causative virus is classified under family picornaviridae, genus aphthovirus (Francki *et al.*, 1991). It occurs as seven serotypes viz., O, A, C, Asia-1, South African Territories (SAT) 1, SAT2 and SAT3 and over 65 subtypes (Pereira, 1977). The virus is icosahedral in shape and is nonenveloped. It is 30 nm in diameter and has a molecular weight of 8.3 to 8.9 x 10^6 daltons. The intact virus particle has a sedimentation co-efficient of 146S. Its genome consists of a positive sense, single stranded RNA molecule about 8500 nucleotides long (Forss *et al.*, 1984), with a small protein (VPg) attached to its 5' end and a poly (A) tract at the 3' terminus. The 5' untranslated region is very long (nearly 1,200 bases) and includes a poly (C) tract whose exact function is not known. The genome is translated into a single polyprotein chain, which is subsequently cleaved into four structural and ten non-structural proteins (Fig.1). The four structural proteins VP1, VP2, VP3 and VP4 are coded by the P1 or capsid coding region where these genes are arranged in the order VP4, VP2, VP3 and VP1. Each of these four proteins together make up a protein subunit or protomer. Five protomers join together to form a pentamer, and a full capsid is an assembly of 12 of these pentamers (Fig. 2). X-ray diffraction (Acharya *et al.*, 1989)

Fig. 1 Aphthovirus Genome and polyprotein processing

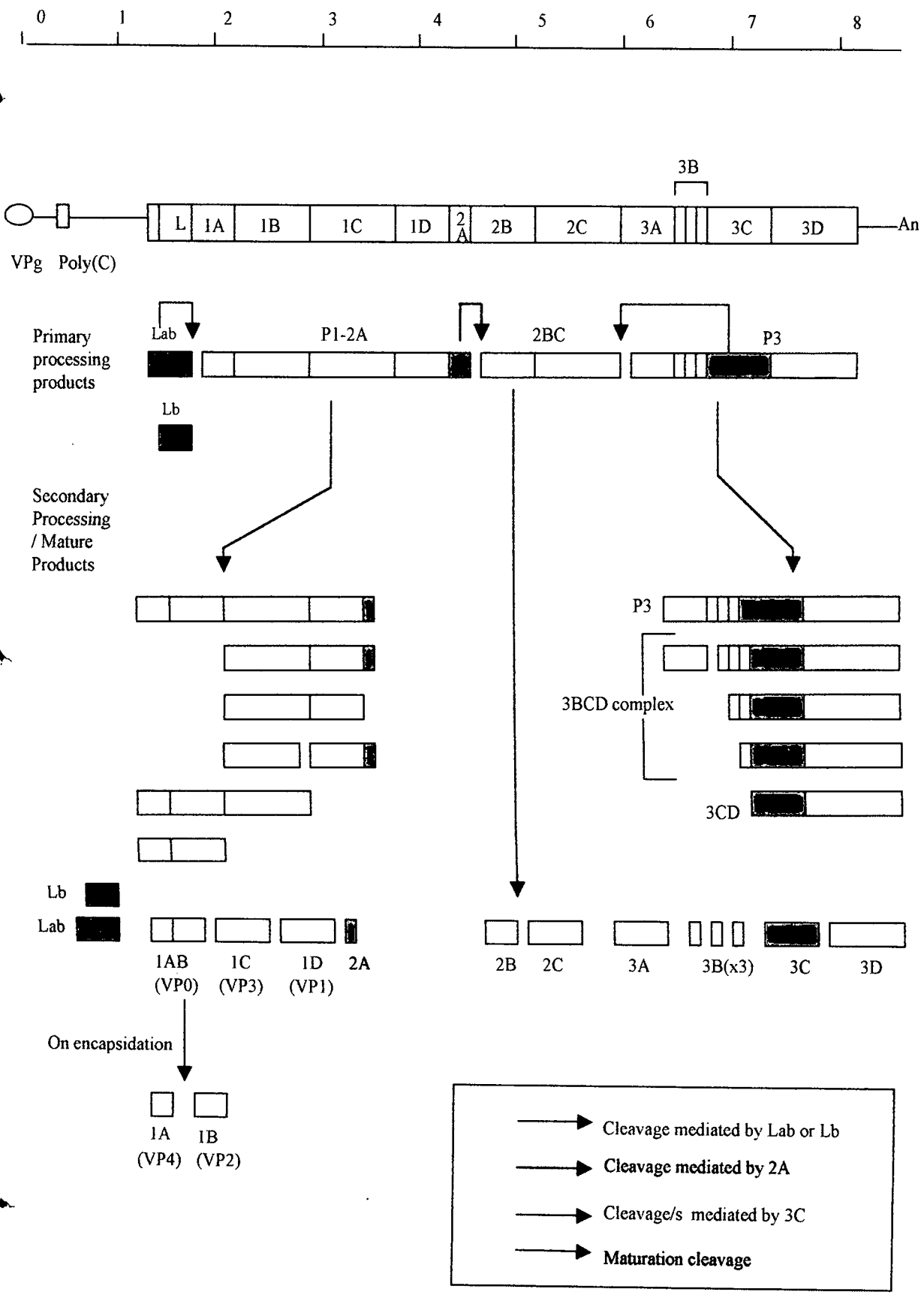
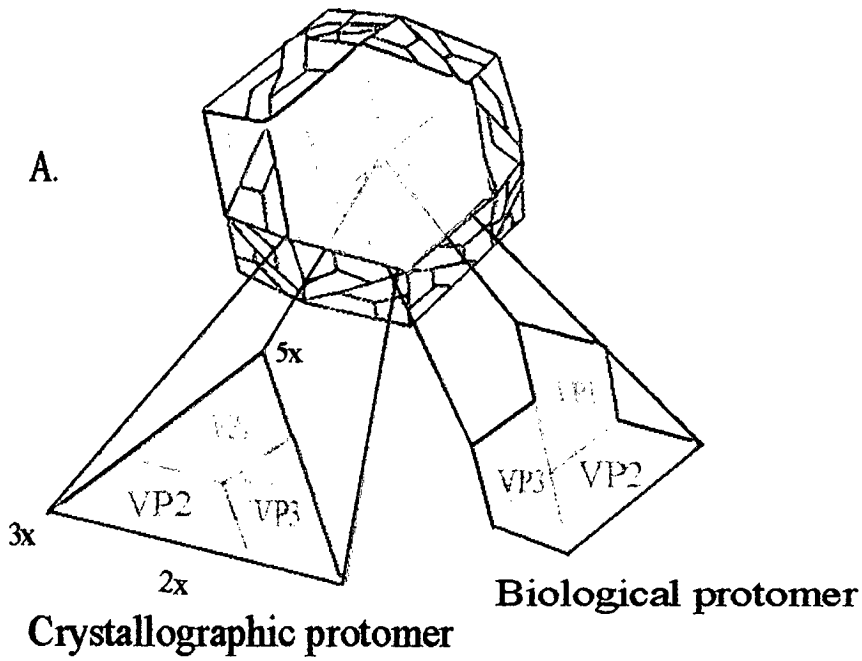
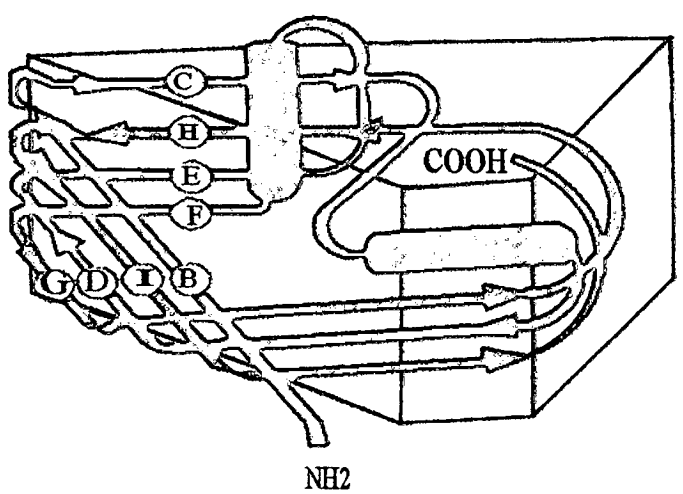


Fig.2. Schematic diagram of FMDV capsid



B. Schematic diagram showing beta barrel structure of FMDV capsid proteins VP1, VP2 and VP3



and immunological studies have shown that VP1, VP2 and VP3 have surface components, while VP4 is entirely internal. VP1 has been shown to be antigenically most important of the four structural proteins.

At present, FMD does not exist in North America, Central America, Australia, New Zealand and Japan. Although most of Western Europe has been successful in eradicating the disease, it is still endemic in Africa, most of South America and Asia, including India. In countries free of FMD, the effective means of controlling spread of the disease 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). Whatever be the control strategy, its efficient implementation is constrained by the inherent antigenic diversity of the causative virus.

Of the seven serotypes, O, A, and C are the most commonly isolated and are found in South America, parts of Africa, the Middle East and Asia. The Asia1 serotype occurs mainly in the Far East and in the Indian Subcontinent. The South African Territories serotypes SAT 1, 2 and 3 are primarily restricted to the African continent, apart from sporadic outbreaks of SAT 1 and 2 in the Middle East. In India, outbreaks of FMD are attributed to serotypes O, Asia 1, A and C. The disease occurs year-round and in all parts of the country. The work carried out by Indian Council of Agricultural Research, through All India Coordinated Research Project for Epidemiological Studies on Foot and Mouth disease indicates that type O is the most prevalent of FMDV serotypes in India and is responsible for more than 88% of outbreaks during the last 5 years. After O, the disease due to serotype Asia1 comes next in the rate of incidence. During the last 5 years Asia1 accounted for the large number of outbreaks in Western India accounting for about 26% followed by 3.7% in Southern India, 1.5% in Eastern India and 0.6% in Northern India. Disease due to type A was only 3.5% of the total outbreaks recorded in the country between 1995 and

1999, whereas type C is the least prevalent and the disease due to type C has not been recorded in the country since 1995.

Viruses with RNA genomes, like FMDV, exhibit very high mutation rates due to lack of proof reading by their replicative enzymes, the RNA polymerases (Weddell *et al.*, 1985). These viruses are thought to exist in nature as a "quasi-species'' which is defined as dynamic genetic organization of a virus population, consisting of complex mixtures of related, non-identical genomes (Domingo *et al.*, 1992). The high genetic diversity exhibited by viral quasi-species allows viable mutant viruses to survive almost any selection pressure, whether *in vivo* or *in vitro* (Holland *et al.*, 1992). The capsid proteins, in naked viruses like FMDV, which are directly involved in neutralization by the host antibodies, too are subject to a variety of selection pressures and show a range of antigenic reactivities. That such a high degree of antigenic variation imposes constraints in efficient disease control is well documented (Domingo, 1989). Hence it is important that the extent of genetic and antigenic diversification attained by these viruses during their circulation in the field be understood to facilitate formulation of strategies for disease control. For this purpose, it is necessary that the antigenic make-up of a representative virus, with which other field isolates can be compared, be known.

Among the various approaches towards this end, nucleic acid sequencing of capsid coding genes of viruses that differ in their antigenic features is one of the widely used methods to map the antigenic sites on the virus surface. Such studies involve i) Subjecting a representative virus to selection pressures in the form of neutralizing monoclonal antibodies (raised against it) directed against individual epitopes; (ii) isolating those viruses which are able to escape neutralization; (iii) sequencing the capsid coding genes of these and the parent virus in order to identify amino acid changes that enabled escape from neutralization. If it is possible to use monoclonal antibodies directed against all possible epitopes on the virus, and also to

isolate all possible neutralization resistant mutants, theoretically, by sequencing the capsid coding region of the parent virus and mutants, it should be possible to pinpoint all the antigenic sites.

Studies of a similar nature, using neutralizing monoclonal antibodies and Mab resistant mutants (MAR mutants) have been carried out extensively in the case of FMDV serotypes O, A & C (Bolwell *et al.*, 1989, Pfaff *et* al., 1988, Barnett *et al.*, 1989, Baxt *et al.*, 1989, Mateu *et al.*, 1990, Xie *et al.*, 1987, Thomas *et al.*, 1988a), and several antigenic sites have been identified in these viruses. Little work (Sanyal *et al.*,1997, Butchiah and Morgan, 1997, Marquardt *et al.*, 2000) has been done on the antigenic sites of FMDV type Asia 1 and not much information is available on them.

This work tittled **"Studies on the antigenic sites of Foot-and-Mouth disease virus serotype Asia-1 "** is undertaken with the following objective :

To identify the antigenic sites of Foot-and-Mouth disease virus serotype Asia-1 so that the field isolates can be compared and variations taking place in the capsid coding region at the level of different antigenic sites can be identified precisely. This will be helpful in identifying the dominant strains suitable for inclusion in the vaccine.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

FMD is an acute and highly contagious disease of domestic and wild cloven-hooved animals. Due to its ability to remarkably reduce productivity and adversely affect trade in animals and animal products it is considered the most important disease of farm animals. Disease control is greatly influenced by many factors like the high contagiousness of the disease, its wide geographical distribution and host spectrum, protracted convalescence and carrier status, plurality of antigenic forms of the causative virus and the relatively short duration of immunity after infection or vaccination.

2.1 THE VIRUS AND ITS PROPERTIES

Foot-and-mouth disease virus is classified under family picornaviridae, genus apthovirus (Francki *et al.*, 1991). The virus is an icosahedron, having a diameter of about 30nm and a sedimentation coefficient of 146S; it is unstable at acidic pH. The virus occurs as seven immunologically distinct serotypes viz., O, A, C, Asia1, SAT1, SAT2 and SAT 3 and over 65 subtypes (Pereira , 1977).

2.1.1 Viral Genome and Proteins

The FMDV genome consists of a single-stranded, positive sense RNA of about 8.5 Kb length with a small protein (VPg) linked covalently to its 5' end. This protein is followed by a non-coding region (NCR) of about 1250 bases which is composed of an S (small) fragment, 400 bases long, a polycytidylic acid (poly-C) tract of 80-200 nucleotides, and a 750 nucleotide L (large) fragment containing the internal ribosome entry site. The single open reading frame follows the 5' NCR and codes for a polyprotein of about 2332 amino acid residues. Another NCR of 92 bases is found at the 3' end of the coding region followed by a poly-A tract of about 100 bases.

The single monocistronic open reading frame codes for a polyprotein that gets cleaved post-translationally into four structural and ten non-structural proteins. The non-structural leader protein (L) which is towards the N-terminus of the polyprotein gets cleaved co-translationally and the remaining polyprotein undergoes primary cleavage to give P1, P2 and P3 polyproteins (Fig. 1). Upon further cleavage, P1 gives four structural proteins (VP1, VP2, VP3 and VP4) and P2 and P3 regions give rise to non-structural proteins (2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C and 3D). The proteins VP1, VP2, VP3 and VP4 form the viral capsid, whereas the non-structural proteins are implicated in viral RNA replication and polyprotein processing, though the precise functions of 2B and 3A are not known.

2.1.2 CAPSID STRUCTURE

Antigenic characteristics of foot and mouth disease virus is determined by the structure of its capsid which is formed of sixty copies each of the four capsid proteins, VP1, VP2, VP3 and VP4. X-ray diffraction (Acharya *et al.*, 1989) and immunological studies have shown that VP1, VP2 and VP3 have surface components, while VP4 is entirely internal. VP1 has been shown to be the most antigenically important and the most exposed protein (Strohmaier *et al.*, 1982); loss of integrity of this protein results in a drastic reduction in infectivity as well as immunizing activity (Wild *et al.*, 1969). Proteins VP2 and VP3 also contribute to antigenicity but to a lesser extent. All the four proteins are coded by the capsid coding or the P1 region composed of 2196-2199 nucleotides (VP4 gene consists of 255 bases, the protein has 85 amino acids ; VP2, 654 bases and 218 amino acids; VP3, 657 bases and 219 amino acids and VP1 with 630 or 633 bases and 210 or 211 amino acids respectively in serotype Asia-1) and situated towards the 5' end of the FMDV genome, next to the leader protein gene.

A common folding pattern known as a" beta-barrel" or " jelly roll" is found with proteins VP1, VP2 and VP3 and essentially consists of a wedge-shaped core of

eight strands of amino acids arranged to form a sandwich of two 4 stranded β- sheets (Fig.2). The intervening loops connect each strand and are identified by the β- strands they connect ie., the G-H loop connects the β-G and β-H sheets. The VP1 proteins are found around the five fold axis of symmetry whilst VP2 and VP3 are around the two and three fold axes of symmetry. The VP4 protein, effectively an N-terminal extension of VP2, does not have residues exposed on the virion surface. X-ray crystallographic studies (Acharya *et al.*, 1989 and Lea *et al.*, 1994) have shown that FMDV has a virion structure similar to that of the other picornaviruses (Rossmann *et al.*, 1985, Hogle *et al.*, 1985, Luo *et al.*, 1987) though its capsid proteins are shorter due to the presence of shorter connecting loops. The surface of the FMDV virion is relatively smooth and does not contain the canyon (as in Rhinoviruses and Enteroviruses) or pits (as in Cardioviruses) around the five-fold axis of symmetry.

2.2 ANTIGENIC VARIATION IN FMD VIRUS

Valee and Carre in 1922 recognized 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 was the first instance of demonstration of antigenic differences between strains of any animal virus. The two serotypes were labeled 'O' (Oise) and 'A' (Allemagne). In 1926, Waldmann and Trautwein reported serotype 'C'. In 1948, at the 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 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 programs. Pringle (1964) reported that 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 selection and size of infecting inocula are important factors in the rate of evolution of FMD virus (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).

Rates of mutation for FMD viral capsid protein genes were found to be six fold higher than for non-structural genes (Sobrino *et al.*, 1986). They also observed genetic heterogeneity among three viruses isolated on the same day. It has been suggested that persistent inapparent infections of ruminants may promote the rapid selection of antigenically variant viruses (Gebauer *et al.*, 1988). Nucleotide and amino acid sequence analysis of VP1 showed that serologically related viruses differ

less in the 143-150 region compared to immunologically distinct viruses (Cheung *et al.*, 1984).

2.3 Methods for Studying Antigenic Variation

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 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 studies 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 complement fixation test, 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.

2.3.1 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, 1982, 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 relevent to protection. This test has also been criticized for its lack of sensitivity and specificity (Rweyemamu et al., 1978, Pay, 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-dimensional 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 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 convalescent sera for characterization of type A FMD virus isolates and the results tallied with conventional virus neutralization test (Samuel and Kitching, 1987).

2.3.2 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 also 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 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.

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.

2.4 ANTIGENIC SITES & METHODS FOR SITE-ANALYSIS

The structure of the capsid proteins determines the antigenic characteristics of FMDV and is responsible for the antigenic differences seen between the seven FMDV serotypes. Not all residues and/or portions of the virus contribute equally to its antigenicity: rather regions (or residues) called "antigenic sites" are found to predominantly elicit an immune response. An antigenic site may be defined as those parts of the capsid or envelope proteins that are specifically recognized by the binding sites or paratopes of antibody molecules (Van Regenmortel, 1990). In a virus like FMDV, the antigenic sites have been found to be distributed over the three capsid proteins, VP1, VP2 and VP3, comprising of overlapping and non-overlapping, conformation-dependent and conformation-independent epitopes.

A lot of work has been done on the antigenic sites of FMDV serotypes O, A and C (Bolwell *et al.*, 1989, Pfaff *et* al., 1988, Barnett *et al.*, 1989, Baxt *et al.*, 1989, Mateu *et al.*, 1990, Xie *et al.*, 1987, Thomas *et al.*, 1988a & b). Several tools and methods have been used for antigenic site-analysis like studies using overlapping and/or non-overlapping synthetic peptides made from known amino acid sequence of proteins considered immunodominant, anti-peptide sera, trypsin treated viruses, 12S and 146S particles, monoclonal antibodies raised against intact FMDV and subunit proteins and most importantly, studies using monoclonal antibody resistant mutants (Xie *et al.*, 1987, Bolwell *et al.*, 1989, Pfaff *et* al., 1988, Thomas *et al.*, 1988 a & b, Barnett *et al.*, 1989, Baxt *et al.*, 1989, Mateu *et al.*, 1990). The advent of monoclonal

antibodies, which recognize specific epitopes, almost revolutionized the way antigenic sites were studied. A typical study is as follows: i) Subjecting a representative virus to selection pressures in the form of neutralizing monoclonal antibodies (raised against it) directed against individual epitopes; (ii) isolating those viruses which are able to escape neutralization; (iii) sequencing the capsid coding genes of these and the parent virus in order to identify amino acid changes that enabled escape from neutralization. If it is possible to use monoclonal antibodies directed against all possible epitopes on the virus, and also to isolate all possible neutralization resistant mutants, theoretically, by sequencing the capsid coding region of the parent virus and mutants, it should be possible to pinpoint all the antigenic sites. Many such studies have been done with viruses of serotype O, A and C.

2.5 ANTIGENIC SITES ON FMDV

a) Serotype O

Meloen and co-workers (1979) found that of the four capsid proteins VP1 alone is located on the outer surface of the virus and hence plays an important role in the antigenicity of the virus. This was also indicated by the fact that trypsin treatment of the virus results in cleavage of only VP1, with concomitant decrease in infectivity and, depending on the virus strain, a loss of immunizing activity (Wild *et al.*, 1969). In another study, Meloen *et al.* (1983) raised neutralizing monoclonal antibodies (Mabs) against intact FMDV serotype O_1 and found that they reacted with intact virus and trypsin treated particles. They also found that some of the Mabs showed a slight but definite reaction with the 12S subunit, but none of them reacted with isolated VP1 or other viral proteins indicating that the neutralizing antigenic determinants exposed on intact virus particle are different from that on isolated VP1 protein.

Ouldridge and co-workers (1984), isolated monoclonal antibodies against the trypsin-sensitive site on the 140S particle of FMDV O_1BFS and used them to probe

the structure of this site. They were able to identify the presence of at least three distinct epitopes within this site: all of which appeared to be absent in the 12S particles and one of the neutralizing epitope was sensitive to even mild configurational changes of the particle.

Stave and co-workers (1986) produced Mabs against inactivated, purified FMDV type O_1 Brugge (140S) and 12S subunits and tested each Mab for its ability to bind to 140S, 12S and purified VP1 by radioimmunoassay (RIA) and to neutralize viral infectivity in mouse protection assays. They found that Mabs that reacted only with 12S subunits in RIA did not neutralize infectious virus. Using synthetic peptides, the binding site of a Mab which reacted with 140S, 12S and purified VP1 and also neutralized infectious virus, was localized between residues 135 and 172 of VP1. In addition, they also identified a Mab whose binding to 140S and 12S was conformation-dependent.

Xie *et al.* (1987) used seven neutralizing Mabs to characterize 30 escape mutants of type O_1 Kaufbeuren and identified three non-overlapping antigenic sites, within two of which, sites for two or more Mabs overlapped. Their studies revealed that two of these sites were conformation-dependent, while the third was not. On sequencing of the VP1 genes of 10 MAR mutants and three parent viruses, they localized the sites to be in three regions: a) the region involving residues 144 to 154 of VP1, b) the region including residue 208 from the C-terminus of VP1 and c) unidentified residues in VP2 or VP3 proteins.

Pfaff *et al.* (1988) characterized neutralizing Mabs against complete virus by Western blotting, enzyme immunoassay and competition experiments with a synthetic peptide, isolated coat protein VP1 and viral particles as antigens. They found that two of the four Mabs reacted with each of these antigens, while the other Mabs recognized only complete viral particles and reacted poorly with the peptide. They also compared the cDNA derived VP1 protein sequences of different viruses that showed different

neutralization patterns with the four Mabs and found that the first two Mabs recognized overlapping sequential epitopes in the previously identified major antigenic site, a hexadecapeptide between amino acids (aa) 144 and 159 in VP1 protein which is able to induce neutralizing antibodies in animals, whereas the other two recognized conformational epitopes. This group showed for the first time that structural proteins other than VP1 are also involved in neutralization of FMDV.

Barnett and co-workers (1989) raised and characterized eleven monoclonal antibodies against FMDV O_1 BFS for their ability to bind viral and subviral antigens in different ELISA tests and to neutralize heterologous type O isolates. They also raised Mab resistant (MAR) mutants using five of the Mabs and used them in cross-neutralization tests with all the eleven Mabs and thus identified three functionally independent, conformational, neutralizing sites, the latter of which appeared to be immunodominant. Isoelectrofocussing and sequencing studies of the mutants strongly suggested that protein VP2 also contributes to the immunodominant site.

Parry and co-workers (1989) used inhibition ELISA techniques along with peptide antigens and antipeptide sera to block Mab binding to virus particles so as to identify those portions of VP1 that they bound to, since the four Mabs that recognized three functionally independent, conformational sites did not react with immobilized capsid proteins or peptides. Their work brought to light several important facts: i) one Mab-binding site had components within regions 146 to 150 and 200 to 213 of VP1 with a critical involvement of the amino acids at positions 146 and 206 or 207 ii) the other site identified, to which two of the four Mabs bound, non-identically, comprised of residues 200 to 213 and 143 to 146 regions with aa 143 and 144 critical for inhibition of virus binding, iii) The fourth Mab was found to bind to residues 160 to 180 and 200-213 of VP1 and iv) immunogenic tracts of VP1 which are physically distant in the primary sequence are brought into proximity in the quaternary structure of the virion to form an antigenic domain containing several conformational epitopes, some of which are functionally independent.

McCahon *et al.* (1989) tested the mutants raised by Xie *et al.* (1987) in their reactivity with Mabs produced against type O viruses in different laboratories and then used those Mabs that reacted, to raise single and multiple mutants. By characterization of the single and multiple mutants they were able to show the existence of a fourth antigenic site. Though they had two more Mabs that still neutralized all the mutants, they were not successful in isolating resistant mutants to them. Also, since all the mutants reacted well with polyclonal bovine sera, they suggested that further sites are involved in virus neutralization and would need to be modified to abolish polyclonal reactivity.

Kitson *et al.* (1990) sequenced the previously described MAR mutants (Xie *et al.*, 1987) and reported the presence of distinct clusters of amino acid substitutions conferring resistance to neutralization at each of the previously defined antigenic sites (Xie *et al.*, 1987, McCahon *et al.*, 1989). Amino acid positions that were altered in the MAR mutants sequenced were as follows: a) In site 1MAR mutants: VP1 144, 148, 154, 152, 171 and 208 ; of these, changes at 152 and 171 were antigenically silent where as changes at 148 resulted in complete resistance to neutralization and none of these changes affected the ability of Mabs recognizing sites 2, 3, or 4 to neutralize these viruses. b) In site 2 MAR mutants: substitutions were found in VP2 at positions 70, 71, 72, 73, 75, 77 and 131; different patterns of substitutions were observed involving one or more residues in mutants against each of the four site 2 Mabs. Substitutions at positions 73 or 75 were found to confer resistance to all four Mabs, while those at other positions gave variable resistance to the four site 2 Mabs. So also, they found that different amino acid substitutions at the same position resulted in different antigenic phenotypes. c) In site 3 MAR mutants: Amino acids substitutions at positions 43 and 44 in VP1 was found to confer resistance to the two site 3 Mabs. d) In site 4 MAR mutants: Residue 58 of VP3 was found to be substituted. Multiple mutants analysed were found to have accumulated mutations which had been identified separately as conferring resistance at individual antigenic sites. The same group also mapped these antigenic sites on the 3D-structure obtained for FMDV

O_1BFS 1860 by X-ray crystallography (Acharya *et al.*, 1989): site 1 on the VP1 βG-βH loop, site 2 on the VP2 β B- β C loop at the 3-fold axis (also involving VP2 131 on the adjacent β E- β B loop), site 3 on the VP1 β B- β C loop at the 5-fold axis, and site 4 on the top of the insertion in VP3 β B sheet.

Krebs *et al.* (1993) raised antiserum to a peptide corresponding to the 135-154 (βG - βH loop) sequence of the FMDV O_1Kaufbeuren in a pig, and though the serum contained neutralizing antibodies, the pig showed clinical signs after challenge and the mutant virus isolated from it had changes at positions 50, 198 and 211 of VP1 and position 209 of VP2. This mutant and a plaque isolate of it which differed from the challenge virus at positions 198 on VP1 and 209 on VP2 was also found to resist neutralization by anti-peptide serum *in vitro,* though it had no sequence changes at the region corresponding to the peptide. The same was observed in the case of a virus related to O_1K isolated from cattle, which had substitutions only at positions 43 and 101 on VP1. Since in both cases the βG- βH loop itself had not changed, they suggested that the relevant epitopes may have become inaccessible to antibodies due to the substitutions that were noted at a distance from it.

A fifth site in FMDV type O was identified by Crowther *et al.* (1993). They found that the four-site multiple mutant G67 (McCahon *et al.*, 1989) reacted with Mab C3 and so they raised a quintuple mutant by subjecting mutant G67 to Mab pressure using Mab C3 and also raised a single C3 escape mutant from the parental O_1 Kaufbeuren virus. Since polyclonal post-vaccinated and infected cattle sera as well as polyclonal mouse and guinea-pig sera which neutralized the four-site mutant did not neutralize the quintuple mutant, they assumed the existence of a fifth site which eliminated all neutralization. They also characterized this site serologically and found it to be conformationally dependent, trypsin-sensitive and independent of previously characterized sites. On sequence analysis of the quintuple mutant, a single change (Q to H) was found at position 149 of VP1 protein.

Pattnaik *et al.* (1996) used a Mab-binding inhibition assay to analyze variation in the trypsin sensitive antigenic site of type O field isolates of Indian origin. They found that though variations were present in the trypsin-sensitive antigenic site of some of the field isolates, a strong neutralizing activity in all the heterologous polyclonal sera against the vaccine virus strain indicated that the antigenic divergence of field isolates from the vaccine virus is subtle.

Barnett *et al.* (1998) used eight neutralizing and two non-neutralizing anti-FMDV bovine IgG1 and IgG2 Mabs which recognize conformationally dependent epitopes in a competition-based ELISA against mouse Mabs which represent five independent neutralizing epitopes on O_1 FMDV. Their studies suggested that though bovine and murine anti-FMDV repertoires may not be identical, they recognized similar antigenic features.

b) Serotype A

As compared to serotype O, antigenic sites on serotype A are more variable and between subtypes A_5, A_{10}, A_{12} and A_{22}, there are considerable differences in the antigenic sites.

Meloen and Barteling (1983) produced Mabs against A_{10} Holland and characterized them in microneutralization test, radioimmunoassay and ELISA with different preparations and categorized neutralizing Mabs into 4 categories. Two of these groups reacted with 12S, but only one of them reacted with trypsin treated 140S. Two other groups reacted only with 140S particle, while one of them reacted well with trypsin-treated 140S; all groups of antibodies showed similar neutralizing activities.

Baxt *et al.* (1984) with the use of monoclonal antibodies raised against type A_{12} virus, isolated A_{12} virus, isolated A_{12} VP1 and CNBr-generated A_{12} VP1 fragment studied the epitopes involved in neutralization and cell-attachment. Based on the

different degrees of viral aggregation and inhibition of cell-adsorbtion observed with the Mabs, they identified the presence of at least three antigenic areas on the viral surface involved in neutralization and one of these was also thought to be important in cell-attachment.

Barteling *et al.* (1986) with a panel of strongly neutralizing anti-A_{10} Holland Mabs directed against four overlapping antigenic domains located on VP1, VP2 and VP3, in a double antibody sandwich ELISA showed that the A_{10} Holland vaccine strain was completely different from the European A_5 vaccine strain.

Thomas *et al.* (1988a) used a set of Mabs against FMDV A_{10} to isolate MAR mutants and sequenced the RNAs of variants. By cross-neutralization and mapping of amino acid changes they identified two major and two minor antigenic sites in FMDV type A_{10}: the first was trypsin-sensitive and included the VP1 140-160 sequence; the second, was trypsin-insensitive and included VP3 residues, the minor sites were located near VP1 169 and on the C-terminus of VP1.

Thomas *et al.* (1988b) investigated whether neutralizing Mabs against FMDV A_{10} were able to compete with polyclonal antibodies, and if so to what extent, so as to assess the relevance of each of the antigenic site identified. Towards this end they performed competition assays with several polyclonal sera from susceptible animals and members of a panel of eleven neutralizing Mabs; the binding of some Mabs were found to be affected by the competition, others were partially affected, while that of the third group of Mabs were unaffected. They suggested that important neutralizing sites of FMDV, as defined by polyclonal sera are not restricted to trypsin-sensitive areas, such as site 140-160 in VP1, but also may be found elsewhere on VP1 or on the proteins VP2 or VP3.

Bolwell *et al.* (1989), used an indirect ELISA with an overlapping set of peptides and MAR mutants to map epitopes on A_{22} Iraq 24/64 and identified the

presence of at least three overlapping linear neutralizing epitopes within the major antigenic site on VP1and also indicated a second, conformational site whose position they could not locate. Based on their studies they also concluded that the major neutralization site of type A viruses show the characteristics of a linear determinant and is less conformationally-constrained when compared to type O viruses.

Saiz *et al.* (1991) used five neutralizing Mabs against type A_5 Spain-86 FMDV and identified two neutralizing antigenic sites; one on VP1 and the second on VP2 based on cross-neutralization and binding assays of mutants generated against these Mabs. Nucleotide sequence comparison of these mutants and the parent viruses revealed that the residues VP1 198 and VP2 72 and 79 were involved in the formation of these antigenic sites.

c) Serotype C

Duchesne *et al.* (1984) raised neutralizing Mabs against the whole virus particle of C_1 Vosges. Reactivity of Mabs with intact virus, isolated VP1 and trypsin treated whole virus reflected the presence of a neutralization epitope in the central region of VP1. Mabs against type C virus was produced and characterized by Capucci *et al.* (1984) using cross-neutralization, ELISA and Western blot. They identified five groups of Mabs, three of which were neutralizing. Of the three sites identified by them, one was trypsin-sensitive and another was a trypsin-resistant one.

Neutralizing Mabs produced against FMDV serotype C_1 were tested with field isolates and variants in several immunoassays (Mateu *et al.*, 1990). Out of a total of 36 neutralizing Mabs tested, 23 recognized capsid protein VP1 and distinguished at least 13 virion conformation-independent epitopes involved in virus neutralization. Eleven epitopes of FMDV C-S8c1 were located in segments 138-156 or 192-209 of VP1 by quantifying the reactivity of neutralizing Mabs with synthetic peptides and with neutralizing Mab-resistant mutants of FMDV C-S8c1 carrying defined amino acid

substitutions. They also suggested that the main antigenic site of FMDV C-S8c1 consited of multiple (at least 10), distinguishable, overlapping epitopes; while some amino acid replacements abolished one of the epitopes, others affected several epitopes in this region. A conservative substitution His (146) Arg found in many mutants abolished the reactivity of the virus with all the Mabs that recognized epitopes in the main antigenic site of FMDV C-S8c1. This site in the G-H loop was designated Site A in type C virus. Other sites in this virus include site C which is located in the carboxy-terminal segment of VP1 and includes about 15 residues (Mateu *et al.*, 1990). The third site, Site D is the major antigenic site of type C (considered equivalent to sites 2 and 4 in type O) and includes the B-C loop of VP2 (residues 70 to 80) and the B-B knob of VP3 (residues 58-61), it also includes a part of the carboxy terminus (residue 193) of VP1 (Mateu *et al.*, 1994).

Martinez *et al.* (1991) with a panel of Mabs that recognized the VP1 C-terminus of serotype C showed that there were predominantly two mechanisms of antigenic diversification. The amino acid replacements that underlay the diversification of the main antigenic site (VP1 residues 138 to 150) were identified by reactivities of the Mabs with VP1 in Western blotting.

Saiz *et al.* (1994) constructed a foot-and-mouth disease virus cDNA cassette containing sequences encoding the capsid precursor P1, proteinase 2A and truncated 2B (which they called P1-2A) of type C FMDV, and modified it to generate the authentic amino acid terminus and the myristoylation signal. This construct was used to generate a recombinant baculovirus which on infection of *Spodoptera frugiperda* insect cells produced the recombinant precursor at high levels. They found that the polyprotein reacted with neutralizing Mabs that bind to continuous epitopes of the major antigenic site of capsid protein VP1 as well as neutralizing Mabs that define complex, discontinuous epitopes previously identified on FMDV particles. Their studies indicated that the capsid precursor could fold in such a way so as to maintain discontinuous epitopes involved in virus neutralization present on the virion surface.

Mateu *et al.* (1998) expressed an unprocessed capsid precursor (P1) of foot-and-mouth disease virus in mammalian cells to study discontinuous epitopes involved in viral neutralization and found that when amino acid replacements found in escape mutants were engineered in the P1 precursor by site-directed mutagenesis, they abolished recognition of unprocessed P1 by the relevant Mabs, parelleling the effects of the corresponding substitutions in neutralization of infectious FMDV. They also suggested on the basis of their findings that site-directed mutagenesis of constructs encoding capsid precursors could be used to probe the structure of viral discontinuous epitopes.

d) Serotype Asia-1

Marquardt *et al.* (2000) sequenced the capsid protein coding genes of five recent type Asia-1foot-and-mouth disease virus isolates representing three genotypes and found sequence differences suggesting different properties of the isolates. They used one of the isolates to generate Mabs which were analysed for neutralizing activity and reactivity with trypsinized virus and found that the five virus isolates formed three reaction patterns with the Mabs irrespective of their genotype. They further suggested the location of the Mab-binding sites to be on the VP1 G-H loop and VP2 B-C loop, the VP3 B-B knob and the N-terminus of VP2 respectively.

e) Serotype SAT2

Rowe (1993) described the characterization of Mab escape mutants of serotype SAT2. They only characterized epitopes located within the G-H loop: some of the Mabs recognized linear epitopes and others appeared to recognize conformational epitopes involving, in part, the G-H loop. A single Mab was also found to recognize an epitope outside VP1, though this Mab was not fully studied. Mapping of antigenic sites has not been done yet for FMD viruses of serotypes SAT1 and SAT3, though

monoclonal antibodies have been produced against isolates of SAT1 (Thevasagayam, 1996).

Much of the knowledge on antigenic sites of different serotypes of FMDV owe itself to studies employing Mabs and Mab-resistant mutants. However, structural and other analyses of epitopes point to some limitations of approach based on mutants (Van Regenmortel, 1990). a) Though amino acid substitutions that affect recognition of a protein by an antibody are generally limited to the surface area in contact with the antibody, MAR mutants may also show substitutions at residues probably located outside the antibody-binding site (Parry *et al.*, 1989); these substitutions are supposed to act by by forcing the involved loops into different positions, thus disrupting epitopes (Parry *et al.*, 1989, Krebs *et al.*, 1993). The effects of such substitutions are often quite difficult to interpret in the absence of structural data. b) Another limitation is the supposition that antigenic sites and epitopes identified using murine Mabs could differ from those recognized by antibodies of the natural hosts. In the case of type O FMDV, studies with bovine Mabs have shown that this is not the case (Barnett *et al.*, 1998). The same amino acid substitutions are seen to behave as antigenically critical with regard to both recognition by Mabs and recognition by antibodies from natural hosts (Mateu *et al.*, 1990, Mateu ,1995 , Martinez *et al.*, 1991). c) The third important point is that sampling limitations are imposed when only a restricted number of Mabs are available against each virus. Collaborative studies with panels of Mabs raised against different virus isolates of the same serotype, in different laboratories can counter this to a large extent as was done with serotype O (McCahon *et al.*, 1989). d) Limitations are also in the form of the restrictions to variation found in capsid proteins (Mateu , 1995). Though 15-20 residues on the capsid surface are in contact with the antibody, only very few positions are tolerant to replacements and changes are seen in MAR mutants only at these places. Better insights into the contributions of different capsid residues towards the formation of antigenic sites can be got by X-ray crystallographic studies of capsid proteins alone and complexed with antibody or with Fab fragments. Verdaguer *et al.* (1998) determined the three-dimensional structures of

the Fab fragment of a neutralizing antibody raised against serotype C1, alone and complexed to an antigenic peptide representing the major antigenic site A (G-H loop of VP1) and found that the receptor recognition motif Arg-Gly-Asp and some residues from an adjacent helix participate directly in the interaction with the complementarity-determining regions of the antibody. Information obtained from MAR mutant studies when analyzed in conjunction with that available on the crystal structure of FMD viruses have helped to arrive at more accurate interpretations of results (Kitson *et al.*,1990). Antigenic sites on FMDV are summarized in Table.1

2.6 WORK DONE IN INDIA

There are two reports on characterization of Mabs and antigenic site analysis of type Asia-1 FMDV, both using monoclonal antibody-resistant mutants and cross-neutralization tests. Sanyal *et al.*, (1997) identified four independent trypsin sensitive neutralizable antigenic sites on FMDV type Asia-1 using a panel of 32 Mabs and mutants against six neutralizing Mabs. Studies employing MAR mutants identified four sites, the first of which was found to contain four different neutralization epitopes. Reactivity of the Mabs for untreated, trypsin-treated virus and subviral antigens in ELISA revealed that the sites are trypsin-sensitive and conformation dependent.

The other study was by Butchiah and Morgan (1997) which also employed similar techniques, but with a different Asia-1(IND 66/86) virus. Their study revealed the presence of three independent antigenic sites with evidence for the occurrence of possibly a fourth site on the virus surface. Site 1 was present on 146S, 12S and VP1 and was thus conformation-independent. Sites 2 and 3 were restricted to the intact virion (140S) and thus were more conformation-dependent. Site 4 was present on 140S virions and 12S protein subunits and was less conformation-dependent. In both these studies the region of the genome coding for these sites has not been sequenced.

Table1. Summary of antigenic sites in different serotypes of FMDV

Serotype	Site	Proteins and Residues Involved*	Reference
O	1	1144, 1148, 1154 and 1208	Xie *et al.*, 1987, Parry *et al.*, 1989, Kitson *et al.*, 1990
	2	2070-2077,2188	Kitson *et al.*, 1990, Barnett *et al.*, 1998
	3	1044 and 1048	Kitson *et al.*, 1990
	4	3058	Kitson *et al.*, 1990
	5	1149	Crowther *et al.*, 1993
A_5	1	1198	Saiz *et al.*, 1991
	2	2072 and 2079	Saiz *et al.*, 1991
A_{10}	1	1142-1147 and 2132	Thomas *et al.*, 1988b
	2	1169 and 1200-1212	
	3	3058-3061, 3069, 3070, 3136, 3139, 3195, 2080 and 2196	
A_{12}	1	1151 and 1152	Baxt *et al.*, 1989
	2	3175, 3178 and 1201	
	3	1173	
	4	1152 and 1209	
A_{22}	1	1138, 1140, 1142, 1149, 1150, 1153 and 1154	Bolwell *et al.*, 1989
C	A	1138-1149	Mateu *et al.*, 1990 and
	C	15 residues in the carboxy terminus of VP1	
	D	1193, 3058-3061 and 2070-2080	Mateu *et al.*, 1994

* First digit (1,2 or 3) refers to the capsid protein (VP1, VP2 or VP3), subsequent digits refer to the position of the amino acid residue.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Monoclonal antibodies

Mouse monoclonal antibodies (Table.2) developed (Venkataramanan et al., 1990-92) and characterized (Sanyal 1995) against complete virion particles of type Asia 1 vaccine virus strain at the Central Laboratory, All India Coordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar, were used in the study. The Monoclonal antibodies (Mabs) in the form of cell culture supernatant were used. The characteristics of the Mabs are given in Table 3.

