DEVELOPMENT OF A MULTIPLEX LATEX AGGLUTINATION ASSAY WITH SEROTYPE SPECIFIC PEPTIDES OF FMD VIRUS FOR DIFFERENTIAL DIAGNOSIS

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY MICROBIOLOGY
(Minor Subject: Veterinary Pathology)

By

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(L-2009-V-21-M)

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CERTIFICATE - I

This is to certify that the thesis entitled, **"Development of multiplex latex agglutination assay with serotype specific peptides of FMD virus for differential diagnosis"** submitted for the degree of M.V.Sc., in the subject of **Veterinary Microbiology** (Minor Subject: **Veterinary Pathology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Dilpreet Kaur** (L-2009-V-21-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE - II

This is to certify that the thesis entitled, **"Development of multiplex latex agglutination assay with serotype specific peptides of FMD virus for differential diagnosis"** submitted by **Dilpreet Kaur** (L-2009-V-21-M), to Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of M.V.Sc. in the subject of **Veterinary Microbiology** (Minor Subject: **Veterinary Pathology**) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

In this study 200 serum samples were collected from cattle (141) and buffaloes (59) of different ages and sex from Foot and Mouth Disease (FMD) vaccinated and unvaccinated animals. Serotype specific peptides and non structural peptide (NSP 2B) of FMDV as reported in published literature and confirmed by bioinformatics tool BLAST, were commercially synthesized and conjugated to latex beads of different colors. LAT was employed for all the samples . Out of 200 serum samples , 22 samples were positive for antibodies to FMDV type O, 14 for type A, 14 for type Asia 1, 12 for both type O and A, 26 for type O and Asia 1, 4 for A and Asia 1, 14 were positive for all the three serotypes (O, A and Asia 1) and 94 were negative for antibodies to all the three serotypes. Sixty nine samples showed agglutination of beads conjugated with NSP 2B peptide. On multiplexing, agglutinates of different types were seen only with the aid of microscope. However clumps of type A were not clearly visible. Agglutination was enhanced by using anti bovine immunoglobulin. On comparison of LAT with LPB ELISA it was found that LPB ELISA was more sensitive as it could detect more samples positive for different serotypes. On comparison of commercially available DIVA-ELISA with NSP peptide based agglutination, the latter could detect more positive samples than DIVA-ELISA kit.

Keywords: Foot and Mouth Disease Virus, Serotype specific peptides, NSP 2B, BLAST, Latex agglutination test, DIVA-ELISA, LPB-ELISA.

Signature of Major Advisor **Signature of the student**

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LIST OF ABBREVIATIONS

-	:	Negative
%	:	Per cent
+	:	Positive
++	:	Strong positive
≥	:	More than equal to
µl	:	Microlitre
^{0}C	:	Degree Celcius
Ab	:	Antibody
BLAST	:	Basic Local Alignment Search Tool
cys	:	Cysteine
DIVA ELISA	:	Differentiate infected and vaccinated animals enzyme linked immunosorbent assay
FMDV	:	Foot and Mouth Disease Virus
g	:	Gram
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
gly	:	Glycine
h	:	Hour
LAT	:	Latex agglutination test
LPB ELISA	:	Liquid phase blocking enzyme linked immunosorbent assay
M	:	Molar
mg/ml	:	Milligram per milliliter
ml	:	Milliliter
MLAT	:	Multiplex latex agglutination test
NCBI	:	National Centre for Biotechnology Information
Neg C	:	Negative control
NSP 2B	:	Non structural protein 2B
OD	:	Optical density
OD_{corr}	:	Optical density corrected
PBST	:	Phosphate buffer saline tween buffer
PP	:	Percent positivity
pro	:	Proline
Pvt ltd	:	Private limited
SAT 3	:	South African Territory 3
ser	:	Serine
SL+	:	Slight positive
VNA	:	Virus neutralizing antibodies
VP1	:	Viral protein 1
w/v	:	Weight by volume