3.1.2 Vaccine Virus (Reference Virus)

Foot-and-mouth disease virus type Asia 1 vaccine strain (IVRI Vaccine strain IND 63/72) adapted in BHK-21 clone 13 cell line and maintained at the repository of the Central Laboratory, All India Coordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar was used as the parent virus in the present study.

3.1.3 Asia-1 Field Isolates

A total of 18 field isolates of type Asia 1 FMD virus recovered from outbreaks in different parts of the country and available at the Central Laboratory, All India Coordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar in the form of cell culture supernatants were used in this study. The detailed history of the field isolates is given in Table 4.

Table .2 Monoclonal Antibodies (Mabs) used in the study

Serial No.	Mab Identification code	Mab designation followed
1	7A8.B1	B3
2	7C3.D6	D
3	1C7.H4	E
4	1C2.A8	H
5	3G7.C6	W
6	1F7.D4	1A
7	2D4.C3	2A
8	1E6.B4	3A
9	7C3.F12	7C3
10	7C4.A6	7
11	3G4.D2	8A
12	1C8.B3	8B
13	1F7.B4	9
14	2E8.E11	10
15	1F7.A6	13
16	2B3.B4	16
17	5C2.D7	34
18	7C1.G5	40
19	5C2.E1	54
20	2A2.F4	61
21	2A6.B4	62
22	3A4.G6	63
23	3A7.F4	64
24	2F3.F6	66
25	15D2.F11	71
26	2H10.D6	78
27	2E8.H7	76
28	2E8.H12	82
29	7D5.A2	72

Table. 3 Characteristics of Monoclonal antibodies used in the study.

Serial No:	Mab	Isotype	Reactivity with			
			146S	TT146S	12S	D.V.
1	B3	IgG2a	+	+	-	-
2	D	IgG2b	+	+	-	-
3	E	IgG2a	+	+	-	-
4	H	IgG2a	+	+	-	-
5	W	IgG2a	+	+	-	-
6	1A	IgG2b	+	+	-	-
7	2A	IgG2b	+	+	-	-
8	3A	IgG2b	+	+	-	-
9	7C3	IgG2a	+	+	-	-
10	7	IgG2a	+	+	-	-
11	8A	IgG2a	+	+	-	-
12	8B	IgG2b	+	+	-	-
13	9	IgG2b	+	+	-	-
14	10	IgG2a	+	+	-	-
15	13	IgG2b	+	+	-	-
16	16	IgG2b	+	+	-	-
17	34	IgG2b	+	+	-	-
18	40	IgG2b	+	+	-	-
19	54	IgG2a	+	+	-	-
20	61	IgG2b	+	+	-	-
21	62	IgG2a	+	+	-	-
22	63	IgG2a	+	+	-	-
23	64	IgG2b	+	+	-	-
24	66	IgG2a	+	+	-	-
25	71	IgG2b	+	+	-	-
26	78	IgG2a	+	+	-	-
27	76	IgG2a	+	+	-	-
28	82	IgG2a	+	+	-	-
29	72	IgG2a	+	+	-	-

146s- Whole Virus , TT 146s - trypsin treated virus, D.V. -disrupted virus,
+ reactive,
- nonreactive

Table.4 History of FMDV Asia1 Field Isolates used in the study

Sl. No:	Isolate No	Year of Isolation	Place	Species
1	IND 132/85	1985	Ramakrishna Dairy , Calcutta	Cattle
2	IND 4/86	1986	Samchi, Bhutan,	Cattle
3	IND 9/90	1990	VBRI, Hyderabad	Bullock
4	IND 49/93	1993	Mathura, U.P.	Buffalo
5	IND 187/94	1994	Calcutta	Cattle
6	IND 234/95	1995	Anand , Gujarat	Cattle
7	IND 339/96	1996	Adoor, Kerala	Cattle
8	IND 125/98	1998	OUAT, Dairy farm, Bhubaneswar	Cattle
9	IND 126/98	1998	Guchguda, Kalahandi, Orissa	Buffalo
10	IND 130/98	1998	Unit B, Delta Area, Bhubaneswar	Cattle
11	IND 324/98	1998	Deoban, Kaithal, Haryana	Cattle
12	IND 445/98	1998	Punia, Hissar	Cattle
13	IND470/98	1998	Pune,Maharashtra	Cattle
14	IND 68/99	1998	Belgaum, Karnataka	Buffalo
15	IND 69/99	1998	Belgaum, Karnataka	Cattle
16	IND 103/99	1999	Basti, Barwala, Hissar	Buffalo
17	IND 192/99	1999	Sodepur, Pinjrapole, WB	Cattle
18	IND 235/99	1999	Dhiktana, Hissar	Buffalo

9. ALFexpress ™ Autocycle ™ Sequencing Kit (Pharmacia Biotech, Cat No:27-2693- 02), for ALFexpress cycle sequencing.

3.2 Equipments

1. Inverted microscope (Olympus).
2. Cell Production Roller Apparatus (BELLCO, USA).
3. Refrigerated Centrifuge (Sorvall).
4. Microfuges (Hettich, Heraeus (refrigerated).
5. Laminar Air Flows (Holten).
6. CO_2 Incubator.(Forma Scientific)
7. ELISA Reader (Spectra Classic, Tecan).
8. Circulating Water baths (Heto).
9. Thermal Cycler with heated lid (Multi Block System, Hybaid), without heated lid (Omnigene, Hybaid).
10. Horizontal Electrophoresis apparatus (Atto).
11. Transilluminator (Ultra Lum).
12. Shaker Incubator (New Brunswick)
13. Ice Flaker (Sarit)
14. Vertical electrophoresis apparatus and power supply (Pharmacia)
15. MilliQ water plant
16. ALFExpress II Automated Sequencer (Pharmacia Biotech)
17. Reproset (Pharmacia Biotech)
18. -80°C freezers (Forma Scientific and Heraeus)
19. Microwave Oven (BPL)
20. Spectrophotometer (Unicam)

3.1.4 Anti-146S sera

Anti-146S sera raised in guinea pigs and rabbits against the parent Asia 1 virus strain available at the Central Laboratory, All India Coordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar was used.

3.1.5 Cell culture

BHK-21 clone 13 cell line maintained at the Central Laboratory, All India Coordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar was used for propagation of virus isolates.

3.1.6 Nucleic acid manipulation kits

1. Rneasy Total RNA Kit (Qiagen, Cat.No:74104), for RNA Extraction.
2. SuperScript ™ One-Step ™ RT-PCR System (Life Technologies , Cat. No: 10928-026), For RT-PCR.
3. Access RT-PCR system (Promega, Cat. No.A1250), For Reverse Transcription and Polymerase Chain Reaction.
4. QIAquick Gel Extraction Kit (Qiagen, Cat.No: 28704), For gel-purification of PCR Products.
5. CloneAmp^{R} pAMP1 & pAMP10System (Life Technologies, Cat No: 18381-012), for rapid cloning of PCR products.
6. Wizard plus minipreps DNA purification system (Promega, Cat No: a 7500) , for plasmid Isolation.
7. fmol^{R} DNA Cycle Sequencing System (Promega,CatNo:Q4100), For cycle sequencing.
8. Silver sequencing ™ DNA staining reagents (Promega, Cat No: Q4132), for silver staining of sequencing gels.

9. ALFexpress ™ Autocycle ™ Sequencing Kit (Pharmacia Biotech, Cat No:27-2693- 02), for ALFexpress cycle sequencing.

3.2 Equipments

1. Inverted microscope (Olympus).
2. Cell Production Roller Apparatus (BELLCO, USA).
3. Refrigerated Centrifuge (Sorvall).
4. Microfuges (Hettich, Heraeus (refrigerated).
5. Laminar Air Flows (Holten).
6. CO_2 Incubator.(Forma Scientific)
7. ELISA Reader (Spectra Classic, Tecan).
8. Circulating Water baths (Heto).
9. Thermal Cycler with heated lid (Multi Block System, Hybaid), without heated lid (Omnigene, Hybaid).
10. Horizontal Electrophoresis apparatus (Atto).
11. Transilluminator (Ultra Lum).
12. Shaker Incubator (New Brunswick)
13. Ice Flaker (Sarit)
14. Vertical electrophoresis apparatus and power supply (Pharmacia)
15. MilliQ water plant
16. ALFExpress II Automated Sequencer (Pharmacia Biotech)
17. Reproset (Pharmacia Biotech)
18. -80°C freezers (Forma Scientific and Heraeus)
19. Microwave Oven (BPL)
20. Spectrophotometer (Unicam)

3.3 METHODS

3.3.1 Roller culture propagation of the parent virus

The parent virus used was inoculated on to BHK monolayers in a roller culture bottle (Costar, USA) in order to have a high-titred virus stock that contains sufficient variant genomes to infect cells. This is required since exerting of selection pressure in the form of a monoclonal antibody merely selects and amplifies mutant viruses that already exist in the parent stock.

The confluent monolayer in a roller bottle was washed twice after which 200 ml of maintenance medium was added to it. To this, 5 ml of the parent virus, once passaged in a 25cm^2 flask was added, mixed and the bottle was incubated at 37°C, at an rpm of 5 in a Cell Production Roller Apparatus (BELLCO,USA). Once cytopathic effect (CPE), as evidenced by rounding and detachment of cells from the surface, was complete, the virus was harvested and centrifuged at 1000 rpm for 10 minutes to remove the debris, after which it was aliquoted in appropriate volumes and stored at -40° C.

3.3.2 Propagation of Field Isolates

The field isolates of FMDV serotype Asia 1 preserved at the Central Laboratory, All India Coordinated Research Project for Epidemiological Studies on FMD, IVRI, Mukteswar, in the form of BHK-21 cell culture infected fluid were used for propagation. The working stock of all the field isolates was obtained by infecting them in 75 cm^2 cell culture flasks (Nunc, Denmark). Briefly, the confluent monolayer in a culture flask was washed with maintenance medium, inoculated with 0.4 ml of virus and allowed it to adsorb at 37°C for 45 minutes. The unadsorbed virus was discarded and the cell-sheet was washed twice. After addition of 18 ml of maintenance medium, it was incubated at 37°C. When complete CPE was observed, the infected

cell-culture fluid was collected and centrifuged at 1000 rpm for 10 minutes to sediment the cell debris, the supernatants were stored at -70°C in aliquots for subsequent use in the study.

3.3.3 Micro-neutralization test

Micro-neutralization test (MNT) was carried out in 96-well flat bottomed tissue culture plates (Nunc, Denmark) to asses the neutralizing ability of Mabs. The procedure followed is described below.

Serial $\log_{10}$ dilutions of the parent virus (from 2 $\log_{10}$ to 8 $\log_{10}$) was prepared in maintenance medium and dispensed to wells of rows A to G in 50 µl quantities. Maintenance medium (100µl) was added to row H as cell control. Each Mab (50µl) was added to two columns (total 5 Mabs per plate) leaving columns 1 and 2 as virus controls to which 50µl of maintenance medium was added in the place of Mabs.

The plates were properly shaken for thorough mixing of Mabs and virus and kept for incubation at 37°C for 1 hour. 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 all the wells in 50 µl quantities. The plates were shaken thoroughly, sealed with adhesive tape and incubated at 37°C for 48 hours under 5% CO_2 tension. After 48 hours incubation, the plates were read for the presence or absence of CPE. The reduction in virus titre in the presence of Mabs was taken as the $\log_{10}$ neutralizing index of the Mab.

3.3.4 Isolation of Monoclonal Antibody Resistant (MAR) mutants

The mutants against some of the Mabs already available (Sanyal *et al.*, 1997) in the laboratory were revived and tested for their stability in their reactivity against the Mab panel. Since many of them showed some residual reactivity with the

homologous Mabs, fresh mutants were isolated for all the Mabs. Variant viruses resistant to neutralization by monoclonal antibodies were isolated by the technique described by Samuel (1997). The high titred virus grown in roller culture was mixed with the undiluted Mab in the ratios of 1:1, 5:1 and 10:1 and incubated for 30 min at 37°C. The virus-Mab mixture was inoculated onto BHK cells in a 6 well plate and 2ml of maintenance medium containing Mab at 1:50 concentration was added to it. The plate was incubated at 37°C until complete CPE was observed. The supernatants were clarified and the viruses were assayed for their binding with the homologous Mab in the Sandwich ELISA procedure described. Those viruses which showed low or no reactivity were again grown in presence of the Mab as described above to ensure complete loss of reactivity with the selecting Mab. Viruses obtained in this way were tested again in Mab profiling ELISA and the mutants obtained were further plaque purified as described.

3.3.5 Isolation of Multiple MAR Mutants

For isolation of double and multiple mutants, the same procedure detailed above was followed with slight modifications. Here, instead of the parent virus, a single site mutant virus (isolated as described) was passaged in the presence of the Mab that was used to isolate it, as well another Mab with which it was reactive, so as to isolate mutant viruses that were resistant to both Mabs. Both the Mabs were included in the maintanance medium of infected cells in the concentration of 1:50. The harvested viruses were then tested against both the Mabs used for their isolation in Mab-profiling sandwich ELISA. The isolated multiple MAR mutants were then plaque purified as described below.

3.3.6 Plaque Purification of MAR Mutants

Fifty µl of diluted virus (10^{-2} to 10^{-6}) was incubated with 150 µl of diluted Mab for 1 hour at 37°C and then added to a confluent BHK cell monolayer in a six-well tissue-culture plate. It was left to adsorb for 30 minutes at 37°C. The inoculum was discarded and the cell-sheet was washed twice with GMEM, 2ml of agar overlay medium containing the appropriate dilution of Mab was added when it was sufficiently cold. The plates were incubated at 37°C in a CO_2 incubator for 24 hours and well-isolated plaques were picked and resuspended in 1ml GMEM. The whole 1ml thus collected was used to infect confluent BHK monolayers in 24-well plates. Once CPE was evident, the supernatant was collected and tested in sandwich ELISA to check the reactivity of the mutant against the corresponding Mab. A second round of plaquing was also done (procedure as above), the plaques (viruses) picked were again tested for their Mab-reactivity. For each mutant, one selected plaque was amplified in a 175cm^2 flask in presence of the selecting Mab. The virus thus produced was clarified by centrifugation, (1000 rpm, 10 minutes), aliquoted in 2ml tubes and stored at -70°C till use.

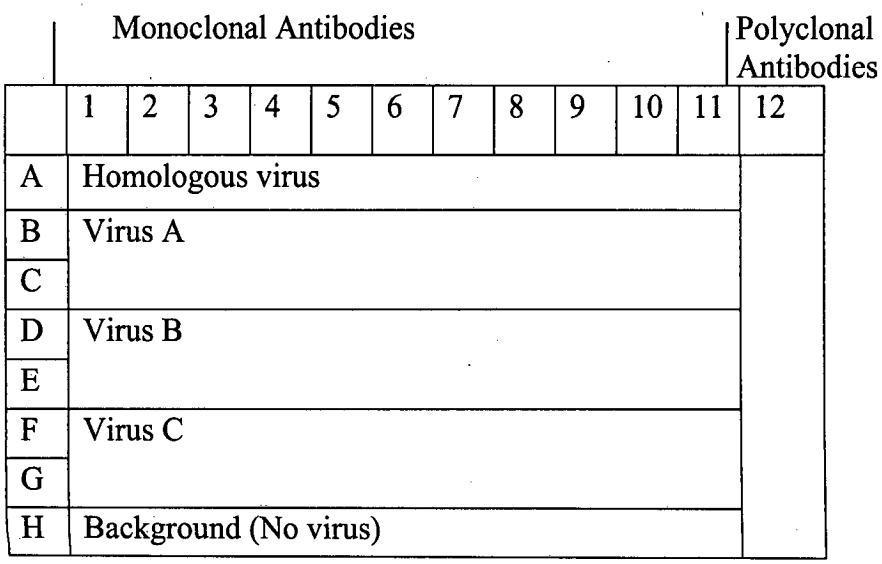
3.3.7 Mab profiling of Mutants and Field Isolates

The sandwich ELISA procedure described by Samuel et al., 1991 was followed for Mab profiling of the generated mutants as well as the 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 overnight.

(ii) The wells were washed 3 times each at 5 minute-intervals using PBS Tween 20 washing buffer.

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



(iii) Type Asia1 vaccine strain virus in the form of infected BHK-21 cell culture fluid was added to the wells of row A of each plate. Three mutants/isolates also in the form of infected BHK-21 cell culture fluid were dispensed into the wells of row B to G (two rows/virus) in 50 µl volumes. Row H was kept as background control and to these wells 50 µl of blocking buffer (3% skimmed milk powder in washing buffer) in place of antigen was dispensed. The virus was allowed to trap at 37°C for 1 hour and then washed as in step (ii).

(iv) Eleven Mabs were dispensed (50 µl per well) at a single pre-titrated dilutions (this dilution was obtained from titration of Mabs against vaccine virus in sandwich ELISA) in blocking buffer (3% skimmed milk powder in washing buffer) to columns 1 to11. To the column 12, 50 µl type Asia 1 anti-146S guinea pig serum in ELISA blocking buffer at the pre-titrated 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, USA, Cat. No. A-4416) diluted to 1:1000 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 10 minutes for the enzyme substrate reaction to take place.

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

(viii) The optical density was taken at 492 nm in a Tecan Spectra ELISA reader.

Calculations:

Corrected OD values of each Mab with each mutant/isolate was obtained by subtracting background OD values from those of test proper. The OD values of each Mab with each mutant/isolate was expressed as percentage OD of polyclonal antibody reactivity with each mutant/isolate. The percent reactivity was expressed as per

Samuel et al. (1991) with minor modifications and qualified by 3 ranges of reaction, viz. 60% and above reflects equal reaction to the homologous virus, 40-59% reflects reduced affinity, 20 to 39% reflects still lower reactivity and below 20% reflects no reaction.

3.3.8 Designing of Primers for cloning of P1 regions into pAmp vectors.

To design primers flanking the P1 region, first the nucleotide sequence of L, 2A & 2B coding region of FMDV Asia1 IND 63/72 was obtained by sequencing of the PCR product made using primers designed for expression of these non-structural genes (available in the laboratory). Based on the sequence of Asia 1 63/72, a total of 6 primers were designed manually and obtained commercially from Life Technologies, their location and other details are given in Table 5. These primers contained either CAU CAU CAC CAU or CUA CUA CUA CUA at their 5' ends to enable them to be cloned into pAmp vectors. Schematic diagram of primer location in P1 region is given in the Fig.4.

3.3.9 Extraction of RNA

Extraction of viral RNA from infected tissue culture fluid was done by Guanidine thiocyanate method using RNeasy Total RNA kit. Equal volume (1.38 ml) of infected tissue culture fluid and Lysis buffer containing 1% 2-mercaptoethanol (RLT- supplied with the kit) was mixed. To this was added 1.38 ml of 70% ethanol in 1% DEPC treated water. After proper mixing, 700 µl of the mixture was passed through the RNeasy spin column by centrifuging it at 10,000 rpm for 15 seconds in a microfuge. The flow-through was discarded and 700 µl of wash buffer (RW1, supplied with the kit) was applied to the column and centrifuged as before. Then, 500 µl wash buffer (RPE, supplied with the kit) was applied to the column and centrifuged as before. The spin column was finally washed with 500 µl of wash buffer RPE and

Table.5 Details of primers used for RT-PCR and cloning into pAmp Vectors.

Primer Name	Sequence (5'-3')	Location	Length	Polarity
MG51	CAUCAUCAUCAUGACATGTCCTCCTGCATCTG	$2B^{58-77}$	32	-ve
MG52	CUACUACUACUAACCTCCAACGGGTGGTACGC	$L^{463-482}$	32	+ve
MG50	CUACUACUACUACCCCTGGACGCCGGACCCGTC	$L^{519-539}$	33	+ve
MG53	CAUCAUCAUCAUGAAGGGCCCAGGGTTGGACTC	$2A^{34}$-$2B^{6}$	33	-ve
MGP1CR	CUACUACUACUAGTCCACCAGTTTGGAGAAGTT	$2B^{28-48}$	33	-ve
MG17C	CAUCAUCAUCAUGTGCCCCAGTTTAAAAAGCTT	5'noncoding region ($^{-78}$ to $^{-57}$)	33	+ve

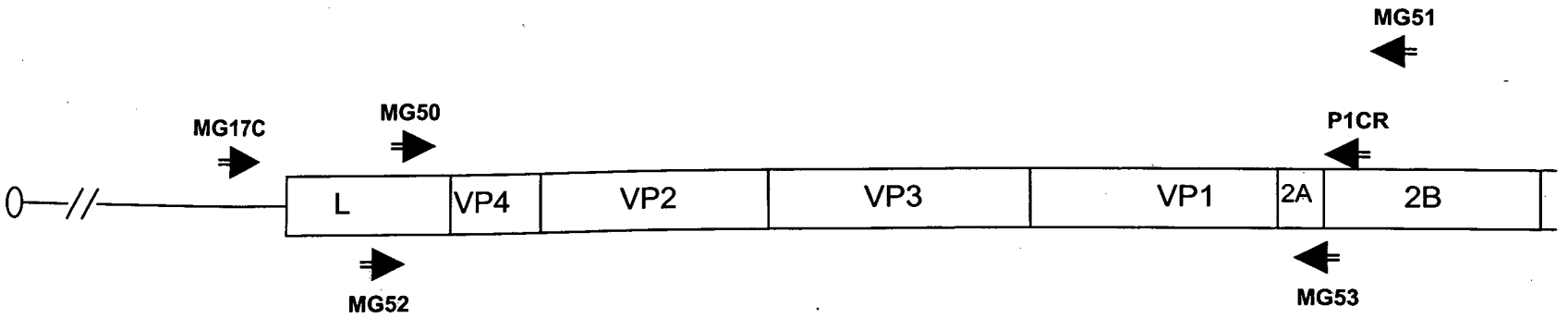
Table. 6 Primer combinations and RT-PCR conditions used

Primer Combination	Reaction Conditions	Length of Amplicon (No. of bases)
MG51-52*	53°C ,30 m,1 cycle; 94°C, 20 s, 56.5°C, 30 s, 68°C , 2.2 m, 40 cycles; 68°C, 10 m, 1 cycle.	2486
MG50-53*	53°C , 30 m,1 cycle; 94°C, 20 s56.5°C, 30 s 68°C , 2.2 m, 40 cycles; 68°C, 10 m, 1 cycle.	2374
MG50-P1CR#	48°C ,30 m,1 cycle; 94°C, 20 s, 55°C, 30 s, 68°C , 2.2 m, 40 cycles; 68°C, 10 m, 1 cycle.	2416
MG17C-P1CR*	48°C ,30 m,1 cycle; 94°C, 20 s, 53°C, 30 s, 68°C , 3 m, 40 cycles; 68°C, 10 m, 1 cycle.	2997

* For PCR-Cloning into pAMP 1 Vector

For PCR-Cloning into pAMP 10 Vector

Fig.4. Location of Primers used for RT-PCR



centrifuged at 12,000 rpm for 2 minutes to dry the membrane. Following this, RNA in the spin column was eluted in 50 µl nuclease-free water (supplied with the kit) by centrifugation at 10,000 rpm for 60 seconds; the RNA was stored at -70°C.

3.3.10 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

The extracted viral RNA was subjected to RT-PCR using the SuperScript One-Step RT-PCR system (Life Technologies) or Access RT-PCR system (Promega) following manufacturers' instructions. For SuperScript system, a 100 µl reaction mixture contained 50 µl of 2X reaction mix, 6 µl of template RNA, 20 µM each of positive and negative sense primers (details of primers used are given in Table.5 a genome map showing their location is given in Fig 4), 2 µl of RT/*Taq* mix and autoclaved distilled water upto 100 µl. The reaction mix was overlaid with mineral oil and the amplification reaction was performed on an Omnigene thermal cycler (Hybaid, UK). The reaction conditions used for RT-PCR are given in Table. 6)

For Access RT-PCR system (Promega), the reaction mix in 100 µl volume contained 20µl AMV/Tfl 5x Reaction buffer, 2 µl of 10 mM dNTP mix, 4µl of 25 mM $MgSo_4$, 2µl AMV Reverse transcriptase (5U/µl), 1µl Tfl DNA polymerase (5U/µl), 6µl RNA sample 20 µM each of positive and negative sense primers (Table.5) and nuclease-free water upto 100 µl. The reaction mixture was overlaid with PCR grade mineral oil and mixed for 2 minutes by centrifugation. The tubes were loaded onto a thermalcycler block (Hybaid, Omnigene) and RT-PCR amplification was done as given in Table. 6 but RT for these reactions was done at 48 °C.

3.3.11 Confirmation of RT-PCR product

The PCR products were electrophoresed on a 1% agarose gel containing 0.5 µg/ml ethidium bromide in 1X TBE buffer. Five microlitre of each PCR product was

PCR product was mixed with 1 µl of 6Xgel loading solution (Promega). Samples were loaded alongside known molecular weight markers and electrophoresed at 100 V for 20 minutes. The bands were viewed using a transilluminator (UV wavelength of 320 nanometer) and the product size was estimated by comparing with the markers.

3.3.12 Gel-Extraction of PCR Products

The positive RT-PCR products were electrophoresed in a 1% low-melting point agarose gel in 1X TAE buffer containing 0.5 µg/ml of ethidium bromide and run in 1XTAE buffer for 30 minutes at 100 volts. The right-sized bands were cut from the gel after they were viewed under a UV-transilluminator and gel purified using the QIAquick Gel Extraction Kit following the manufacturer's protocol. The procedure was as follows: The DNA fragment was excised from the agarose gel with a clean sharp scalpel and the weight of the gel slice was measured. Three volumes of Buffer QX1 (supplied with the kit) added to the gel slice and incubated at 50°C for 10 minutes in a water bath to solubilize it. After complete dissolving of the gel, one volume of isopropanol was added to the sample and mixed, then it was loaded into a QIAquick column and centrifuged at 10,000 rpm for 1 minute so as to allow DNA binding to the column. The flow-through was discarded and the column was washed with 0.75 ml of wash buffer PE and centrifuged as before. The flow-through was discarded and the column spun for one more minute. After placing the column in a new 1.5ml tube, to elute DNA 30 µl of water (pH adjusted to 8.3) was added to the center of the column, allowed to stand for 1 minute and centrifuged for 1 minute. Appropriate volumes of the eluted gel-purified DNA (sample DNA) and control DNA (of known quantity) were electrophoresed side by side in a 1% agarose gel (as described previously) and the sample DNA was quantified by visual comparison. The quantified, gel-purified DNA was stored at -20°C till further use.

3.3.13 Annealing and Cloning of Gel-Purified PCR Products

The PCR products having modified (uracil) bases at their 5' termini were cloned into pAMP1 or pAMP10 vectors. Uracil DNA glycosylase enzyme (UDG) treatment of these PCR products renders dUMP residues abasic and unable to base-pair resulting in 3' protruding termini. The ready-to-use vector (supplied with the kit) contains 5' protruding termini to enable directional (pAMP1) or non-directional (pAMP10) annealing of the UDG treated PCR products. In the reaction mixture, selective deglycosylation of dUMP residues and annealing of the PCR product to the vectors occur simultaneously. Outline of cloning procedure into pAmp vectors is given in Fig.5a and the vector map in Fig. 5b.

The annealing reactions were performed as follows: To a 0.5 ml microcentrifuge tube, 10-50 ng of gel-purified PCR product, 15µl of 1X annealing buffer (20mM Tris HCl (pH 8.4), 50mM KCl, 1.5 mM $MgCl_2$), 1 unit of UDG and 50 ng of vector were added and volume was adjusted to 20 µl. The components were mixed and incubated at 37°C for 30 minutes to enable vector insert annealing. Five µl of annealing mixture was used for transformation of competent *E.coli*.

3.3.14 Transformation of Annealed products

(A) Preparation of competent cells: A single colony of JM 109 or DH5 α strain of *E.coli* was picked up from overnight growth on LB agar plate and grown in 10 ml of SOB medium overnight at 37°C. From the overnight growth, 200 µl of bacteria was inoculated into 20 ml SOB and allowed to grow with gentle shaking. After 2.5 to 3 hours, when the culture was in the log phase of growth cycle, and cell density was enough to give an OD of 0.3 to 0.35 at A_{600} (absorbance at 600 nm), the culture was cooled on ice. The cooled cells (20 ml) were centrifuged at 2000 rpm for 15 minutes

Fig. 5a. Outline of the rapid cloning procedure followed

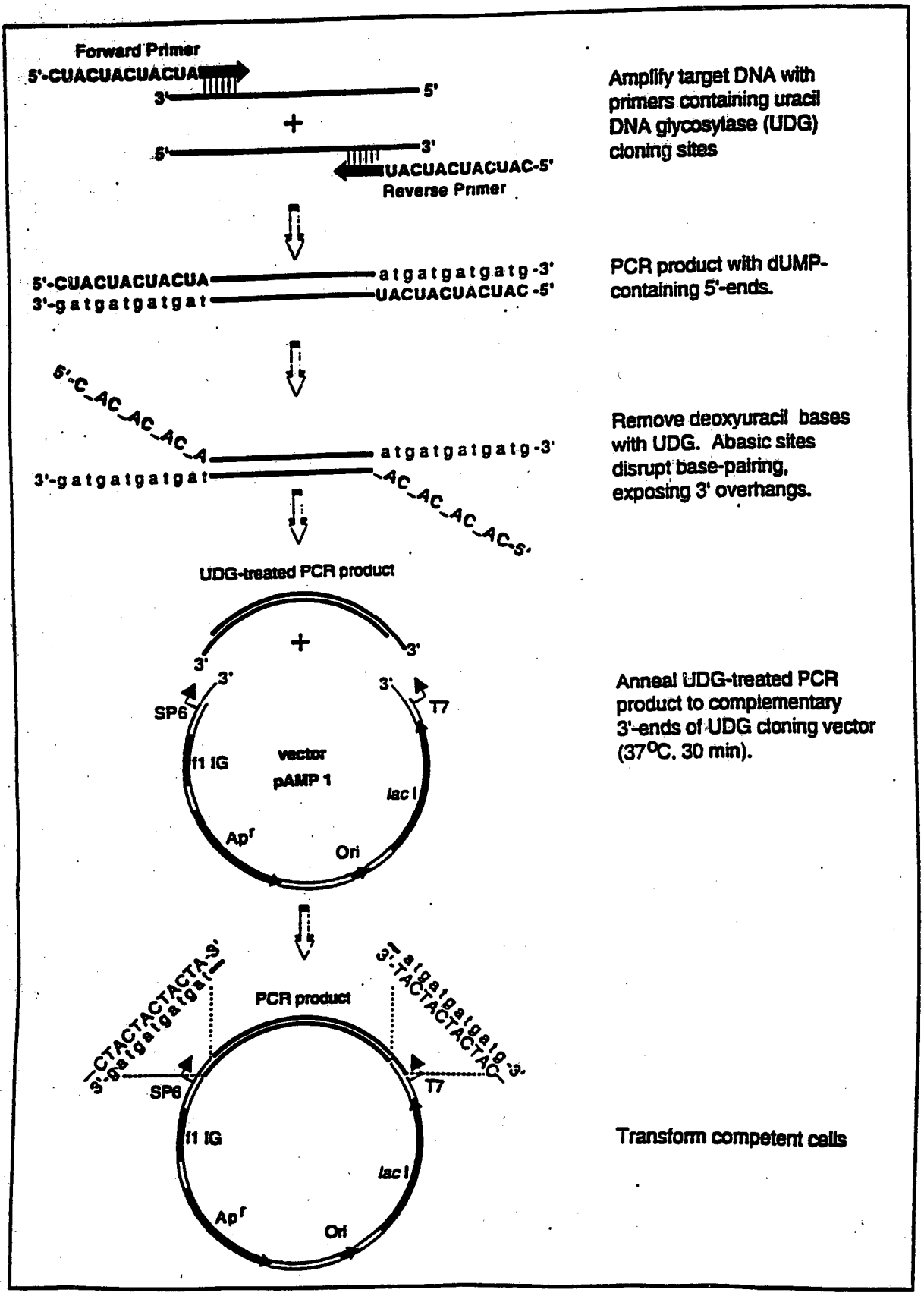
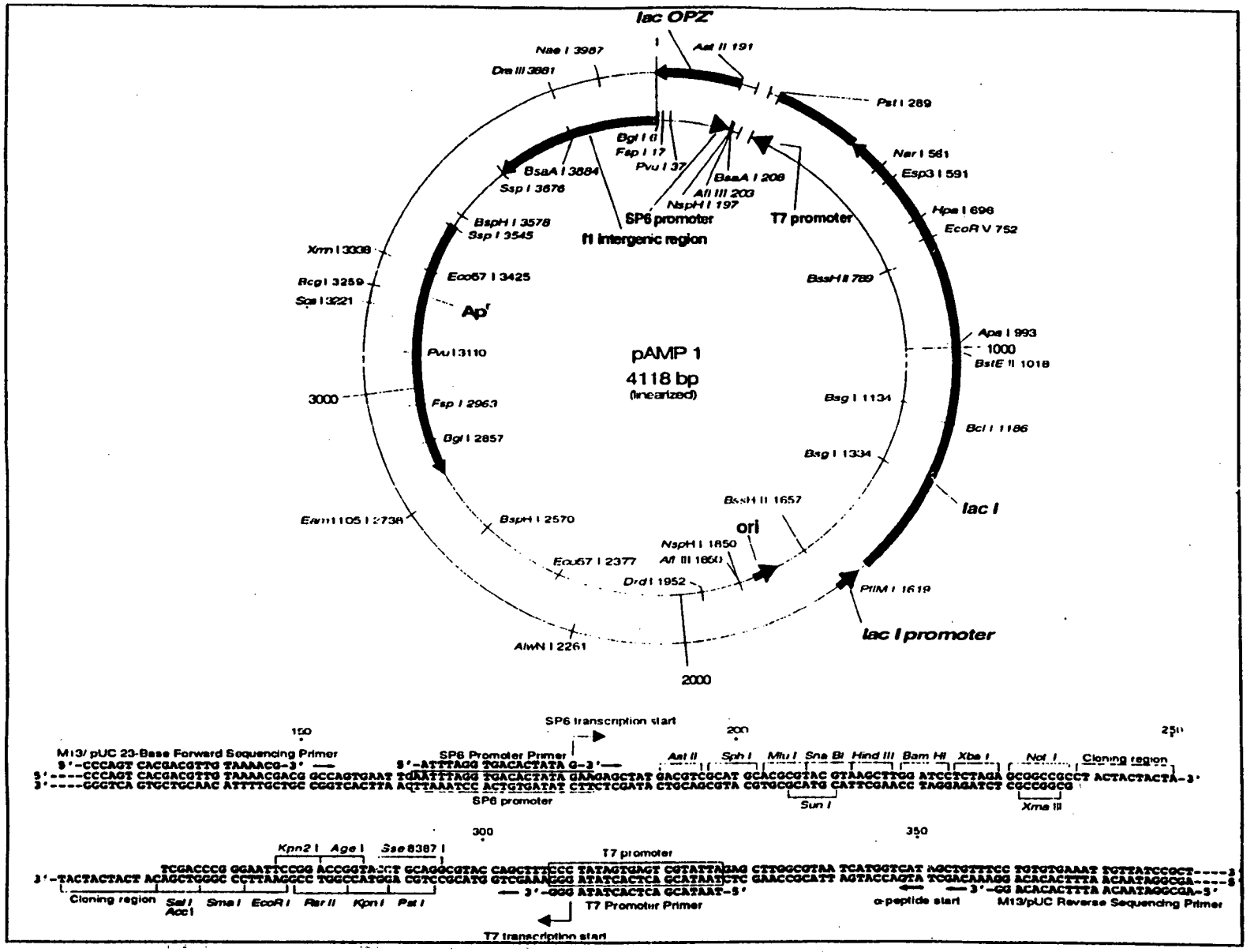


Fig. 5b. Vector Map of pAmp 1 vector used for rapid cloning



to pellet them. The bacterial pellet was resuspended in 1/10th volume of transformation and storage solution (TSS) and allowed to cool on ice for 30 minutes. The cells are now competent.

(B) Transformation of competent cells: Five µl of annealing reaction was added to 200 µl ice cold competent cells It was mixed gently and thoroughly and left on ice for 15 minutes. Heat shocking was done at 42.5°C for 55 seconds following which, the mixture was kept on ice for 5 minutes. Eight hundred µl of SOC medium was then added, mixed and incubated with shaking at 37°C for 1 hour. The whole of it was then spread on IPTG-X-Gal Ampicillin LB agar (80µg/ml X-Gal, 0.5 mMIPTG and 100µg/ml Ampicillin) plates and incubated at 37°C till the appearance of blue/white colonies.

3.3.15 Growth of Recombinant Bacteria and Plasmid Isolation

White recombinant colonies which appeared by 12-14 hours on IPTG-X-Gal Ampicillin LB agar plates were picked up and grown overnight in 10 ml LB broth containing 100 µg/ml ampicillin for 12-14 hours with shaking at 150 rpm.

Plasmid isolation from 10 ml overnight culture was carried out using Wizard plus minipreps DNA purification system (Promega). Briefly, 5 ml culture was centrifuged at 2500 rpm for 20 minutes to pellet bacteria. The pellet was then re-suspended in 300 µl cell resuspension solution. To the bacterial suspension, 300 µl of cell lysis solution was added to lyse the cells. After complete lysis, 300 µl neutralization solution was added to it. The lysate was centrifuged at 12,000 rpm in a microfuge for 5 minutes and the supernatant was collected; this was mixed with 1 ml of DNA purification resin containing 7 M Guanidine hydrochloride. One Wizard prep minicolumn syringe assembly was prepared on the vacuum manifold for each plasmid

DNA and the resin and supernatant mix was transferred into the syringe barrel. The mix was slowly pushed through the minicolumn to waste. The column was washed with 2 ml Column Wash Solution by pipetting the same into the syringe barrel and pushing it down through the minicolumn. The minicolumn was then centrifuged at 12,000 rpm for 20 seconds to dry the resin. The plasmid DNA was eluted in 50 µl DEPC treated water put on top of the resin, by centrifuging the column at 12,000 rpm for 20 seconds. Five µl of the plasmid DNA was electrophoresed on a 0.8% agarose gel in order to check the purity of the preparations.

3.3.16 Confirmation of presence of insert by Restriction Endonuclease digestion

The extracted plasmids were treated with EcoR1 and BamH1 in a 10 µl reaction. The reaction mixture contained 1 µl of 10X React buffer 3 (Life Technologies), 1 unit each of EcoR1and BamH1 (Life Technologies) and 6 µl of plasmid DNA. Restriction digestion was carried out in a 37°C water bath for 1hour. The reaction was stopped by adding 6X gel loading solution (Promega) and electrophoresed on a 1% agarose gel (in TBE) containing 0.5 µg/ml of ethidium bromide together with known molecular weight markers.

3.3.17 Isolation of Ultra-Pure Plasmids

For use in automated cycle sequencing reactions, one of every set of positive colonies were grown again in 5ml LB-Ampicillin broth and ultra-pure plasmid was extracted using QIAGEN Plasmid Mini Kits. The procedure followed is given below.

The bacterial pellet obtained following centrifugation was resuspended in 0.3 ml Buffer P1 containing Rnase A (supplied with the kit). To the resuspended bacteria, 0.3 ml of Buffer P2 (supplied with the kit) was added, mixed gently but throughly and incubated at room temperature for 5 minutes. Following the 5 minute incubation, 0.3

ml of chilled Buffer P3 (supplied with the kit) was added, mixed immediately but gently, and incubated on ice for 5 minutes after which it was centrifuged in a microfuge at maximum speed for 10 minutes. While the centrifugation was in progress, a QIAGEN-tip 20 was equilibrated by applying 1ml of Buffer QBT (supplied with the kit), and allowing it to empty by gravity flow. As soon as the centrifugation was over, the supernatant was applied on the pre-equilibrated QIAGEN-tip 20 and allowed to enter the resin by gravity flow. The tip was then washed with 4 x 1 ml Buffer QC (supplied with the kit). After the washing step, the DNA in the resin was eluted with 0.8 ml Buffer QF (supplied with the kit). The eluted DNA was then precipitated with 0.7 volumes of room-temperature isopropanol and centrifuged immediately at >10,000 rpm for 30 minutes, after which the supernatant was carefully decanted. The DNA pellet in the tube was washed twice with 70% ethanol, air-dried and redissolved in a suitable volume of Tris-EDTA (TE) buffer. The purity and concentration of the extracted plasmid was checked by gel-electrophoresis.

3. 3.18 Designing of Primers for Sequencing of P1 Region

All primers used for sequencing were designed manually. Initially, three primers viz., MG39, MG40 and 41 were designed based on the conserved regions after comparing sequences of serotype O and A. These primers were found to work in the case of Asia1 viruses also and the sequences generated using them became useful for designing the rest of the primers listed in Table 7a & 7b. A total of 12 primers used in manual sequencing were obtained commercially from Life Technologies and 6 Cy5 labelled primers used in Automated sequencing were purchased from Operon, USA.

3.3.19 Manual Sequencing

Recombinant plasmids harboring the right-sized inserts as evident from RE digestion were sequenced using the primers designed in the study (Table.7a,

Table .7a Primers used in manual sequencing

Primer Name	Sequence (5' - 3')	Position	Polarity	Length	T_A °C
MG39	GGTGGTGAGGATGCGGTCTTC	VP2, 31-51	-ve	21	50
MG40	AACGGGTGGGACATTGAGGT	VP2, 307-326	+ve	20	45
MG41	GTAGGTGTTGGACATGTGCCCCGC	VP3, 244-267	-ve	24	50
MG42	ATCATCAACAACTACTACATGCA	VP4, 61-84	+ve	24	45
MG43	CCCAACACCTCAGGCTTGGAGAC	VP2, 155-178	+ve	24	50
MG44	GGTGCTGCATTCATGTAAACCTT	VP2, 594-616	-ve	23	45
MG45	GGCAACATGGTGACCACAGACCC	VP3, 34-56	+ve	23	50
MG46	GCGGGGCACATGTCCAACACCTAC	VP3, 244-267	+ve	24	50
MG47	GAGAACTACGGAGGAGAGACTCA	VP1, 75-97	+ve	23	45
MG48	CTCCCCTACACCGCCCCCCA	VP1, 348-367	+ve	20	50
MG1D180	ATGCAGATCCCCTCACACACGCTG	VP1, 180-203	+ve	24	50
AS1C505	TACACTGCTTCTGACGTGGC	VP3, 505-524	+ve	20	50

T_A °C, Annealing Temperature in cycle sequencing

Table .7b Primers used in Automated sequencing

Primer Name	Sequence (5' - 3')	Position	Polarity	Length	T_A °C
MG41	GTAGGTGTTGGACATGTGCCC CGC	VP3, 244-267	-ve	24	50
MG45	GGCAACATGGTGACCACAGACCC	VP3, 34-56	+ve	23	50
DH1	AACAACTACTACATGCA	VP4, 67-83	+ve	17	45
MG47	GAGAACTACGGAGGAGAGACTCA	VP1, 75-97	+ve	25	45
MGP1RS	GTCCACCAGTTTGGAGAAGTT	2B, 28-48	-ve	21	50
DH6	TTGTTCTGAGTGTTGGTTGTGTG	VP4, 171-193	-ve	23	50

T_A °C, Annealing Temperature in cycle sequencing

Table. 8 Cycling conditions used in sequencing reactions

Temperature	Time	Number of Cycles	Remarks
95°C	2 minutes	1	Denaturation
95°C T_A (Table .7) 72°C	30 seconds 30 seconds 1 minute	60	Cycle Sequencing reaction
4°C	Hold		

Fig. 6a. Location of Primers used in Manual sequencing

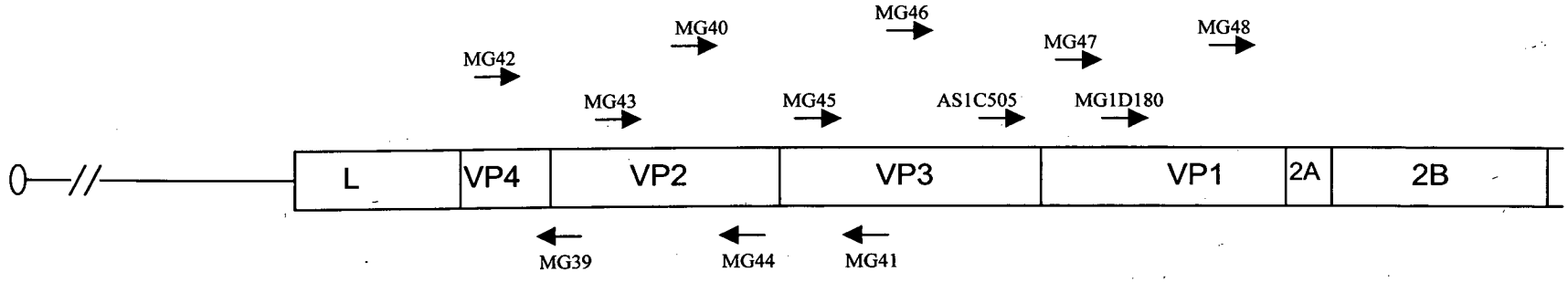


Fig. 6b. Location of Cy5 labelled Primes used in Automated sequencing

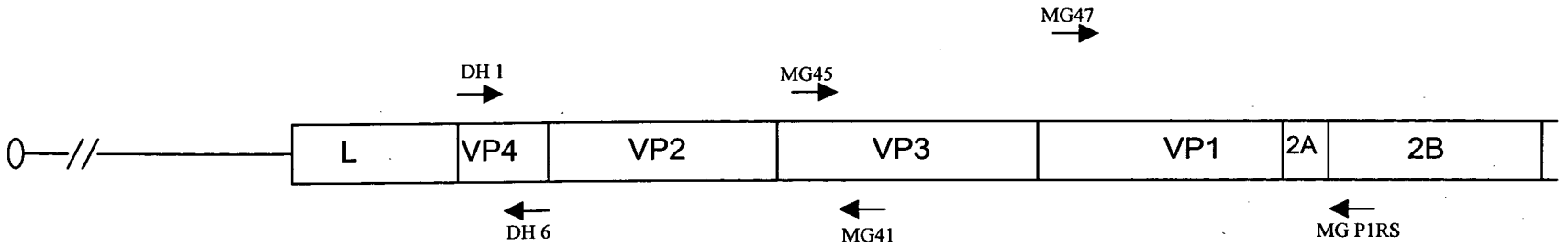


Fig.6).The "fmol^R DNA cycle sequencing system" (Promega, Cat. No. Q4100) was used for the purpose.

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

A sequencing reaction mixture contained 7 µl template plasmid, 4 µl 5X sequencing buffer (250 mM Tris Hcl pH 9.0, 10 mM $MgCl_2$), 25 pmol primer (Table.7a), 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 20 µl) of PCR grade mineral oil was dispensed into the tubes and they were briefly centrifuged to collect the contents at the bottom of the tubes. The sequencing tubes were loaded on to a thermocycler (Omnigene, Hybaid) and it the cycling was performed according to the program given in Table 8. The sequencing reactions were stopped by adding 3 µl of sequencing stop solution and the reactions were stored at -20°C till loading onto a sequencing gel.

3.3.20 Polyacrylamide Gel Electrophoresis of Cycle Sequencing products

The glass plates used for making the gel-sandwich were first thoroughly cleaned with warm water and detergent, rinsed with de-ionized water to remove detergent residues and finally cleaned with ethanol-soaked tissue papers. The shorter glass plate was pretreated with 1ml of binding solution (1ml 95% ethanol, 0.5% glacial acetic acid and 3µl of Bind silane (Promega)) while pre-treatment of the larger glass plate was accomplished using Sigmacote^R (Sigma) solution. The pre-treatments

were done so as to enable the gel to remain with the shorter glass plate on which it will be stained to visualize the DNA bands.

The cleaned and treated glass plates were taped together with 0.2 mm spacers on either side and 6% Sequencing Gels (Promega) was poured into the assembled gel cassette after adding 400 µl 10% ammonium persulfate solution. After polymerization was complete, the tapes were removed and the gel sandwich was mounted on to the sequencing apparatus. Wells formed after inserting the comb were washed to remove urea and the gel was pre-electrophoresed for 30 minutes at a set wattage of 70. After cleaning the wells again, heat-denatured (85°C for 2 minutes) cycle sequencing samples were loaded (2.5µl/well) onto the gel in the order T, C, G, A. The gel was run at a set wattage of 70 for the required time (2-5 hours).

3.3.21 Fixing, Staining and Developing of the Sequencing Gel

After the electrophoresis was over the sequencing device was disassembled; spacers and comb were removed fom the gel sandwich and the glass plates were pried apart. For fixing the gel, the shorter glass plate along with the gel was immersed (gel side up) in a tray containing 10% glacial acetic acid (Sigma, Cat. No. A-0808) in ultra-pure water and agitated gently for 20 minutes; the gel was stored in the fixing solution for an additional 3 hours without shaking. Following fixing, the gel was stained using "Silver Sequencing ™ DNA staining reagents" (Promega, Cat. No. Q4132). For staining, the glass plate with the gel was first washed thrice in ultra-pure water and then immersed in the staining solution (prepared by dissolving 2g of silver nitrate in 2 liters of ultra-pure water to which 3ml of 37% formaldehyde was added) and agitated for 30 minutes. To develop the gel the following solutions were made: Developing solution was prepared by dissolving 60g of Sodium Carbonate (Na_2CO_3) in 2 liters of ultra-pure 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). Following staining, the gel on the glass plate was washed briefly by immersing it in a tray containing ultra-pure water and immediately transferred into the first developing-tray and gently agitated till the tracks appeared. Immediately, it was transferred to the second developing tray and agitated till all bands became sufficiently visible. 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 ultra-pure water for 2 minutes each, it was dried at 37°C and viewed on a light box to read the sequence.

3.3.22 Autocycle sequencing for ALFexpress II Automatic Sequencer

Sequencing of the ultra-pure plasmids was done using the ALFexpress™ Autocycle ™ sequencing kit. A reaction master mix was prepared by adding 1μg of plasmid DNA, 2μl of reaction buffer, 5μl of dNTP solution, 2μl of DMSO, 10 picomols of Cy5 (carbo cyanine) labeled primer (Table. 7b) and 2 l of diluted *Taq* DNA polymerase (1.25 units /μl in enzyme dilution buffer); the volume was adjusted to 18 μl with de-ionized water. The reaction components were mixed thoroughly and four μl of the master mix was added to four 0.2ml tubes labeled A, C, G, T and containing 2 μl of the respective ddNTPs. The tubes were spun to mix the contents and loaded onto a Hybaid Multiblock System thermocycler (Hybaid, U.K) and 40 cycles of the sequencing reactions were performed according to the program given in Table 8. At the end of the reaction, they were stopped by adding 4 μl of stop solution (deionized formamide containing 5mg/ml blue dextran) and stored at 4° C until use.

3.3.23 Preparation of the gel cassette and Electrophoresis

For use in the ALFexpress sequencer, the gel solution is to be poured into a gel cassette made of a special thermoplate and a glass plate. First, both the thermoplate

and the glass plate were cleaned with detergent and tepid water and thoroughly rinsed with Milli Q water. Starting from the bottom, both the plates were cleaned with ethanol and polished dry. A few drops of bind silane solution (1ml absolute alcohol, 3 µl Bind Silane (Pharmacia Biotech), 250 µl of 10% acetic acid) was applied sparingly to the upper 2-3 cm of the thermoplate and 5 cm of the glass plate and polished dry. Excess binding solution was removed with MilliQ soaked tissue and the plates were cleaned with ethanol.

Clean spacers (0.3 or 0.5 mm) were placed on the indented edges of the thermoplate and the glass plate was lowered on to the thermoplate; both plates were clamped together using four pairs of gel clips. A clean comb of appropriate thickness (0.3 or 0.5mm) was inserted into the gel cassette on the top and the assembled cassette was placed in the Reproset (UV-polymerizer, Pharmacia Biotech). The two components of the ReproGel™ Long Read (Pharmacia Biotech) were mixed (final gel concentration, 7%) and the gel was cast from the bottom of the gel cassette as per the manufacturers instructions, and exposed to UV light for 10 minutes. The cassette with the polymerized gel was then mounted on to the ALFexpress II sequencer, buffer reservoirs filled with 0.5X TBE, comb removed, and the wells flushed clean. Denatured and snap cooled samples were loaded in the order A, C, G, T and electrophoresced at 25 watts for 700 minutes. The data obtained at the end of the run was processed using the ALFwin™ 2.1 software and exported as an ASCII text file.

3.4 Analysis of Sequence Data

The nucleotide sequences were read manually (in manual sequencing procedure) and sequences for all the mutants were compared with the parent virus. The sequences were aligned and translated using the program EditSeq of the DNASTAR package.

A total of 5 primers were sufficient in ALF express sequencer to obtain the entire P1 region. The data from the electrophoresed gel was processed by the ALFWin™ 2.1 software and exported as ASCII text files. The sequence data from 5 individual files, for each complete P1 region, were analyzed manually to remove the overlapping sequence stretches obtained using different primers. Sequences of all the viruses generated this way were aligned and translated using the program EditSeq of the DNASTAR package.

X-ray crystallographic data submitted to the Brookhaven Protein Databank in the form of a Protein DataBank (PDB) file can be visualized with the help of Molecular Graphics programs like RasMol. In the case of closely related proteins, where crystallographic data is not available, to help in understanding the spatial location of protein subunits and particular amino acid residues, the amino acid sequences of the protein of interest can be aligned with the amino acid sequences of that protein whose PDB file is available. In the case of FMD virus, since the anti-parallel β -barrel or jelly-roll structure of the capsid proteins is largely conserved between serotypes, such alignment can help in highlighting the corresponding residues in the known structure so that it may be assumed within reasonable limits that the amino acid residues on the protein of interest might also have a probability of being located in the same place.

In order to have a better understanding of the location of those residues that have changed with respect to the parent virus in the three-dimensional structure of the FMDV capsid, 1FOD.PDB, a Protein Data Bank (PDB) file which contains the atomic coordinates of FMDV O_1 BFS 1860 capsid was down-loaded from the Brookhaven Protein DataBank at www.rcsb.org. Also, the amino acid sequence of FMDV O_1 BFS 1860 capsid proteins were down-loaded. They were aligned with that of the Asia1 parent virus using the Megalign program of Dnastar, the corresponding positions in the O1BFS sequence with respect to residues changed in the mutants were noted and highlighted in the capsid structure using RASMOL 2.6 B2 (Roger Sayle, 1996).

RESULTS

4. RESULTS

4.1 Roller Culture Propagation of Parental virus stocks

The parent virus once passaged in 25 cm^2 flasks was inoculated onto a BHK cell monolayer in a roller bottle to obtain a high-titred virus stock for use throughout the study. This was also to avoid any variation in using virus stocks of different passages grown in different batches. Complete CPE was noticed in 18 hours post-infection and the virus harvested had a titre of $10^{6.5}TCID_{50}$/ml.

4.2 Selection of Monoclonal Antibodies

All the 29 Mabs were tested for their ability to neutralize the infectivity of the parent virus in a microneutralization test. All Mabs were found to neutralize the parent virus to varying degrees (Table.9) and were used to isolate Mab-resistant mutants and for further screening of mutants isolated.

4.3 Isolation of Single Monoclonal Antibody Resistant (MAR) Mutants

Isolation of single monoclonal antibody resistant mutants against all the 29 neutralizing Mabs were tried and it was possible to isolate complete mutants against all of them. Depending on the Mab-virus ratios, in some instances mutants with partial reactivity against the selecting Mabs, in addition to complete mutants, were also obtained. All mutants listed could be isolated in a maximum of two trials, except in the case of MAR10 that needed more number of attempts in which the Mab: virus ratio was to be standardized more critically. In this case, the diluted virus (to 10^{-6} $TCID_{50}$/ml) was mixed with the undiluted Mab at 10 different ratios (1:1 to 1:10) and

Table .9 Nuetralization titres of Mabs Used

Serial No	Mab	Log_{10} Nuetralizing Index
1	B3	2.5
2	D	2.0
3	E	2.5
4	H	2.0
5	W	2.5
6	1A	1.5
7	2A	3.0
8	3A	3.0
9	7C3	3.0
10	7	2.5
11	8A	2.5
12	8B	2.5
13	9	2.5
14	10	3.5
15	13	2.5
16	16	2.0
17	34	2.0
18	40	3.0
19	54	2.5
20	61	3.0
21	62	2.0
22	63	2.5
23	64	2.0
24	66	3.0
25	71	3.0
26	72	3.0
27	78	1.5
28	76	3.0
29	82	3.0

infected to BHK cells in 6 well plates as described. The viruses from wells in which the CPE was evident were analyzed for their reactivity with the Mab. The entire procedure was repeated once more to get a stable mutant population. As this was the only Mab in this group (see below), unlike in other groups where atleast three mabs were used for mutant generation, this mab was used to generate mutants in two more independent trials. This was to confirm the mutations occuring due to pressure from Mab 10.

4.4 Mab profiling of MAR mutants

All the 29 single site mutants isolated were subjected to Mab-profiling ELISA to know their reactivity pattern. The profiling results of mutants with different Mabs are given in Table 10 & Fig.7. Profiling ELISA indicated that there were a maximum of three different reactivity patterns. The grouping of MAR mutants is designated by I, II and III and that of corresponding Mabs is designated by 1, 2 and 3.

I The three mutants (MAR 72, MAR 76 and MAR 82) showed similar reactivities with the entire Mab panel and were included in Group I. These mutants did not react with the corresponding Mabs (72, 76 and 82) but retained homologous (60% and above) reactivity with rest of the Mabs. Such a reactivity pattern of these three mutants indicated that the selecting Mabs recognize a distinct epitope or antigenic site on the virion surface. All the three mutants of this group were chosen for cloning and sequencing.

II All the single Mab mutants other than 72,76,82 and 10 (a total of 25 mutants) formed the second group. They showed reduced or no reactivity with the Mabs of group 2 but reacted with Mabs of the other two groups. Based on the degree of reactivity with homologous Mabs, this group was subdivided into two groups (Group IIa and IIb).

Table.10.Percentage reactivity values of mutants against the Mab panel

Mab / Mutant	Monoclonal Antibodies															Group
	10	82	72	76	7C3	62	64	61	W	63	78	71	7	54	34	
MAR 72	74	0	0	0	101	93	94	92	93	75	100	88	94	91	120	
MAR 76	69	0	10	0	102	107	103	99	105	98	100	101	103	97	118	I
MAR 82	61	0	10	0	90	89	85	88	91	86	92	93	89	84	108	
MAR 1A	71	57	54	37	71	71	75	82	78	76	63	69	68	62	62	
MAR B3	79	128	103	105	50	48	50	48	48	48	35	40	40	31	32	
MAR 66	60	75	71	74	64	60	58	58	59	60	53	54	52	42	58	
MAR 13	72	100	83	88	108	120	100	84	99	97	75	69	69	54	70	IIa
MAR E	76	92	99	97	49	40	58	49	54	60	28	47	45	43	54	
DMAR 1A-B3	89	112	107	110	37	55	53	52	47	57	40	44	40	30	39	
MAR W	75	100	96	101	25	31	34	30	29	34	34	25	23	21	30	
DMAR 13-16	78	102	101	104	21	36	33	33	28	36	24	25	23	24	22	
MAR 3A	60	92	63	70	0	0	0	0	0	0	0	0	1	0	4	
MAR 7C3	90	90	100	98	0	0	0	0	0	0	0	0	0	0	0	
MAR 62	100	100	100	99	0	0	0	0	0	0	0	0	0	0	0	
MAR 64	62	68	68	71	0	0	0	0	0	0	0	0	0	0	0	
MAR 61	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	
MAR 71	100	100	90	91	0	0	0	0	0	0	0	0	0	0	0	
MAR 63	100	100	90	92	0	0	0	0	0	0	0	0	0	0	0	
MAR 78	100	100	90	94	0	1	2	2	2	0	1	2	1	0	1	
MAR 7	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	IIb
MAR 54	100	100	100	100	0	0	1	1	1	0	1	1	1	0	0	
MAR 34	64	92	100	100	0	0	0	0	0	0	2	5	2	2	0	
MAR 8A	66	100	97	98	6	7	8	7	8	8	5	6	6	4	6	
MAR 9	95	90	100	100	0	0	0	0	0	0	0	0	0	0	0	
MAR D	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	
MAR 8B	60	69	72	72	12	15	10	8	6	6	6	5	3	0	8	
MAR H	72	87	96	97	2	2	1	0	0	0	0	0	0	0	0	
MAR 2A	47	69	52	58	0	0	0	0	0	0	0	0	0	0	0	
MAR 40	33	60	53	56	0	0	0	0	0	0	0	0	0	0	0	
MAR 16	31	48	37	14	12	6	6	4	4	4	4	0	0	0	8	
DMAR B3-10	23	20	36	24	18	19	13	13	11	10	8	7	6	0	9	
DMAR 7-10	12	12	16	18	19	19	19	17	17	16	14	13	10	8	14	
MAR 10	0	0	0	0	16	18	12	10	8	8	8	8	6	0	2	III
DMAR 66-72	8	8	12	10	0	2	2	2	3	4	4	4	4	1	4	
DMAR 72-B3	2	2	2	2	0	2	2	3	3	4	6	4	4	2	4	

Continued

Table.10.Percentage reactivity values of mutants against the Mab panel

Mab / Mutant	Monoclonal Antibodies														Group
	3A	40	E	16	D	B3	H	2A	13	66	9	8A	8B	1A	
MAR 72	97	121	118	94	98	125	70	107	72	106	94	91	95	97	
MAR 76	109	108	106	122	102	104	97	96	98	121	117	122	102	101	I
MAR 82	96	104	91	109	93	102	94	105	82	109	98	110	100	97	
MAR 1A	61	70	44	50	54	65	65	42	43	98	57	73	73	34	
MAR B3	65	52	36	68	81	49	51	29	16	22	53	149	120	12	
MAR 66	50	60-	46	55	65	55	50	30	27	29	45	42	40	18	
MAR 13	59	72	50	61	70	70	72	40	41	35	54	57	59	21	IIa
MAR E	45	44	17	48	52	43	57	16	20	24	42	40	42	7	
DMAR 1A-B3	46	55	16	48	49	55	61	29	10	24	35	35	35	10	
MAR W	27	25	9	29	33	3	31	9	9	13	25	21	22	3	
DMAR 13-16	23	33	8	28	26	31	33	15	6	12	19	19	18	14	
MAR 3A	9	4	14	43	47	0	0	0	1	3	11	14	13	3	
MAR 7C3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
MAR 62	0	0	0	0	0	0	0	0	0	0	0	0	0	3	
MAR 64	0	0	0	0	0	0	0	0	0	0	0	0	0	3	
MAR 61	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
MAR 71	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
MAR 63	0	0	0	0	0	0	0	0	0	0	0	0	0	2	
MAR 78	0	2	1	0	0	2	2	1	1	1	0	0	0	2	
MAR 7	0	2	0	0	0	0	1	0	0	0	0	0	0	1	
MAR 54	0	2	1	0	0	0	0	1	1	1	0	1	1	1	IIb
MAR 34	1	0	0	0	1	0	0	1	3	0	0	0	0	3	
MAR 8A	5	7	8	4	7	6	7	5	4	3	4	4	3	2	
MAR 9	0	0	0	0	0	0	0	0	0	0	0	0	0	3	
MAR D	0	0	0	0	0	0	0	0	0	0	0	0	0	3	
MAR 8B	5	12	3	11	13	5	3	3	0	1	3	3	3	0	
MAR H	1	0	0	2	2	0	7	0	0	0	1	1	1	3	
MAR 2A	0	0	0	0	0	0	0	0	0	0	0	0	0	3	
MAR 40	0	0	0	0	0	0	0	0	0	0	0	0	0	3	
MAR 16	3	8	0	10	19	6	2	0	0	0	1	0	0	0	
DMAR B3-10	6	9	5	12	18	6	17	4	1	3	4	6	6	2	
DMAR 7-10	10	15	10	15	17	13	19	9	9	11	9	11	12	8	
MAR 10	2	2	2	3	4	2	3	1	0	0	0	0	0	1	III
DMAR 66-72	3	7	3	9	17	5	0	2	2	1	2	2	2	0	
DMAR 72-B3	3	8	4	3	10	5	0	2	3	1	14	7	6	0	

Fig. 7. Mab-profiling results of mutants

MUTANTS	MONOCLONAL ANTIBODIES															GROUP
	10	82	72	76	7C3	62	64	61	W	63	78	71	7	54	34	
MAR 72	●	○	○	○	●	●	●	●	●	●	●	●	●	●	●	I
MAR 76	●	○	○	○	●	●	●	●	●	●	●	●	●	●	●	
MAR 82	●	○	○	○	●	●	●	●	●	●	●	●	●	●	●	
MAR 1A	●	◆	◆	▲	●	●	●	●	●	●	●	●	●	●	●	IIa
MAR B3	●	●	●	●	◆	◆	◆	◆	◆	◆	▲	◆	◆	▲	▲	
MAR 66	●	●	●	●	●	●	◆	◆	◆	●	◆	◆	◆	◆	◆	
MAR 13	●	●	●	●	●	●	●	●	●	●	●	●	●	◆	●	
MAR E	●	●	●	●	◆	◆	◆	◆	◆	●	▲	◆	◆	◆	◆	
DMAR 1A-B3	●	●	●	●	▲	◆	◆	◆	◆	◆	◆	◆	◆	▲	▲	
MAR W	●	●	●	●	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	
DMAR 13-16	●	●	●	●	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	
MAR 3A	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	IIb
MAR 7C3	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 62	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 64	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 61	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 71	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 63	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 78	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 7	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 54	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 34	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 8A	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 9	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR D	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 8B	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR H	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	
MAR 2A	◆	●	◆	◆	○	○	○	○	○	○	○	○	○	○	○	
MAR 40	▲	●	◆	◆	○	○	○	○	○	○	○	○	○	○	○	
MAR 16	▲	◆	▲	○	○	○	○	○	○	○	○	○	○	○	○	
DMAR B3-10	▲	▲	▲	▲	○	○	○	○	○	○	○	○	○	○	○	III
DMAR 7-10	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 10	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
DMAR 66-72	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
DMAR 72-B3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	

● 60% and above; ◆ 40-59%; ▲20-39%; ○ 20% and below

(Continued)

Fig. 7. Mab-profiling results of mutants

MUTANTS	MONOCLONAL ANTIBODIES														GROUP
	3A	40	E	16	D	B3	H	2A	13	66	9	8A	8B	1A	
MAR 72	●	●	●	●	●	●	●	●	●	●	●	●	●	●	I
MAR 76	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
MAR 82	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
MAR 1A	●	●	◆	◆	◆	●	●	◆	◆	●	◆	●	●	▲	IIa
MAR B3	●	◆	▲	●	●	◆	◆	▲	○	▲	◆	●	●	○	
MAR 66	◆	●	◆	◆	●	◆	◆	▲	▲	▲	◆	◆	◆	○	
MAR 13	◆	●	◆	●	●	●	●	◆	◆	▲	◆	◆	◆	▲	
MAR E	◆	◆	○	◆	◆	◆	◆	○	▲	▲	◆	◆	◆	○	
DMAR 1A-B3	◆	◆	○	◆	◆	◆	●	▲	○	▲	▲	▲	▲	○	
MAR W	▲	▲	○	▲	▲	○	▲	○	○	○	▲	▲	▲	○	
DMAR 13-16	▲	▲	○	▲	▲	▲	▲	○	○	○	○	○	○	○	
MAR 3A	○	○	○	◆	◆	○	○	○	○	○	○	○	○	○	IIb
MAR 7C3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 62	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 64	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 61	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 71	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 63	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 78	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 7	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 54	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 34	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 8A	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 9	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR D	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 8B	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR H	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 2A	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 40	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 16	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
DMAR B3-10	○	○	○	○	○	○	○	○	○	○	○	○	○	○	III
DMAR 7-10	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MAR 10	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
DMAR 66-72	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
DMAR 72-B3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	

● 60% and above; ◆ 40-59%; ▲20-39%; ○ 20% and below

Six mutants (MAR 1A, MAR B3, MAR 66, MAR 13, MAR E and MAR W) which showed reduced or low reactivity with the selecting Mab were included in Group IIa. These mutants also had homologous reactivity (60% and above) with Mabs of the other two groups. Two of the mutants (MAR 1A and MAR 13) in this group had retained homologous reactivity with 17 of the Mabs of this group and hence they were used to isolate double mutants using a different Mab (Mab B3 in the case of MAR 1A and Mab 16 in the case of MAR 13) from the same set. After pressure from a second Mab also they retained the same reactivity pattern except a slight reduction in reactivity with all the Mabs. However these two mutants were not selected for further studies.

A total of 19 mutants included in group IIb were complete mutants against the group 2 Mabs and showed homologous reactivity with the Group 1 and Group 3 Mabs. In this way, these mutants can be considered as true mutants of Group 2 Mabs. From Group II a total of eight mutants, (five from Group IIa and three from Group IIb) were selected for sequence analysis. This was to find out if differences in the nature of substitutions exist in the case of partial and complete mutants against the same set of Mabs.

III MAR mutant isolated against Mab 10 (group 3 Mab) did not react with any Mab and formed the third group, This mutant behaved like a multiple mutant since it was non-reactive to all the Mabs. All the three mutans isolated using this Mab (in three independent trials) were non-reactive to any of the Mabs. Inspite of repeated trials, a mutant non-reactive to only to Mab 10 could not be isolated.

4.5 Isolation of Multiple Monoclonal Antibody Resistant Mutants and Their Mab profiling

Based on the above reactivity pattern of single mutants, one mutant (MAR 72) from the first group and three mutants (MAR B3, MAR 7 and MAR 66) from second group were selected and subjected to Mab pressure with Mabs from the other two groups. A total of four double mutants were isolated in this way viz. DMAR B3-10, DMAR 7-10 (Mab pressure from Group 3 Mabs against the mutants of Group 2 Mabs), DMAR 66-72 (Mab pressure from Group 1 Mab against the mutant of Group 2 Mabs) and DMAR 72-B3 (Mab pressure from Group 2 Mab against the mutant of Group 1 Mabs). Strangely, in their Mab reactivities these multiple mutants were similar to MAR10, ie., they were non-reactive to all the Mabs and hence were included in group III. Inspite of repeated trials, mutants resistant to all possible combination of Mabs could not be isolated. Four mutants (MAR 10, DMAR B3-72, DMAR 7-10, DMAR 66-72) from this group were chosen for sequencing.

The Mab profiling results of the 15 mutants selected for sequencing studies are given in Table.11 and represented in Fig. 8 and 9.

All the selected mutants were plaque purified twice and tested again in profiling ELISA. The reactivity pattern was same as before. These plaque purified viruses were used for all further studies.

4.6 Mab Profiling of Field Isolates

Eighteen field isolates of serotype Asia-1 collected from outbreaks that occurred in different parts of the country between 1985 and 1999 were propagated and tested in Mab Profiling ELISA to see their reactivity with the Mab-panel and also to identify viruses with a reactivity pattern different from that obtained with

Table. 11 Percentage reactivity values of selected mutants against selected Mabs

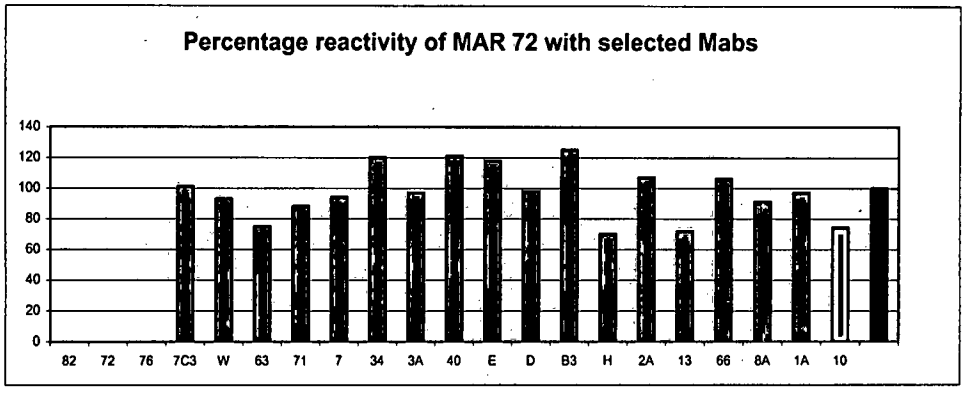
Mabs	1			2																	3	Grouping of mutants
	82	72	76	7C3	W	63	71	7	34	3A	40	E	D	B3	H	2A	13	66	8A	1A	10	
MAR 72	0	0	0	101	93	75	88	94	120	97	121	118	98	125	70	107	72	106	91	97	74	
MAR 76	0	10	0	102	105	98	101	103	118	109	108	106	102	104	97	96	98	121	122	101	69	I
MAR 82	0	10	0	90	91	86	93	89	108	96	104	91	93	102	94	105	82	109	110	97	61	
MAR 66	75	71	74	64	59	60	54	52	58	50	60-	46	65	55	50	30	27	29	42	18	60	
MAR B3	128	103	105	50	48	48	40	40	32	65	52	36	81	49	51	29	16	22	149	12	79	
MAR 13	100	83	88	108	99	97	69	69	70	59	72	50	70	70	72	40	41	35	57	21	72	IIa
MAR E	92	99	97	49	54	60	47	45	54	45	44	17	52	43	57	16	20	24	40	7	76	
MAR W	100	96	101	25	29	34	25	23	30	27	25	9	33	3	31	9	9	13	21	3	75	
MAR 3A	92	63	70	0	0	0	0	1	4	9	4	14	47	0	0	0	1	3	14	3	60	
MAR 7	100	100	100	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0	1	100	IIb
MAR H	87	96	97	2	0	0	0	0	0	1	0	0	2	0	7	0	0	0	1	3	72	
MAR 10	0	0	0	16	8	8	8	6	2	2	2	2	4	2	3	1	0	0	0	1	0	
DMAR 7-10	12	16	18	19	17	16	13	10	14	10	15	10	17	13	19	9	9	11	11	8	12	III
DMAR66-72	8	12	10	0	3	4	4	4	4	3	7	3	17	5	0	2	2	1	2	0	8	
DMAR72-B3	2	2	2	0	3	4	4	4	4	3	8	4	10	5	0	2	3	1	7	0	2	

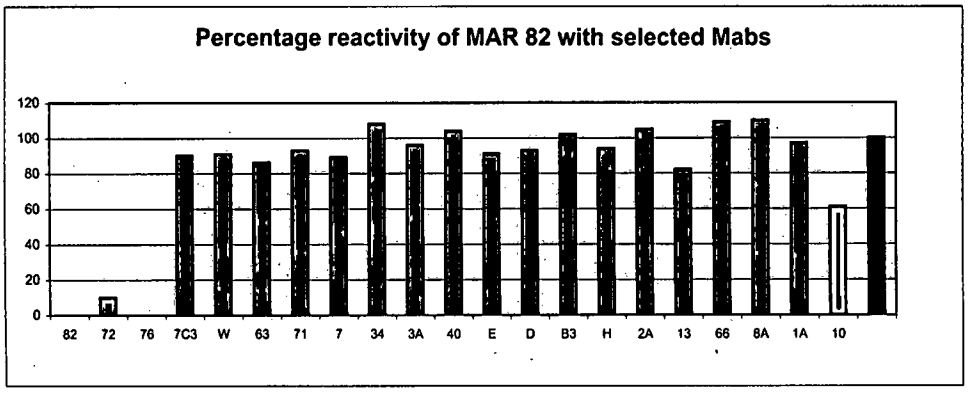
Fig.8. Mab-Profiling Results of selected mutants

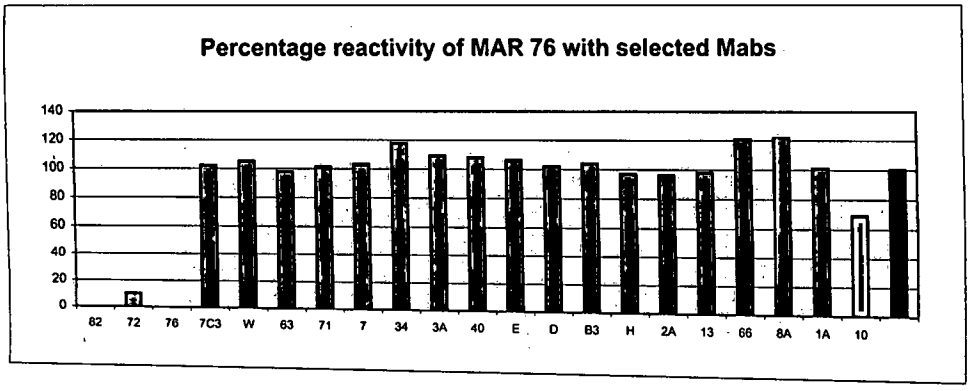
Grouping of Mabs	1		2																	3		Grouping of mutants
	82	72	76	7C3	W	63	71	7	34	3A	40	E	D	B3	H	2A	13	66	8A	1A	10	
MAR 72	○	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	I
MAR 76	○	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
MAR 82	○	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
MAR 66	●	●	●	●	◆	●	◆	◆	◆	◆	●	◆	●	◆	◆	▲	▲	▲	◆	○	●	IIa
MARB3	●	●	●	◆	◆	◆	◆	◆	▲	●	◆	▲	●	◆	◆	▲	○	▲	●	○	●	
MAR 13	●	●	●	●	●	●	●	●	●	◆	●	◆	●	●	●	◆	◆	▲	◆	▲	●	
MAR E	●	●	●	◆	◆	●	◆	◆	◆	◆	◆	○	◆	◆	◆	○	▲	▲	◆	○	●	
MAR W	●	●	●	▲	▲	▲	▲	▲	▲	▲	▲	○	▲	○	▲	○	○	○	▲	○	●	
MAR 3A	●	●	●	○	○	○	○	○	○	○	○	○	◆	○	○	○	○	○	○	○	●	IIb
MAR 7	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	
MAR H	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	
MAR 10	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	III
DMAR 7-10	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
DMAR 66 -72	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
DMAR 72-B3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	

● 60% and above; ◆ 40-59%; ▲20-39%; ○ 20% and below

Fig.9.Percentage reactivity of selected mutants with selected Mabs

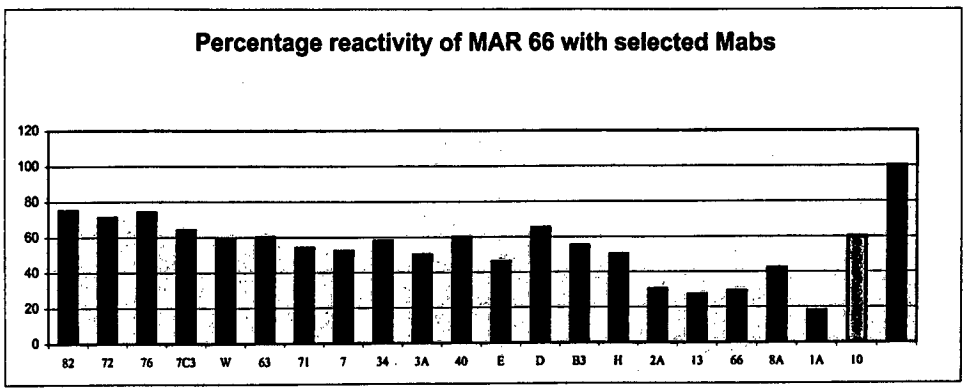


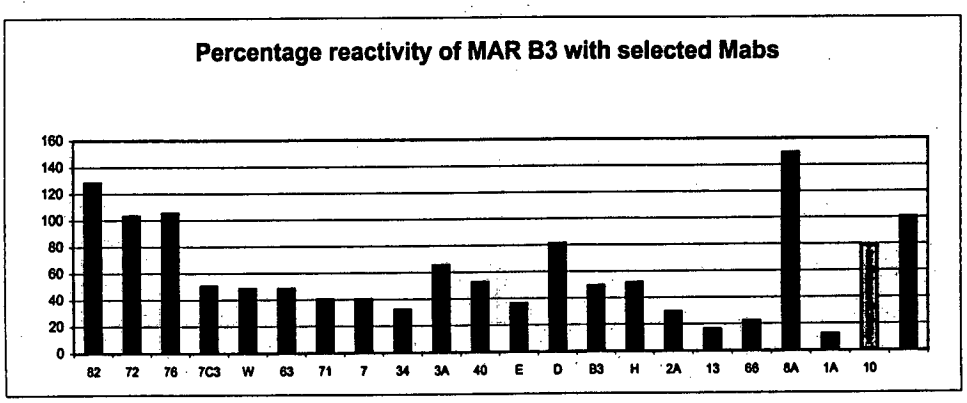


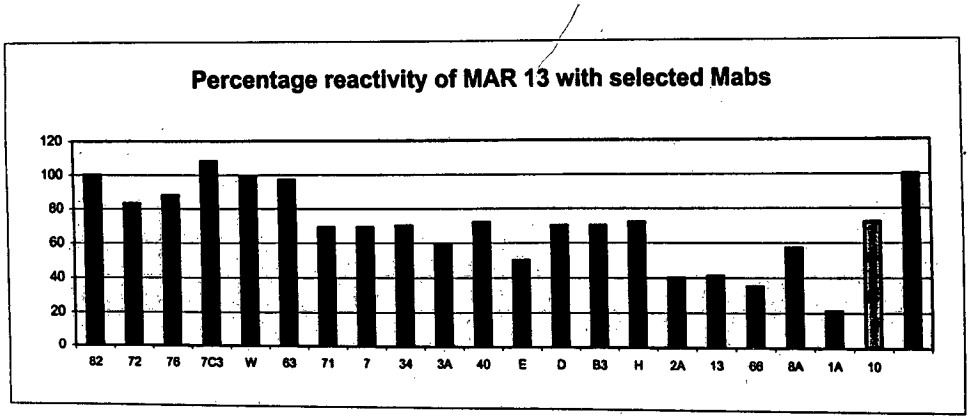


(Continued)

Fig.9. Percentage reactivity of selected mutants with selected Mabs

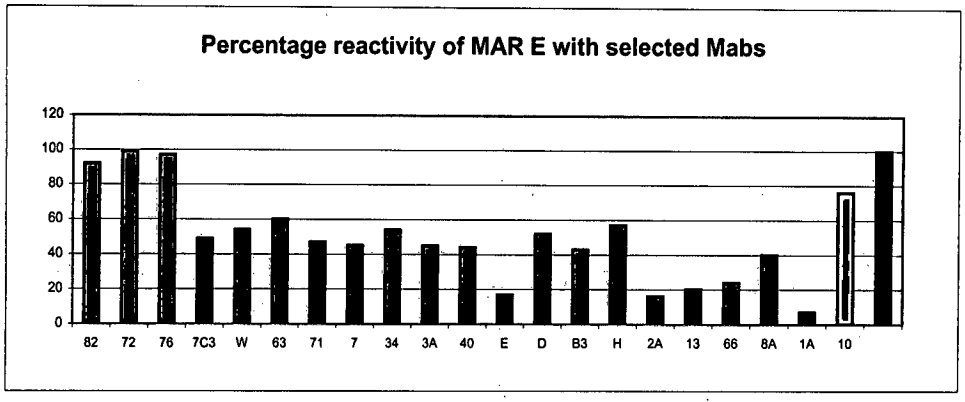


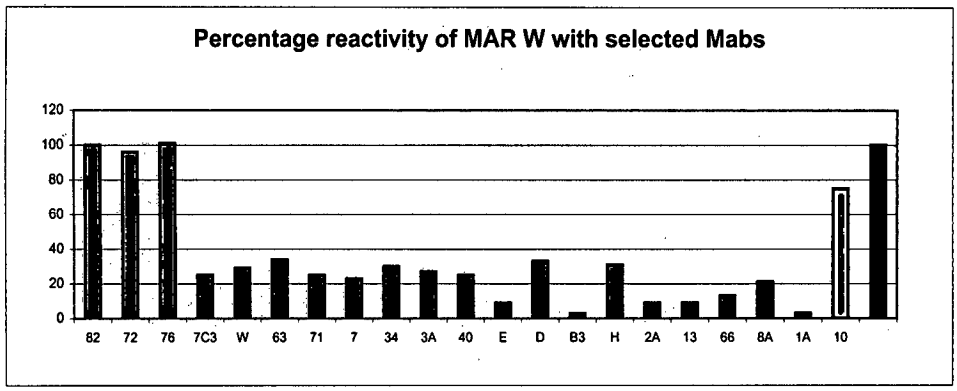


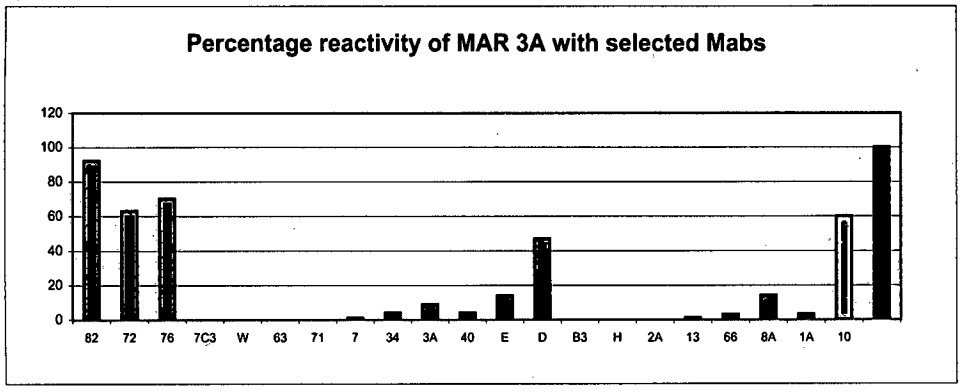


(Continued)