CHAPTER I

INTRODUCTION

Livestock production plays an important role in the country's economy. The contribution of livestock industry to the national economy is 25 to 30 percent. The diseases caused by micro-organisms in livestock are the major hindrance to their performance. Among the viral infections, Foot and mouth disease (FMD) is a highly contagious disease in cloven footed animals and economically the most important disease of livestock worldwide (Bachrach 1968 and Kitching 1999). Outbreaks of FMD can severely disrupt livestock production and trade of animals, and require significant resources to control. Direct and indirect economic losses due to FMD equivalent to several billion US dollars annually are common worldwide. Approximately 470 million domestic livestock are susceptible to FMD. The annual loss due to FMD in India is estimated to be US$ 800 million.

Foot and Mouth Disease is caused by a single stranded, positive-sense RNA virus belonging to the *Aphthovirus* genus of *Picornaviridae.* The virus consists of seven serotypes (O, A, C, Asia1, SAT1, SAT2, and SAT3) with multiple subtypes within each serotype (Domingo *et al* 2003 and Knowles and Samuel 2003). Out of seven serotypes, only four serotypes (O, A C, Asia-1) are present in Asia. There is no cross-protection among the serotypes of the virus. Also, it has been seen that the serotypes of FMDV exhibit some regionality. The high mutation rate and antigenic variability of the virus complicates the control of the disease further.

The viral genome of FMDV encodes a single polypeptide that is cleaved to form mature polypeptide products that include four structural proteins (VP1, VP2, VP3, and VP4) and eight non structural proteins (2B, 2C, 3A, 3D, L^{pro}, 2A, and $3C^{pro}$) (Mason *et al* 2003, Belsham 2005 and Carrillo *et al* 2005). Out of these structural proteins VP1 is a immunodominant protein of the virus.

VP1 is the most frequently studied protein owing to its significant roles in virus attachment, protective immunity and serotype specificity. It is known that the main cell attachment site and the immunodominant region of FMD virus are both located on a solvent exposed region at the surface of the virion, namely in trypsin-sensitive areas of VP1 (Laporte and Lenoir 1973 and Cavanagh *et al* 1977). This immunodominant region corresponds to the loop which connects β-sheets G and H of the VP1 β-barrel, named the *GH loop (*Lea *et al* 1994*).* Important antigenic sites on FMD virus are located on the sequence between amino acids 140 and 160 and that of the C terminus of VP1 (Bittle *et al* 1982, Strohmaier *et al* 1982 and Pfaff *et al* 1988). Several overlapping B-cell epitopes are located within this region and are able to induce both neutralising and non-neutralising antibody responses (Grubman *et al* 1987). The high sequence variability found in this region accounts for the low crossreactivity observed among different serotypes (Grubman *et al* 1987).

The main route of infection of ruminants with FMDV is the inhalation of airborne virus, but infection *via* the alimentary tract or skin lesions is also possible, although requiring higher doses of virus. After primary replication in the pharynx, the virus enters the bloodstream and following a 3 to 5 days period of febrile viræmia, it spreads throughout the organs and tissues where new sites for secondary infection are established. Some clinical symptoms of FMD are fever, anorexia, weight loss, lameness, salivation and vesicular lesions (mouth and skin). Although FMD only rarely causes death in adult animals, the virus can cause severe lesions in the myocardium of young animals, leading to high mortality rates (Woodbury 1995).

One of the constrictive factors in the control of foot and mouth disease in developing countries is the long delay in diagnosis period. Due to rapid spreading nature of the disease, any suspicious case in the field should be considered as FMD

after the confirmation with a laboratory test. Although several reliable diagnostic tests such as complement fixation test (CFT), enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) already exist; none of them are applicable in the field conditions due to their cumbersome nature, high costs and requirement of skilled personnel.