Fig.9. Percentage reactivity of selected mutants with selected Mabs

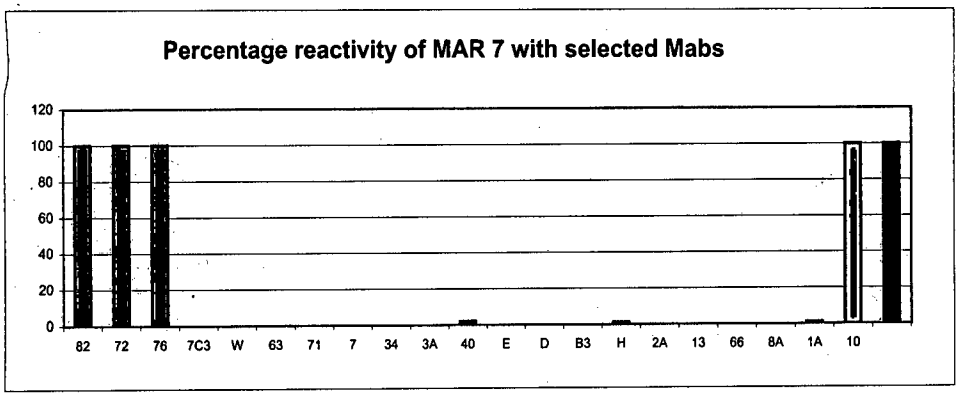


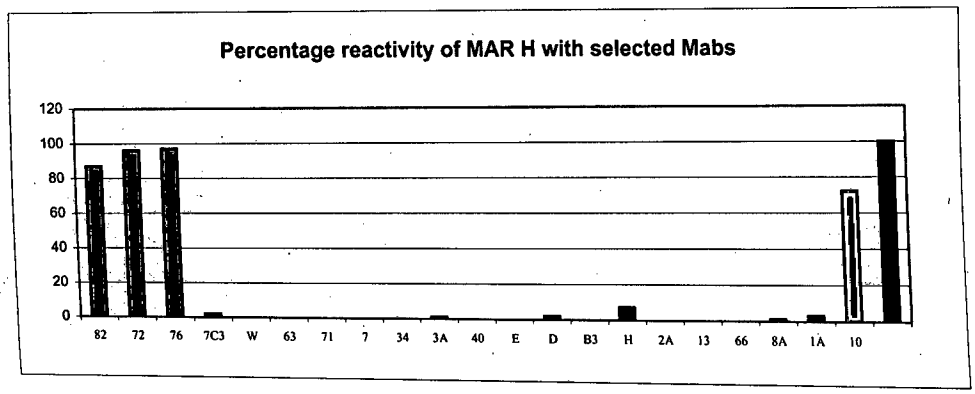


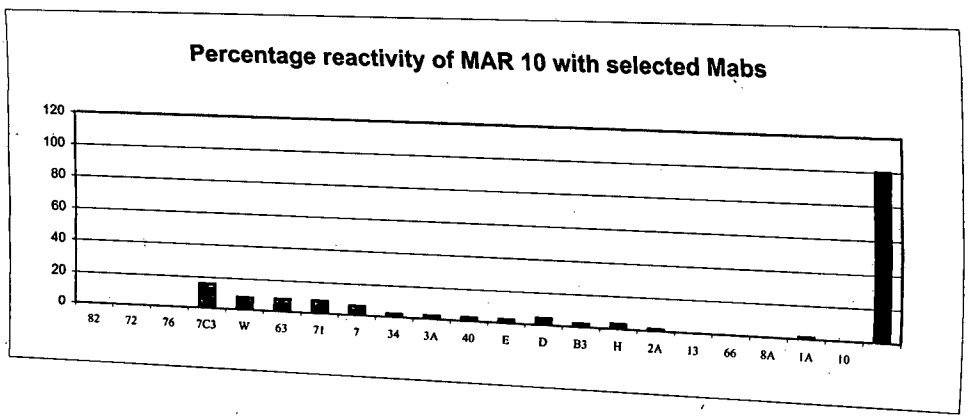


(Continued)

Fig.9. Percentage reactivity of selected mutants with selected Mabs

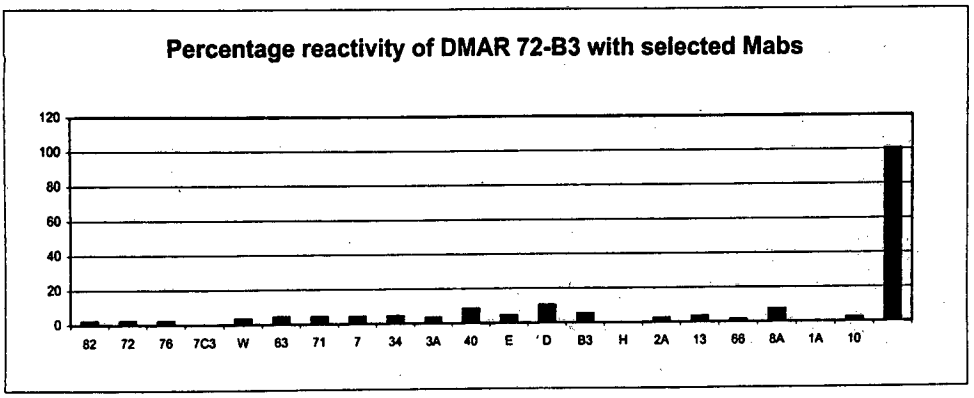


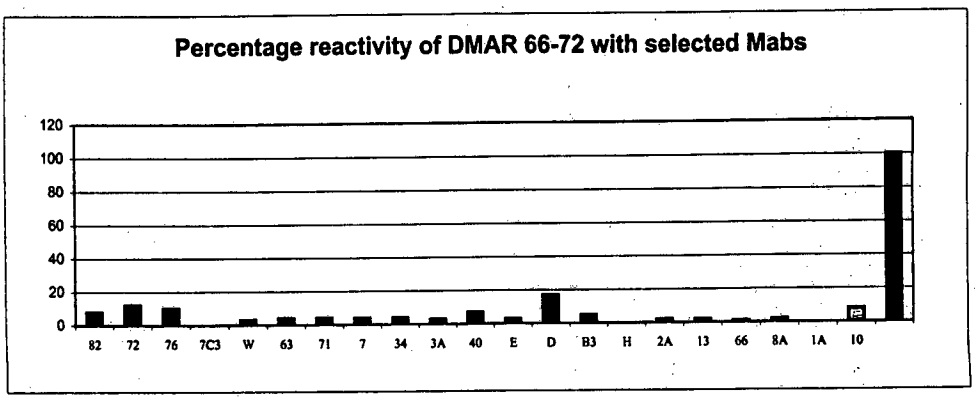


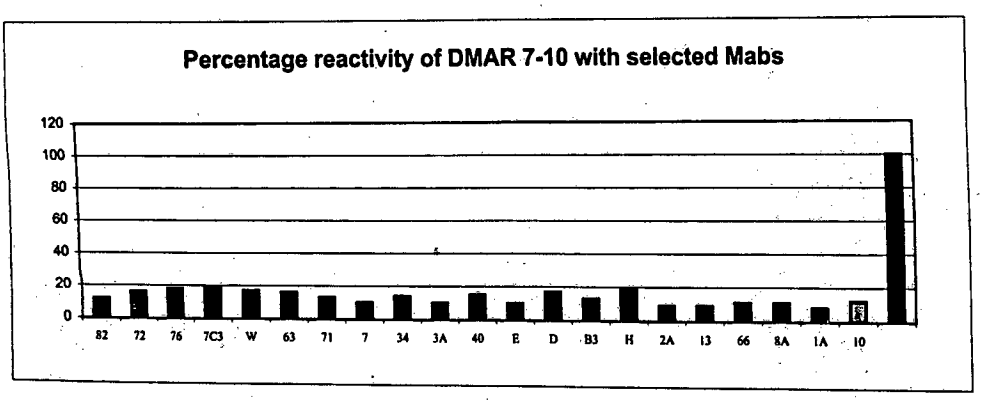


(Continued)

Fig.9. Percentage reactivity of selected mutants with selected Mabs







MAR mutants. The Mab profiling results of field isolates are given in Fig. 10, the percentage reactivity values are given in Table. 12. Based on the reactivity pattern the 18 field isolates were clustered into 5 groups (designated as group A, B, C, D and E) as follows.

A) Isolates 4/86,9/90,187/94 & 234/95 reacted (85% and above) with all the Mabs in the panel and formed the first group indicating that these isolates are very similar to the vaccine virus in their antigenic profile.

B) The second group consisted of two isolates 132/85 and 339/96 which showed 44 to 75% reactivity with group 1 Mabs (72, 76 & 82) reduced (37 &42%) reactivity with Mab 10. They showed low or reduced (22 to 48%) reactivity with Mabs 66, 40, 63, E, B3, 2A, 7C3, D,and 3A (group 2 Mabs) and very low reactivity (16% & less) with rest of the Mabs of this group.

C) Six isolates (ie., 235/99, 445/98,324/98, 103/99, 192/99and 126/98) of the third group showed homologous reactivity (60 to 75%) or reduced (39 to 51%) with group 1 Mabs and reduced reactivity with Mab 10 (24 to 48%). These viruses showed very low reactivity (<20%) with most of the group 2 Mabs while they retained reduced reactivity (21 to 32%) with Mabs 66, 40, 63, E and B3 of this group of Mabs.

D) The fourth group consisted of 5 isolates (IND 125/98, IND 130/98, IND 470/98, IND 68/99 and IND 69/99) all of which did not react with any Mabs of the panel.

E) Only one isolate, 49/93 formed this group. This virus did not react with Mabs 72, 76, 81 & 10 showed reduced reactivity with Mab 1A

Table.12. Percentage reactivity values of field isolates against selected Mabs

FIELD ISOLATES	MONOCLONAL ANTIBODIES																				Grouping of isolates
	72	76	82	10	66	40	63	E	B3	2A	7C3	D	3A	34	7	13	71	8A	W	H	1A	
IND 4/86	117	99	108	93	104	102	96	93	95	91	85	90	96	94	169	172	105	98	88	85	98	
IND 9/90	117	98	103	91	119	105	102	88	94	92	90	89	90	110	170	166	101	103	88	93	92	
IND 187/94	137	122	129	117	93	100	89	113	114	119	104	108	105	126	110	107	100	115	109	105	136	A
IND 234/95	134	127	128	124	94	101	90	115	121	121	109	115	102	122	101	110	101	116	106	106	126	
IND 132/85	68	44	59	37	52	37	42	31	32	23	22	25	25	4	15	12	14	3	16	15	3	B
IND 339/96	69	55	64	42	48	39	47	29	35	28	27	28	31	2	14	10	17	10	17	17	4	
IND 235/99	69	54	60	40	31	22	26	22	22	17	14	17	15	1	4	3	7	1	7	7	1	
IND 445/98	75	56	63	48	26	24	30	20	18	10	18	15	17	1	3	4	7	6	10	8	3	
IND 324/98	44	30	38	27	22	21	28	17	19	9	16	13	14	4	2	4	7	7	8	6	1	
IND 103/99	39	45	36	24	28	23	29	16	14	14	21	13	18	7	5	6	8	10	11	12	3	C
IND 192/99	49	35	39	26	32	17	21	10	10	7	10	8	10	0	3	2	4	1	5	5	0	
IND 126/98	55	39	51	35	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	
IND 125/98	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	
IND 130/98	4	2	3	3	1	1	4	5	2	1	2	3	2	4	0	0	1	5	3	3	1	
IND 470/98	0	0	0	0	0	0	0	0	0	1	0	0	0	3	0	0	0	0	0	0	0	D
IND 68/99	4	7	6	3	4	6	4	4	5	5	1	4	4	17	3	3	4	9	4	4	2	
IND 69/99	6	8	7	5	11	11	12	2	2	2	8	6	7	11	4	3	4	5	5	4	1	
IND 49/93	0	0	0	10	100	88	92	94	88	90	86	84	83	23	67	68	79	48	80	78	47	E

Fig. 10 Mab-Profiling Results of Field Isolates

FIELD ISOLATES	MONOCLONAL ANTIBODIES																					Grouping of isolates
	72	76	81	10	66	40	63	E	B3	2A	7C3	D	3A	B	7	13	71	8A	W	H	1A	89	
4/86	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	A
9/90	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
187/94	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
234/95	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
132/85	●	◆	◆	▲	◆	▲	◆	▲	▲	▲	▲	▲	▲	○	○	○	○	○	○	○	○	○	B
339/96	●	◆	●	◆	◆	▲	◆	▲	▲	▲	▲	▲	▲	○	○	○	○	○	○	○	○	○	
235/99	●	◆	●	◆	▲	▲	▲	▲	▲	○	○	○	○	○	○	○	○	○	○	○	○	○	C
445/98	●	◆	●	◆	▲	▲	▲	▲	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
324/98	◆	▲	▲	▲	▲	▲	▲	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
103/99	○	○	○	▲	▲	▲	▲	○	○	▲	▲	○	○	○	○	○	○	○	○	○	▲	▲	
192/99	◆	▲	▲	▲	▲	○	▲	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
126/98	◆	◆	◆	◆	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
125/98	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	D
130/98	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
470/98	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
68/99	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
69/99	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
49/93	○	○	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	◆	○	E

● 60% and above; ◆ 40-59%; ▲20-39%; ○ 20% and below

and reacted with all other Mabs. This virus, in its reactivity to the Mab panel behaved like a double mutant of Group 1 (72, 76, 82) and 3 Mabs (10). Unlike MAR10, this isolate while mutated against Mab 10 and Group 1 Mabs, appeared to have retained its reactivity against Group 2 Mabs.

4.7 Cross-Neutralization of MAR mutants

In order to check whether the cross reactivity pattern of the mutants with Mabs from heterologous groups as revealed in ELISA, is similar in cross-neutralizing ability of the heterologous Mabs, cross-neutralization of the selected mutants (from each group) was done with the representative Mabs from all the 3 groups. The results shown in Table. 13 indicate that MAR 72 was neutralized by Mabs of group 2 & 3 but was no longer neutralized by the homologous Mab (ie.,Mab 72). Similarly the complete mutants of group II (MAR 7, 3A & H) were neutralized by heterologous (group 1 & 3) Mabs but not by the group 2 Mabs. Since MAR B3 and 66 were not complete mutants of group 2 Mabs they were neutralized to some extent (log_{10} NI of 1) by the homologous Mabs & were neutralized to a greater extent (log_{10} NI of 3) by Mab 10 (group 3 Mab). The non-reactivity of MAR10 with any Mab in ELISA is also evident in cross-neutralization i.e. it is not neutralized by the Mabs of all the 3 groups.

4.8 Viral RNA Extraction & Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Viral RNA was extracted from the parent virus, all mutants and isolates as described in Materials and Methods. The RNA was then subjected to RT-PCR amplification (using the primers and procedure described) to amplify the entire P1 region (in mutants) or LP1 region (in isolates), in a single step RT-PCR using SuperScript RT-PCR system (Life Technologies) or Access RT-PCR system

Table. 13. Cross-neutralization results of the selected MAR mutants with the representative Mabs.

Viruses(mutants) tested in Cross Neutralization		Mab Group I	Mabs of Group II			Mab Group III
		Mab 72	Mab B3	Mab 66	Mab 7	Mab 10
Group I mutant	MAR 72	-	++	++	++	++
Group II mutants	MAR B3	++	+	+	+	+++
	MAR 66	++	+	+	+	+++
	MAR 7	++	-	-	-	++
	MAR 3A	++	-	-	-	++
	MAR H	++	-	-	-	++
Group III mutant	MAR 10	-	-	-	-	-

+; The number of "+" correspond to the number of log_{10} neutralizing units.

- Not neutralized

(Promega). The four different combination of primers tried viz. MG 51-52, MG 50-53, MG 50-P1CR and MG17C-P1CR, gave amplicons of calculated sizes ie., 2486, 2374, 2416 and 2997 bases respectively. All viral P1 or LP1 regions could be thus amplified; in a few instances nonspecific amplicons of about 0.5 and 1Kb were also noticed. These fragments did not interfere with cloning of the desired region since the right-sized fragment was gel purified for cloning. The primer combinations and conditions were found to work efficiently with all templates tried. A sample photograph of gel purified PCR products is given in Fig. 11.

4.9 Cloning of Gel-Purified PCR Products into pAmp vectors

The gel purified PCR products were rapid-cloned into either pAMP1 or pAMP10 vectors (depending on the primer combination used for PCR: Table. 6). Rapid cloning was a relatively fast and easy procedure, the number of right-sized recombinants obtained were much higher, background colonies were negligible. Altogether this procedure is much more efficient when compared to routine cloning (T/A cloning or RE digested fragments cloning). The number of colonies screened for right-sized inserts ranged from 1-5 (in most cases at least two of the three colonies picked contained the right-sized inserts, but for MAR3A five colonies were screened to obtain two positive recombinants with right-sized inserts). A photograph showing PCR products, positve colonies and their RE analysisis is given in Fig. 12.

4.10. Sequencing of recombinant plasmids

Plasmid DNA extracted using Wizard plus minipreps DNA purification system (Promega) was found to perform satisfactorily in manual sequencing. However, for automated sequencing, Plasmid DNA was extracted using the QIAGEN Plasmid Mini Kits (Qiagen) so as to obtain ultra-pure plasmid DNA free of salts and other impurities for sequencing. Ultra-pure plasmid DNA gave better results and read lengths

Fig.11. Photograph showing Agarose gel electrophoresis of Gel-purified PCR products.

Lane 1	DRIgest Marker
Lane 2	PCR product MG 50-53
Lane 3	PCR product MG 50-53
Lane 4	PCR product MG 51-52
Lane 5	PCR product MG 51-52
Lane 6	PCR product MG 50-P1CR
Lane 7	PCR product MG 50-P1CR
Lane 8	PCR product MG 17C-P1CR
Lane 9	PCR product MG 17C-P1CR
Lane 10	PCR product MG 17C-P1CR
Lane 11	PCR product MG 17C-P1CR

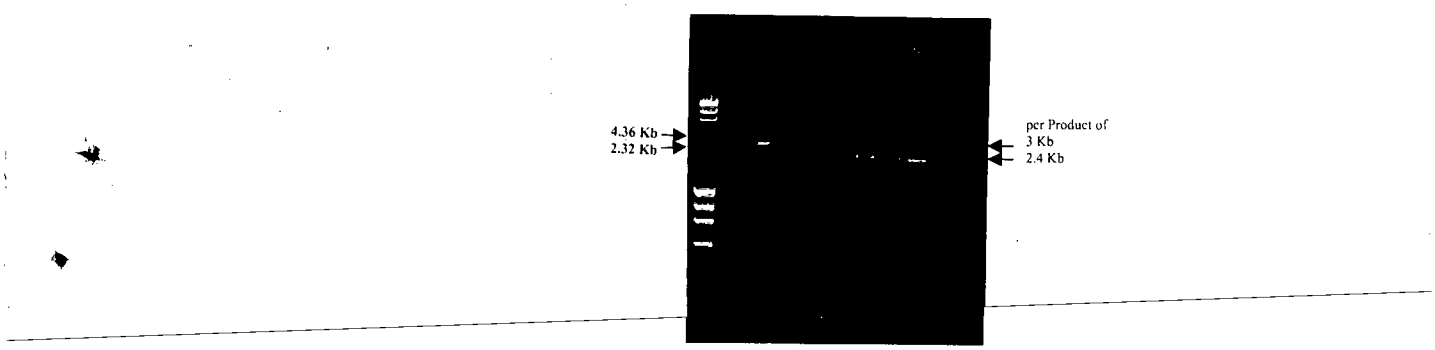
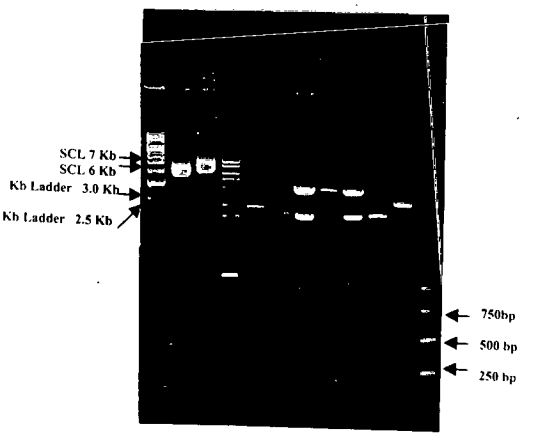

4.36 Kb
2.32 Kb
per Product of
3 Kb
2.4 Kb

Fig. 12. Photograph showing Agarose gel Electrophoresis of Gel-purified PCR products, recombinant plasmids and their RE digestion

Lane 1	**PCR marker**
Lane 2	**Gel-purified MG17C-P1CR PCR product**
Lane 3	**Bam H1 & EcoR1 cut MG17C-P1CR PCR product**
Lane 4	**Bam H1 & EcoR1 cut plasmid with the insert loaded in Lane 2**
Lane 5	**Linearized vector**
Lane 6	**Bam H1 & EcoR1 cut plasmid with the insert loaded in Lane 8**
Lane 7	**Bam H1 & EcoR1 cut MG50-P1CR PCR product**
Lane 8	**Gel-purified MG 50-P1CR PCR product**
Lane 9	**Kilobase ladder**
Lane 10	**Plasmid containing insert loaded in Lane 2**
Lane 11	**Plasmid containing insert loaded in Lane 8**
Lane 12	**Supercoiled ladder**


SCL 7 Kb
SCL 6 Kb
Kb Ladder 3.0 Kb
Kb Ladder 2.5 Kb
750bp
500 bp
250 bp

compared plasmids extracted in the rapid protocol. The 12 primers listed in Table. 7a. were used for sequencing the entire 2.2Kb P1 region. Of them, 9 primers, (viz., MG 39, 40, 41, 42, 43, 1C505, 46, 47 & 48) were found sufficient to sequence the entire P1 region. The parent virus and all mutants were sequenced manually. The parent and all the mutant viruses were again sequenced using the automated sequencer, with the same templates to confirm the substitutions. Since the precise location of antigenic sites of FMDV serotype Asia1 is not available, this became necessary. In automated sequencing, five primers, viz., MG41, 45, P1RS, DH1 and DH6 (Table.7b.) were found sufficient to obtain the entire P1 region. However, an additional primer (MG47) was used to sequence the opposite strand in the VP1 region to confirm the changes observed in the sequence obtained by sequencing the sense strand. In the case of VP2 region, the sequence for both the strands was covered by the primers DH1 and MG 41. These two regions (VP1 andVP2) were found to have many substitutions and hence both strands were sequenced as described. No difference was noticed in the sequence data generated by sequencing both the strands.

4.11 Analysis of Sequence Data

4.11.1Sequences of MAR mutants

All the 15 mutants (comprising of 12 single and 3 double mutants) were sequenced to get the entire P1 region sequence. The sequences thus obtained were compared and the results are presented as the nucleotide sequences in Fig. 13 and deduced amino acid sequences in Fig. 14 and Tables.14a, 14b and 14c. Nucleotide sequence comparison of the mutants showed absence of sequence changes in the VP4 gene, but base substitutions ranging from 1 (in MAR 66, B3, H and 81) to 6 (in MAR 72) were seen in the other three genes. Only the mutants MAR 3A, MAR 13, MAR 10 and DMAR 66-72 showed few (1 to 2 per mutant, Fig. 13) silent mutations in their sequences.

Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants. The amino acid sequence of the parent virus is given in the first line. The nucleotide changes leading to amino acid substitutions are shown in bold and their codons are shaded. Synonymous mutations are italicised.

VP4 Gene, 255nucleotides[85 Amino acids]

```
                1                                                                                                   20
                G   A   G   Q   S   S   P   A   T   G   S   Q   N   Q   S   G   N   T   G   S
IND63/72        GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MAR72           GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MAR76           GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MAR82           GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MAR66           GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MARB3           GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MAR13           GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MARE            GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MAR3A           GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MAR7            GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MARH            GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
MAR10           GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
DMAR7-10        GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
DMAR66-72       GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
DMAR72-B3       GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC

                21                                                                                                  40
                I   I   N   N   Y   Y   M   Q   Q   Y   Q   N   S   M   D   T   Q   L   G   D
IND 63/72       ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MAR72           ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MAR76           ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MAR82           ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MAR66           ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MARB3           ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MAR13           ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MARE            ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MARW            ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MAR3A           ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MAR7            ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MARH            ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
MAR10           ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
DMAR7-10        ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
DMAR66-72       ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
DMAR72-B3       ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC

                41                                                                                                  60
                N   A   I   S   G   G   S   N   E   G   S   T   D   T   T   S   T   H   T   N
IND 63/72       AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MAR72           AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MAR76           AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MAR82           AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MAR66           AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MARB3           AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MAR13           AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MARE            AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MARW            AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MAR3A           AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MAR7            AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
MARH            AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
DMAR10          AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
DMAR7-10        AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
DMAR66-72       AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
DMAR72-B3       AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
```

Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP4 Gene

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           61                                                                  80
            N   T   Q   N   N   D   W   F   S   R   L   A   S   S   A   F   T   G   L   F
IND 63/72  AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MAR72      AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MAR76      AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MAR82      AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MAR66      AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MARB3      AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MAR13      AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MARE       AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MARW       AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MAR3A      AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MAR7       AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MARH       AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
MAR10      AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
DMAR7-10   AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
DMAR66-72  AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
DMAR72-B3  AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT

           81                  85
            G   A   L   L   A
IND 63/72  GGC GCT CTT TTG GCC
MAR72      GGC GCT CTT TTG GCC
MAR76      GGC GCT CTT TTG GCC
MAR82      GGC GCT CTT TTG GCC
MAR66      GGC GCT CTT TTG GCC
MARB3      GGC GCT CTT TTG GCC
MAR13      GGC GCT CTT TTG GCC
MARE       GGC GCT CTT TTG GCC
MARW       GGC GCT CTT TTG GCC
MAR3A      GGC GCT CTT TTG GCC
MAR7       GGC GCT CTT TTG GCC
MARH       GGC GCT CTT TTG GCC
MAR10      GGC GCT CTT TTG GCC
DMAR7-10   GGC GCT CTT TTG GCC
DMAR66-72  GGC GCT CTT TTG GCC
DMAR72-B3  GGC GCT CTT TTG GCC
```

Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP2 gene

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            121                                                 130                                                 140
              L   L   V   A   L   V   P   E   L   K   E   L   D   T   R   Q   K   Y   Q   L
IND 63/72   CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR72       CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR76       CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR82       CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR66       CTC CTC GTC GCA CTC GTC CCG GAG CTG ACA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MARB3       CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR13       CTC CTC GTC GCA CTC GTC CCG GAG CTG ACA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MARE        CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MARW        CTC CTC GTC GCA CTC GTC CCG GAG CTG ACA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR3A       CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR7        CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MARH        CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR10       CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
DMAR7-10    CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
DMAR66-72   CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
DMAR72-B3   CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG

            141                                                                                                 160
              T   L   F   P   H   Q   F   I   N   P   R   T   N   M   T   A   H   I   N   V
IND 63/72   ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR72       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR76       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR82       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR66       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MARB3       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR13       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MARE        ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MARW        ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR3A       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR7        ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MARH        ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR10       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
DMAR7-10    ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
DMAR66-72   ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
DMAR72-B3   ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG

            161                                                                                                 180
              P   F   V   G   V   N   R   Y   D   Q   Y   K   L   H   K   P   W   T   L   V
IND 63/72   CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR72       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR76       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR82       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR66       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MARB3       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR13       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MARE        CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MARW        CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR3A       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR7        CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MARH        CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR10       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
DMAR7-10    CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
DMAR66-72   CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
DMAR72-B3   CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
```

Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP2 gene

```
            181                                                                 200
             V   M   V   V   A   P   L   T   V   K   T   G   G   S   E   Q   I   K   V   Y
IND 63/72 GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR72     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR76     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR82     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR66     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MARB3     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR13     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MARE      GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MARW      GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR3A     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR7      GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MARH      GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR10     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
DMAR7-10  GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
DMAR7-10  GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
DMAR66-72 GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
DMAR72-B3 GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC

            201                                                         218
             M   N   A   A   P   T   H   V   H   V   A   G   E   L   P   S   K   E
IND 63/72 ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR72     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR76     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR82     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR66     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MARB3     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR13     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MARE      ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MARW      ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR3A     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR7      ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MARH      ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR10     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
DMAR7-10  ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
DMAR66-72 ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
DMAR72-B3 ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP3 Gene, 657nucleotides[219 Amino acids]

```
            1                                                 8  9                                                 20
            G   I   V   P   V   A   C   A   A   G   Y   G   N   M   V   T   T   D   P   K
IND 63/72   GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR72       GGG ATA GTA CCC GTT GCG TGT GCG GTC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR76       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR82       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR66       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MARB3       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR13       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MARE        GGG ATA GTA CCC GTT GCG TGT GCG GTC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MARW        GGG ATA GTA CCC GTT GCG TGT GTG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR3A       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR7        GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MARH        GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR10       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
DMAR7-10    GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
DMAR66-72   GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
DMAR72-B3   GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG

            21                                                                                                  40
            T   A   D   P   V   Y   G   K   V   F   N   P   P   R   T   N   L   P   G   R
IND 63/72   ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR72       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR76       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR82       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR66       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MARB3       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR13       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MARE        ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MARW        ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR3A       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR7        ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MARH        ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR10       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
DMAR7-10    ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
DMAR66-72   ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
DMAR72-B3   ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC

            41                                                                                                  60
            F   T   N   F   L   D   V   A   E   A   C   P   T   F   L   R   F   G   E   V
IND 63/72   TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR72       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR76       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR82       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR66       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MARB3       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR13       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MARE        TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MARW        TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR3A       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR7        TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MARH        TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR10       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
DMAR7-10    TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
DMAR66-72   TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
DMAR72-B3   TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP3 gene

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            61                                  66                                  75                                  80
             P   F   V   K   T   G   N   S   G   D   R   L   L   A   K   F   D   V   S   L
IND 63/72   CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR72       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR76       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR82       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR66       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MARB3       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR13       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MARE        CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC CAG TTT GAC GTG TCG CTC
MARW        CCA TTT GTG AAG ACG GTG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR3A       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR7        CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MARH        CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR10       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
DMAR7-10    CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
DMAR66-72   CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
DMAR72-B3   CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC

            81                                                                                                              100
             A   A   G   H   M   S   N   T   Y   L   A   G   L   A   Q   Y   Y   T   Q   Y
IND 63/72   GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR72       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR76       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR82       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR66       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MARB3       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR13       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MARE        GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MARW        GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR3A       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR7        GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MARH        GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR10       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
DMAR7-10    GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
DMAR66-72   GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
DMAR72-B3   GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC

            101                                                                                                             120
             S   G   T   M   N   I   H   F   M   F   T   G   P   T   D   A   K   A   R   Y
IND 63/72   AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR72       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR76       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR82       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR66       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MARB3       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR13       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MARE        AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MARW        AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR3A       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR7        AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MARH        AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR10       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
DMAR7-10    AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
DMAR66-72   AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
DMAR72-B3   AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP2 Gene, 654nucleotides [218 Amino acids]

	1																		19	20
	D	K	K	T	E	E	T	T	L	L	E	D	R	I	L	T	T	R	N	G
IND 63/72	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MAR72	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MAR76	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MAR82	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MAR66	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MARB3	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MAR13	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MARE	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MARW	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MAR3A	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MAR7	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MARH	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
MAR10	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
DMAR7-10	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	GAC	GGC
DMAR66-72	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC
DMAR72-B3	GAC	AAG	AAA	ACG	GAA	GAG	ACA	ACC	CTG	CTT	GAA	GAC	CGC	ATC	CTC	ACC	ACC	AGG	AAC	GGC

	21																			40
	H	T	T	S	T	T	Q	S	S	V	G	V	T	Y	G	Y	A	V	A	E
IND 63/72	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MAR72	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MAR76	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MAR82	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MAR66	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MARB3	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MAR13	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MARE	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MARW	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MAR3A	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MAR7	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MARH	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
MAR10	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
DMAR7-10	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
DMAR66-72	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA
DMAR72-B3	CAC	ACG	ACG	TCG	ACG	ACA	CAG	TCA	AGC	GTC	GGC	GTG	ACT	TAC	GGT	TAC	GCT	GTG	GCC	GAA

	41																			60
	D	A	V	S	G	P	N	T	S	G	L	E	T	R	V	T	Q	A	E	R
IND 63/72	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MAR72	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MAR76	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MAR82	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MAR66	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MARB3	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MAR13	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MARE	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MARW	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MAR3A	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MAR7	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MARH	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
MAR10	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
DMAR7-10	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
DMAR66-72	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTG	ACA	CAG	GCT	GAA	CGG
DMAR72-B3	GAC	GCT	GTT	TCT	GGG	CCC	AAC	ACC	TCA	GGC	TTG	GAG	ACC	CGC	GTT	ACA	CAG	GCT	GAA	CGG

Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP2 gene

```
            61                                                                  80
             F   F   K   K   H   L   F   D   W   T   P   N   L   S   F   G   H   C   H   Y
IND 63/72   TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MAR72       TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MAR76       TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MAR66       TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MARB3       TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MAR13       TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MARE        TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MARW        TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TGC
MAR3A       TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MAR7        TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MARH        TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
MAR10       TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
DMAR7-10    TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
DMAR66-72   TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC
DMAR72-B3   TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT GGA CAC TGT CAC TAC

            81                                                                  100
             L   E   L   P   S   E   H   K   G   V   F   G   S   L   M   D   S   Y   A   Y
IND 63/72   CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MAR72       CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC ACA
MAR76       CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MAR82       CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MAR66       CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MARB3       CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MAR13       CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MARE        CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MARW        CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MAR3A       CTG GAA CTC CCC TCG GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MAR7        CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MARH        CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
MAR10       CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
DMAR7-10    CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
DMAR66-72   CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC
DMAR72-B3   CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG GAC TCC TAC GCC TAC

            101                                                                 120
             M   R   N   G   W   D   I   E   V   T   A   V   G   N   Q   F   N   G   G   C
IND 63/72   ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MAR72       ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MAR76       ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MAR82       ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MAR66       ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MARB3       ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MAR13       ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MARE        ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MARW        ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MAR3A       ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MAR7        ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MARH        ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
MAR10       ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
DMAR7-10    ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
DMAR66-72   ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
DMAR72-B3   ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG TTC AAT GGT GGT TGC
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP2 gene

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            121                                                 130                                                 140
              L   L   V   A   L   V   P   E   L   K   E   L   D   T   R   Q   K   Y   Q   L
IND 63/72   CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR72       CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR76       CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR82       CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR66       CTC CTC GTC GCA CTC GTC CCG GAG CTG ACA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MARB3       CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR13       CTC CTC GTC GCA CTC GTC CCG GAG CTG ACA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MARE        CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MARW        CTC CTC GTC GCA CTC GTC CCG GAG CTG ACA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR3A       CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR7        CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MARH        CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
MAR10       CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
DMAR7-10    CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
DMAR66-72   CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG
DMAR72-B3   CTC CTC GTC GCA CTC GTC CCG GAG CTG GAA GAA CTT GAC ACG CGG CAG AAG TAC CAG TTG

            141                                                                                                 160
              T   L   F   P   H   Q   F   I   N   P   R   T   N   M   T   A   H   I   N   V
IND 63/72   ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR72       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR76       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR82       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR66       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MARB3       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR13       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MARE        ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MARW        ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR3A       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR7        ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MARH        ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
MAR10       ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
DMAR7-10    ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
DMAR66-72   ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG
DMAR72-B3   ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG GCT CAC ATC AAC GTG

            161                                                                                                 180
              P   F   V   G   V   N   R   Y   D   Q   Y   K   L   H   K   P   W   T   L   V
IND 63/72   CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR72       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR76       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR82       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR66       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MARB3       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR13       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MARE        CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MARW        CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR3A       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR7        CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MARH        CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
MAR10       CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
DMAR7-10    CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
DMAR66-72   CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
DMAR72-B3   CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG CCG TGG ACG CTT GTT
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP2 gene

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          181                                                                 200
           V   M   V   V   A   P   L   T   V   K   T   G   G   S   E   Q   I   K   V   Y
IND 63/72 GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR72     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR76     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR82     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR66     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MARB3     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR13     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MARE      GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MARW      GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR3A     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR7      GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MARH      GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
MAR10     GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
DMAR7-10  GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
DMAR7-10  GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
DMAR66-72 GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC
DMAR72-B3 GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA CAG ATC AAG GTT TAC

          201                                                           218
           M   N   A   A   P   T   H   V   H   V   A   G   E   L   P   S   K   E
IND 63/72 ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR72     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR76     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR82     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR66     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MARB3     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR13     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MARE      ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MARW      ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR3A     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR7      ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MARH      ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
MAR10     ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
DMAR7-10  ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
DMAR66-72 ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC TCG AAA GAG
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP3 Gene, 657nucleotides[219 Amino acids]

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                1                                   8  9                                  20
                G   I   V   P   V   A   C   A   A   G   Y   G   N   M   V   T   T   D   P   K
IND 63/72       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR72           GGG ATA GTA CCC GTT GCG TGT GCG GTC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR76           GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR82           GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR66           GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MARB3           GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR13           GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MARE            GGG ATA GTA CCC GTT GCG TGT GCG GTC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MARW            GGG ATA GTA CCC GTT GCG TGT GTG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR3A           GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR7            GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MARH            GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
MAR10           GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
DMAR7-10        GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
DMAR66-72       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG
DMAR72-B3       GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA GAC CCG AAG

                21                                                                      40
                T   A   D   P   V   Y   G   K   V   F   N   P   P   R   T   N   L   P   G   R
IND 63/72       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR72           ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR76           ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR82           ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR66           ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MARB3           ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR13           ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MARE            ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MARW            ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR3A           ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR7            ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MARH            ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
MAR10           ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
DMAR7-10        ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
DMAR66-72       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC
DMAR72-B3       ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC CCT GGG CGC

                41                                                                      60
                F   T   N   F   L   D   V   A   E   A   C   P   T   F   L   R   F   G   E   V
IND 63/72       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR72           TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR76           TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR82           TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR66           TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MARB3           TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR13           TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MARE            TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MARW            TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR3A           TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR7            TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MARH            TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
MAR10           TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
DMAR7-10        TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
DMAR66-72       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
DMAR72-B3       TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC GGA GAA GTA
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP3 gene

```
            61                  66                                  75                  80
             P   F   V   K   T   G   N   S   G   D   R   L   L   A   K   F   D   V   S   L
IND 63/72   CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR72       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR76       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR82       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR66       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MARB3       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR13       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MARE        CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC CAG TTT GAC GTG TCG CTC
MARW        CCA TTT GTG AAG ACG GTG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR3A       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR7        CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MARH        CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
MAR10       CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
DMAR7-10    CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
DMAR66-72   CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC
DMAR72-B3   CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC GTG TCG CTC

            81                                                                      100
             A   A   G   H   M   S   N   T   Y   L   A   G   L   A   Q   Y   Y   T   Q   Y
IND 63/72   GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR72       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR76       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR82       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR66       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MARB3       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR13       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MARE        GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MARW        GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR3A       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR7        GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MARH        GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
MAR10       GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
DMAR7-10    GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
DMAR66-72   GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC
DMAR72-B3   GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC ACA CAG TAC

            101                                                                     120
             S   G   T   M   N   I   H   F   M   F   T   G   P   T   D   A   K   A   R   Y
IND 63/72   AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR72       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR76       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR82       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR66       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MARB3       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR13       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MARE        AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MARW        AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR3A       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR7        AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MARH        AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
MAR10       AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
DMAR7-10    AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
DMAR66-72   AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
DMAR72-B3   AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA GCT CGC TAC
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP3 gene

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          121                                                                 140
            M   V   A   Y   V   P   P   G   M   E   P   P   T   E   P   E   R   A   A   H
IND 63/72 ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MAR72     ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MAR76     ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MAR82     ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MAR66     ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MARB3     ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MAR13     ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MARE      ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MARW      ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MAR3A     ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MAR7      ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MARH      ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
MAR10     ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
DMAR7-10  ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
DMAR66-72 ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC
DMAR72-B3 ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG GCC GCG CAC

          141                                                                 160
            C   I   H   S   E   W   D   T   G   L   N   S   K   F   T   F   S   I   P   Y
IND 63/72 TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MAR72     TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MAR76     TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MAR82     TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MAR66     TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MARB3     TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MAR13     TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MARE      TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MARW      TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MAR3A     TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MAR7      TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MARH      TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
MAR10     TGT ATA CAT TCT GAG TGG GAC ACT GGC CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
DMAR7-10  TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
DMAR66-72 TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC
DMAR72-B3 TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC ATT CCT TAC

          161                                                                 180
            L   S   A   A   D   Y   A   Y   T   A   S   D   V   A   E   T   T   S   V   Q
IND 63/72 CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MAR72     CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MAR76     CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MAR82     CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MAR66     CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MARB3     CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MAR13     CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MARE      CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MARW      CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MAR3A     CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MAR7      CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MARH      CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
MAR10     CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
DMAR7-10  CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
DMAR66-72 CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
DMAR72-B3 CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG AGT GTG CAG
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP3 gene

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          181                                                                 200
            G   W   V   C   I   Y   Q   I   T   H   G   K   A   E   G   D   A   L   V   V
IND 63/72 GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MAR72     GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MAR76     GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MAR82     GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MAR66     GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MARB3     GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MAR13     GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MARE      GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MARW      GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MAR3A     GGA TGG GTG TGC ATT TAT CAG ATC ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MAR7      GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MARH      GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
MAR10     GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
DMAR7-10  GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
DMAR66-72 GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG
DMAR72-B3 GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG CTG GTC GTG

          201                                                                 219
            S   V   S   A   G   K   D   F   E   F   R   L   P   V   D   A   R   R   E
IND 63/72 TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MAR72     TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MAR76     TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MAR82     TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MAR66     TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MARB3     TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MAR13     TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MARE      TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MARW      TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MAR3A     TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MAR7      TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MARH      TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
MAR10     TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
DMAR7-10  TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
DMAR66-72 TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
DMAR72-B3 TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC CGA GAG
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP1 gene, 630nucleotides[210 Amino acids]

	1																			20
	T	T	T	A	G	E	S	A	D	P	V	T	T	T	V	E	N	Y	G	G
IND 63/72	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MAR72	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MAR76	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MAR82	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MAR66	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MARB3	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MAR13	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MARE	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MARW	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MAR3A	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MAR7	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MARH	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
MAR10	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
DMAR7-10	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
DMAR66-72	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA
DMAR72-B3	ACT	ACC	ACC	GCT	GGC	GAG	TCC	GCA	GAC	CCA	GTC	ACC	ACC	ACA	GTT	GAG	AAC	TAC	GGA	GGA

	21																		39	40
	A	T	Q	S	A	R	R	L	H	T	D	V	A	F	V	L	D	R	F	V
IND 63/72	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MAR72	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	CTT	GTG
MAR76	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MAR82	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MAR66	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MARB3	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MAR13	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MARE	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MARW	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MAR3A	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MAR7	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MARH	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
MAR10	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
DMAR7-10	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
DMAR66-72	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG
DMAR72-B3	GAG	ACT	CAG	TCG	GCC	CGA	CGG	CTA	CAC	ACT	GAC	GTT	GCT	TTT	GTT	CTC	GAC	AGG	TTT	GTG

	41					46		48												60
	K	L	T	P	K	N	T	Q	I	L	D	L	M	Q	I	P	S	H	T	L
IND 63/72	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MAR72	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MAR76	AAA	CTC	ACC	CCC	AAG	GAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MAR82	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAC	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MAR66	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MARB3	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MAR13	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MARE	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MARW	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MAR3A	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MAR7	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MARH	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
MAR10	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
DMAR7-10	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
DMAR66-72	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG
DMAR72-B3	AAA	CTC	ACC	CCC	AAG	AAC	ACC	CAG	ATT	CTT	GAT	CTC	ATG	CAG	ATC	CCC	TCA	CAC	ACA	CTG

Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP1 gene

	61											72								80
	V	G	A	L	L	R	S	A	T	Y	Y	F	S	D	L	E	V	A	L	V
IND 63/72	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MAR72	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MAR76	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MAR82	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MAR66	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MARB3	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MAR13	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MARE	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TCC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MARW	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MAR3A	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MAR7	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MARH	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
MAR10	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
DMAR7-10	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
DMAR66-72	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT
DMAR72-B3	GTT	GGA	GCG	TTA	CTC	CGG	TCC	GCG	ACG	TAC	TAC	TTC	TCG	GAC	CTG	GAG	GTT	GCG	CTT	GTT

	81																	98		100
	H	T	G	S	V	T	W	V	P	N	G	A	P	K	D	A	L	D	N	H
IND 63/72	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MAR72	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MAR76	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MAR82	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MAR66	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MARB3	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MAR13	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAT	GCC	TTG	GAC	AAC	CAC
MARE	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MARW	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MAR3A	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GGC	AAC	CAC
MAR7	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MARH	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
MAR10	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
DMAR7-10	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
DMAR66-72	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC
DMAR72-B3	CAC	ACA	GGC	TCA	GTC	ACA	TGG	GTG	CCC	AAT	GGC	GCG	CCC	AAG	GAC	GCC	TTG	GAC	AAC	CAC

	101																			120
	T	N	P	T	A	Y	Q	K	K	P	I	T	R	L	A	L	P	Y	T	A
IND 63/72	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MAR72	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MAR76	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MAR82	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MAR66	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MARB3	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MAR13	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MARE	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MARW	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MAR3A	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MAR7	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MARH	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
MAR10	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
DMAR7-10	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
DMAR66-72	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT
DMAR72-B3	ACC	AAC	CCG	ACT	GCC	TAC	CAG	AAG	AAA	CCC	ATC	ACC	CGC	CTG	GCG	CTC	CCC	TAC	ACC	GCT

Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants (continued).