The potential for the use of peptides corresponding to immunodominant sites on VP1 for detection of antibodies against FMD virus in blood from convalescent animals (Petrov *et al* 1996) and detection of FMD virus non structural protein antibody using a chemically synthesized 2B peptide as antigen (Inoue *et al* 2006) encouraged us to investigate the possibility for the use of structural as well as non-structural peptides to detect FMD through Latex Agglutination Test (LAT) that can be ultimately used with minimum expertise in the field conditions.

At present three FMD virus serotypes namely O, A and Asia 1 are prevalent in India. Synthesizing the type specific viral peptides and their conjugation with coloured latex beads can make them more easily visible with the naked eyes and enable clear cut differentiation between serotypes based on their reactivity with serum samples in multiplex LAT.

Keeping in view the above facts, the present work was aimed to investigate the following :

1. Identification of non structural proteins of FMDV and serotype specific viral peptides of VP1 protein of FMDV type O, A, Asia 1 for the diagnosis and their conjugation with latex beads of different colours.
2. Differentiation of various FMDV serotypes by immunoassay employing latex bead conjugated peptides.

CHAPTER II

REVIEW OF LITERATURE

2.1 Foot and Mouth disease

Foot-and-mouth disease (FMD) is an economically important disease of livestock worldwide. Loeffler and Frosch (1897) demonstrated that a filterable infectious agent smaller than bacteria caused the disease. This was the first description of a virus producing an animal disease. FMD is the highly transmissible viral disease of animals and ranks first in the list A of OIE (Nunez *et al* 1998). It is highly contagious and affects artiodactylae mostly cattle, swine, sheep and goat . Besides farm animals, FMD also affects more than 30 wild ruminant species (Snowdon 1968). Despite low mortality rates, FMD severely decreases livestock production and leads to trade restrictions on animals and livestock products.

In natural infections, the main route of transmission of virus is through respiratory tract and as few as one to ten infective particles can produce the disease (Sellers 1971 and Donaldson et al 1987). The virus can be mechanically transmitted by animals, farmers, farming equipment, animal transport(Brooksby 1982) and long distance airborne transmission has also been reported (King et al 1981).

The initial virus multiplication usually takes place in the pharyngeal epithelium producing primary vesicles or “aphthae” (Burrows *et al* 1981). The virus can also penetrate through skin lesions and it can be experimentally produced by intradermal injection into the tongue or in the interdigital space of foot. Within 24–48 h after epithelium infection, fever and viraemia develops and the virus enters the blood stream and spreads to different organs and tissues producing secondary vesicles preferentially in the mouth and feet. Little is known on the mechanism

mediating the viral spread observed during the viraemia, although the involvement of macrophages in this process has been suggested (Yilma 1980 and Baxt and Mason 1995). The acute phase of disease lasts about one week and declines gradually coinciding with the appearance of a strong humoral immune response (Bachrach 1977). In some cases, mortality can be observed among young animals, associated with lesions in the myocardium. The vesicles produced by FMD virus generally affect cells from the epithelial stratum spinosum (Beard and Mason 2000). However, the ability of the virus to replicate in different internal organs and tissues, in which it replicates in high titres (Burrows *et al* 1981), remains to be studied in detail. A high viral amplification frequently takes place in infected animals particularly in pigs, for which up to 1012 infectious units per infected animal have been recorded (Sellers 1971).

In ruminants, an asymptomatic persistent infection can be established (Bekkum *et al* 1959) during which virus can be isolated from the oesophagus and throat fluids of the animals from a few weeks up to several years of the initial infection (Salt 1993). Both naive and vaccinated animals can become persistently infected (Sutmoller and Gaggero 1965). The mechanisms that mediate this persistence are unclear but they are likely to result from equilibrium between the host immune response and the selection of viral antigenic variants at the mucosae of the upper respiratory tract (Gebauer *et al* 1988 and Salt 1993). There is epidemiological evidence to support that carrier animals may be the origin of outbreaks of acute disease, when brought into contact with susceptible animals (Hedger and Condy 1985).