VP1 gene

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          121                                                                 140
            P   H   R   V   L   A   T   V   Y   N   G   K   T   T   Y   G   T   Q   P   T
IND 63/72 CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MAR72     CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MAR76     CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MAR82     CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MAR66     CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MARB3     CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MAR13     CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MARE      CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MARW      CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MAR3A     CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MAR7      CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MARH      CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
MAR10     CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
DMAR7-10  CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
DMAR66-72 CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG
DMAR72-B3 CCC CAC CGT GTG CTG GCA ACA GTG TAC AAC GGG AAG ACA ACG TAC GGG ACA CAA CCC ACG

          141                                                                 160
            R   R   G   D   L   A   V   L   A   Q   R   V   S   N   R   L   P   T   S   F
IND 63/72 CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MAR72     CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MAR76     CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MAR82     CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MAR66     CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MARB3     CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MAR13     CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MARE      CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MARW      CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MAR3A     CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MAR7      CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MARH      CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
MAR10     CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
DMAR7-10  CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
DMAR66-72 CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC
DMAR72-B3 CGG CGT GGT GAC CTT GCT GTT CTT GCA CAG CGG GTA AGC AAC AGG CTG CCC ACC TCC TTC

          161                       168                                 178     180
            N   Y   G   A   V   K   A   D   T   I   T   E   L   L   I   R   M   T   R   A
IND 63/72 AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MAR72     AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MAR76     AAC TAC GGT GCT GTG AAG GCT GGC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MAR82     AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MAR66     AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MARB3     AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MAR13     AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MARE      AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MARW      AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MAR3A     AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MAR7      AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG AAG CGT GCG
MARH      AAC TAC GGT GCT GTG AAG GCT GAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
MAR10     AAC TAC GGT GCT GTG AAG GCT AAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
DMAR7-10  AAC TAC GGT GCT GTG AAG GCT AAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
DMAR66-72 AAC TAC GGT GCT GTG AAG GCT GGC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
DMAR72-B3 AAC TAC GGT GCT GTG AAG GCT AAC ACC ATC ACG GAG CTG TTG ATC CGC ATG ACG CGT GCG
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Figure 13. Aligned nucleotide sequences of the parent virus (IND 63/72) and MAR mutants.

VP1 gene

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            181                                                                 200
             E   T   Y   C   P   R   P   L   L   A   L   D   T   T   H   D   R   R   K   Q
IND 63/72   GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MAR72       GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MAR76       GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MAR82       GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MAR66       GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MARB3       GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MAR13       GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MARE        GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MARW        GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MAR3A       GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MAR7        GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MARH        GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
MAR10       GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
DMAR7-10    GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
DMAR66-72   GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG
DMAR72-B3   GAG ACA TAC TGC CCC AGG CCT TTG CTA GCT CTT GAC ACC ACC CAC GAC CGC CGT AAG CAG

            201                                 210
             E   I   I   A   P   E   K   Q   V   L
IND 63/72   GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MAR72       GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MAR76       GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MAR82       GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MAR66       GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MARB3       GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MAR13       GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MARE        GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MARW        GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MAR3A       GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MAR7        GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MARH        GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
MAR10       GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
DMAR7-10    GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
DMAR66-72   GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
DMAR72-B3   GAG ATC ATT GCA CCT GAG AAG CAA GTT TTG
```

Fig.14 Aligned amino acid sequence of P1 region of parent virus (IND 63/72) and mutants, - indicates same as in IND 63/72

VP4

```
                1       10        20        30        40        50        60        70        80   85
IND 63/72       GAGQSSPATGSQNQSGNTGSIINNYYMQQYQNSMDTQLGDNAISGGSNEGSTDTTSTHTNNTQNNDWFSRLASSAFTGLFGALLA
MAR 72          ----------------------------------------------------------------------------------
MAR 76          ----------------------------------------------------------------------------------
MAR 82          ----------------------------------------------------------------------------------
MAR 66          ----------------------------------------------------------------------------------
MAR B3          ----------------------------------------------------------------------------------
MAR 13          ----------------------------------------------------------------------------------
MAR E           ----------------------------------------------------------------------------------
MAR W           ----------------------------------------------------------------------------------
MAR 3A          ----------------------------------------------------------------------------------
MAR 7           ----------------------------------------------------------------------------------
MAR H           ----------------------------------------------------------------------------------
MAR 10          ----------------------------------------------------------------------------------
DMAR 7-10       ----------------------------------------------------------------------------------
DMAR 66-72      ----------------------------------------------------------------------------------
DMAR 72-B3      ----------------------------------------------------------------------------------
```

(continued)

Fig.14 Aligned amino acid sequence of P1 region of parent virus (IND 63/72) and mutants, - indicates same as in IND 63/72

VP2

```
                1         10        20        30        40        50        60        70        80        90        100       110
IND 63/72       DKKTEETTLLEDRILTTRNGHTTSTTQSSVGVTYGYAVAEDAVSGPNTSGLETRVTQAERFFKKHLFDWTPNLSFGHCHYLELPSEHKGVFGSLMDSYAYMRNGWDIEVTAV
MAR 72          ----------------------------------------------------------------------------------------------------T-----------
MAR 76          ----------------------------------------------------------------------------------------------------------------
MAR 82          ----------------------------------------------------------------------------------------------------------------
MAR 66          ----------------------------------------------------------------------------------------------------------------
MAR B3          ----------------------------------------------------------------------------------------------------------------
MAR 13          ----------------------------------------------------------------------------------------------------------------
MAR E           ----------------------------------------------------------------------------------------------------------------
MAR W           ------------------------------------------------------------------------------------C---------------------------
MAR 3A          ----------------------------------------------------------------------------------------------------------------
MAR 7           ----------------------------------------------------------------------------------------------------------------
MAR H           ----------------------------------------------------------------------------------------------------------------
MAR 10          ----------------------------------------------------------------------------------------------------------------
DMAR 7-10       ----------------D-----------------------------------------------------------------------------------------------
DMAR 66-72      ----------------------------------------------------------------------------------------------------------------
DMAR 72-B3      ----------------------------------------------------------------------------------------------------------------

                        120       130       140       150       160       170       180       190       200       210       218
IND 63/72       GNQFNGGCLLVALVPELKELDTRQKYQLTLFPHQFINPRTNMTAHINVPFVGVNRYDQYKLHKPWTLVVMVVAPLTVKTGGSEQIKVYMNAAPTHVHVAGELPSKE
MAR 72          ----------------------------------------------------------------------------------------------------------
MAR 76          ----------------------------------------------------------------------------------------------------------
MAR 82          ----------------------------------------------------------------------------------------------------------
MAR 66          ----------------T-----------------------------------------------------------------------------------------
MAR B3          ----------------E-----------------------------------------------------------------------------------------
MAR 13          ----------------T-----------------------------------------------------------------------------------------
MAR E           ----------------------------------------------------------------------------------------------------------
MAR W           ----------------T-----------------------------------------------------------------------------------------
MAR 3A          ----------------E-----------------------------------------------------------------------------------------
MAR 7           ----------------E-----------------------------------------------------------------------------------------
MAR H           ----------------E-----------------------------------------------------------------------------------------
MAR 10          ----------------E-----------------------------------------------------------------------------------------
DMAR 7-10       ----------------E-----------------------------------------------------------------------------------------
DMAR 66-72      ----------------E-----------------------------------------------------------------------------------------
DMAR 72-B3      ----------------E-----------------------------------------------------------------------------------------
```

(continued)

Fig.14 Aligned amino acid sequence of P1 region of parent virus (IND 63/72) and mutants, - indicates same as in IND 63/72

VP3

```
                1         10        20        30        40        50        60        70        80        90        100       110
IND 63/72       GIVPVACAAGYGNMVTTDPKTADPVYGKVFNPPRTNLPGRFTNFLDVAEACPTFLRFGEVPFVKTGNSGDRLLAKFDVSLAAGHMSNTYLAGLAQYYTQYSGTMNIHFMFTG
MAR 72          --------V-------------------------------------------------------------------------------------------------------
MAR 76          ---------------------------------------------------------------------------------------------------------------
MAR 82          ---------------------------------------------------------------------------------------------------------------
MAR 66          ---------------------------------------------------------------------------------------------------------------
MAR B3          ---------------------------------------------------------------------------------------------------------------
MAR 13          ---------------------------------------------------------------------------------------------------------------
MAR E           --------V-------------------------------------------------------------------Q-------------------------------
MAR W           -------V--------------------------------------------------------V-------------------------------------------
MAR 3A          ---------------------------------------------------------------------------------------------------------------
MAR 7           ---------------------------------------------------------------------------------------------------------------
MAR H           ---------------------------------------------------------------------------------------------------------------
MAR 10          ---------------------------------------------------------------------------------------------------------------
DMAR 7-10       ---------------------------------------------------------------------------------------------------------------
DMAR 66-72      ---------------------------------------------------------------------------------------------------------------
DMAR 72-B3      ---------------------------------------------------------------------------------------------------------------

                      120       130       140       150       160       170       180       190       200       210       219
IND 63/72       PTDAKARYMVAYVPPGMEPPTEPERAAHCIHSEWDTGLNSKFTFSIPYLSAADYAYTASDVAETTSVQGWVCIYQITHGKAEGDALVVSVSAGKDFEFRLPVDARRE
MAR 72          ---------------------------------------------------------------------------------------------------------
MAR 76          ---------------------------------------------------------------------------------------------------------
MAR 82          ---------------------------------------------------------------------------------------------------------
MAR 66          ---------------------------------------------------------------------------------------------------------
MAR B3          ---------------------------------------------------------------------------------------------------------
MAR 13          ---------------------------------------------------------------------------------------------------------
MAR E           ---------------------------------------------------------------------------------------------------------
MAR W           ---------------------------------------------------------------------------------------------------------
MAR 3A          ---------------------------------------------------------------------------------------------------------
MAR 7           ---------------------------------------------------------------------------------------------------------
MAR H           ---------------------------------------------------------------------------------------------------------
MAR 10          ---------------------------------------------------------------------------------------------------------
DMAR 7-10       ---------------------------------------------------------------------------------------------------------
DMAR 66-72      ---------------------------------------------------------------------------------------------------------
DMAR 72-B3      ---------------------------------------------------------------------------------------------------------
```

(continued)

Fig.14 Aligned amino acid sequence of P1 region of parent virus (IND 63/72) and mutants, - indicates same as in IND 63/72

VP1

```
                1         10        20        30        40        50        60        70        80        90        100       110
IND 63/72       TTTAGESADPVTTTVENYGGATQSARRLHTDVAFVLDRFVKLTPKNTQILDLMQIPSHTLVGALLRSATYYFSDLEVALVHTGSVTWVPNGAPKDALDNHTNPTAYQKKPIT
MAR 72          ----------------------------------------L---------------------------------------------------------------------
MAR 76          ---------------------------------------------D-----------------------------------------------------------------
MAR 82          ------------------------------------------------H--------------------------------------------------------------
MAR 66          ----------------------------------------------------------------------------------------------------------------
MAR B3          ----------------------------------------------------------------------------------------------------------------
MAR 13          ----------------------------------------------------------------------------------------------------------------
MAR E           ------------------------------------------------------------------------S---------------------------------------
MAR W           ----------------------------------------------------------------------------------------------------------------
MAR 3A          ----------------------------------------------------------------------------------------------------------G-----
MAR 7           ----------------------------------------------------------------------------------------------------------------
MAR H           ----------------------------------------------------------------------------------------------------------------
MAR 10          ----------------------------------------------------------------------------------------------------------------
DMAR 7-10       ----------------------------------------------------------------------------------------------------------------
DMAR 66-72      ----------------------------------------------------------------------------------------------------------------
DMAR 72-B3      ----------------------------------------------------------------------------------------------------------------

                        120       130       140       150       160       170       180       190       200       210
IND 63/72       RLALPYTAPHRVLATVYNGKTTYGTQPTRRGDLAVLAQRVSNRLPTSFNYGAVKADTITELLIRMTRAETYCPRPLLALDTTHDRRKQEIIAPEKQVL
MAR 72          ----------------------------------------------------------------------------------------------------
MAR 76          ----------------------------------------------------G-----------------------------------------------
MAR 82          ----------------------------------------------------------------------------------------------------
MAR 66          ----------------------------------------------------------------------------------------------------
MAR B3          ----------------------------------------------------------------------------------------------------
MAR 13          ----------------------------------------------------------------------------------------------------
MAR E           ----------------------------------------------------------------------------------------------------
MAR W           ----------------------------------------------------------------------------------------------------
MAR 3A          ----------------------------------------------------------------------------------------------------
MAR 7           ------------------------------------------------------------------K---------------------------------
MAR H           ----------------------------------------------------------------------------------------------------
MAR 10          ----------------------------------------------------N-----------------------------------------------
DMAR 7-10       ----------------------------------------------------N-----------------------------------------------
DMAR 66-72      ----------------------------------------------------G-----------------------------------------------
DMAR 72-B3      ----------------------------------------------------N-----------------------------------------------
```

Table. 14a. Amino acid changes in mutants with respect to the parent virus* in VP2 protein (codons are given in brackets).

Viruses	Amino acid Positions				Grouping of Mutants
	VP2-19	VP2-80	VP2-100	VP2-130	
IND 63/72*	*N(AAC)*	*Y(TAC)*	*Y(TAC)*	*K(AAA)*	
MAR 72	-	-	T(ACA)	-	I
MAR 76	-	-	-	-	
MAR 82	-	-	-	-	
MAR 66	-	-	-	T(ACA)	IIa
MAR B3	-	-	-	E(GAA)	
MAR 13	-	-	-	T(ACA)	
MAR E	-	-	-	-	
MAR W	-	C(TGC)	-	T(ACA)	
MAR 3A	-	-	-	E(GAA)	IIb
MAR 7	-	-	-	E(GAA)	
MAR H	-	-	-	E(GAA)	
MAR 10	-	-	-	E(GAA)	III
DMAR 7-10	D(GAC)	-	-	E(GAA)	
DMAR 66-72	-	-	-	E(GAA)	
DMAR 72-B3	-	-	-	E(GAA)	

Table. 14b. Amino acid changes in mutants with respect to the parent virus* in VP3 protein (codons are given in brackets).

Viruses	Amino acid Positions				Grouping of Mutants
	VP3-8	VP3-9	VP3-66	VP3-75	
IND 63/72*	*A (GCG)*	*A (GCC)*	*G (GGG)*	*K (AAG)*	
MAR 72	-	V (GTC)	-	-	I
MAR 76	-	-	-	-	
MAR 82	-	-	-	-	
MAR 66	-	-	-	-	IIa
MAR B3	-	-	-	-	
MAR 13	-	-	-	-	
MAR E	-	V (GTC)	-	Q (CAG)	
MAR W	V (GTG)	-	V (GTG)	-	
MAR 3A	-	-	-	-	IIb
MAR 7	-	-	-	-	
MAR H	-	-	-	-	
MAR 10	-	-	-	-	III
DMAR 7-10	-	-	-	-	
DMAR 66-72	-	-	-	-	
DMAR 72-B3	-	-	-	-	

Table. 14c. Amino acid changes in mutants with respect to the parent virus* in VP1 protein (codons are given in brackets).

Viruses	Amino acid Positions							Grouping of Mutants
	VP1-39	VP1-46	VP1-48	VP1-72	VP1-98	VP1-168	VP1-178	
IND 63/72*	*F (TTT)*	*N (AAC)*	*Q (CAG)*	*F (TTC)*	*D (GAC)*	*D (GAC)*	*T (ACG)*	
MAR 72	L (CTT)	-	-	-	-	-		I
MAR 76	-	D (GAC)	-	-	-	G (GGC)		
MAR 82	-	-	H (CAC)	-	-	-		
MAR 66	-	-	-	-	-	-		IIa
MAR B3	-	-	-	-	-	-		
MAR 13	-	-	-	-	-	-		
MAR E	-	-	-	S (TCC)	-	-		
MAR W	-	-	-	-	-	-		
MAR 3A	-	-	-	-	G (GGC)	-		IIb
MAR 7	-	-	-	-	-	-	K (AAG)	
MAR H	-	-	-	-	-	-		
MAR 10	-	-	-	-	-	N (AAC)		III
DMAR 7-10	-	-	-	-	-	N (AAC)		
DMAR 66-72	-	-	-	-	-	G (GGC)		
DMAR 72-B3	-	-	-	-	-	N (AAC)		

Since the antigenic sites involve the capsid proteins the detailed comparison of the mutants to identify the sites were made using the deduced amino acid sequences as shown in Fig. 14 & Table. 14. The results revealed that all the mutants had some changes in the amino acids coding for the 3 structural proteins namely VP1, VP2 and VP3. No changes were observed in the protein VP4.

The amino acid substitutions in VP2 protein were seen at four (19, 80, 100 and 130) positions. At position 19 the residue asparagine (N) was changed to aspartic acid (D) in DMAR 7-10. The amino acid tyrosine (Y) at position 80 was substituted by cysteine (C) in MAR W and there was tyrosine (Y) to threonine (T) change in MAR 72 at position 100. Out of 15 mutants, 11 mutants showed difference in their amino acids at position 130. The mutants MAR 66, MAR 13 and MAR W had lysine to threonine (T) replacement whereas MAR B3, MAR 3A, MAR 7, MAR H, MAR 10, DMAR 7-10, DMAR 66-72 and DMAR 72-B3 showed lysine (K) to glutamic acid (E) substitution.

In the case of VP3 protein also changes were seen at four (8, 9, 66 and 75) positions. Except at position 75, all the changes led to a valine (V) substitution. At position 75, lysine (K) was changed to glutamine (Q) in MAR E. The residues alanine (A) and glycine (G) at position 8 and 66 were changed (to valine, V) in MAR W. The residue alanine (A) at position 9 was changed to valine (V) in MAR 72 and MAR E.

The highest number of residues changed were seen in the protein VP1. A total of 7 positions viz,. 39, 46, 48, 72, 98,168 and 178 showed substitutions in different mutants. All the changes were unique to different mutants except at position 168 where 5 mutants showed substitutions. The residue phenylalanine (F) was changed to leucine (L) in MAR 72 at residue 39 and there were N→D and Q→H replacements at positions 46 and 48 in MAR 76 and MAR 82 respectively. At position 72 MAR E showed phenylalanine (F) to Serine (S) replacement and D→G change was noticed in MAR 3A at position 98. The residue 168 aspartic acid (D) was changed to asparagine

(N) in MAR 10, DMAR 7-10 and DMAR 72-B3 and to glycine (G) in MAR 76 and DMAR 66-72. The last change in VP1 protein was at position 178 (T→K) in MAR 7.

The amino acid changes in each group of mutants are summarized below.

Group I mutants viz., MAR 72, MAR 76 and MAR 82 showed substitutions at VP1 39(F→L), 46 (N→D) and 48 (Q→H) respectively. In addition, MAR 76 had a D→G substitution at VP1 168 and MAR 72 had changes at VP2 100(Y→T) and VP3 9(A→V).

Among Group II mutants, all viruses had a change at VP2 130 (either K→E or K→T) except MAR E. MAR E had substitutions at VP3, 9 (A→V), VP3, 75 (K→Q) and VP1, 72 (F→S). Besides a change at VP2 130, MAR 3A had a substitution at VP1, 98 D→G and MAR W showed VP2 80 (Y→C) and A→V & G→V substitutions at positions 8 and 66 of VP3 respectively. MAR 7 showed a T→K substitution at VP1 178.

Mutants of Group III viz., MAR 10 and all the double mutants had two amino acid substitutions each (one at VP1 168 and the other at VP2 130). The substitution at VP2 130 was K→E in all these mutants whereas the change at VP1 168 was D→N, except in DMAR 66-72 where it was D→G. In MAR 76 also, the nature of substitution at VP1 168 was D→G. The substitutions at these two positions were common for all the mutants that were non-reactive to Mabs of group 2 and 3. The mutants generated in three trials against Mab 10 did not differ in their nucleotide sequence. All the three mutants had the same changes at positions at VP1 168 and VP2 130. In addition DMAR 7-10 showed N→D substitution at VP2 19.

4.11.2 RasMol Images

To know the probable location of positions mutated, the corresponding residues on the structure of O_1 BFS 1860 were highlighted by displaying them in the "space-fill" form using RasMol as described in Materials and Methods. The residues where the substitutions were noticed in MAR mutants were highlighted on the structural backbone of O1 BFS protomer comprising of one copy each of the four structural proteins. This proposed structure could be taken as a rough sketch as the X-ray crystallographic data for Asia1 virus is not yet available. The residues unique to each group of mutants are shown in Fig. R1 to R3 & and Fig. R4 depicts the overall changes observed in mutants. For better understanding of the spatial location of the residues highlighted, the side view image of RasMol showing all the residues is represented in Fig. R5.

In Fig. R1 the highlighted residues of VP1 (i.e. 39, 46 and 48) appear to be situated closely to each other and near the 5-fold axis of capsid symmetry. The substitutions in these 3 residues were noticed in group I mutants (MAR 72, 76 and MAR 82).

The only common residue (VP2 130) substituted in all the mutants that did not react with group 2 Mabs is highlighted in Fig. R2. This residue, as found important in type A_{10}, is situated near the 2-fold axis of symmetry. Unlike all the mutants MAR E which also did not react with this group of Mabs did not have any change at VP2 130 but had two substitutions elsewhere (VP3, 75 & VP1, 72). These two residues, in addition to VP2 130, are highlighted in Fig. R2b. Of the two residues, VP3 75 appears to be more closely associated with VP2 130 residue, especially when two protomeric subunits are placed together though they appear far away from each other on a single protomer. The other residue i.e. VP1 72 though appears closer to VP2 130 when compared to VP3 75, its association with VP2 130 in the formation of antigenic site remains doubtful as in the side view of the protomer depicting all the residues

(Fig.R5), this (VP1 72) residue appears to be situated a little inside in the protomer structure. All the other residues appear to be located well exposed on the capsid. As said before, how far the structural similarities (especially in variable loops or residues) exist between these two viruses (O1 BFS and Asia 1) is not discernable in the absence of X-ray crystallographic structural data for Asia 1 virus.

The residue 168 that showed variations in MAR 76, MAR 10 and all the double mutants is highlighted in figure R3 which is also situated very close to the 5-fold axis adjacent to the residues 39, 46 & 48 (Fig. R1).

4.11.3 Sequences of field isolates

The complete P1 sequences of 18 field isolates which were subjected to Mab profiling using selected 22 Mabs were derived to i) confirm the residues identified as important for antigenic site using mutants studies ii) to examine whether these sites are represented in naturally-occurring viruses and iii) to detect other variable locations that might be having a role in antigenicity of the virus. The nucleotide sequence alignment and the deduced amino acid alignments of field isolates along with the vaccine virus sequences are given in Fig. 15 & 16 respectively. The total changes in nucleotide and amino acid in isolates compared to vaccine virus are given in table 15 & 16.

Nucleotide sequence comparison of field isolates revealed that unlike in the case of mutants where there were few changes, many positions in the capsid coding region of isolates were found capable of accepting substitutions. There were a minimum of nine nucleotide changes in isolate IND 234/95 and ten each in IND 4/86 and IND 187/94 and fifteen in the case of IND 9/90. These four isolates were very similar to vaccine virus in their reactivity pattern with the entire Mab panel. The *isolate IND 9/90* had four substitutions in VP4 gene, while the other three did not have any changes in this region. The base substitutions in these isolates in the other three genes varied from two to five.

Fig. 15. Aligned nucleotide sequences of vaccine virus(IND 63/72) and field isolates. .indicates same as in vaccine virus. - indicates absence of nucleotides at these positions. Consensus sequence nucleotides differing from vaccine virus are given in the last line. VP4 Gene, 1 to 255; VP2 Gene, 256 to 909; VP3 Gene, 910 to 1566; VP1 gene, 1567 to 2199.

```
          1                                                                            60
IND63/72  GGA GCC GGG CAA TCC AGC CCG GCA ACC GGG TCG CAG AAC CAG TCA GGC AAC ACT GGA AGC
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ... ... ... ... ..T ... ..G ..T ... ..A ... ... ..A ... ... ... ... ... ..T
IND132/85 ... ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ... ... ... ... ... ..T
IND339/96 ... ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ... ... ... ... ... ..T
IND125/98 ..C ... ... ... ... ... ... ..G ..T ... ... ... ... ... ... ... ... ... ... ..T
IND126/98 ..C ... ... ... ... ... ..A ..G ..T ... ... ... ... ... ... ... ... ... ... ..T
IND130/98 ..C ... ... ... ... ... ... ..G ..T ... ... ... ... ... ... ... ... ... ... ..T
IND69/99  ... ... ... ... ... ... ... T.G ..T ... ..A ... ... ... ... ... ... ... ... ...
IND68/99  ... ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ... ... ... ... ... ...
IND103/99 ... ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ... ... ... ... ... ..T
IND192/99 ... ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ... ... ... ... ... ..T
IND445/98 ... ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ... ... ... ... ... ..T
IND235/99 ... ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ... ... ... ... ... ..T
IND324/98 ... ... ... ... ... ... ... ..G ..T ... ..A ... ... ... ... ... ... ... ... ..T
IND470/98 ... ... ... ... ... ..T ... ... ... ... ..A ... ... ... ..T ... ... ... ... ...
Consensus                             G   T       A                                   T

          61                                                                          120
IND63/72  ATC ATT AAC AAC TAC TAC ATG CAG CAA TAC CAG AAT TCC ATG GAC ACA CAA CTT GGT GAC
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ..C ... ... ..T ... ... ... ... ... ..A ..C ... ... ... ... ... ... ... ...
IND132/85 ... ..C ... ... ... ... ... ..A ..G ... ..A ..C ... ... ... ... ... ... ... ...
IND339/96 ... ..C ... ... ... ... ... ..A ..G ... ..A ..C ... ... ... ... ... ... ... ...
IND125/98 ... ... ... ... ..T ... ... ... ... ... ..A ..C ... ... ... ... ..G ... ... ...
IND126/98 ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ..G ... ... ...
IND130/98 ... ... ... ... ..T ... ... ... ..G ... ..A ..C ... ... ... ... ..G ... ... ...
IND69/99  ... ..C ... ... ... ... ... ..A ..G ... ..A ... ... ... ... ... ... ... ... ..T
IND68/99  ... ..C ... ... ... ... ... ..A ..G ... ..A ..C ... ... ... ... ..G ... ... ..T
IND103/99 ... ..C ... ... ... ... ... ..A ..G ... ... ... ... ... ... ... ... ... ... ...
IND192/99 ... ..C ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ...
IND445/98 ... ..C ... ... ... ... ... ..A ..G ... ... ... ... ... ... ... ... ... ... ...
IND235/99 ... ..C ... ... ... ... ... ..A ..G ... ... ... ... ... ... ... ... ... ... ...
IND324/98 ... ..C ... ... ... ... ... ..A ..G ... ... ... ... ... ... ... ... ... ... ...
IND470/98 ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ...
Consensus       C                         G

          121                                                                         180
IND63/72  AAC GCT ATT AGC GGA GGC TCC AAC GAA GGT TCC ACG GAC ACC ACT TCC ACA CAC ACA AAC
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ..T ... ...
IND132/85 ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... G.. ... ... ...
IND339/96 ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..C ... ... ... ... ...
IND125/98 ... ..C ... ... ... ... ... ... ... ... ..T ..T ... ... ... ... ... ..T ... ...
IND126/98 ... ..C .C. ... ... ... ... ... ... ... ..T ..T ... ... ... ... ... ... ... ...
IND130/98 ... ... ... ... ... ... ... ... ... ... ..T ..T ... ... ... ... ... ..T ... ...
IND69/99  ... ..C ..C ... ... ..T ... ... ... ... ... ..T ... ... ..C ... ... ... ... ...
IND68/99  ... ..C ..C ... ... ..T ... ... ... ... ... ..T ... ... ..C ..T ... ... ... ...
IND103/99 ... ..C ..C ... ... ... .T. ... ... ... ... ..T ... ... ..C ... ... ... ... ...
IND192/99 ... A.C ..C ... ... ... ... ... ... ... ... ..T ... ... ..C ... ... ... ... ...
IND445/98 ... ..C ..C ... ... ... ... ... ... ... ... ..T ... ... ..C ... ... ... ... ...
IND235/99 ... ..C ..C ... ... ... ... ... ... ... ... ..T ... ... ..C ... ... ... ... ...
IND324/98 ... ..C ..C ... ... ... ... ... ... ... ... ..T ... ... ..C ... ... ... ... ...
IND470/98 ... ... ... ... ..G ... ... ... ..G ... ... ... ..T ... ..C ... ... ... ... ...
Consensus                                             T
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
            181                                                                      240
IND63/72    AAC ACC CAA AAC AAC GAT TGG TTC TCG CGC CTA GCC AGT TCG GCC TTC ACC GGA CTG TTT
IND4/86     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90     ... ... ... ... ... ... ... ... ... ... ... ... ... ..C .G. ... ... ... ... ...
IND187/94   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93    ... ... ... ... ..T ... ... ..T ... ... ..G ... ..C ..T ... ... .G. ... ... ...
IND132/85   ... ... ... ... ... ... ... ..T ... ... ..G ..T ... ..C ... ... .G. ... ... ..C
IND339/96   ... ... ... ... ..T ... ... ..T ... ... ..G ..T ... ..C ... ... .G. ... ... ...
IND125/98   ... ... ... ... ..T ... ... ..T ... ... ..G ..T ... ..C ... ... .G. ... T.. ...
IND126/98   ... ... ... ... ..T ... ... ..T ... ... ..G ..T ... ..C ... ... .G. ... T.A ...
IND130/98   ... ... ... ... ..T ... ... ..T ... ... ..G ..T ... ..C ... ... .G. ... T.. ...
IND69/99    ... ... ... ... ..T ... ... ..T ... ... ..G ..T ... ..C ... ... .G. ... ... ...
IND68/99    ... ... ... ... ..T ... ... ..T ... ... ..G ..T ... ..C ... ... .G. ... ... ...
IND103/99   ... ... ... ... ..T ... ... ..T ... ... T.G ..T ... ..C ... ..T .G. ... ... ...
IND192/99   ... ... ... ... ..T ... ... ..T ... ... T.G ..T ... ..C ... ..T .G. ... ... ...
IND445/98   ... ... ... ... ..T ... ... ..T ... ... T.G ..T ... ..C ... ..T .G. ... ... ...
IND235/99   ... ... ... ... ..T ... ... ..T ... ... T.G ..T ... ..C ... ..T .G. ... ... ...
IND324/98   ... ... ... ... ..T ... ... ..T ... ... T.G ..T ... ..C ... ..T .G. ... ..T ...
IND470/98   ... ... ... ... ..T ... ... ..T ... ... ..G ..T ... ..T ..T ... .G. ... ... ...
Consensus                     T           T           G   T       C          G
```

```
            241                                                                      300
IND63/72    GGC GCT CTT TTG GCC GAC AAG AAA ACG GAA GAG ACA ACC CTG CTT GAA GAC CGC ATC CTC
IND4/86     ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ...
IND9/90     ... ... ... ... ... ... ... ... ... ... ... ... ... T.. ... ... ... ... ... ...
IND187/94   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93    ... ..G ... C.. ..T ... ... ... ... ... ... ... ..T ... ... ... ... ..T ..T ...
IND132/85   ... ..G ... C.. ..T ... ... ... ..C ... ..A ... ..T T.. ... ... ... ... ..T ...
IND339/96   ... ..G ... C.. ..T ... ... ... ... ... ..A ... ..T T.. ... ... ... ... ..T ..T
IND125/98   ... ..G ... C.. ..T ... ... ... ... ... ..A ... ..T ... ... ... ... ... ..T ..T
IND126/98   ... ..G ... C.. ..T ... ... ... ... ... ..A ... ..T ... ... ... ... ... ..T ..T
IND130/98   ... ..G ... C.. ..T ... ... ... ... ... ..A ... ..T ... ... ... ... ... ..T ..T
IND69/99    ... ..G ... C.. ..T ... ... ... ... ... ... ... ..T T.. ... ... ... ... ..T ...
IND68/99    ... ..G ... C.. ..T ... ... ... ... ... ... ... ..T T.. ... ... ... ... ..T ...
IND103/99   ... ..G ... C.. ..T ... ... ... ... ... ..A ... ..T T.. ... ... ... ..T ..T ...
IND192/99   ... ..G ... C.. ..T ... ... .G. ... ... ..A ... ..T T.. ... ... ... ..T ... ...
IND445/98   ... ..G ... C.. ..T ... ... ... ... ... ..A ... ..T T.. ... ... ... ..T ..T ...
IND235/99   ... ..G ... C.. ..T ... ... ... ... ... ..A ... ..T T.. ... ... ... ..T ..T ...
IND324/98   ... ..G ... C.. ..T ... ... ... ... ... ..A ... ..T T.. ... ... ... ..T ..T ...
IND470/98   ... ..G ... C.. ..T ... ... ... ... ... ... ... ..T T.. ... ... ... ... ..T ...
Consensus         G     C     T                       A       T T                     T
```

```
            301                                                                      360
IND63/72    ACC ACC AGG AAC GGC CAC ACG ACG TCG ACG ACA CAG TCA AGC GTC GGC GTG ACT TAC GGT
IND4/86     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93    ... ... ... ... ... ... ..A ... ... ... ... ... ..G ... ..T ... ... ..G ... ...
IND132/85   ... ... ... ..T ... ... ..A ... ... ... ..G ... ... ... ..T ... ... ..A ... ...
IND339/96   ... ... ... ..T ... ... ..A ... ... ... ..G ... ..G ... ..T ... ... ..A ... ...
IND125/98   ... ... ... ..T ... ... ..A ... ... ... ... ... ..G ... ..T ... ... ..A ... ...
IND126/98   ... ... ... ..T ... ... ..A ... ..A ... ... ... ..G ... ..T ... ... ..A ... ...
IND130/98   ... ... ... ..T ... ... ..A ... ... ... ... ... ..G ... ..T ... ... ..A ... ...
IND69/99    ... ... ... ... ... ... ..A ... ... ... ..G ... ..G ... ..T ... ... ..C ..T ...
IND68/99    ... ... ... ... ... ... ..A ... ... ... ..G ... ..G ... ..T ... ... ..C ..T ...
IND103/99   ... ... ... ..T ... ... ..A ... ... ... ..G ... ..G ... ..T ... ... ..A ..T ...
IND192/99   ... ... ... ..T ... ... ..A ... ... ... ..G ... ... ... ..T ... ... ..A ..T ...
IND445/98   ... ... ... ..T ... ... ..A ... ... ... ..G ... ..G ... ..T ... ... ..A ..T ...
IND235/99   ... ... ... ..T ... ... ..A ... ... ... ..G ... ..G ... ..T ... ... ..A ..T ...
IND324/98   ... ... ... ..T ... ... ..A ... ... ... ..G ... ..G ... ..T ... ... ..A ..T ...
IND470/98   ... ... ... ... ... ... ..A ... ... ... ... ... ..G ... ..T ... ..A ..A ... ...
Consensus                 T           A                       G       T           A
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
           361                                                                             420
IND63/72   TAC GCT GTG GCC GAA GAC GCT GTT TCT GGG CCC AAC ACC TCA GGC TTG GAG ACC CGC GTG
IND4/86    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93   ... ... ... ..T ..G ..T ..G ..G ..A ..A ..T ... ... ... ..T C.. ... ... ..T ..T
IND132/85  ... ... ... ..T ..G ... ..G ..G ..A ..A ..T ... ... ... ... C.. ... ... ..T A.T
IND339/96  ... ... ... ..T ..G ... ..G ..G ..A ..A ..T ... ... ... ... C.. ... ... ..T ..T
IND125/98  ... ... ... ..T ..G ... ..A ..G ..A ..A ..T ... ..T ... ... C.. ... ... ... ..T
IND126/98  ... ... ... ..T ..G ... ..A ..G ..A ..A ..T ... ..T ... ... C.. ... ... ... ..T
IND130/98  ... ... ... ..T ..G ... ..A ..G ..A ..A ..T ... ..T ... ... C.. ... ... ... ..T
IND69/99   ... ... ... ..T ..G ..T ..G ..G ..A ... ..T ... ... ... ... C.. ... ... ..T ..T
IND68/99   ... ... ... ..T ..G ..T ..G ..G ..A ... ..T ... ... ... ... C.. ... ... ..T ..T
IND103/99  ... ... ... ..T ..G ... ..G ..G ..A ..A ..T ... ... ... ... C.. ... ... ..T ..T
IND192/99  ... ... ... ..T ..G ... ..G ..G ..A ..A ..T ... ... ... ... C.. ... ... ..T ..T
IND445/98  ..T ... ... ..T ..G ... ..G ..G ..A ..A ..T ... ..T ... ... C.. ... ... ..T ..T
IND235/99  ... ... ... ..T ..G ... ..G ..G ..A ..A ..T ... ... ... ... C.. ... ... ..T ..T
IND324/98  ... ... ... ..T ..G ... ..G ..G ..A ..A ..T ... ... ... ... C.. ... ... ..T ..T
IND470/98  ... ... ... ... ..G ... ..G ..G ..C ..A ..T ... ... ... ..T C.. ... ... ..T ..T
Consensus              T   G       G   G   A   A   T                 C             T   T

           421                                                                             480
IND63/72   ACA CAG GCT GAA CGG TTT TTC AAG AAA CAC CTG TTT GAT TGG ACA CCA AAT CTA TCG TTT
IND4/86    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95  G.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93   CAC ..A ..A ... ... ..C ... ..A ..G ... T.. ... ..C ... ... ..G ... T.G G.A ...
IND132/85  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ... T.G G.A ...
IND339/96  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ... T.G G.A ...
IND125/98  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ... T.G G.A ...
IND126/98  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ... T.. G.A ...
IND130/98  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ... T.G G.A ...
IND69/99   CA. ..A ..G ... ... ..C ... ..A ..G ... T.. ... ..C ... ... ... G.. T.G G.A ...
IND68/99   CA. ..A ..G ... ... ..C ... ..A ..G ... T.. ... ..C ... ... A.. G.. T.G G.A ...
IND103/99  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ..C T.G G.A ...
IND192/99  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ..C T.G G.A ...
IND445/98  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ..C T.G G.A ...
IND235/99  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ..C T.G G.A ...
IND324/98  CA. ..A ..G ... ... ..C ..T ..A ..G ... T.. ... ..C ... ... ..G ..C T.G G.A ...
IND470/98  CA. ... ..A ... ... ... ..T ..A ..G ..T T.. ... ..C ... ... ..G G.C T.G G.A ...
Consensus  CA    A   G           C   T   A   G     T           C           G     T G G A