In vitro, FMD virus productively infects several primary cultures as well as established cell lines such as BHK-21, IBRS-2 or BK cells. The intraperitoneal

inoculation of FMD virus produces death in suckling mice and this has been extensively exploited to titrate virus infectivity. Likewise, FMDvirus can be adapted by serial passages to produce clinical symptoms in guinea-pigs (Francis *et al* 1987).

2.2 Natural distribution of the disease

The earliest outbreaks of FMD were in Northern Italy in 1514 and in Southern Africa in 1780. It is endemic in many regions of South America, Africa and Asia and it can readily cross international boundaries to cause epidemics in previously disease-free areas (Sumption K *et al* 2008). FMD distribution is associated mainly with the developing countries and contributes to their severe economic problems. FMD control in endemic areas is implemented through regular vaccination which resulted in the eradication of the disease in some areas of the world like the European Union and more recently Uruguay, Argentina, Paraguay and the South of Brazil.

Seven immunologically different serotypes of the FMD virus are known namely A, O, C, Asia-1, South-African Territories (SAT) 1, SAT 2 and SAT 3, which comprise more than 65 subtypes. Globally, FMD virus serotypes O and A are the most prevalent. However, Asia has its own unique serotype i.e. Asia 1. It was first detected in samples collected in India in 1951 - 1952 (Dhanda *et al* 1957). The primary serotype-endemic region for Asia 1 is the Indian subcontinent which includes Afghanistan, India, Pakistan, Bhutan and Nepal where outbreaks occur regularly and it is speculated that this distribution is related to that of the Asian water buffalo (*Bubalus bubalis*). The serotype has been more sporadically reported from countries to the west or east and it has spread into the Middle East and occasionally to Europe (Ansell *et al* 1994, Islam *et al* 2000, Schumann *et al* 2008 and Valarcher *et*

al 2008) but it has not been reported from Africa or the America. However, even in its endemic heartland, the Asia 1 serotype has normally been the cause of only a small proportion of cases compared with the proportion caused by serotypes O and A.

2.3 Incidence in India

Foot and mouth disease is an endemic disease of national importance. Every year approximately 5000 outbreaks of FMD disease are reported in India affecting nearly 3,00,000 animals. The distribution of FMD virus types in North-West regions of India has been reported from time to time (Tewari et al 1975, Prasad et al 1978, 1992, and Ahuja et al 1986, 1996). In North-West region type O had predominated other virus types over the years except in 1976 and 1984 when Asia-1 was the dominant type (Prasad et al 1978, Ahuja et al 1986). During 1985 and 1986 type O and Asia-1 were the main serotypes predominating, however, in 1987 type O started predominating (Prasad et al 1992) and the phenomenon continued during the following years.

Goel (1989) studied an outbreak of FMD in a herd of Sahiwal*Friesian cattle with various degrees of Friesian inheritance. Out of 695 cattle, 408 were affected and highest incidence was in crossbreds with high percentage of Friesian inheritance and in young animals. The herd was vaccinated annually against FMD and had been free of infection since 1978.

Sporadic incidence of FMD was recorded in 19 of the 37 villages of district Ludhiana. The disease affected 147 animals with morbidity rate of 1.14 per cent. Epidemiological studies revealed that both vaccinated and unvaccinated animals were affected though the severity of symptoms and incidence of disease was lower in vaccinated animals (Saini et al 1991).

There were 120 outbreaks of foot and mouth disease in north eastern parts of India in 1988-1990, of which 97 were in Assam and 13 were in Meghalaya. 106 samples were taken for virus typing. Type O was recovered from 58, subtype A22 from 11 and type C and Asia-1 from 10 and 2 respectively. Of the 120 outbreaks, 32 were recorded in cattle vaccinated with FMD polyvalent vaccine (Sarma et al 1991).

Sharma and Singh (1993) studied the cyclic behaviour of foot and mouth disease in India. They found that overall FMD seemed to follow a 6 year epidemic cycle over the whole country. It is suggested that periodic fluctuations in the size of FMD susceptible livestock populations may be a factor I cyclic development and decline of FMD epidemics by altering the herd immunity and changing the probability of contact.

A study by Saxena in 1995 estimates the average annual rate of FMD incidence in India to be about 23 per cent. As regards the incidence in different regions of the country, the FMD incidence is found to be the highest in the Eastern region (35%) and lowest in the Southern region (11%) with Northern (20%) and Western (24%).

Five outbreaks of foot and mouth disease in North-Eastern states of India in 1994 were studied by Sarma and Hazarika (1996). The outbreaks were recorded in Assam, Arunachal Pradesh, Meghalaya, Tripura and West Bengal. Apart from the outbreaks in Meghalaya, all the outbreaks involved cattle vaccinated against FMD. The prevalence of affected animals in the outbreaks varied from 45 to 100 per cent. The outbreaks in Arunachal Pradesh were caused by the Asia-1 virus type and in the rest of the regions all the outbreaks were caused by FMD virus type O.

In an outbreak of FMD at an organized farm in Rajasthan 93.3 per cent unvaccinated goats were affected. From the vesicular fluid and tongue epithelium FMD virus type O was identified by ELISA (Mishra et al 1997).

Maan et al (1998) studied prevalance of foot and mouth disease virus types in North-West India during period of 1994-96. A total of 196 FMD specimens were processed and overall percentage prevalence was found to be 64.06. Type O was the most predominant type (79.6%) followed by A22 (17.89%) and Asia-1 (2.44%). They found that the disease occurred in all susceptible domestic livestock species with cattle and buffaloes contributing to the majority of the cases.

Singh and Singh (1998) reported that among the prevalent strains of FMD virus in India, type O occupies the peak position (81.89%) followed by Asia-1 (12.4%), type A (3.3%) and type C (2.4%).

2.4 Genomic organization

FMDV belongs to the genus aphthovirus of the *Picornaviridae* family (Rodrigo and Dopazo 1994). The viral particle contains a positive-strand RNA genome of about 8 500 nucleotides (nt) enclosed within a protein capsid. The viral RNA consists of a single open reading frame (ORF), flanked by two non coding regions (NCR). A small viral protein, VPg, is covalently linked to the 5' end of the molecule (Sangar *et al* 1977). The 5'NCR (about 1200 nt) is divided by a poly C tract located about 400 nt from the 5' end (Brown *et al* 1974). Little is known about the RNA region upstream of the poly C, except that a clover leaf structure is predicted at the 5' end (Clarke *et al* 1987). The translation initiation of the FMDV RNA starts at two AUG codons separated by 84 nt (Beck et al 1983).

Replication and translation of FMDV RNA (Sangar 1979) occur in the cytoplasm of infected cells (Arlinghaus and Polatnick 1969) and these biochemical processes are associated with cell membranes (Newman *et al* 1979). FMDV RNA is infectious by itself, when transfected into susceptible cells (Belsham and Bustock

1988). This feature has made possible the construction of infectious cDNA clones (Zibert *et al* 1990) and their use is a powerful tool to study different genes and functional motives of the viral RNA by the analysis of derivatives bearing mutations or deletions at preselected genomic sites. A continuous 7802 nucleotide sequence spanning the 94% of foot and mouth disease virus RNA between the 5 -proximal poly(C) tract and the 3'-terminal poly(A) was obtained from cloned cDNA and the total size of the RNA genome was corrected to 8450 nucleotides. A long open reading frame was identified within this sequence starting about 1300 bases from the 5 end of the HNA genome and extending to a termination codon 92 bases from its polyadenylated 3' end. The protein sequence of 2332 amino acids deduced from this coding sequence was correlated with the 260 K FMDV polyprotein (Fross *et al* 1984).