           481                                                                             540
IND63/72   GGA CAC TGT CAC TAC CTG GAA CTC CCC TCC GAA CAC AAA GGC GTG TTC GGC AGC CTC ATG
IND4/86    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94  ... ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ... ... ... ...
IND234/95  ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93   ... ... ... ... ... ... ... ... ..T A.T ... ... ... ... ... .A. ..T ..T ... ...
IND132/85  ... ..T ... ... ... ... ... ... ... A.T ... ... ... ... ... .A. ... ..T ... ...
IND339/96  ... ..T ... ... ... ... ... ... ... A.T ... ... ... ... ... .A. ... ..T ... ...
IND125/98  ... T.T ... ... ... ... ... ... ... A.T ... ... ... ... ... .A. ... ..T ... ...
IND126/98  ... T.T ... ... ... ... ... ... ... A.T ... ... ... ... ... .A. ... ..T ... .C.
IND130/98  ... T.T ... ... ... ... ... ... ... A.T ... ... ... ... .C. .A. ... ..T ... ...
IND69/99   ... ... ... ... ... ..A ... ... ... A.T ... ... ..G ..T ... .A. ... ..T ... ...
IND68/99   ... ..T ... ... ... ..A ... ... ... A.T ... ... ..G ..T ... .A. ... ..T ... ...
IND103/99  ..G ..T ... ... ... ... ... ... ... A.T ... ... ... .C. ... .A. ... ..T ... ...
IND192/99  ..G ..T ... ... ... ..T ... ... ... A.T ... ... ... ... ... .A. ... ..T ... ...
IND445/98  ..G ..T ... ... ... ... ... ... ... A.T ... ... ... ... ... .A. ... ..T ... ...
IND235/99  ..G ..T ... ... ... ... ... ... ... A.T ... ... ... ... ... .A. ... ..T ... ...
IND324/98  ..G ..T ... ... ... ... ... ... ... A.T ... ... ..G ... ..T .A. ..T ..T ... ...
IND470/98  ... ... ... ... ..T ..T ... ... ... A.T ... ... ... ... ... .A. ... ..T ... ...
Consensus        T                             A T                     A         T
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
            541                                                                                                                 600
IND63/72    GAC TCC TAC GCC TAC ATG AGG AAC GGG TGG GAC ATT GAG GTG ACC GCT GTT GGA AAC CAG
IND4/86     ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
IND9/90     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93    ... ..G ... ... ... ... ... ... ... ... ... ..A ... ... ..T ... ... ... ... ..A
IND132/85   ..T ..G ... .G. ... ... ..A ... ... ... ... ..A ... ... ..T ... ... ... ... ...
IND339/96   ..T ..G ... ... ... ... ..A ..T ... ... ... ..A ... ... ... ... ... ... ... ...
IND125/98   .CT ..G ... ... ... ... ..A ..T ... ... ... ..A ... ... ..T ... ... ... ... ...
IND126/98   .CT ..G ... ... ... ... ..A ..T ... ... ... ..A ... ... ..T ... ... ... ... ...
IND130/98   .CT ..G ... ... ... ... ..A ..T ... ... ... ..A ... ... ..T ... ... ... ... ...
IND69/99    .CT ..G ... ... ... ... ..A ..T ... ... ... ..A ... ... ..T ... ... ... ... ..A
IND68/99    .CT ..G ... ... ... ... ..A ..T ... ... ... ..A ... ... ..T ... ... ... ... ..A
IND103/99   .CT ..G ... ... ... ... ..A ... ... ... ... ..A ... ... ..T ... ... ... ... ..A
IND192/99   .CT ..G ... ... ... ... ..A ... ... ... ... ..A ... ... ..T ... ... ..G ... ...
IND445/98   .CT ..G ... ... ... ... ..A ... ... ... ... ..A ... ... ..T ... ... ... ... ..A
IND235/99   .CT ..G ... ... ... ... ..A ... ... ... ... ..A ... ... ..T ... ... ... ... ..A
IND324/98   .CT ..G ... ... ... ... ..A ... ... ... ... ..A ... ... ..T ... ... ... ... ...
IND470/98   ... ..G ... ... ... ... ... ..T ..A ... ... ..A ... ... ..T ... ... ... ... ...
Consensus    CT   G                    A                   A           T

            601                                                                                                                 660
IND63/72    TTC AAT GGT GGT TGC CTC CTC GTC GCA CTC GTC CCG GAG CTG AAA GAA CTT GAC ACG CGG
IND4/86     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90     ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ...
IND187/94   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93    ... ..C ..C ... ..T ... ..T ..T ... ..T ..G ..A ... ... ..G A.C ... ... ..A ...
IND132/85   ... ..C ..C ... ..T ... ..T ..T ... ..T ..G ..A ... ..A ..G AGC ... ... ..C ...
IND339/96   ... ..C ..C ... ..T ... ..T ..T ... ..T ..G ..A ... ... ..G AGC ... ... ... ...
IND125/98   ... ..C ..C ... ... ... ..T ..T ... ..T ..G ..A ... ... ..G AGC ... ... ... ...
IND126/98   ... ..C ..C ... ..T ... ..T ..T ... ..T ..G ..A ... ... ..G AGC ... ... ... ...
IND130/98   ... ..C ..C ... ... ... ..T ..T ... ..T ..G ..A ... ... ..G AGC ... ... ... ...
IND69/99    ... ..C ..C ... ..T ... ..T ..T ... ..T ..G ..A ... ... ..G AGC ... ... ... ...
IND68/99    ... ... ..C ... ..T ... ..T ..T ... ..T ..G ..A ... ... ..G AGC ... ... ... ...
IND103/99   ... ..C ..C ..C ..T ... ..T ..T ... ..T ..G ..A ..A T.. ..G AGC ... ... ... ...
IND192/99   ..T ..C ..C ..C ..T ... ..T ..T ... ..T ..G ..A ..A T.. ..G AGC ..C ... ... ..A
IND445/98   ... ..C ..C ..C ..T ... ... ..T ... ..T ..G ..A ..A T.. ..G AGC ... ... ... ..A
IND235/99   ... ..C ..C ..C ..T ... ..T ..T ... ..T ..G ..A ... T.. ..G AGC ... ... ... ...
IND324/98   ... ..C ..C ... ..T ... ..T ..T ... ..T ..G ..A ..A T.. ..G AGC ... ... ... ..A
IND470/98   ..T ..C ..C ... ..T ... ..T ... ... ... ..G ... ... ... ..G AGG ..C .G. .T. ..A
consensus         C   C       T       T   T       T   G   A           G AGC

            661                                                                                                                 720
IND63/72    CAG AAG TAC CAG TTG ACC CTC TTC CCA CAC CAG TTC ATC AAC CCA CGC ACC AAC ATG ACG
IND4/86     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93    ... ..A ... ... C.. ... ... ..T ..C ... ... ... ... ... ... ..T ... ... ... ...
IND132/85   ... G.. ... ..A C.. ... ... ... ..C ... ... .A. ... ... ... ..G ... ... ... ...
IND339/96   ... ... ... ..A C.. ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ...
IND125/98   ... ... ... ..A C.. ... ..T ..T ..C ... ... ... ... ... ... ... ... ... ... ...
IND126/98   ... ... ... ..A C.. ... ..T ... ..C ... ... ... ..T ... ... ... ... ... ... ...
IND130/98   ... ... ... ..A C.. ... ..T ..T ..C ... ... ... ... ... ... ... ... ... ... ...
IND69/99    ... ..A ... ..A C.. ... ..T ... ..C ... ... ... ... ... ... ... ... ... ... ...
IND68/99    ... ..A ... ..A C.. ... ..T ... ..C ... ... ... ... ... ... ... ... ... ... ...
IND103/99   ... ... ... ..A C.T ..T ... ... ..C ... ... ... ... ... ... ... ... ... ... ...
IND192/99   ... ... ... ..A C.. ..T ... ... ..C ... ... ... ... ... ... ... ... ... ... ...
IND445/98   ... ... ... ..A C.. ..T ... ... ..C ... ... ... ... ... ... ... ... ... ... ...
IND235/99   ... ... ... ..A C.. ..T ... ... ..C ... ... ... ... ... ... ... ... ... ... ...
IND324/98   ... ... ... ..A C.. ..T ... ... ..C ... ... ... ... ... ... ... ... ... ... ...
IND470/98   ... ..A ... ... ... ..T ... ..T ..C ... ... ... ... ... ... ... ... ... ... ...
Consensus                 A C                 C
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
          721                                                                                                                  780
IND63/72  GCT CAC ATC AAC GTG CCG TTC GTG GGT GTC AAC AGG TAC GAC CAA TAC AAG CTC CAC AAG
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ... ... ... ... ..T ..T ... ... ..T ... ... ... ... ..G ... GC. ..T ... ..A
IND132/85 ..C ... ..T ... ... ..T ..T ... ... ..T ... ... ..T ... ..G ... GC. ... ... ..A
IND339/96 ..C ... ... ... ... ..T ..T ... ... ..T ... ... ..T ... ..G ... GC. ... ... ..A
IND125/98 ... ... ... ... ... ..T ... ... ... ..T ... ... ..T ..T ..G ... GC. ... ... ..A
IND126/98 ... ... ... ... ... ..T ... ... ... ..T ... ... ..T ... ..G ... GC. ... ... ..A
IND130/98 ... ... ... ... ... ..T ... ... ... ..T ... ... ..T ..T ..G ... GC. ... ... ..A
IND69/99  ... ... ... ... ... ... ..T ... ... ..T ... ... ..T ... ..G ... GT. ... ... ..A
IND68/99  ... ... ... ... ... ... ..T ... ... ..T ... ... ..T ... ..G ... G.. ... ... ..A
IND103/99 ..C ... ... .GT ..A ..T ..T ... ... ..T ... ... ..T ..T ..G ... GG. ... ... ..A
IND192/99 ..C ... ... .GT ... ..T ..T ... ... ..T ... ... ..T ..T ..G ... GC. ... ... ..A
IND445/98 ..C ... ... .GT ..A ..T ..T ... ... ..T ... ... ..T ..T ..G ... GC. ... ... ..A
IND235/99 ..C ... ... .GT ..A ..T ..T ... ... ..T ... ... ..T ..T ..G ... GC. ... ... ..A
IND324/98 ..C ... ... .GT ... ..T ..T ... ... ..T ... ... ..T ..T ..G ... GC. ... ... ..A
IND470/98 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... GT. ..T. ... .A
Consensus                       T   T           T           T       G     GC         A

          781                                                                                                                  840
IND63/72  CCG TGG ACG CTT GTT GTG ATG GTG GTG GCT CCA CTT ACC GTC AAA ACC GGT GGT TCC GAA
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ... ... ..C ... ... ... ... T.. ..C ... ... ... ..T ..G ..T ... ... ..T ...
IND132/85 ... ... ... ..C ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ..C ..C ..T ...
IND339/96 ... ... ..A ..C ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ..C ..C ..T ...
IND125/98 ... ... ... ..C ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ..C ..C ..T ...
IND126/98 ... ... ... ..C ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ..C ..C ..T ...
IND130/98 ... ... ... ..C ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ..C ..C ..T ...
IND69/99  ... ... ... ..C ... ... ... ... ... ..C ..G ..C ..T ... ..G ..A ... ..C ..T ...
IND68/99  ... ... ... ..C ... ... ... ... ... ..C ..G ..C ..T ... ..G ..A ... ..C G.T ...
IND103/99 ... ... ... G.. ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ... ..C ..T ...
IND192/99 ... ... ... ... ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ... ..C ..T ...
IND445/98 ... ... ... ... ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ... ..C ..T ...
IND235/99 ... ... ... ... ... ... ... ... ... ..C ... ..C ..T .C. ..G ..T ... ..C ..T ...
IND324/98 ... ... ... ... ... ... ... ... ... ..C ... ..C ..T ... ..G ..T ... ..C ..T ...
IND470/98 ... ... ... ..C ... ... ... ... ... ..C ... ..C ... ... ..G ..T ... ... ..T ...
Consensus                                       C       C   T       G   T       C   T

          841                                                                                                                  900
IND63/72  CAG ATC AAG GTT TAC ATG AAT GCA GCA CCA ACC CAC GTG CAT GTG GCA GGG GAA CTG CCC
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ... .G. ... ... ... ... ..T ... ... ... T.. ... ..C ... ... ... ..G ... ...
IND132/85 ... ... ... ..C ... ... ... ... ... ..G ... T.. ... ..C ... ... ... ..G ... ...
IND339/96 ... ... ... ..C ... ... ... ..G ... ..G ... T.. ... ..C ... ... ... ..G ... ...
IND125/98 ... ..T ... ..C ... ... ... ... ... ..G ... T.. ... ..C ... ... ... ..G ... ...
IND126/98 ... ... ... ..C ... ... ... ... ... ..G ... T.. ... ..C ... ... ... ..G ... ...
IND130/98 ... ... ... ..C ... ... ... ... ... ..G ... T.. ... ..C ... ... ... ..G ... ...
IND69/99  ... ... ... ..C ... ... ... ... ... ..G ... T.. ... ..C ... ... ... ..G ... ...
IND68/99  ... ... ... ..C ... ... ... ... ... ..G ... T.. ... ..C ... ... ... ..G ... ...
IND103/99 ... ..T ... ... ... ... ... ... ... ..G ... T.. ... ..C ... ... ... ..G ... ...
IND192/99 ... ..T ... ... ... ... ... ... ... ... ... T.. ..A ..C ... ... ... ..G ... ...
IND445/98 ... ..T ... ... ... ... ... ... ... ... ... T.. ... ..C ... ... ... ..G ... ..T
IND235/99 ... ..T ... ... ... ... ... ... ... ... ... T.. ... ..C ... ... ... ..G ... ...
IND324/98 ... ..T ... ... ... ... ... ... ... ... ... T.. ... ..C ... ... ... ..G ... ...
IND470/98 ... ... ... ... ... ... ... ... ... ... ... T.. ... ..C ... ... ... ..G ... ...
Consensus                                             T       C             G  ... ..G ... ...
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
           901                                                                            960
IND63/72   TCG AAA GAG GGG ATA GTA CCC GTT GCG TGT GCG GCC GGT TAT GGC AAC ATG GTG ACC ACA
IND4/86    ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ...
IND9/90    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94  ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ...
IND234/95  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93   ... ... ... ... ... ..T ... ... ... ... ... .A. ... ..C ... ... ... ... ... ..G
IND132/85  ... ... ... ..A ... ..C ... ... ... ... ... .A. ... ... ... ... ... ... ... ..G
IND339/96  ... ... ... ..A ... ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..G
IND125/98  ... ... ... ..A ..T ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..G
IND126/98  ... ... ... ..A ..T ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..G
IND130/98  ... ... ... ..A ..T ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..G
IND69/99   ... ..G ... ..A ... ..T ... ... ... ... ... .A. ... ... ..T ... ... ... ... ..G
IND68/99   ... ..G ... ..A ... ..T ... ... ... ... ... .A. ... ... ..T ... ... ... ... ..G
IND103/99  ... ... ... ..A ... ..T ... A.. ... ... ... .A. ..A ... ... ... ... ... ... ..C
IND192/99  ... ... ... ..A ... ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..C
IND445/98  ... ... ... ..A ... ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..C
IND235/99  ... ... ... ..A ... ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..C
IND324/98  ... ... ... ..A ... ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..C
IND470/98  ... ... ... ..A ... ..T ... ... ... ... ... .A. ... ... ... ... ... ... ... ..G
Consensus              A       T                       A                                G

           961                                                                           1020
IND63/72   GAC CCG AAG ACG GCT GAC CCC GTT TAC GGG AAA GTG TTC AAC CCC CCC AGA ACA AAT CTC
IND4/86    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93   ... ... ... ..T ... ... ..A ..G ... ... ... ... ... ... ... ... ..G ..G ..C ..T
IND132/85  ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..T ... C.G ... ... ..T
IND339/96  ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..T ... C.G ..G ..C ..T
IND125/98  ... ... ..A ..T ... ... ..A ..G ... ... .G. ... ... ... ..A ... C.G ..G ... ..A
IND126/98  ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..A ... C.G ..G ... ..T
IND130/98  ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..A ... C.G ..G ... ..T
IND69/99   ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..T ... C.G ..G ... ...
IND68/99   ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..T ... C.G ..G ... ...
IND103/99  ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..T ... C.G ... ... ...
IND192/99  ... ... ..A ..T ... ... ..A ..G ... ... ... ..A ... ... ..T ... C.G ... ..C ...
IND445/98  ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..T ... C.G ... ... ...
IND235/99  ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..T ... C.G ... ... ...
IND324/98  ... ... ..A ..T ... ... ..A ..G ... ... ... ... ... ... ..T ... C.G ... ... ...
IND470/98  ... ... ... ..T ..C ..T ..A ... ... ... ... ... ..T ... ... ... ..G ... ..C ...
Consensus            A   T           A   G                           T       C G

           1021                                                                          1080
IND63/72   CCT GGG CGC TTC ACA AAC TTC CTT GAT GTA GCG GAG GCA TGC CCA ACC TTC CTC CGC TTC
IND4/86    ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
IND9/90    ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
IND187/94  ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
IND234/95  ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
IND49/93   ..C ... ... ... ... ... ... ... ... ..C ... ... ..G ..T ... ... ... ... ... ...
IND132/85  ..C ... ... ... ..G ... ... ... ... ..C ... ... ..G ..T ... ... ... ... ... ...
IND339/96  ..C ... ... ... ..G ... ... ... ... ..C ... ..C ..G ..T ... ... ... ... ... ...
IND125/98  ..G ... ..G C.. ..G ... ... ... ... ..T ... ... ..G ..T ... ... ... ... ... ...
IND126/98  ..C ... ... ... ..G ... ... ... ... ..T ... ... ..G ..T ... ... ... ... ... ...
IND130/98  ..C ... ... ... ..G ... ... ... ... ..T ... ... C.G ..T ... ... ... ... ..A ...
IND69/99   ..C ... ... ... ..C ... ... ... ... ..T ... ... ..G ..T ... ... ... ... ... ...
IND68/99   ..C ... ... ... ..C ... ... ... ... ..C ... ... ..G ..T ... ... ..T ... ... ...
IND103/99  ..C ... ... ... ..G ... ... ... ... ..C ... ... ..G ..T ... ... ..T ... ... ...
IND192/99  ..C ... ... ... ..G ... ... ... ... ..T ... ... ..G ..T ..C ... ... ... ... ...
IND445/98  ..C ... ... ... ..G ... ... ... ... ..T ... ... ..G ..T ..C ... ... ... ... ...
IND235/99  ..C ... ... ... ..G ... ... ... ... ..T ... ... ..G ..T ..C ... ... ... ... ...
IND324/98  ..C ... ... ... ..G ... ... ... ... ..T ... ... ..G ..T ..C ... ... ... ... ...
IND470/98  ... ... ... ... ... ... ... ..G ... ..T ... ... ..G ..T ..C ... ... ... ... ...
Consensus    C               G                   ..T ... ... ... ..T ... ... ... ... ... ...
                                                   T           G   T
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
          1081                                                                                                                1140
IND63/72  GGA GAA GTA CCA TTT GTG AAG ACG GGG AAC TCT GGT GAC CGC TTG CTT GCC AAG TTT GAC
IND4/86   ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ... ..G ... ... ... ... ..A .T. ... ... ..C ... ..T C.. ..G ... ... ..C ..T
IND132/85 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T C.. ..A ... ... ... ..T
IND339/96 ... A.. ... ... ... ... ..A ..A .T. ... ... ... ... ..T C.. ..A ... ... ... .TT
IND125/98 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T C.. ..A ... ... ... ..T
IND126/98 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T C.. ..A ... ... ... ..T
IND130/98 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T C.. ..A ... ... ... ..T
IND69/99  ... ... ... ... ... ... ... ..A .TA ... ... ... ... ..T C.. ..A ..T ... ... ..T
IND68/99  ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T C.. ..A ..T ... ... ..T
IND103/99 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T ... ..G ... ... ... ..T
IND192/99 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T ... ..G ... ... ... ..T
IND445/98 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T ... ..G ... ... ... ..T
IND235/99 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T ... ..G ... ... ... ..T
IND324/98 ... ... ... ... ... ... ... ..A .T. ... ... ... ... ..T ... ..G ... ... ... ..T
IND470/98 ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... T.G ... ... ..C ...
Consensus                             A  T                       T       A               T

          1141                                                                                                                1200
IND63/72  GTG TCG CTC GCT GCG GGG CAC ATG TCC AAC ACC TAC TTG GCA GGC TTG GCG CAG TAC TAC
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ..C ... ... ..C ... ... ..T ... ... ... ... ... ... ..C ..A C.. ..A ... ... ...
IND132/85 ..C ... ... ... ... ... ... ... ... ... ... ... ... ..T ..T C.. ..A ... ... ...
IND339/96 ..C ... ... ... ... ... ... ... ... ... ... ... ... ..C ..T C.. ..A ... ... ...
IND125/98 ..C ... ... ... ... ..A .GA ... ... ... ... ... C.. ..T ..G C.. ... ... ... ...
IND126/98 ..C ... ... ... ... ..A ... ... ... ... ... ... C.. ..T ..T C.. ..A ... ... ...
IND130/98 ..C ... ... ... ... ..A ... ... ... ... ... ... C.. ..T ..T C.. ... ... ... ...
IND69/99  ..C ... ..T ... ... ... ... ... ..T ... ... ... ... ..T ... C.. ..A ... ... ...
IND68/99  ..C ... ..T ... ... ... ... ... ..T ... ... ... ... ..T ... C.. ..A ... ... ...
IND103/99 ..C ... ..T ... ... ... ... ... ... ... ... ... ... ..T ..T C.. ..A ... ... ...
IND192/99 ..C ... ..T ... ... ... ... ... ... ... ... ... ... ..T ..T C.. ..A ... ... ...
IND445/98 ..C ... ..T ... ... ... ... ... ... ... ... ... ... ..T ..T C.. ..A ... ... ...
IND235/99 ..C ... ..T ... ... ... ... ... ... ... ... ... ... ..T ..T C.. ..A ... ... ...
IND324/98 ..C ... ..T ... ... ... ... ... ... ... ..T ... ... ..T ..T C.. ..A ... ... ...
IND470/98 ... ... ... ..C ..A ... ... ... ... ... ... ... ... ..T ..T ... ... ... ..T ...
Consensus   C                                                       T   T C     A

          1201                                                                                                                1260
IND63/72  ACA CAG TAC AGC GGC ACC ATG AAC ATC CAC TTC ATG TTC ACC GGG CCC ACG GAT GCC AAA
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ... ... ..T ... ... ..A ..T G.T ... ... ... ... ... ... ... ... ..C ... ..G
IND132/85 ... ... ... ..T ... ... ... ... G.. ... ... ... ... ... ... ... ... ..A ..C ... ...
IND339/96 ... ... ... ..T ... ... ... ... G.. ... ... ... ... ... ... ... ... ..A ..C ... ...
IND125/98 ... ... ... ..T ... ... ... ..T G.. ... ... ... ... ... ... ... ... ..A ..C ... ...
IND126/98 ... ... ... ..T ... ... ... ..T G.. ... ... ... ... ... ... ... ... ..A ..C ... ...
IND130/98 ... ... ... ..T ... ... ... ..T G.. ... ... ... ... ... ... ... ... ..A ..C ... ...
IND69/99  ... ... ... ..T ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ...
IND68/99  ... ... ... ..T ... ... ... ... G.. ... ... ... ... ... ... ... ... ... ... ...
IND103/99 ... ... ... ..T ... ... ... ..T G.T ... ... ... ... ... ... ... ... ..A ... ... ...
IND192/99 ... ... ... ..T ... ... ... ... G.T ... ... ... ... ... ... ... ... ..A ... ... ...
IND445/98 ... ... ... ..T ... ... ... ... G.T ... ... ... ... ... ... ... ... ..A ... ... ...
IND235/99 ... ... ... ..T ... ... ... G.. G.T ... ... ... ... ... ... ... ... ..A ... ... ...
IND324/98 ... ... ... ..T ... ... ... ... G.T ... ... ... ... ... ... ... ... ..A ... ... ...
IND470/98 ... ... ... ... ... ... ..A ... G.T ... ... ... ... ... ... ... ... ... ..C ... ...
Consensus               T                   G                                           A
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
          1261                                                                                                                1320
IND63/72  GCT CGC TAC ATG GTG GCT TAC GTA CCT CCT GGT ATG GAG CCA CCC ACA GAA CCC GAG CGG
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ..T ... ... ... ..C ... ACT ..C ..C ..C ... ACA ..G ... ..T ..C ..T ... ..C
IND132/85 ... ..G ... ... ... ..C ... A.T ... ..C ..C ... ACA ..G ... ... ..C ..T ... ..C
IND339/96 ... ..G ... ... ... ..C ... A.T ... ..C ..C ... ACA ..G ... ... ..C ..T ... ..C
IND125/98 ... ..G ... ... ... ..C ... A.T ... ... ..C ... ACA ..G ... ..G ..C ..T ... ..C
IND126/98 ... ..G ... ... ... ..C ... A.T ... ... ..C ... ACA ... ... ..G ..C ..T ... ..C
IND130/98 ... ..G ... ... ... ..C ... A.T ... ... ..C ... ACA ... ... ..G ..C ..T ... ..C
IND69/99  ..C ..G ..T ... ... ..C ... A.T ... ..C ..C ... ACA ... ... ... ..C ..T ... .AC
IND68/99  ..C ..G ..T ... ... ..C ... A.T ... ..C ..C ... ACA ... ... ... ..C ..T ... .AC
IND103/99 ... ..G ... ... ..A ..C ... A.C ... ..C ..C ... ACA ..G ... ..G ..C ... ... .AC
IND192/99 ... ..G ... ... ..A ..C ... A.C ... ..C ..C ... ACA ..G ... ..G ..C ... ... .AC
IND445/98 ... ..G ... ... ..A ..C ... A.C ... ..C ..C ... ACA ..G ... ..G ..C ... ... .AC
IND235/99 ... ..G ... ... ..A ..C ... A.C ... ..C ..C ... ACA ..G ... ..G ..C ... ... .AC
IND324/98 ... ..G ... ... ..A ..C ... A.C ... ..C ..C ... ACA ..G ... ..G ..C ... ... .AC
IND470/98 .TC ... ... ... ... ... ... A.T ..C ..C ..C ... ACA ..G ... ... ..C ..T ... .CC
Consensus       G               C     A T       C   C     ACA   G           C             C

          1321                                                                                                                1380
IND63/72  GCC GCG CAC TGT ATA CAT TCT GAG TGG GAC ACT GGT CTT AAT TCC AAG TTC ACC TTT TCC
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ..T ..C ..T ..C ..T ..C ... ... ... ..T ... ... ... ..C ... .T. ... ... C.. ...
IND132/85 ... ... ... ..C ..C ..C ... ... ... ... ... ... ... ..C ... ... ... ... ... ...
IND339/96 ... ... ... ..C ..C ..C ... ... ... ... ... ... ... ..C ... ... ... ... ... ...
IND125/98 ... ... ... ... ..C ..C ... ..A ... ... ... ... ... ... ... ... ... ... ... ...
IND126/98 ... ... ... ... ..C ..C ... ..A ... ... ... ... ... ... ... ... ... ... ... ...
IND130/98 ... ... ... ... ..C ..C ... ..A ... ... ... ... ... ... ... ... ... ... ... ...
IND69/99  ... ... ... ..C ..C ..C ... ... ... ... ... ... ..C ..C ... ... ... ... ... ...
IND68/99  ... ... ... ..C ..C ..C ... ... ... ... ... ... ..C ..C ... ... ... ... ... ...
IND103/99 ... ... ... ..C ..C ..C ... ... ... ... ... ..C ... ..C ... ... ... ... ... ...
IND192/99 ... ... ... ..C ..C ..C ... ... ... ... ... ..C ... ..C ... ... ... ... ... ...
IND445/98 ... ... ... ..C ..C ..C ... ... ... ... ... ..C ... ..C ... ... ... ... ... ...
IND235/99 ... ... ... ..C ..C ..C ... ... ... ... ... ..C ... ..C ... ... ... ... ... ...
IND324/98 ... ... ... ..C ..C ..C ... ... ... ... ... ..C ... ..C ... ... ... ... ... ...
IND470/98 ... ... ... ..C ..T ..C ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
Consensus               C   C   C                       C

          1381                                                                                                                1440
IND63/72  ATT CCT TAC CTC TCT GCT GCT GAC TAC GCT TAC ACT GCT TCT GAC GTG GCC GAG ACC ACG
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ..A ..C ... ... ... ... ... ... ... ..G ... ... ... ... ... AC. ..G ... ... ..A
IND132/85 ..A ..C ... ... ... ... ... ... ... ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND339/96 ..A ..C ... ... ... ... ... ... ... ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND125/98 ..A ..C ... ... ... ... ... ... ..T ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND126/98 ..A ..C ... ... ... ... ... ... ..T ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND130/98 ..A ..C ... ... ... ... ... ... ..T ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND69/99  ..A ..C ... ... ... ... ... ... ... ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND68/99  ..A ..C ... ... ... ... ... ... ... ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND103/99 ..A ..C ... ... ... ... ... ... ... ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND192/99 ..A ..C ... ... ... ... ... ... ... ..A ... ... ... ... ... AC. ..G ... ... ..A
IND445/98 ..A ..C ... ... ... ... ... ... ... ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND235/99 ..A ..C ... ... ... ... ... ... ... ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND324/98 ..A ..C ... ... ... ... ... ... ... ..A ... ..C ... ... ... AC. ..G ... ... ..A
IND470/98 ..A ... ... ... ... ... ... ... ..T ..C ... ... ... ... ... ... ..G ... ... ..A
Consensus   A   C                               A       C             AC    G           A
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
          1441                                                                                                                1500
IND63/72  AGT GTG CAG GGA TGG GTG TGC ATT TAT CAG ATT ACG CAC GGC AAA GCT GAA GGC GAC GCG
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ... ... ..A ... ... ... ... ..C ... ... ..C ..C ... ... ..G ... ... ..A ... ..C
IND132/85 ... ... ..A ... ... ... ... ..C ..C ... ..C ..C ... ... ... ... ... ..A ... ..A
IND339/96 ... ... ..A ... ... ... ... ..C ..C ... ..C ..C ... ... ... ... ... ..T ... ..A
IND125/98 ... ... ..A ... ... ... ... ..C ..C ... ..C ..C ... ... ... ... ... ..A ... ..A
IND126/98 ... ... ..A ..T ... ... ... ..C ..C ... ..C ..C ... ... ... ... ... ..A ... ..A
IND130/98 ... ... ..A ... ... ... ... ..C ..C ... ..C ..C ... ... ... ... ... ..A ... ..A
IND69/99  ... ... ..A ... ... ... ... ..C ..C ... ..C ..C ... ... ... ... ... ..A ... ...
IND68/99  ... ... ..A ... ... ... ... ..C ..C ... ..C ..C ... ... ... ... ... ..A ... ...
IND103/99 ... ... ..A ... ... ... ... ..C ... ... ..C ..C ... ... ... ... ... ..A ... ..A
IND192/99 ... ... ..A ... ... ... ... ..C ... ... ..C ..C ... ... ... ... ... ..A ... ..A
IND445/98 ... ... ..A ... ... ... ... ..C ... ... ..C ..C ... ... ... ... .G. ..A ... ..A
IND235/99 ... ... ..A ... ... ... ... ..C ... ... ..C ..C ... ... ... ... ... ..A ... ..A
IND324/98 ... ... ..A ... ... ... ... ..C ... ... ..C ..C ... ... ... ... ... ..A ... ..A
IND470/98 ... ... ... ..T ... ... ... ..C ... ... ..C ..C ... ... ..G ... ... ..A ... ..A
Consensus           A                   C           C   C                       A       A

          1501                                                                                                                1560
IND63/72  CTG GTC GTG TCT GTC AGT GCC GGC AAG GAC TTT GAG TTT CGA CTG CCA GTG GAT GCT CGC
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  T.. ... ..T ... ... ..C ... ... ..A ..T ... ... ..C ... ..T ..C ..C .AA ..G
IND132/85 ... ... ..T ... ... ..C ... ... ..A ... ... ... ..C ... ..C ..C ..C ..G ...
IND339/96 ... ... ..T ... ... ..C ... ... ..A ... ... ... ..C ... ..C ..C ..C ..G ...
IND125/98 ... ... ..T ... ... ..C ... ..A ..A ... ... ... ..C ... ..C ..C ..C ..A ..G
IND126/98 ... ..T ..T ... ... ..C ... ..A ..A ... ... ... ..C ... ..C ..C ..C ..A ...
IND130/98 ... ... ..T ... ... ..C ... ..A ..A ... ... ... ..C ... ..C ..C ..C ..A ...
IND69/99  ... ... ..T ..C A.. ..C ... ... ..A ... ... ... ..T ... ..C ..C ..C ..G ..A
IND68/99  ... ... ..T ..C A.. ..C ... ... ..A ... ... ... ..T ... ..C ..C ..C ..G ..A
IND103/99 ... ... ..T ... ... ..C ... ... ..A ... ... ... ..C ... ..C ..T ..C ..G ...
IND192/99 ... ... ..T ... ... ..C ... ... ..A ... ... ... ..C ... ..C ..T ..C ..G ...
IND445/98 ... ... ..T ... ... ..C ... ... ..A ... ... ... ..C ... ..C ..T ..C ..G ...
IND235/99 ... ... ..T ... ... ..C ... ... ..A ... ... ... ..C ... ..C ..T .GC ..G ...
IND324/98 ... ... ..T ... ... ..C ... ... ..A ... ... ... ..C ... ..C ..T ..C ..G ...
IND470/98 ... ... ..T ... ... ..C ... ... ..A ..T ... ... ... ..C T.. ..T ..T .GC ..G ...
Consensus           T           C           A                   C       C   C   C   G

          1561                                                                                                                1620
IND63/72  CGA GAG ACT ACC ACC GCT GGC GAG TCC GCA GAC CCA GTC ACC ACC ACA GTT GAG AAC TAC
IND4/86   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94 ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93  ..G C.A ..C ... ..A A.C ... ... ..G ..G ... ..G ..A ..A ... ..G ..C ... ..T ...
IND132/85 ..G C.A ... ... ..T A.C ... ... ..A ..G ... ..G ..A ..A ... ..G ... ... ... ...
IND339/96 ..G C.A ... ... ..T A.C ... ... ..A ..G ... ..G ..A ..A ... ..G ... ... ... ...
IND125/98 ..G C.. ..C ... ..G A.C ... ... ..G ..G ... ..G ..A ..A ... ..G ... ... ... ...
IND126/98 ..G C.. ... ... ..G A.C ... ... ..G ..G ... ..G ..A ..A G.. ..G ... ... ... ...
IND130/98 ..G C.. ..C ... ..G A.C ... ... ..G ..G ... ..G ..A ..A ... ..G ... ... ... ...
IND69/99  ..G C.. ... ... G.. A.C ... ... ..A ..G ... ..C ..G ..A ... ..T ... ... ... ...
IND68/99  ..G C.. ... ... G.. A.C ... ... ..A ..G ... ..G ..G ..A ... ..T ... ... ......
IND103/99 ... C.A ..C ... ..T A.C ... ... ..A ..G ... ..G ..A ..A ... ..G ... ... ... ...
IND192/99 ..G C.A ..C ... ..T A.C ... ... ..A ..G ... ..G ..A ..A ... ..G ... ... ... ...
IND445/98 ... C.A ..C ... ..T A.C ... ... ..G ..G ... ..G ..A ..A G.. ..G ... ... ... ...
IND235/99 ... C.A ..C ... ..T A.C ... ... ..A ..G ... ..G ..A ..G ... ..G ... ... ... ...
IND324/98 ..G C.A ..C ... ..T A.C ... ... ..A ..G ... ..G ..A ..A ... ..G ... ... ... ...
IND470/98 ..G C.A ..C ... ..A A.C ... ... ..A ... ... ... ..A ..A ... ..G ..C ... ... ...
Consensus   G C             T A C           A  G       G   A   A       G
```

Fig. 15. Aligned nucleotide sequences of vaccine virus and field isolates. (continued)

```
           1621                                                                         1680
IND63/72   GGA GGA GAG ACT CAG TCG GCC CGA CGG CTA CAC ACT GAC GTT GCT TTT GTT CTC GAC AGG
IND4/86    ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ...
IND9/90    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93   ... ... ..A ... ... A.. ... A.. ... ..T ... ... ..T ... ..C .CC ... ..T ... ...
IND132/85  ... ... ..A ... ... A.A ... A.. ... ..T ... ... ... ... ..C ..C ... ... ... ...
IND339/96  ... ... ..A ... ... A.A ... A.. ... ..T ... ... ... ... ..C ..C ... ..T ... ...
IND125/98  ... ... ... ... ... A.A ... A.. ... ..T ... ... ... ... ..C ..C ... ..T ... ...
IND126/98  ... ... ... ... ... A.A ... A.. ... ..T ... ... ... .G. ..C ..C ... ..T ... ...
IND130/98  ... ... ... ... ... A.A ... A.. ... ..T ... ... ... ... ..C ..C ... ..T ... ...
IND69/99   ... ... ..A ... ... A.A ... A.. ... ... ... ... ... ... ..C ..C ... ..T ..T ...
IND68/99   ... ... ..A ... A.. A.A ... A.. ... ... ... ... ... ... ..C ..C ... ..T ..T ...
IND103/99  ..G ... ..A ... ... G.A ... A.. ... ..T ... ... ... .GC ..C ..C ..C T.G ... ...
IND192/99  ..G ... ..A ... ... A.A ... A.. ... ..T ... ... ... ..C ..C ..C ... ..T ... ...
IND445/98  ... ... ..A ... ... A.A ... A.. ... ..T ... ... ... ..C ..C ..C ... ..T ... ...
IND235/99  ... ... ..A ... ... A.A ... A.. ... ..T .G. ... ... ..C ..C ..C ... ..T ... ...
IND324/98  ... ... ..A ... ... A.A ... A.. ... ..T ... ... ... ..C ..C ..C ... ..T ... ...
IND470/98  ... ... ..A ... ... A.. ... A.. ... ..T ... ... ... ... ..C ..C ... ..T ... ...
Consensus            A         A A     A          T                 C   C           T
```

```
           1681                                                                         1740
IND63/72   TTT GTG AAA CTC ACC --- CCC AAG AAC ACC CAG ATT CTT GAT CTC ATG CAG ATC CCC TCA
IND4/86    ... ... ... ... ... --- ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90    ... ... ... ... ... --- ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94  ... ... ... ... ... --- ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND234/95  ... ..T ... ... ... --- ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93   ... ... ... ... ..T GCA ... ... .G. ... ... .CC ..A ..C ..T ... ... ... ... CT.
IND132/85  ... ... ... ... ..T GCA ... ... ... ..T ... .CC ... ..C ... ... ..A ... ... ...
IND339/96  ... ... ... ... ..T GCA ... ... ... .TT ... .CC ... ..C ... ... ..A ... ... ...
IND125/98  ... ... ... ... ..T GCA ... ..A ... ..T ... .CC ... ..C ... ... ..A ... ..T G..
IND126/98  ... ... ... ... ..T GCA ... ... ... ..T ... .CC ... ..C ... ... ..A ... ..T G..
IND130/98  ... ... ... ... ..T GCA ... ..A .G. ..T ... .CC ... ..C ... ... ..A ... ..T G..
IND69/99   ... ... ... ..T ..T GCA ... ... ... .TT ... .C. ... ... ... ... GTA ... ... GG.
IND68/99   ... ... ... ... ..T GCA ... ... ... .TT ... .C. ... ... ... ... GCA ... ... G..
IND103/99  ... ... ... ..T ..T GCA ... ... ... .TT ... .C. ... ..C ... ... ..A ... ..T GA.
IND192/99  ..C ... ... ... ..T GCA ... ... ... .TT ... .C. ... ..C ... ... ..A ... ..T G..
IND445/98  ... ... ... ... ..T ACA ... ... ... .TT ... .C. ... ..C ... ... ..A ... ..T G..
IND235/99  ... ... ... ... ..T GCA ... ... ... ..T ... .C. ... ..C ... ... ..A ... ..T G..
IND324/98  ... ... ... ... ..T GCA ... ... ... .TT ... .C. ... ..C ... ... ..A ... ..T G..
IND470/98  ... ... ... ... ..T GCA ... ... .G. ... ... .CC ... ... ... ... ... ... ... ...
Consensus                    T                      T      C        C           A       G
```