Newton *et al* (1985) determined the nucleotide sequence of foot-and-mouth disease virus (FMDV) RNA to the 5' side of the poly(C) tract (S fragment) for representatives of the A and O serotypes of the virus. The two S fragments differed in length by five nucleotides (nt), with 367 nt for O_1 compared with 362 nt for A_{10}, due to a number of insertions or deletions. However, the two sequences showed 86% homology.

In RNA replication, the role of p56a as the viral polymerase has been confirmed and the involvement of another non-structural protein (p34) has been demonstrated (McCahon 1986).

Sanyal *et al* (2004) described the complete nucleotide sequences except the poly (C) tract and poly (A) tail of a vaccine strain (IND 491/97) and an atypical field isolate (IND 321/01) of Foot-and-mouth disease virus (FMDV) serotype Asia1. Amino acid (aa) sequence analysis of the VP1 protein of the field isolate revealed

that the latter has 212 instead of 210 or 211 aa found in the so far available sequences of other FMDV isolates of Asia1 serotype. The insertion was localized in the hypervariable region of aa 130–160 of VP1 protein. Nucleotide sequencing of the entire genome was therefore carried out to detect changes in other parts of the genome, if any, besides VP1, which could contribute to its fitness. An 8.16 kb sequence of IND 491/97 and an 8.162 kb sequence of IND 321/01 were compared with each other and also with the known sequence of IND 63/72, another vaccine strain of serotype Asia1. Comparison of the entire polyprotein coding (L to 3D) region of IND 321/01 with those of the two Asia1 vaccine strains (IND 63/72 and IND 491/97) revealed no significant differences. A similar comparison of IND 491/97 with IND 63/72 revealed variability across the entire length of the genome. In addition to the capsid-coding region, sequence variability was also observed in non-structural proteins albeit to different extent. This study shows that in the gene pool of serotype Asia1 at least three groups of isolates/strains are present with respect to the length of VP1 protein.

Carrillo *et al* (2005) present complete genome sequences, including a comparative analysis, of 103 isolates of foot and mouth disease virus (FMDV) representing all seven serotypes and including the first complete sequences of the SAT1 and SAT3 genomes. The data revealed novel highly conserved genomic regions, indicating functional constraints for variability as well as novel viral genomic motifs with likely biological relevance. Previously undescribed invariant motifs were identified in the 5_ and 3_ untranslated regions (UTR), as was tolerance for insertions/deletions in the 5_ UTR. Fifty-eight percent of the amino acids encoded by FMDV isolates are invariant, suggesting that these residues are critical for virus biology. Novel, conserved sequence motifs with likely functional

significance were identified within proteins Lpro, 1B, 1D, and 3C. An analysis of the complete FMDV genomes indicated phylogenetic incongruities between different genomic regions which were suggestive of interserotypic recombination. Additionally, a novel SAT virus lineage containing nonstructural protein encoding regions distinct from other SAT and Euroasiatic lineages was identified. Insights into viral RNA sequence conservation and variability and genetic diversity in nature will likely impact our understanding of FMDV infections, host range, and transmissifon.

The results of a simple pairwise-scanning analysis designed to identify inter-serotype recombination fragments, applied to genome data from 156 isolates of Foot-and-mouth disease virus (FMDV) representing all seven serotypes, were reported by Jackson *et al* (2007). Large numbers of candidate recombinant fragments were identified from all parts of the FMDV genome, with the exception of the capsid genes, within which such fragments are infrequent. As expected, intertypic fragment exchange is most common between geographically sympatric FMDV serotypes. After accounting for the likelihood of intertypic convergence in highly conserved parts of the FMDV genome, it is concluded that intertypic recombination is probably widespread throughout the non-structural genes, but that recombination over the 2B/C and 3B/C gene boundaries appears to be less frequent than expected, given the large numbers of recombinant gene fragments arising in these genes.

Klein *et al* (2007) sequenced and analysed the complete coding sequence of three subtype A/IRN/2005 isolates collected in Pakistan in 2006, the complete coding sequence of one subtype A/IRN/2005 isolate collected during the first outbreak in Turkey in 2005 and, in addition, the partial 1D coding sequence derived from 4 epithelium samples and 34 swab-samples from Asian buffaloes or cattle subsequently found to be infected with the A/IRN/2005 subtype. Potential recombination events

have been detected in parts of the genome region coding for the non-structural proteins of FMDV. In addition, amino acid substitutions have been detected in the VP1 protein sequence, potentially related to clinical or subclinical outcome of FMD. Furthermore, hitherto unknown insertions of 2 amino acids before the second start codon, as well as sublineage specific amino acids have been detected in the genome region encoding for the leader proteinase of A/IRN/2005 sublineage. It was indicated that the A/IRN/2005 sublineage has undergone two different paths of evolution for the structural and non-structural genome regions. The structural genome regions have had their evolutionary starting point in the A22 sublineage. It can be assumed that, due to the quasispecies structure of FMDV populations and the error-prone replication process, advantageous mutations in a changed environment have been fixed and lead to the occurrence of the new A/IRN/2005 sublineage. Together with this mechanism, recombination within the non-structural genome regions, potentially modifying the virulence of the virus, may be involved in the success of this new sublineage. The possible origin of this recombinant virus may be a co-infection with Asia1 and a serotype A precursor of the A/IRN/2005 sublineage potentially within Asian Buffaloes, as these appears to relatively easy become infected, but usually without developing clinical disease and consequently showing not a strong acute inflammatory immune response against a second FMDV infection.

Dong *et al* (2011) examined the genomic differences between foot-and-mouth disease virus (FMDV) R strain and its attenuated, chick-passaged (R_{304}) strain. Eleven pairs of primers were used to amplify the complete genome of FMDV R and R_{304} by RT-PCR. Each fragment was cloned into pMD18-T vector and sequenced. Nucleotide analyses showed that the genome encoding regions of R and R_{304} strains open reading frame (ORF) were both 6966 nucleotides (nt) in length, encoding 2322

amino acids. One hundred and ten nucleotides or 32 amino acids were found to be mutated most frequently were in the 3A gene. The next highest rates of mutation were observed in the LP and 1D genes. No mutations were found in either the 2A or 2C genes. The length of 5′IRES region and 3′UTR were 450 nt and 94 nt, respectively. The 5′IRES region and 3′UTR had only 4 nt and 3 nt mutation, respectively after attenuation. The R_{304} poly(A) tail length of 18 nt, while that of the R strain was 30 nt. This result demonstrated the primary genomic changes of a FMDV and its attenuated strain, which has important implications in understanding the molecular epidemiology and functional genomics of FMDV.

Saravanan et al (2011) constructed a genome length cDNA clone of FMDV Asia 1 vaccine strain IND 63/72. The functionality of the cDNA was checked in BHK 21 cells and it did not yield any viable virus particle. The genome-length cDNA contained a single ORF of 6902 nucleotides terminating at a UAA codon 95 bases from the 3' poly (A) tail sequence. The 8167 base pair sequence and the deduced amino acid sequence (2330 aa) were compared with the published FMDV sequence of Chinese strain YNBS/58 showed 5.3% variation at amino acid level.

2.5 Protein processing and antigenic Structure

The FMDV genome encodes a polyprotein from which four different structural and eight different non-structural proteins are formed by the viral proteases. After translation, the four primary cleavage products are formed: (i) the amino terminal L protease which cleaves at its own carboxy terminus, P1- 2A, (ii) the precursor of the capsid proteins (iii) 2BC and (iv) P3 which is cleave to make the NSPs (Belsham 1993). FMDV has two proteinases. The NSP leader proteinases (Lpro) located in the N-terminal region of the polyprotein acts both intra and