```
           1741                                                                         1800
IND63/72   CAC ACA CTG GTT GGA GCG TTA CTC CGG TCC GCG ACG TAC TAC TTC TCG GAC CTG GAG GTT
IND4/86    ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND9/90    ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND187/94  ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A..
IND234/95  ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
IND49/93   ... ..G T.. ..C ..G ..A C.G ..T ... ..T ... ... ... ... ... ..A ... ... ... ..C
IND132/85  ... ..G ... ... ... ..A C.G ..T ... ..T ... ... ... ... ... ..A ... ... ... ...
IND339/96  ... ..G ... ... ... ..A C.G ..T ... ..T ... ... ... ... ... ..A ... ... ... ...
IND125/98  ... ..T ... ... ... ..A C.G ..T ... ..T ... ... ... ... ... ..A ... ... ... ...
IND126/98  ... ..T ... ... ... ..A C.G ..T ... ..T ... ... ... ... ... ..A ... ... ... ...
IND130/98  ... ..T ... ... ... ..A C.G ..T ... ..T ... ... ... ... ... ..A ... ... ... ...
IND69/99   ... ... ... ... ... ..A ..G ..T ... ..T ... ... ... ... ... ..A ... ... ... ...
IND68/99   ... ... ... ... ... ..A ..G ..T ... ..T ... ... ... ... ... ..A ... ... ... ...
IND103/99  ... ..G ... ... ... ..A C.G ..T ... ..T ..A ... ... ... ..T ..A ... ... ... ...
IND192/99  ... ..G ... ... ... ..A C.G ..T ... ..T ... ... ... ... ..T ..A ... ... ... ...
IND445/98  ... ..G ... ... ... ..A C.G ..T ... ..T ..A ... ... ... ... ..A ... ... ... ...
IND235/99  ... ..G ... ... ... ..A C.G ..T ... ..T ..A ... ... ... ... ..A ... ... ... ...
IND324/98  ... ..G ... ... ... .AA C.G ..T ... ... ... ... ... ... ... ..A ... ... ... ...
IND470/98  ... ..G ... ... ... ..A C.G ... ... ..T ... ... ... ... ... ..A ... ..A ... ..C
Consensus      G                     A C G   T       T
                                                                    A
```

Fig.16 Aligned amino acid sequence of P1 region of vaccine virus (IND 63/72) and field isolates, - indicates same as in IND 63/72

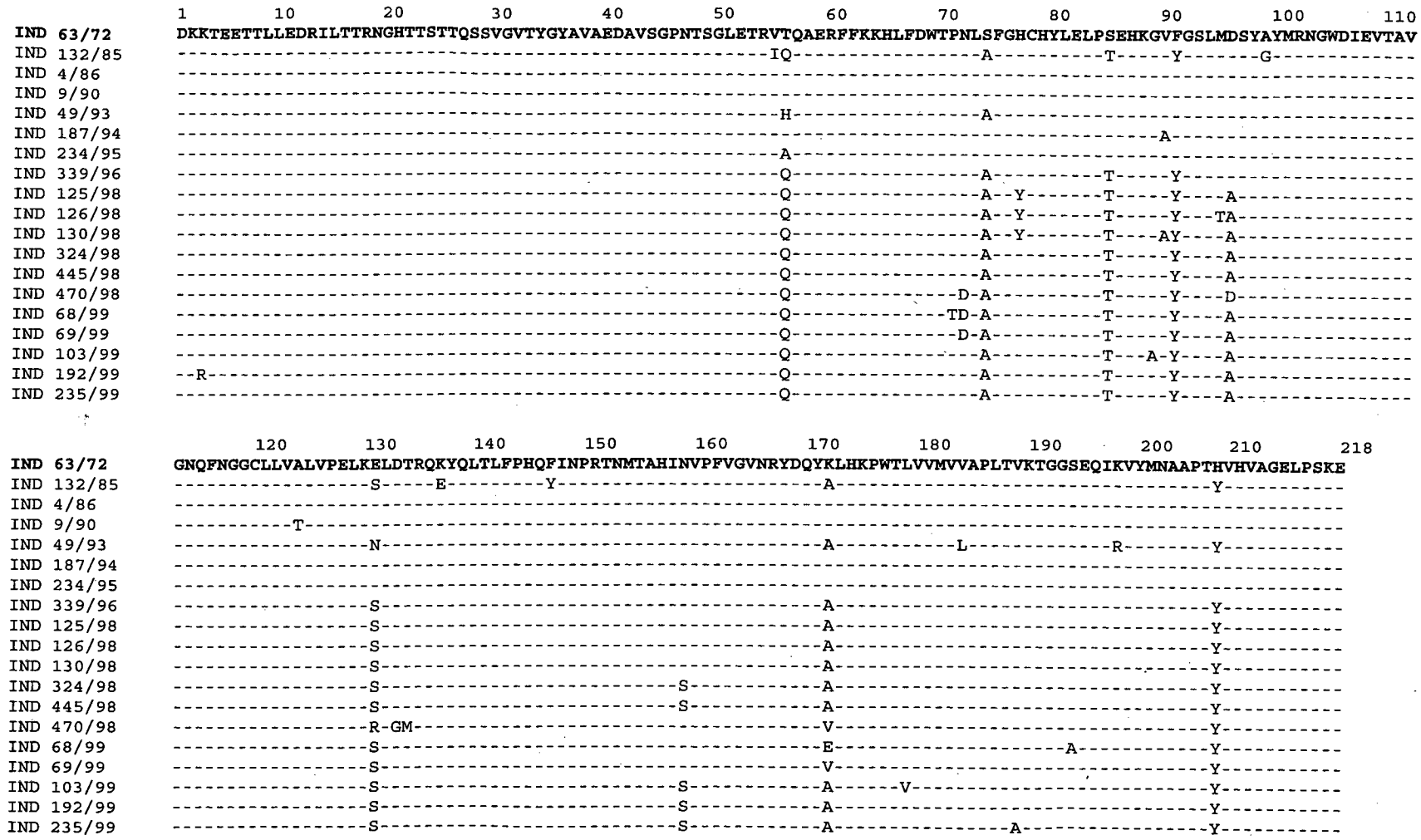
VP4

```
                1       10        20        30        40        50        60        70        80   85
IND 63/72       GAGQSSPATGSQNQSGNTGSIINNYYMQQYQNSMDTQLGDNAISGGSNEGSTDTTSTHTNNTQNNDWFSRLASSAFTGLFGALLA
IND 132/85      ----------------------------------------------------A-------------------S--------
IND 4/86        ------------------------------------------------------------------------------------
IND 9/90        ------------------------------------------------------------------------G-----------
IND 49/93       ------------------------------------------------------------------------S--------
IND 187/94      ------------------------------------------------------------------------------------
IND 234/95      ------------------------------------------------------------------------------------
IND 339/96      ------------------------------------------------------------------------S--------
IND 125/98      ------------------------------------------------------------------------S--------
IND 126/98      ------------------------------------------T---------------------------S--------
IND 130/98      ------------------------------------------------------------------------S--------
IND 324/98      ------------------------------------------------------------------------S--------
IND 445/98      ------------------------------------------------------------------------S--------
IND 470/98      ------------------------------------------------------------------------S--------
IND 68/99       ------------------------------------------------------------------------S--------
IND 69/99       -------S----------------------------------------------------------------S--------
IND 103/99      ------------------------------------------F---------------------------S--------
IND 192/99      ------------------------------------------T---------------------------S--------
IND 235/99      ------------------------------------------------------------------------S--------
```

(continued)

Fig.16 Aligned amino acid sequence of P1 region of vaccine virus (IND 63/72) and field isolates, - indicates same as in IND 63/72

VP2



(continued)

Fig.16 Aligned amino acid sequence of P1 region of vaccine virus (IND 63/72) and field isolates, - indicates same as in IND 63/72

VP3

```
                1       10        20        30        40        50        60        70        80        90       100       110
IND 63/72       GIVPVACAAGYGNMVTTDPKTADPVYGKVFNPPRTNLPGRFTNFLDVAEACPTFLRFGEVPFVKTGNSGDRLLAKFDVSLAAGHMSNTYLAGLAQYYTQYSGTMNIHFMFTG
IND 132/85      --------D-------------------------------------------------------------V-----------------------------------V------
IND 4/86        -------V--------------------------------------------------------------V-----------------------------------------
IND 9/90        -----------------------------------------------------------------------V-----------------------------------------
IND 49/93       --------D-------------------------------------------------------------V-----------------------------------I-V------
IND 187/94      -------V--------------------------------------------------------------V-----------------------------------------
IND 234/95      -----------------------------------------------------------------------V-----------------------------------------
IND 339/96      --------D-----------------------------------------D---------K------V----------V-----------------------------V------
IND 125/98      --------D-----------------R-------------L-----------------------------V-----------------R-----------------V------
IND 126/98      --------D-------------------------------------P---------------------V-----------------------------------V------
IND 130/98      --------D-------------------------------------------------------------V-----------------------------------V------
IND 324/98      --------D-------------------------------------------------------------V-----------------------------------V------
IND 445/98      --------D-------------------------------------------------------------V-----------------------------------V------
IND 470/98      --------D-------------------------------------------------------------V-----------------------------------V------
IND 68/99       --------D-------------------------------------------------------------V-----------------------------------I-V------
IND 69/99       --------D-------------------------------------------------------------V-----------------------------------V------
IND 103/99      ----I---D-------------------------------------------------------------V-----------------------------------V------
IND 192/99      --------D-------------------------------------------------------------V-----------------------------------V------
IND 235/99      --------D-------------------------------------------------------------V-----------------------------------DV-----

                     120       130       140       150       160       170       180       190       200       210       219
IND 63/72       PTDAKARYMVAYVPPGMEPPTEPERAAHCIHSEWDTGLNSKFTFSIPYLSAADYAYTASDVAETTSVQGWVCIYQITHGKAEGDALVVSVSAGKDFEFRLPVDARRE
IND 132/85      ------------I----T---D-------------------------------------------T-------------------------------------------Q
IND 4/86        ------------------------------------------------------------K-----------------------------------------------
IND 9/90        ------------------------------------------------------------------------------------------------------------
IND 49/93       ------------T----T---D-------------------M--L----------------T-----------------------------------------E--Q
IND 187/94      ------------------------------------------------------------------------------------------------------------
IND 234/95      ------------------------------------------------------------------------------------------------------------
IND 339/96      ------------I----T---D-------------------------------------------T-------------------------------------------Q
IND 125/98      ------------I----T---D-------------------------------------------T-------------------------------------------Q
IND 126/98      ------------I----T---D-------------------------------------------T-------------------------------------------Q
IND 130/98      ------------I----T---D-------------------------------------------T-------------------------------------------Q
IND 324/98      ------------I----T---D--H----------------------------------------T-------------------------------------------Q
IND 445/98      ------------I----T---D--H----------------------------------------T-------------------G-----------------------Q
IND 470/98      -----V------I----T---D--P------------------------------------------------------------------------------G---Q
IND 68/99       ------------I----T---D--H----------------------------------------T-----------------------------I-------------Q
IND 69/99       ------------I----T---D--H----------------------------------------T-----------------------------I-------------Q
IND 103/99      ------------I----T---D--H----------------------------------------T-------------------------------------------Q
IND 192/99      ------------I----T---D--H----------------------------------------T-------------------------------------------Q
IND 235/99      ------------I----T---D--H----------------------------------------T-------------------------------------------G---Q
```

(continued)

Fig.16 Aligned amino acid sequence of P1 region of vaccine virus (IND 63/72) and field isolates, - indicates same as in IND 63/72,; indicates absence of amino acid at this position

VP1

```
                1         10        20        30        40        50        60        70        80        90        100       110
IND 63/72       TTTAGESADPVTTTVENYGGATQSARRLHTDVAFVLDRFVKLT;PKNTQILDLMQIPSHTLVGALLRSATYYFSDLEVALVHTGSVTWVPNGAPKDALDNHTNPTAYQKKPI
IND 132/85      ---T-----------------T-----------------A-----T----------------------------------PA------S--H----Q--------Q--
IND 4/86        ----------------------------------------;-------------------------------------------------------------------
IND 9/90        ----------------------------------------;-------------------------------------------------------------------
IND 49/93       ---T-----------------T---------S---------A--S--T-------L-------------------------P-------S--------Q----------
IND 187/94      ---V-----------------------------------;---------------------------------I-----------------------------------
IND 234/95      ----------------------------------------;-------------------------------------------------------------------Y----------
IND 339/96      ---T-----------------T-----------------A---I-T----------------------------------PA------S-------Q--------Q--
IND 125/98      ---T-----------------T-----------F-------A-----T-------A-------------------------PA------S------E-Q--------Q--
IND 126/98      ---T--------A----------T-------G----------A-----T-------A-------------------------PA------S-------Q--------Q--
IND 130/98      ---T-----------------T-------------------A--S--T-------A-------------------------PA------S------E-Q--------Q--
IND 324/98      ---T-----------------T-------------------A---I-T-------A-----E-------------------PA------S-------Q--------Q--
IND 445/98      ---T--------A----------T-----------------T---I-T-------A-------------------------PA------S-------Q--------Q--
IND 470/98      ---T-----------------T-------------------A--S--T---------------------------------P---------------Q--------Q--
IND 68/99       --AT----------------KT------------------A---I-T----A--A-------------------------PA------S-----N-Q--------Q--
IND 69/99       --AT----------------T-------------------A---I-T----V--G-------------------------PA------S-----N-Q--------Q--
IND 103/99      ---T-----------------T-------G----------A---I-T-------E-------------------------PA------S-------Q--------Q--
IND 192/99      ---T-----------------T-------------------A---I-T-------A-------------------------PA------S-------Q--------Q--
IND 235/99      ---T-----------------T----R--------------A-----T-------A-------------------------PA------S-------Q--------Q--

                      120       130       140       150       160       170       180       190       200       211
IND 63/72       TRLALPYTAPHRVLATVYNGKTTYGTQPTRRGDLAVLAQRVSNRLPTSFNYGAVKADTITELLIRMTRAETYCPRPLLALDTTHDRRKQEIIAPEKQVL
IND 132/85      ------------------------ETTP----M-A----L-GQ----------------------K----------------Q-----K-------MM
IND 4/86        ------------------------------------------------------A-----------K--------------------------------
IND 9/90        ------------------------------------------------C---------------K-------------------------L-----
IND 49/93       ------------------------ETTT----M-A----L-G-------------ES--------K----------------Q----------------
IND 187/94      ------------------------------------------------------------K--------------------------------
IND 234/95      ------------------------------------------------------------K--------------------------------
IND 339/96      ------------------------ETTP----M-A----L-GQ----------------------K-------------G--Q------------MM
IND 125/98      ------------------------ETTS----M-A----L-GQ----------------------K----------------Q------------MM
IND 126/98      ----------------------M---ETTS----M-A----L-GQ----------------------K----------------Q------------MM
IND 130/98      ------------------------ETTS----M-A----L-GQ----------------------K----------------Q------------MM
IND 324/98      ----------------------R----ETTS----M-A-T--L-GQ----------------------K----------------Q------------MM
IND 445/98      ----------------------R----ETTS----M-A----L-GQ------------E---------K-----------=IA--Q------------MM
IND 470/98      ------------------------ETTS----MEA----L-I----------------------K----------------Q------------A-
IND 68/99       ----------------G--------ETTP----M-A----L-GQ----------------G-----K----------------Q------------M-
IND 69/99       ------------------------ETTP----M-A----L-GQ----------------------K----------------Q------------M-
IND 103/99      ----------------------R----ETTS----M-A----L-GQ----------------------K----------------Q------------MM
IND 192/99      ----------------------R----ETTS----M-A----L-GQ----------------------K----------------Q------------MM
IND 235/99      ----------------------R----ETTS----M-A----L-GQ----------------------K----------------Q----------G--MM
```

The remaining fourteen isolates showed many base substitutions in all the four genes that ranged from 253 in IND 470/98 to 300 in IND 103/98. These isolates showed 18 to 25 changes in VP4, 77 to 98 in VP2, 71 to 93 in VP3 and 90 to 103 in VP1 gene. In addition, all these isolates had three extra nucleotides in the VP1 gene (at130-132), consequently they had 633 nucleotides in VP1 gene as against 630 in vaccine virus and the four isolates discussed earlier.

Deduced amino acid sequences of isolates also revealed several changes. In VP4 protein, 7 residues (out of 85) were found to be varying. All the changes were unique in different isolates except at position 77 where there was a T→S substitution in IND 132/85, IND 49/93 and all the 12 isolates recovered after the year 1996. The isolate IND 69/99 had A→S change at position 8 and isolate IND 9/90 showed change at position 75(A→ G). Besides these, substitutions were seen at positions 42 (A→T) 43 (I→T), 47 (S→F) and 57(T→A) in isolates IND 192/99, IND126/98, IND103/99 and IND132/85 respectively.

In the VP2 protein there were a total of 28 residues found to be variable with a maximum of 11 changes noticed per isolate (IND132/85, IND68/99 and IND103/99). Of the four isolates that were similar to vaccine virus IND 4/86 did not have any change in VP2 and the other three isolates (IND9/90, IND187/94 and IND234/95) showed substitutions at one position each. The remaining fourteen isolates showed changes at several places, many of them were common in different isolates. All the 14 isolates had common changes at 7 positions (56, 74, 85, 92, 131, 173 and 207). The other residues changed in many isolates were 96 and 168. Other than these, the isolates IND125/98, IND 126/98 and IND 130/98 had H→Y change at 77 and isolates IND 68/99 & IND 69/99 had N→D substitution at 72 as common changes. All the remaining changes noticed in VP2 protein were unique to different isolates.

In the VP3 protein a total of 27 positions were found to accept changes. As in the case of VP2 protein, changes were minimum (1 to 3) in isolates 4/86, 9/90, 187/94

Table. 15. Nucleotide differences in Asia 1 field isolates ***(* These isolates possess additional three nucleotides at position 130-132)***

Serial No.	Field Isolates	Structural Proteins (Coding Genes)				Total Changes
		VP4 (1A)	VP2 (1B)	VP3 (1C)	VP1 (1D)	
1	IND 4/86	0	2	4	4	10
2	IND 9/90	4	2	2	4	14
3	IND 187/94	0	1	4	5	10
4	IND 234/95	0	2	2	5	9
5	IND 49/93*	21	81	93	98*	293
6	IND 132/85*	20	91	85	93*	289
7	IND 339/96*	20	85	81	98*	284
8	IND 125/98*	22	87	85	97*	291
9	IND 126/98*	21	86	83	98*	288
10	IND 130/98*	22	86	78	98*	284
11	IND 69/99*	21	87	82	93*	283
12	IND 68/99*	25	86	81	93*	285
13	IND 103/99*	23	98	79	103*	303
14	IND 192/99*	22	98	78	96*	294
15	IND 445/98*	22	96	77	98*	293
16	IND 235/99*	22	94	78	97*	291
17	IND 324/98*	23	95	79	97*	294
18	IND 470/98*	18	77	71	90*	256

Table. 16.Amino acid differences in Asia 1 field isolates ***(* These isolates possess an additional amino acid at position 44.)***

Serial No.	Field Isolates	Structural Proteins				Total Changes
		VP4	VP2	VP3	VP1	
1	IND 4/86	0	0	3	2	5
2	IND 9/90	1	1	1	3	6
3	IND 187/94	0	1	2	3	6
4	IND 234/95	0	1	1	2	4
5	IND 49/93*	1	9	12	23*	45
6	IND 132/85*	2	11	8	24*	45
7	IND 339/96*	1	7	11	24*	43
8	IND 125/98*	1	9	11	24*	45
9	IND 126/98*	2	10	9	26*	47
10	IND 130/98*	1	10	8	25*	44
11	IND 69/99*	2	9	10	25*	46
12	IND 68/99*	1	11	10	29*	51
13	IND 103/99*	2	11	10	26*	49
14	IND 192/99*	2	10	9	25*	46
15	IND 445/98*	1	9	10	26*	46
16	IND 235/99*	1	10	10	25*	46
17	IND 324/98*	1	9	9	29*	48
18	IND 470/98*	1	10	11	23*	45

& 234/95. All the 18 isolates showed a G-V change at position 66. In VP3 also there were 7 common changes in all the remaining 14 isolates as in the case of VP2. There were also two more locations (137 & 173) where changes were seen in many isolates and all the remaining changes were unique to different isolates.

The highest number of positions (a total of 45) found to be variable were in the VP1 protein. As in the case of VP3, one change (at 178) was common to all the isolates. The isolates IND 4/86, IND 9/90, IND 187/94 and IND 234/85 also showed changes in 2 to 3 positions each. The remaining (14) isolates varied in 23 to 29 positions compared to vaccine virus. The two most variable regions in VP1protein in all FMDV serotypes viz., 40 to 60 and 138 to 154 were also found to be quite variable. All the 14 isolates had an extra residue, an alanine, at position 44 except in IND 445/98 where it was a threonine. The position 58 was found to be the most flexible as there were S→L/A/G/E substitutions in different isolates. There were at least three pattern of changes (ETTP or ETTS or ETAP) at positions 138 to 141, i.e., just before the RGD sequence in different isolates. There were a maximum of 9 positions found varying in GH loop in these isolates. The isolate 470/98 also had 9 changes of which one (147 A→E) was unique to this isolate. The last two amino acids of VP1 were also changed in many isolates, all the substitutions in these cases were leading to methionine except in 470/98 where the substitutions at 210 was valine to alanine.

The overall amino acid changes in isolates are summarized below.

As in nucleotide sequence similarity, the four isolates IND 4/86, IND 9/90, IND 187/94 and IND 234/95 showed very few amino acid changes (a total of 4 to 6) from vaccine virus compared to rest of the 14 isolates. Of the five base substitutions in the VP4 gene in the case of isolate IND 9/90, two changes had led to amino acid substitutions. The other three isolates (IND 4/86, IND 187/94 and IND 234/95) did not have any changes in VP4 while they had 1-3 changes in the other three proteins.

The remaining fourteen isolates varied at 42 (IND 339/96) to 50(IND 103/99) positions from the vaccine virus. Many of the 18-25 base changes showed in VP4 gene by these isolates were apparently synonymous since at the amino acid level there were only 1 to 2 changes. Amino acid changes per isolate were highest in VP1 protein (23-29), in VP2 and VP3 they were almost equal (8-12 in VP3 and 9-11 in VP2). As these isolates had three extra nucleotides in the VP1 gene, an extra amino acid (alanine or threonine) was present at position 44 of VP1 protein.

DISCUSSION

5. DISCUSSION

Foot-and-mouth disease virus is a highly contagious animal pathogen notorious for its antigenic diversity. It occurs as seven serotypes and over 65 subtypes. The basic criterion for such classification is their serological cross-reactivity and antigenic differences. Several studies have shown that only defined amino acid residues in discrete areas on the virion surface contribute towards the antigenicity of the virus and these are termed antigenic sites. Of the seven serotypes of FMDV, the serotypes O, A and C have been encountered in all parts of FMD-prevalent areas of the world, whereas serotype Asia 1 is confined to Asian subcontinent and SAT serotypes are confined to Africa. Since all the seven serotypes of FMDV are distinct from each other antigenically, and their inter-relationships in terms of their antigenicity is difficult to study, their genetic inter-relationships have been studied in detail. Such studies also reveal a great degree of divergence between the serotypes. FMD virus serotypes O, A and C have originated from Europe which make one group. The serotypes SAT 1, SAT 2 and SAT 3 form the second group and all these have originated from Africa. The third group comprises of serotype Asia 1 which has originated from Asia. Genetically each of these three lineages differ from each other by 35 to 40%. Similarly, to understand the antigenic sites, extrapolation of the data on antigenic sites identified in one serotype onto others may not reveal those residues that are specifically important for a serotype.

Antigenic profiles detailing the exact amino acids involved in virus neutralization have been deduced for serotypes O, A and C (Bolwell *et al.*, 1989, Pfaff *et* al., 1988, Barnett *et al.*, 1989, Baxt *et al.*, 1989, Mateu *et al.*, 1990, Xie *et al.*, 1987, Thomas *et al.*, 1988a). The antigenic sites so identified (summarized in Table.1) are shown to differ in these viruses. It is of great importance to know the antigenic make up of FMD virus as it can help in the selection of appropriate vaccine strains and for undertaking necessary control measures. Serotype Asia 1 is responsible for FMDV outbreaks in India, next to type O. Though three reports exist in literature on antigenic

site analysis of Asia-1, the actual residues involved in forming the sites have not yet been mapped. This study is aimed at identifying the key residues which form the antigenic sites of FMDV Asia 1 using the widely acclaimed and cited technique of production of neutralizing monoclonal antibody-resistant (MAR) mutants, comparison of the deduced amino acid sequence of capsid coding proteins of mutants with that of the parent virus and thus identify those residues which mutated enabling neutralization-escape, ie., the exact residues involved in virus neutralization. Towards this end 29 neutralizing Mabs were used to isolate single and multiple Mab resistent mutants and the complete capsid coding region (of 2196 bases) of selected 15 mutants (based on their cross-reactivity & cross-neutralization pattern) and the parent virus were sequenced and their deduced amino acid sequences compared.

As all monoclonal antibody escape mutants have been isolated *in vitro*, and there are variable regions in FMDV capsid protein sequences that have not been correlated with neutralization escape, analysis of field isolates that have faced different selection pressures during replication in the host may provide information on substitutions that are important for antigenic variation under field conditions (Krebs et al., 1993). To undertake such comparison, 18 field isolates of serotype Asia 1 collected from outbreaks that occurred in different parts of the country between 1985 and 1999 have been profiled using monoclonal antibodies. The entire P1 region of these 18 isolates were sequenced and compared.

5.1 Isolation of MAR mutants

Populations of RNA viruses like FMDV consist of multiple variants collectively termed viral quasispecies (Domingo *et al.*,1985). A complex equilibrium between the high rate of mutation and the competitive fitness of each of the arising variants determines, in a given environment and time, the composition of the quasispecies (Eigen and Biebricher, 1998, Domingo *et al.*, 1985, Holland *et*

al.,1992).This property endows these viruses with a high potential for variation and adaptation, hence it is little wonder that frequencies of isolation of Mab-resistant (MAR) mutants of FMDV is close to 10^{-5} (Martinez *et al.*, 1991).In order to have a virus stock that would contain enough variant genomes, roller culture propagation of the parent virus to high titres (>l0 ^{6}) was done. This parent virus was subjected to selection pressures in the form of 29 neutralizing Mabs to isolate 29 single MAR mutants and 6 double MAR mutants. All the Mabs used in the present study are reactive to the whole virus particle (146 S) only and not to 12S and disrupted virus particle and hence can be considered as identifying conformation-dependent epitopes or sites. The mutants thus generated were plaque-purified and were tested again in Mab-profiling ELISA. All mutants were tested against the panel of neutralizing Mabs and reactivity patterns categorized them into 3 distinct groups:

I MAR 72,76 & 82 showed similar reactivities; they did not react with all the group 1 Mabs but reacted with all others,

II The remaining 25 single MAR mutants other than 72,76,82 and 10 formed the second group; they showed reduced or complete absence of reactivity with the corresponding Mab but reacted with Mabs of the other two groups. Based on the degree of reactivity with homologous Mabs, this group was subdivided into Group IIa in which the mutants were partially reactive and Group II b, where the mutants were non-reactive, a few mutants (MAR 13, MAR B3, MAR 2A, MAR 1A, MAR 40 and MAR 16) in Group II b also showed reduced reactivity with group I and III Mabs.

III MAR mutant isolated against Mab 10 did not react with any Mab and formed the third group, (this mutant behaved like a multiple mutant since it was non-reactive to all the Mabs). In spite of repeated attempts, a mutant specific only to Mab 10 could not be isolated.

Difficulty in isolating a neutralization escape mutant against Mab 10 could be due to the presence of such mutants at extremely low frequencies in the parent population. The fact that MAR 10 viruses that were isolated using the modified procedure given in Materials and Methods, in three independent trials had the same phenotype, ie., it was non-reactive against all the Mabs in the panel indicates that while selection pressure is exerted in the form of Mab 10, mutants that were selected were those viruses that already had mutations in the Group 2 and 3 Mab-binding sites, even in the absence of selection pressure from these Mabs. Functional relationships between epitopes as reported by Barnett *et al.* (1989) could be another probable reason for such findings. They had noticed that one particular Mab (14.7.1) used by them appeared to be unique in that it neutralized all the mutants resistant to other Mabs, but attempts to isolate a mutant resistant only to this Mab were consistently unsuccessful.

Based on their Mab reactivity, mutants from Groups I and II were subjected to selection pressure using Mabs from the other groups. A total of 6 double mutants were isolated. On profiling, each of these double mutants were found to have lost their reactivity to the Mab panel; in effect phenotypically all of them behaved as multiple mutants.

5.2 Cloning and Sequencing of the Capsid Coding Region of the Parent virus, Mutants and Isolates.

In order to identify the amino acid changes associated with neutralization escape (or loss of Mab reactivity in the case of isolates), viral RNA extracted from the parent virus, mutants and isolates was RT-PCR amplified in the P1 region, gel-purified and rapid-cloned into pAmp1 or pAmp10 vectors. Primers for PCR were designed in this study (Table.5) and were found to work well with the parent virus, mutants and isolates. The generated PCR products were rapid-cloned into pAmp vectors. This procedure relies on the incorporation of dUMP residues in the place of

dTMP into the 5' end of each amplification primer. After amplification, the PCR products contain the dUMP containing sequence at their 5' termini. Treatment with Uracil DNA Glycosylase (UDG) renders dUMP residues abasic, and unable to base-pair, resulting in 3' protruding termini. Cloning is performed by adding the linearized vector (whose termini are compatible for cloning) and UDG to a portion of the amplification product to be cloned (Fig.5a). Selective deglycosylation of dUMP residues by UDG and annealing of PCR products to vector occur simultaneously. The annealing reaction is complete in 30 minutes producing chimeric molecules which are ready for transformation. This procedure eliminates the time-consuming tasks normally associated with cloning of PCR products, including Restriction Endonuclease (RE) digestion, PCR product purification, end-polishing or ligation. Unlike many REs, UDG functions effectively near nucleic acid termini, thus enabling high efficiency annealing and subsequent cloning. Also UDG will not cleave the PCR products internally. The vectors used contain a pUC origin of replication, the beta-lactamase gene conferring ampicillin resistance and a multiple cloning site (MCS) within the alpha peptide of the *lac*Z gene so that insertion of DNA into the MCS results in white, rather than blue, colonies on medium containing IPTG and X-Gal.

White colonies that appeared on selection plates after transformation of annealing reactions were grown in LB-Ampicillin broth, the plasmid DNA extracted and checked for presence of inserts. One positive plasmid from each clone was sequenced in the P1 region using primers listed (Table.5) Primers for sequencing were designed and evaluated in the study. Since initially no sequence data was available on the P1 region of Asia1 FMDV, the nucleotide sequence of serotype O, and A capsid-coding regions available in the laboratory were compared and three primers (MG39, MG40 and MG41) were designed in those regions that were conserved across serotypes. As more sequence data was generated, it was also included in the comparison to design primers in the conserved regions. All the primers were found to work efficiently in both manual and automatic sequencing. Initially, all the eleven primers were used to sequence the P1 region, but later, after comparison of the

sequence read-lengths generated using these primers, 9 of them were found to be sufficient to sequence the entire P1 region in manual sequencing. Since automated sequencing gave much longer read-lengths, 5 primers were found sufficient to sequence the 2196 nucleotide P1 region. All the primers gave overlapping read lengths at their ends, that assisted in confirming the ambiguities that normally occur towards the termini.

The parent virus and all mutants were first sequenced manually, the changes found in them in relation to the parent virus, were confirmed by sequencing those regions again using the ALFexpress™ Auto Cycle™ Sequencing Kit (Pharmacia Biotech) and running these reactions in the ALFexpress II DNA sequencer. Since sequencing using the automated sequencer was less time consuming, yielded much longer read-lengths and most importantly, the data generated was ready for analysis in a few minutes after the end of the run, all mutants were sequenced and the samples run in the automated sequencer. On an average a single reaction yielded read lengths ranging from 700-800 bases (as against 250-350 bases in manual sequencing). Similarly all the field isolates were sequenced to obtain the full capsid coding region.

5.3 Antigenic sites of FMDV serotype Asia 1.

All the mutants sequenced had changes in their amino acid sequences in one position or the other in the structural proteins VP1, VP2 and VP3. No changes were noticed in any of the mutants in the protein VP4. This was expected as this protein is situated internally in the virion capsid. The amino acid changes that were seen in the other three proteins varied from 1 to 3.

Taking into consideration the pattern of reactivities seen with these mutants in Mab-profiling ELISA and the changes seen in the sequences, the following observations were made.

1. MAR 72, 76 and 82 (Group I mutants) had changes in the VP1 39-48 region. The changes were as follows. In MAR 72, at position 39 F was changed to L, in MAR 76, at position 46 N was changed to D and in MAR 82 Q was changed to H at position 48. Mab profiling results in conjunction with these sequence differences indicate that residues 39, 46 and 48 might be critical for binding of Group 1 Mabs (Mab 72, Mab 76 and 82). These three residues (VP1 39, 46 and 48) are located in and around the βB- βC loop region (loop that connects the beta sheets B and C of VP1 protein. The residues 44 and 48 of VP1 in serotype O, which are also in this region are shown to form an antigenic site (Kitson *et al.*, 1990).

2. Sequence analysis of Group II mutants revealed that seven of the eight mutants showed amino acid difference at position 130 of VP2. The change at VP2 130 were of two types. In the partial mutants MAR66, 13, and W, the residue K was changed to T (basic to neutral amino acid) and in all the complete mutants and the partial mutant B3, K was changed to E (basic to acidic amino acid). It is apparent that the nature of substitution (Kitson *et al.*, 1990), ie., basic or neutral or acidic affects the Mab-binding to different extents. The residue 132 in this region of VP2 is shown to have an important role in forming an antigenic site in type A_{10} along with VP1 142-147 residues (Thomas *et al.*, 1988a). The only mutant that did not show change at VP2 130 in this Group was MAR E, which had unique substitutions at VP1 72 and VP3 75. Since this mutant is also non-reactive to Group 2 Mabs, it may be assumed that these substitutions also inhibited binding of this group of Mabs. Since all the Mabs used in the study were conformation dependent (Sanyal 1995) this observation that residues situated far away from each other are involved in the formation of antigenic sites seems logical. Though amino acid substitutions that affect recognition of a protein by an antibody are generally limited to the surface area in contact with the antibody, MAR mutants may also show substitutions at residues probably located outside the antibody-binding site (Parry *et al.*, 1989); these substitutions are supposed to act by forcing the involved loops into different positions, thus disrupting epitopes (Parry *et al.*, 1989, Krebs *et*

et al., 1993). The effects of such substitutions are often quite difficult to interpret in the absence of structural data.

3. MAR 10 and all double mutants showed amino acid substitutions at VP1 168 (Table.14c) and VP2 130; the residue VP1 168 was assumed to be critical to the binding of Mab 10. These mutants also were mutated against Group 1 Mabs, though sequence analysis did not reveal any substitutions in those residues critical for the binding of Group 1 Mabs (VP1 39, 46 or 48). It may be assumed that since these residues lie in close proximity to VP1 168, in the three-dimensional structure (Fig.10), substitutions at VP1 168 might have in some way inhibited Mab-binding at VP1 39, 46 and 48. But it should also be noted that though the binding sites of Group 1 and 3 Mabs lie so close, mutations at the Group 1 Mab-binding site does not seem to affect Mab-binding at VP1 168 to a great extent as seen in the reverse case. As was expected, Mar 10 and other double mutants showed substitutions at VP2 130, a change well-correlated with their non-reactivity with Group 2 Mabs. Though MAR 76 (Group I) had mutated at VP1 168 (D to G, a change similar to the one shown by DMAR 66-72), it still retained its reactivity to Mab 10. It should be remembered that not only substitutions, but also the nature of substitutions at defined positions affect Mab-binding (Mateu *et al.*, 1990, Kitson *et al.*,1990). In MAR 76, the effect of D to G substitution (VP1 168) appears to be nullified or reduced by the substitution of N to D at VP1 46 and so it has retained its reactivity to Mab 10. This suggests that though the Mab10-binding site appears to be an independent entity, the same cannot be said about the Mab-76 binding site.

Further, the single amino acid changes seen at other positions in the different groups of mutants could not be assigned to any distinct site. Their role, if any, in the formation/ delineation of distinct antigenic sites may become more clear when more Mabs/mutants to Asia1 viruses are characterized.

5.4 Predicted Antigenic Sites on FMDV Asia1

The building blocks of the viral capsid in icosahedral viruses are one or more structural proteins which in their tertiary structure appear as wedge-shaped blocks (Fig. 2). Such blocks are formed due to the adoption of specified secondary structure elements by the amino acid sequences of the component proteins. A common folding pattern seen in these structural proteins is an eight-stranded anti-parallel beta barrel, which is formed by eight beta sheets/strands that are arranged anti-parallely, with loops of variable lengths connecting them, interspersed with 2 or 3 alpha helices. The beta sheets are named B, C, D, E, F, G, H and I, of them, the sheets B, D, I and G are in one direction while C, E, H and F are parallel to them but in the opposite direction. The connecting loops are named depending on the sheets that they connect, for example, the loop that connects the beta sheets G and H is named the G-H loop. The alpha helices are named Z, A and B. Each of the three capsid proteins of FMDV (VP1, VP2 and VP3) are found to follow this folding pattern, while the much smaller VP4 protein behaves as an internal and N-terminal extension of VP2. So in effect, a protomeric unit is composed of VP1, VP2 and VP3, and five of such protomers form a pentamer and 12 pentamers assemble to form the viral capsid. Structural comparisons between serotypes with respect to antigenic sites often involve the protomer which is the simplest structural unit of the capsid. The antigenic differences seen between serotypes do not usually involve the amino acid residues that constitute the beta sheets which are more or less conserved across the serotypes, but have been mapped to the connecting loops which are variable both in the number and nature of the constituent residues. However, caution should be exercised in extrapolating the findings on one serotype to the structure of another serotype. Such kind of comparison should only be regarded as a visual aid to understand the probable location of residues that have been identified as antigenically critical. Predicted structures based on homology modeling (where similarity between two proteins are taken to imply similarity in structure also) provide a better alternative, but ideally the actual localization of critical residues can

be possible only when X-ray crystallographic data is available on the particular serotype.

The residues thought to form the antigenic sites in Asia1 viruses as deduced from MAR mutant studies were highlighted on the protomeric structural data of serotype O using the Molecular Graphics program, RasMol (Sayle R., 1996).

RasMol images (Fig.R1) show that residues 39, 46 and 48 of VP1 are well exposed on the virion surface and are situated towards the 5-fold axis of symmetry. In case of serotype O the residues 44 and 48 of antigenic site 3 also lie in the same location. It is evident in the figure that the identified Mab-binding site of Asia1 virus should be analogous to site 3 in serotype O, though the actual residues involved are different.

The residue found important in Group II mutants viz., VP2 130 is shown in Fig. R2. Adjacent residues (VP2 131, in type O and VP2 132 in type A 10) have been implicated in antigenic site formation in serotypes O and A (Kitson *et al.*, 1990, Thomas *et al.*, 1988a). This site is located towards the two-fold axis of symmetry. One mutant, MAR E, which did not react with these Mabs did not have a change at VP2 130. The changes were seen instead at VP1 72 and VP3 75 and are highlighted in Fig. R2b along with VP2 130. Though VP1 72 appears closer to VP2 130 in the single protomer, when viewed along the side (Fig. R5), this residue appears less accessible on the virion surface. The other residue VP3 75, though located away from VP2 130 on the protomer, these two residues (VP2 130 of one protomer and VP3 75 of another) come close to each other in the viral capsid (Fig.). Similarly, in case of serotype O, the residues VP2 70-77 in one protomer, and VP3 58 in another, that are antigenically important, have been shown to come together in their three-dimensional structure (Barnett *et al.*, 1998).

Fig. R1. Antigenic sites of FMDV Asia1, Site 1 residues

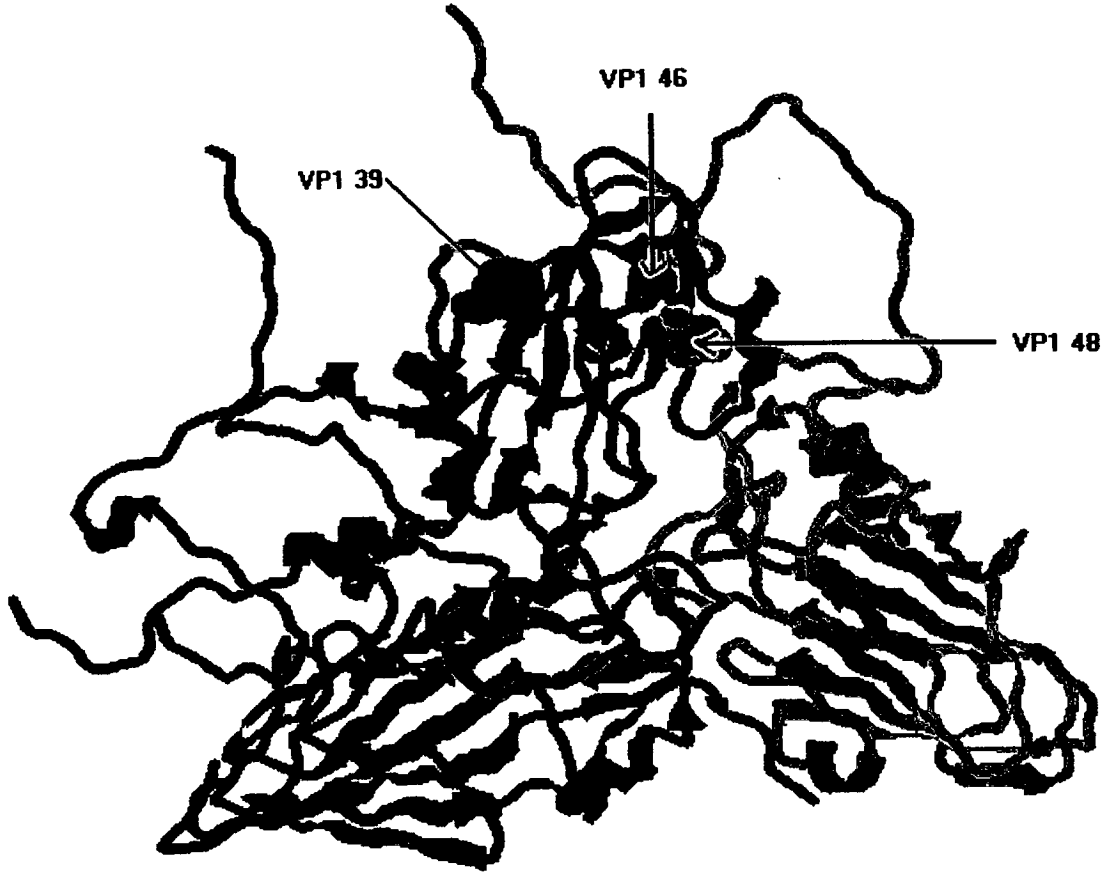


Fig. R2. Antigenic sites of FMDV Asia1, Site 2 (VP2 130)

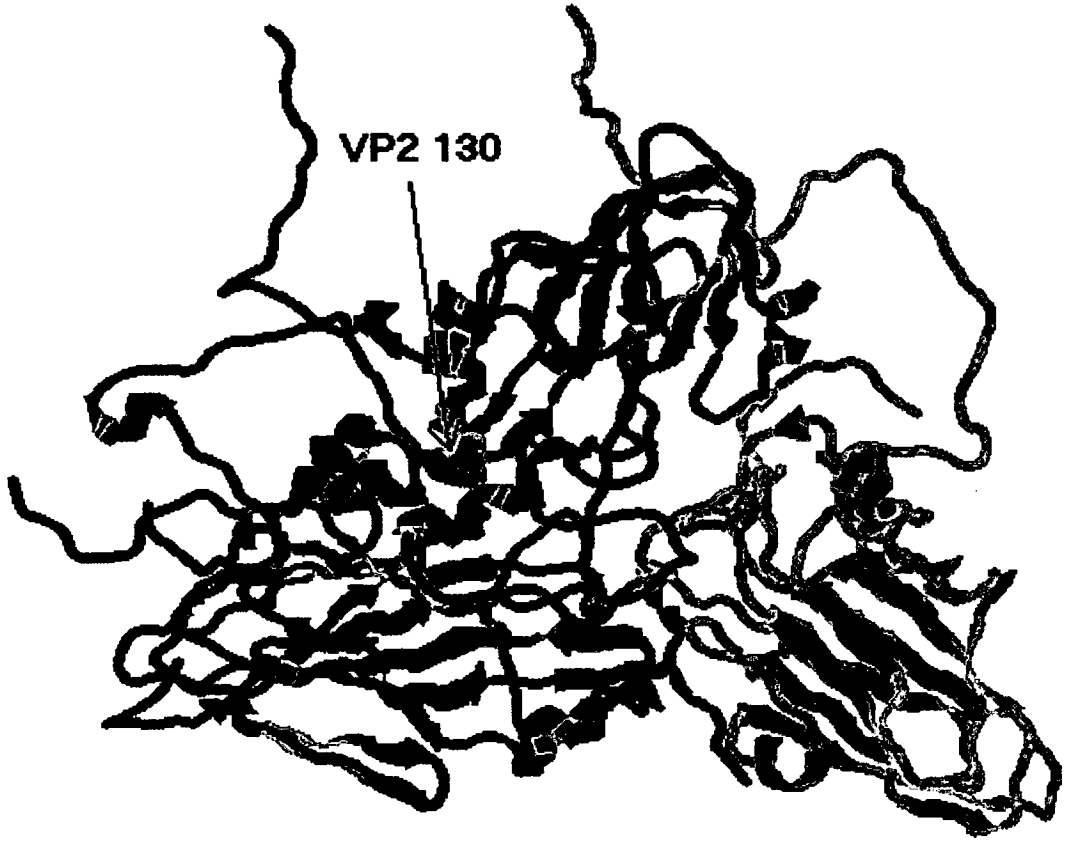
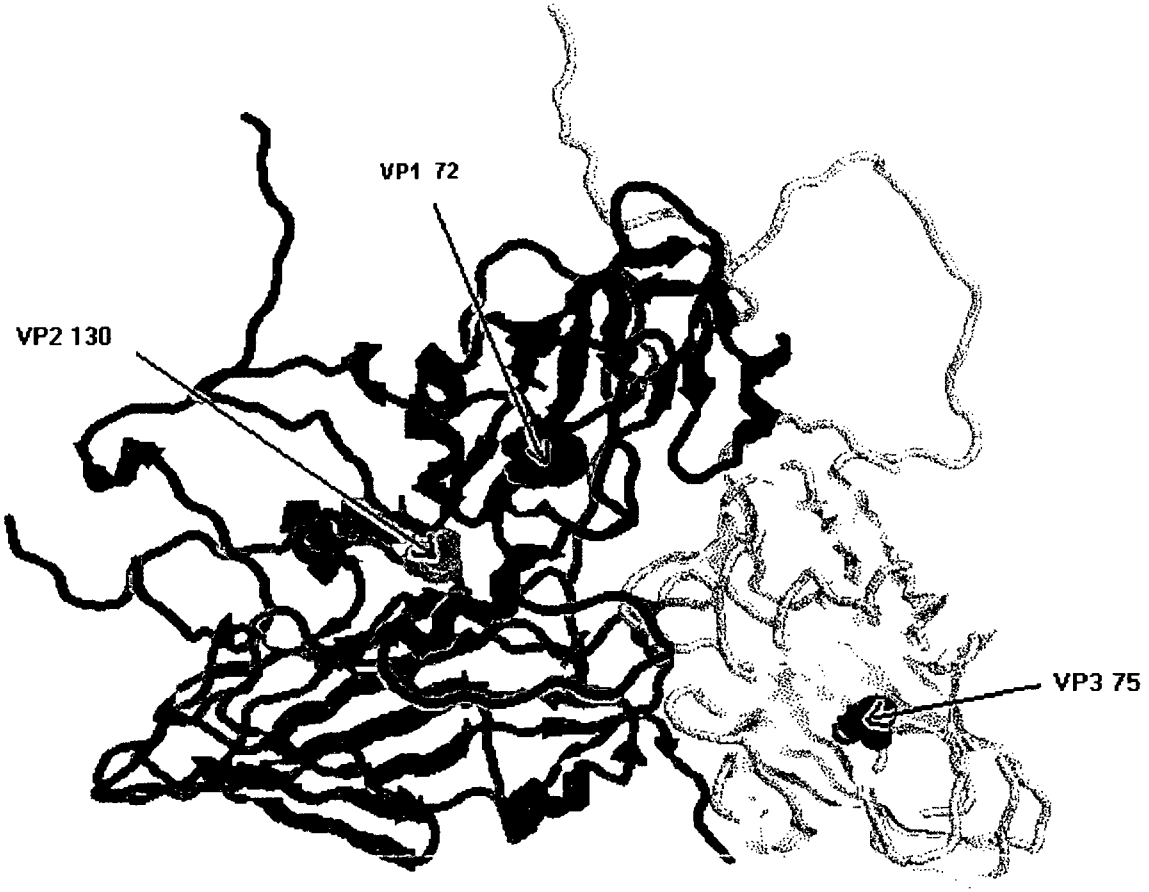


Fig. R2b. Antigenic sites of FMDV Asia1, Site 2 residues



FiçR3. Antigenic sites of FMDV Asia1, Site 3 (VP1 168)

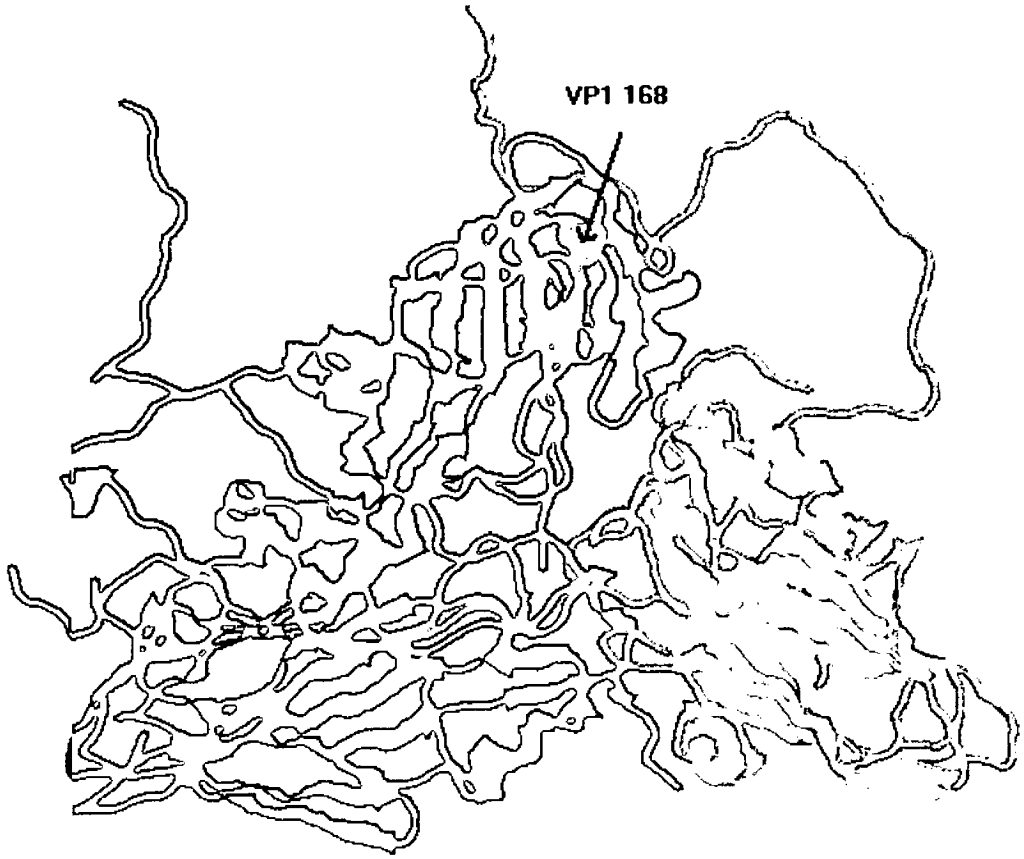


Fig. R4. Antigenically important residues of FMDV serotype Asia 1

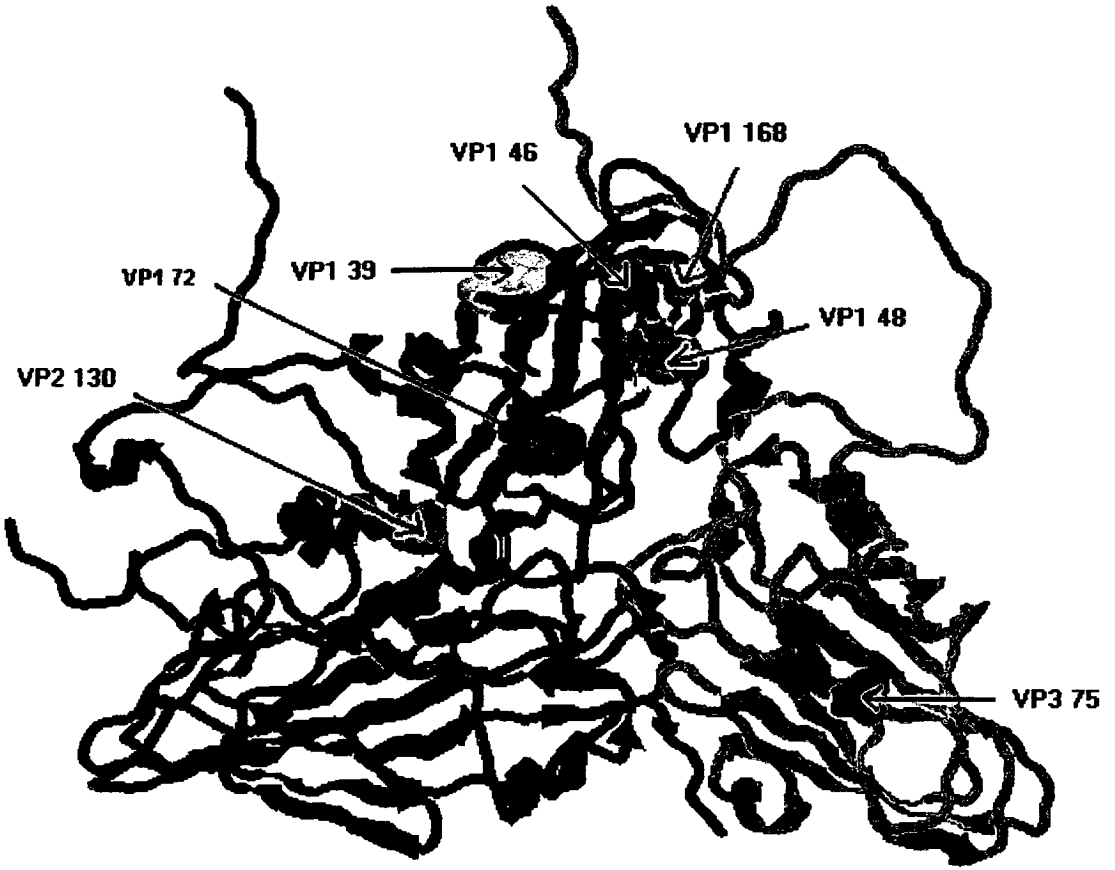
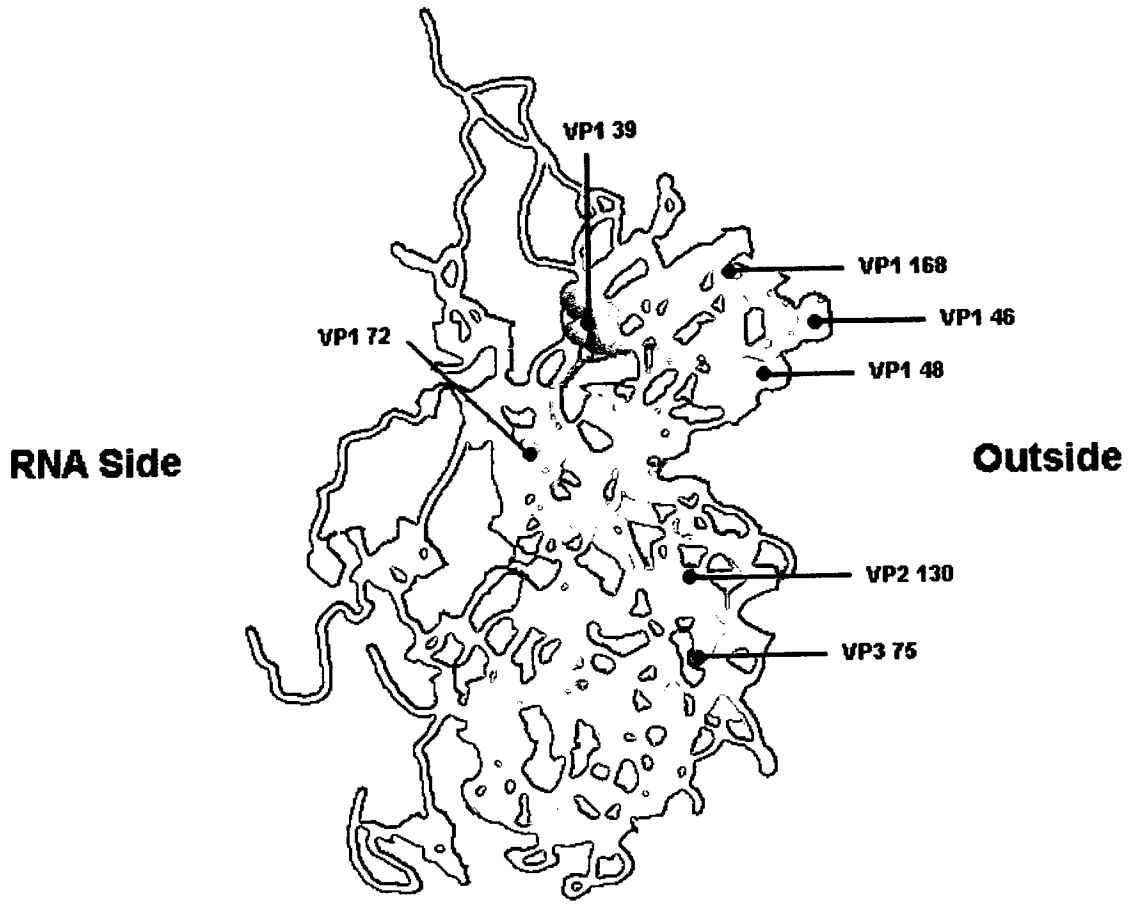


Fig. R5. Side view of RasMol image showing all the antigenically important residues of FMDV serotype Asia1



The VP1 168 residue is alone highlighted in Fig. R3. Surprisingly, though it is distant from residues 39,46 and 48 in the primary sequence, it lies in close proximity to these residues. Analysis of mutants has indicated that change in VP1 168 has affected binding of Mabs both at VP1 168 and VP1 39, 46 and 48. This is clear in the figure where all these residues are found to cluster near the five-fold axis of symmetry.

5.5 Analysis of Field isolates

Amino acid sequences in structural proteins VP1, VP2 and VP3 of FMDV are inherently variable in naturally occurring (field isolates) viruses (Mateu *et al.*, 1989, Saiz *et al.*, 1993, Mateu *et al.*, 1994, Pfaff *et al.*, 1989). Such kind of random substitutions occur generally in residues that are structurally non-critical (Domingo *et al.*, 1990), which may or may not have a role in the antigenicity of the virus (Mateu, 1995). Therefore, studies on antigenic sites have predominantly involved the isolation of monoclonal antibody resistant mutants in vitro, where the occurrence of such random mutations are very much limited (Mateu, 1995). Even in MAR mutants, if amino acid substitutions are observed in more than one place, the residue actually involved in Mab binding is further confirmed by characterizing mutants generated either in independent trials or using Mabs of similar reactivity (Thomas *et al.*, 1988a, Kitson *et al.*, 1990). However, studies on field isolates in conjunction with that of MAR mutants have been useful to verify whether the residues identified by mutant analysis are also antigenically critical in the case of field isolates (Mateu *et al.*, 1990, Krebs *et al.*, 1993, Matue *et al.*, 1994). With this objective, in this study, 18 field isolates of FMDV serotype Asia1 collected between 1985 and 1999 were Mab-profiled and the sequence of their capsid coding regions determined.

Mab-profiling of field isolates to differentiate their antigenic features revealed a varied pattern of reactivities on the basis which they were clustered into 5 groups. The four isolates of Group A (IND 4/86, IND 9/90, IND 187/94 and IND 234/95) that were collected between 1986 and 1995 reacted with all the Mabs. As expected, these

isolates did not show any sequence differences from the vaccine virus in the residues identified as critical for Mab-binding. However these isolates differed from the vaccine virus at 4 to 6 other amino acid positions (Table.16)

Isolates of Group B, C and D had general reduction in their reactivity against Group 2 Mabs which was reflected in their sequence variation from vaccine virus near the binding site of these Mabs. All these isolates showed a change in VP2 131, a residue adjacent to VP2 130 which was identified as important for the binding of Group 2 Mabs in MAR mutant studies. These results show that either VP2 130 (as seen in mutants) or VP2 131(as in isolates) should be having a critical role in Mab-binding. Among these viruses, Group B and C isolates differed from Group D viruses in their reactivity with the Mabs 72, 76 and 82. When the residue substitutions in the VP1 30-50 region were analyzed in these isolates where this group of Mabs were found to bind, isolates of Group D (which did not react with these Mabs) showed changes at VP1 34, 36 and 47. This further confirms that this region of VP1 is involved in Mab binding and the residues 39, 46 and 48 (identified in MAR mutant studies) and , 34, 36 and 47 (as seen in field isolates) may be important in Mab-binding. In this case it is seen that a broader region in the VP1 protein comprising of residues from 34-39 and 46-48, rather than a single amino acid is critical to Mab-binding. Such observations were noted in studies with serotype O viruses, where MAR mutants against Site 2 Mabs were found to have substitutions spread over seven amino acids (VP2 70-77) (Kitson *et al.*, 1990). In addition to the above sequence variations, these isolates varied from the vaccine virus at many other positions (Fig. 16). Amino acid variations normally occur due to the high mutation frequencies of 10^{-3} to 10^{-5} seen in RNA viruses in structurally non-critical residues, and most of such changes seen out side the antigenically important regions seldom contribute towards antigenicity (Domingo *et al.*, 1990, Mateu at al., 1995).

The only isolate that constituted Group E ie., IND 49/93, behaved like a combined mutant of Group 1 and 3 Mabs. Sequence comparison of this isolate further

confirmed the role of VP1 168 in the binding of Mab 10 as this isolate had a D→ E change at this position. This virus also had N→S change in the adjacent residue (VP169). A substitution at VP1 168 was found in all mutants that did not react with Mab 10. But some isolates from Group B, C and D which were also non-reactive to this Mab had no change at this location. The concept of action at a distance (Mateu *et al.*, 1995) explains that amino acid substitutions that occur elsewhere in the capsid proteins might also disrupt Mab-binding, though pin-pointing of which of the many substitutions is directly involved in such distant effects can be done only through site-directed mutagenesis studies (Mateu *et al.*, 1998). Since this virus reacted with all the group 2 Mabs, no change was expected at VP2 131. But this virus showed E to N change as against E to S/R change in the isolates (group B to D) which did not react with these Mabs and it was assumed that such a change (at this location in IND 49/93) has not inhibited the binding of group 2 Mabs

Further the comparison of sequence differences in field isolates revealed a total of 78 mutable amino acid positions spread over the capsid proteins (2 in VP4, 19 in VP2, 22 in VP3 and 45 in VP1). The significance of such changes in the finer antigenic differences of Asia1 viruses will become clear when further studies are undertaken involving more number of Mabs.

The published sequences of Asia1 viruses were also compared to verify the identified antigenic sites. Two reports of Asia1 field isolate sequences are available and one of them (Marquardt *et al.*, 2000) contains the complete P1 region of 7 field isolates (three from Bangladesh, two from Israel, and two from Taiwan). Sequence comparison revealed that substitutions were present at positions in the VP1 protein between 30 to 50 (particularly at 33, 35, 45, 47, 48 and 50). VP1 168 also showed changes in all the seven viruses, while VP2 130 and 131 were different from IND 63/72 in four of the isolates. The other study (Ansell *et al.*, 1994) contained only partial sequences at the 3' end of VP1. Of the 44 isolates in this report, which included

Asia1 viruses from many countries, including 5 from India, 18 showed variation at position 168 compared to IND 63/72.

This study which involved generation and characterization of monoclonal antibody resistant mutants of serotype Asia 1 to map the antigenic sites on the virus has revealed the following findings. Studies employing 29 neutralizing Mabs and 15 mutants generated against them indicated that there were at least three distinct Mab-binding sites on the FMDV Asia1 virus capsid. The 3 sites are as follows:

Site 1 : This site was located on the structural protein VP1 and comprises of three amino acid residues at positions 39, 46 and 48.

Site 2 : This site was located on the structural protein VP2 at the amino acid position 130 which was found critical for this site. In addition the amino acid residues at position 72 of VP1 and position 75 of VP3 also appear to have a role in this site.

Site 3 : This site was located on the structural protein VP1 at the amino acid position 168 .

Since all the Mabs used to identify these sites were reactive against only to intact virion particle and not to 12S or disrupted virus (Sanyal 1995) these sites were considered to be conformation dependent.

Structural comparison of these residues on the X-ray crystallographic data of serotype O revealed that Site 1 and 2 were clustered near the five-fold axis of symmetry and site 3 was found near the two-fold axis of symmetry.

Mab-profiling and sequencing of 18 field isolates revealed that the antigenically critical residues, identified in the MAR mutant studies were found to be antigenically significant in naturally occurring viruses also.

Thus the present study, the first of its kind on FMD virus serotype Asia 1, has revealed the presence of three distinct antigenic sites and made a beginning in understanding the antigenic features of this serotype. Similar studies employing more Mabs and mutants needs to be done in order to identify all the antigenic sites present on this virus. The next step towards this end would be structural studies using X-ray crystallographic techniques.

SUMMARY

SUMMARY

There is probably no infectious disease of livestock that engenders more discussion than foot-and-mouth disease. Even a century after its identification, it still remains a major scourge of livestock industry, especially in the developing world. The control of this disease by vaccination is complicated by the existence of the causative agent, the foot-and-mouth disease virus, in seven distinct immunological types and several subtypes. The high antigenic variability exhibited by the virus necessitates that a close watch be maintained on the antigenic profile of viruses circulating in the field so that new and emerging variants can be detected immediately and current vaccine strains be updated as per the need of the times. Such preparedness demands that the antigenic make-up of a representative virus, with which field isolates can be compared, be known.

Antigenic profiles detailing the exact amino acids involved in the formation of antigenic sites have been deduced for serotypes O, A and C. No such data is available for serotype Asia1, which is responsible for the second largest number of outbreaks of foot-and-mouth disease in India.This study is aimed at identifying the key residues involved in the formation of antigenic sites of FMDV Asia-1 using the widely acclaimed and cited technique of production of neutralizing monoclonal antibody-resistant (MAR) mutants, comparison of the deduced amino acid sequence of capsid proteins of mutants with that of the parent virus and thus identify those residues which mutated enabling neutralization-escape, ie., the exact residues involved in virus neutralization.

A total of 29 neutralizing monoclonal antibodies (Mabs) raised against the whole virus particle (146S) of Asia1 FMDV which recognize conformation-dependent epitopes were used to isolate 29 single and 6 double monoclonal antibody-resistant (MAR) mutants.The isolated mutants were characterized by Mab-profiling ELISA and cross-neutralization tests which divided them into 3 groups. Group I consisted of 3

mutants which did not react with the corresponding Mabs but reacted with all others. Group II consisted of 25 single MAR mutants, these showed reduced or complete absence of reactivity with the corresponding Mabs but reacted with Mabs of the other two groups. Based on the degree of reactivity with homologous Mabs, this group was subdivided into Group IIa in which the mutants were partially reactive and Group II b, where the mutants were non-reactive. Group III consisted of the MAR mutant isolated against Mab 10, this mutant behaved like a multiple mutant since it was non-reactive to all the Mabs. Despite repeated attempts, a mutant specific only to Mab 10 could not be isolated. Based on their Mab reactivity, mutants from Groups I and II were subjected to selection pressure using Mabs from the other groups. A total of 6 double mutants were isolated. On profiling, each of these double mutants were found to have lost their reactivity to the Mab panel; phenotypically all of them behaved as multiple mutants.

In order to identify the amino acid changes associated with neutralization escape, viral RNA extracted from the parent virus and selected 15 mutants (at least 3 from each group) were RT-PCR amplified in the P1 region, gel-purified and rapid-cloned into pAmp1 or pAmp10 vectors. Primers for RT-PCR were designed in the study and contained Uracil residues to facilitate rapid cloning into pAmp vectors. The capsid coding regions of all the viruses used in the study could be successfully amplified. The rapid cloning procedure followed relies on the incorporation of dUMP residues in the place of dTMP into the 5' end of each amplification primer so that PCR products have dUMP containing sequence at their 5' termini. On treatment with Uracil DNA Glycosylase (UDG) dUMP residues were made abasic, and unable to base-pair, resulting in 3' protruding termini. Cloning was performed by annealing the linearized vector (whose termini were compatible for cloning) with the UDG-treated PCR products. The annealing reaction was over in 30 minutes producing chimeric molecules which were ready for transformation. This procedure eliminates the time-consuming tasks normally associated with cloning PCR products, including Restriction Endonuclease (RE) digestion, PCR product purification, end-polishing or

ligation. It was found to be fast and easy, the number of recombinants obtained were much higher and background colonies were negligible, as in most cases two of the three colonies screened contained right-sized inserts.

One positive plasmid from each clone was sequenced in the P1 region using primers designed in the study. All primers were found to work quite well with all the viruses sequenced.

Taking into consideration the pattern of reactivities seen with the mutants in Mab-profiling ELISA and the changes seen in the sequences, the following observations were made.

Group I mutants had changes in the VP1 39-48 region; the positions and residues substituted were 39 F to L (in MAR 72), 46 N to D (in MAR 76) and 48 Q to H (in MAR 82) and since these residues were critical for the binding of Group 1 Mabs they may be regarded as Site 1 of Asia1 FMDV.

Group II mutants had a substitution at position 130 of VP2 and the change was either K to T (in the case of partial mutants) or K to E (in the case of complete mutants). The only mutant that did not show change at VP2 130 in this group was MAR E, instead since substitutions were present at VP1 72 and VP3 75 these positions were also regarded important for binding of this group of Mabs. VP2 130 was regarded as Site 2.

MAR 10 and all double mutants showed amino acid substitutions at VP1 168 and VP2 130; the residue VP1 168 was assumed to be critical to the binding of Mab 10. These mutants also were mutated against Group 1 Mabs, though sequence analysis did not reveal any substitutions in those residues critical for the binding of Group 1 Mabs (VP1 39, 46 or 48). It may be assumed that since these residues lie in close proximity to VP1 168, in the three-dimensional structure substitutions at VP1 168

might have in some way inhibited Mab-binding at VP1 39, 46 and 48. It was also seen that though the binding sites of Group 1 and 3 Mabs lie so closely, mutations at the Group 1 Mab-binding site does not seem to affect Mab-binding at VP1 168 to a great extent as seen in the reverse case.

In order to verify whether the residues identified by mutant analysis are also antigenically critical in the case of field isolates, 18 field isolates of FMDV serotype Asia1 collected between 1985 and 1999 were Mab-profiled and the sequence of their capsid coding regions determined.

Mab-profiling of field isolates to differentiate their antigenic features revealed a varied pattern of reactivities on the basis which they were clustered into 5 groups. The four isolates of Group A that were collected between 1986 and 1995 reacted with all the Mabs, and these isolates did not show any sequence differences from the vaccine virus in the residues identified as critical for Mab-binding.

Isolates of Group B, C and D had general reduction in their reactivity against Group 2 Mabs and all these isolates showed a change in VP2 131, a residue adjacent to VP2 130 which was identified as important for the binding of Group 2 Mabs in MAR mutant studies. These results show that either VP2 130 (as seen in mutants) or VP2 131(as in isolates) should be having a critical role in Mab-binding.

Among these viruses, Group B and C isolates differed from Group D viruses in their reactivity with the Group 1 Mabs and they all showed changes at VP1 32, 34, 36 and 47. This further confirms that this region of VP1 is involved in Mab binding and the residues 39, 46 and 48 (identified in MAR mutant studies) and , 34, 36 and 47 (as seen in field isolates) may be important in Mab-binding.

The only isolate that constituted Group E ie., IND 49/93, behaved like a combined mutant of Group 1 and 3 Mabs and sequence comparison of this isolate

showed a D to E change at VP1 168. This virus also had N to S change in the adjacent residue (VP169) VP1 168 residue was identified as critical for binding of group 3 Mabs and regarded as Site 3. Since this virus reacted with all the group 2 Mabs, no change was expected at VP2 131. But this virus showed E to N change as against E to S/R change in the isolates (group B to D) which did not react with these Mabs and it was assumed that such a change (at this location in IND 49/93) does not inhibit the binding of group 2 Mabs

Sequence comparison of field isolates revealed a total of 78 mutable amino acid positions spread over the capsid proteins (2 in VP4, 19 in VP2, 22 in VP3 and 45 in VP1) the significance of which will become clear only when further studies are undertaken involving more number of Mabs.

The published sequences of Asia1 viruses were also compared to verify the identified antigenic sites and it was seen that substitutions were present at positions in the VP1 protein between 30 to 50 (particularly at 33, 35, 45, 47, 48 and 50), at position 168 and also at 130 and 131 in the VP2 protein, confirming the importance of these regions in antigenicity.

Based on the X-ray crystallographic data of serotype O it was revealed that Site 1 and 2 were clustered near the five-fold axis of symmetry and site 3 was found near the two-fold axis of symmetry. All the Mabs used in this study react with the whole virus particle only and not with subviral particles. Hence it was concluded that all the three sites identified were conformation-dependent. The identified sites were found to be comparable to those of serotypes O and A_{10} though the residues involved were different.

Thus the present study, the first of its kind on FMD virus serotype Asia 1, has revealed the presence of three distinct antigenic sites. Site 1 was located on the protein VP1 and comprised of three amino acid residues at positions 39, 46 and 48. Site 2 was located on the protein VP2 at the amino acid position 130 which was found critical for

this site. In addition the amino acid residues at position 72 of VP1 and position 75 of VP3 also appeared to have a role in this site. Site 3 was located on the protein VP1 at the amino acid position 168. Similar studies employing more Mabs and mutants needs to be done in order to identify all the antigenic sites present on this virus. The next step towards this end would be structural studies using X-ray crystallographic techniques.

लघु सारांश

खुर व मुँह पका रोग पालतू पशुओं का आर्थिक रूप से एक महत्वपूर्ण रोग हैं और कारण हेतु विषाणु, खुर व मुँह पका विषाणु, अपने ऐन्टिजेनिक विविधता के लिए मशहूर हैं । ये रोग हमारे देश में अति प्रचलित रूप में होता हैं और खुर–मुँह रोग सीरम प्रारूप(सीरोटाइप) एशिया 1 दूसरा सर्वाधिक रोग विस्फोट का कारण हैं । उन्तीस उदासीन एक –(पुंजक)प्रतिरक्षियों एन–मैब्स और उनके विरूद्ध उत्पादित प्रतिरक्षित रेसिस्टेन्ट म्यूटेन्टस (मार म्यूटेन्टस) से वर्तमान अध्ययन में खुर–मुँह रोग विषाणु सीरोटाइप ऐशिया 1 के ऐन्टिजेनिक साइटस मैप करने का प्रयास किया हैं ।

उन्तीस एन–मैब्स जो शुद्धिकृत पूर्ण विषाणु करण (146S) के विरूद्ध उत्पादित किया गया था और जो अनुरूप एपिटोपों के पहचान करते हैं, उनको लेकर उन्तीस एकल और द्विलक प्रतिरक्षित रेसिस्टेन्ट मार उत्पादित किया गया । क्रास न्यूट्रलाइजेशन और मैब–प्रोफाइलिंग एलिसा अध्ययन द्वारा म्यूटेन्टस तीन समूहों में विभाजित किया गया । हर समूह से चूना गया(कुल मिला के 15) विषाणुओं का आर0एन0ए0 निकाला गया , काप्सिड कोडिंग रीजियन में RT-PCR किया गया और (pAmpVedor) में क्लोन किया गया । हर म्यूटेन्टस का एक पोजिटिव क्लोन में अध्ययन में विकसित प्राइमरस में मैनुवल और आटोमेटड सीक्वेन्सिंग किया गया जिससे 2196 बेस लंबा काप्सिड कोडिंग रीजियन का सीक्वेन्स पाया गया । प्रोफाइलिंग एवं सीक्वेन्सिंग अध्ययन से प्रत्येक समूहों के म्यूटेन्टस में प्रत्येक अमैनों एसिड श्रेणी में अन्तर पाया गया जिससे तीन साइट के पहचान हो गये । साइट एक वी0पी0 1 प्रोटीन में थे और रेसिड्यूस 39,46 व 48 में पाया गया, साइट दो वी0पी0–2 प्रोटीन में पोजिसन 130 पाया गया और साइट तीन, वी0पी0 1 प्रोटीन के पोजिसन 168 में पाया गया । पहचान किया गया साइटस टाईप ओ और ए–10 के साइटस के तुलनात्मक थे, परन्तु प्रेतेक रेसिड्यूस अलग थे । अठारह आइसोलेटस जो सन् 1985 बाार 1999 के बीच में प्राप्त किये गये, उनको भी मैब–प्रोफाइलिंग और सीक्वेन्सिंग अध्ध्यन में शामिल किया गया और ये पता चला कि जो साइटस कि पहचान किये गये वे आइसोलेटस में भी महत्वपूर्ण हैं । इस विषाणु में जो और साइटस हैं उनको समझने के लिये ज्यादा मैब्स और म्यूटेन्टस को लेकर इस प्रकार के और भी अध्ययन करना हैं । इन रेसिड्यूस के निश्चित स्थान के बारे में तभी पता चलेगा जब इस विषाणु पर एक्स–रे क्रिस्टालोग्राफिक अध्ययन किया जायेगा ।

MINIABSTRACT

Foot-and-mouth disease (FMD) is an economically important disease of cloven-hooved animals and the causative virus, foot-and-mouth disease virus , is known for its high antigenic diversity. This disease is endemic in India and FMDV serotype Asia1 causes the second largest number of outbreaks. The present work was undertaken to map the antigenic sites of FMDV serotype Asia1 using a panel of 29 neutralizing monoclonal antibodies and monoclonal antibody resistant (MAR) mutants isolated against them.

29 neutralizing Mabs, which recognize conformation-dependent epitopes, raised against the purified whole virus particle (146S) were used to isolate 29 single and 6 double MAR mutants. Cross-neutralization and Mab-profiling ELISAs grouped them into 3 groups. RNA from selected viruses of each group (a total of 15) were extracted, RT-PCR amplified in the capsid-coding region and rapid cloned into pAmp vectors. One positive clone each of the 15 mutants was sequenced both manually and using automated sequencer using primers designed in the study, to obtain the base sequence (2196 nucleotides) of the capsid coding region. Mab-profiling results in conjunction with MAR mutant sequencing revealed the presence of specific amino acid changes in each group of mutants, allowing the identification of three sites. Site 1 was located on VP1 protein and involved residues 39,46 and 48. The residue 130 of VP2 formed site 2 and Site 3 was mapped to residue 168 of VP1. All the 3 sites were conformation dependent. The identified sites were found to be analogous to those of serotypes O and A_{10} though the residues involved were different. A total of 18 field isolates, collected between 1985 and 1999, were profiled using the same Mab-panel and were sequenced in the capsid coding region and it was found that the antigenic sites identified were also important in the case of field isolates. Similar studies employing more Mabs and mutants needs to be done in order to identify all the antigenic sites present on this virus. The exact location of these antigenic sites on FMDV serotype Asia 1 will become clear only when the 3 dimensional structure is deduced by X-ray crystallography.

लघु सारांश

खुर व मुँह पका रोग पालतू पशुओं का आर्थिक रूप से एक महत्वपूर्ण रोग हैं और कारण हेतु विषाणु, खुर व मुँह पका विषाणु, अपने ऐन्टिजेनिक विविधता के लिए मशहूर हैं । ये रोग हमारे देश में अति प्रचलित रूप में होता हैं और खुर–मुँह रोग सीरम प्रारूप(सीरोटाइप) एशिया 1 दूसरा सर्वाधिक रोग विस्फोट का कारण हैं । उन्तीस उदासीन एक –(पुंजक)प्रतिरक्षियों एन–मैब्स और उनके विरूद्ध उत्पादित प्रतिरक्षित रेसिस्टेन्ट म्यूटेन्टस (मार म्यूटेन्टस) से वर्तमान अध्ययन में खुर–मुँह रोग विषाणु सीरोटाइप ऐशिया 1 के ऐन्टिजेनिक साइटस मैप करने का प्रयास किया हैं ।

उन्तीस एन–मैब्स जो शुद्धिकृत पूर्ण विषाणु करण (146S) के विरूद्ध उत्पादित किया गया था और जो अनुरूप एपिटोपों के पहचान करते हैं, उनको लेकर उन्तीस एकल और द्विलक प्रतिरक्षित रेसिस्टेन्ट मार उत्पादित किया गया । क्रास न्यूट्रलाइजेशन और मैब–प्रोफाइलिंग एलिसा अध्ययन द्वारा म्यूटेन्टस तीन समूहों में विभाजित किया गया । हर समूह से चूना गया(कुल मिला के 15) विषाणुओं का आर0एन0ए0 निकाला गया , काप्सिड कोडिंग रीजियन में RT-PCR किया गया और (pAmpVector) में क्लोन किया गया । हर म्यूटेन्टस का एक पोजिटिव क्लोन में अध्ययन में विकसित प्राइमरस में मैनुवल और आटोमेटड सीक्वेन्सिंग किया गया जिससे 2196 बेस लंबा काप्सिड कोडिंग रीजियन का सीक्वेन्स पाया गया । प्रोफाइलिंग एवं सीक्वेन्सिंग अध्ययन से प्रत्येक समूहों के म्यूटेन्टस में प्रत्येक अमैनों एसिड श्रेणी में अन्तर पाया गया जिससे तीन साइट के पहचान हो गये । साइट एक वी0पी0 1 प्रोटीन में थे और रेसिड्यूस 39,46 व 48 में पाया गया, साइट दो वी0पी0–2 प्रोटीन में पोजिसन 130 पाया गया और साइट तीन, वी0पी0 1 प्रोटीन के पोजिसन 168 में पाया गया । पहचान किया गया साइटस टाईप ओ और ए–10 के साइटस के तुलनात्मक थे, परन्तु प्रेतेक रेसिड्यूस अलग थे । अठारह आइसोलेटस जो सन् 1985 बार 1999 के बीच में प्राप्त किये गये, उनको भी मैब–प्रोफाइलिंग और सीक्वेन्सिंग अध्ध्यन में शामिल किया गया और ये पता चला कि जो साइटस कि पहचान किये गये वे आइसोलेटस में भी महत्वपूर्ण हैं । इस विषाणु में जो और साइटस हैं उनको समझने के लिये ज्यादा मैब्स और म्यूटेन्टस को लेकर इस प्रकार के और भी अध्ययन करना हैं । इन रेसिड्यूस के निश्चित स्थान के बारे में तभी पता चलेगा जब इस विषाणु पर एक्स–रे क्रिस्टालोग्राफिक अध्ययन किया जायेगा ।

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APPENDIX

APPENDIX

1. Buffers used in ELISA

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)
$Na_2HPO_4.12H_2O$	-2.680g (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)
$Na_2 HPO_4. 12H_2O$	- 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

2. 10nM dNTP mix

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

3. Loading buffer (6X)

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

4. Direct purification buffer

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

5. Purification resin

Guanidine thiocyanate	-6 M

6. dd/dNTPs mixes of fmol Sequencing Kit (Promega)

dd/dATP mix		dd/dCTP mix	
ddA	350 µm	ddC	200 µm
ddG	20 µm	ddA	20 µm
ddC	20 µm	ddG	20 µm
ddT	20 µl	ddT	20 µm

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

includes 7-deaza dGTP1

7. 5X sequencing buffer

Tris Hcl (pH 9.0 at 25°C)	-250 nm
$MgCl_2$	-10 mM

8. Sequencing stop solution

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

9. 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*	- 2.924g
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 NaOH solution)
*(monohydrochloride salt)
Stored at -20°C

10. 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.

11. Trypsin-versene solution

NaCl	- 5.0 g
KCl	- 0.125 g
$Na_2HPO_4.2H_2O$	- 0.950 g
KH_2PO_4	- 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.

12. 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.

13. 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.

14. Agar overlay for plaque purification

Agar	-1g (1%w/v)
DEAE-dextran	-25mg (250 μg/ml)
HEPES	-238.3 mg (10mM)
Maintanance medium	-50ml.

The mixture was autoclaved at 15 lb pressure for 15 minutes and then 50ml of growth medium was added.The pH (7.4) was adjusted by addition of 7% $NaHCO_3$ solution.

15. 1X TBE buffer

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

16. 1X TAE Buffer

Tris acetate	-40mM
EDTA	- 1mM

17. TE Buffer (pH 7.5)

Tris-HCl	- 10mM
EDTA	- 1mM

18. LB broth

Tryptone	- 10g
Yeast extract	-5g
Sodium Chloride	-10g
Distilled water	-1L

Adjusted pH to 7.0 with 5N NaOH.Autoclaved for 20 minutes at 15 lb pressure.

19. LB Agar

LB broth	-1L
Agar	-15g
Autoclaved for 20 minutes at 15lb pressure.	

20. SOC Medium

Tryptone	- 20g
Yeast extract	-5g
Sodium Chloride	-0.5 g
250mM Potassium Chloride	-10 ml.
Distilled water	-1L.
Autoclave ; just before use add	
2M sterile Magnesium Chloride	- 5ml
1M sterile glucose	- 20ml

21. SOB Medium

Tryptone	- 20g
Yeast extract	-5g
Sodium Chloride	- 0.5g
250 mM Potassium Chloride	-10ml
Distilled water	-1L
Autoclave; just before use add	
2M Sterile Magnesium Chloride	- 5ml.

22. 0.1M IPTG Stock Solution

1.2 g IPTG is dissolved in 50 ml deionized water and filtered through a 0.2 mm membrane.

Store at 4°C.

23. X-Gal stock solution(50mg/ml)

X-Gal	-100mg
N,N'-dimethyl formamide	-2ml

24. Transformation and Storage Solution (TSS)

LB 2X	-10ml
Mg++2M	-200ul
DMSO	-1ml

PEG8000 30%	-7ml
Sterile distilled water	-2ml

25. Ethidium bromide

Ethidium bromide	-10mg
Distilled water	-1ml
Mixed and stored in a dark place.	

26. Ampicillin stock

Ampicillin (Sigma)	-500mg
Distilled water	- upto 10ml
Filter-sterilized and stored at -20°C	

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

Full name	Three letter code	One letter code
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
Tryptophan	Trp	W
Tyrosine	Tyr	Y

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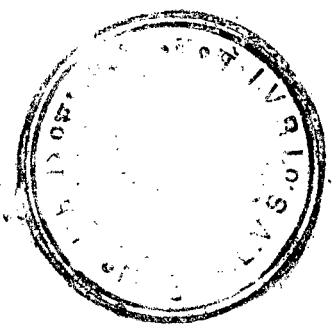

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