intermolecularly. This protease initiates cleavage by separating itself from P1, the precursor of the capsid protein and the remainder of the growing polypeptide chain (Ryan *et al* 1991). In addition, during virus replication Lpro specifically cleaves a host cell protein, the eukaryotic initiation factor 4G (elf-4G), which impair the ability of the host cell to translate to own capped mRNA (Devaney *et al* 1988). The 3C protease is responsible for the cleavage of VP1 into 1AB (VP0), 1C (VP3) and 1D (VP1). The 1A/1B (VP4/VP2) cleavage occurs at the late stage in virus morphogenesis and is associated with maturation of capsid. The 2C/3A primary cleavage is *cis* and subsequent cleavage is also mediated by the 3Cpro and producing processing intermediates and mature proteins (Newman *et al* 1994). In addition to viral protein processing, the 3Cpro cleave the host cell protein histone H3 and may be involved with the shut down of host cell transcription (Capozzo *et al* 2002). The cleavage between 2A/2B junctions is mediated by 2A polypeptide separating itself and P1 away from 2BC/P3 (Belsham 1993). This change is independent of both L and 3C. The FMDV 2A region is very short (about 18 amino acids) and together with the N-terminal residues of protein 2B, represents an autonomous element capable of mediating cleavage at its own Cterminus (Donnelly *et al* 1997).

A major continuous FMDV antigenic site is located in the G-H loop of VP1, as deduced from the immunogenicity of peptides spanning VP1 residues around positions 140 to 160 (Strohmaier *et al* 1982). For serotype C, the antigenic structure of the G-H loop is complex, since different overlapping epitopes, defined by their differential ability to react with individual MAbs, have been mapped within this loop (Mateau *et al* 1990). Site C is located at the C-terminus of VP1 and is apparently continuous and independent from the G-H loop in serotypes A and C (Lea *et al* 1994). In type O, its vicinity with the G-H loop in the structure of the capsid, as well

as competition studies with neutralising MAbs, suggest that sites A and C conform a single site composed of discontinuous epitopes (Barnett *et al* 1989). Site D is discontinuous, comprising residues involving all surface polypeptides, and lies within the C-terminus of VP1 (residue 193), the VP3 B-B knob (residue 58), and VP2 B-C loop (residues 72, 74 and 79). These antigenic sites are located at exposed regions adjacent to each other and close to the 3-fold axis of symmetry in the capsid.

Meloen *et al* (1979) showed that antibody produced against VP_1, one of the four structural polypeptides of foot-and-mouth disease virus, neutralized the virus and reacted with both full and empty particles in radioimmunoassays (RIA). Antiserum against VP_2 reacted with artificial empty particles of the virus but not with full particles. In contrast, none of the individual polypeptides of poliovirus produced antisera which neutralized the virus nor reacted with it in RIA. However, antisera produced with VP_1 and VP_2 reacted with artificial empty particles in RIA.

A segment of 1160 nucleotides of the FMDV genome was sequenced by Kurz *et al* (1981) using three overlapping fragments of cloned cDNA from FMDV strain O_1K. This sequence contained the coding sequence for the viral capsid protein VP1 as shown by its homology to known and newly determined amino acid sequences from this main antigenic polypeptide of the FMD virus. The structural gene for VP1 comprises 639 nucleotides which specify a sequence of 213 amino acids for the VP1 protein. The coding sequence is not flanked by start and stop codons which is consistent with the mode of biosynthesis of VP1 by post-translational processing of a polyprotein precursor.

Francis *et al* (1985) showed that a sub-immunizing dose of a synthetic peptide corresponding to the amino acids 141 to 160 region of protein VP1 from foot-and-mouth disease virus (FMDV), serotype O_1, coupled to keyhole limpet

haemocyanin (141-160KLH) primed the immune system of guinea-pigs for an FMDV serotype-specific neutralizing antibody response to a second sub-immunizing dose of the same peptide. Optimal priming required an interval of 42 days between the priming dose and the booster dose. No priming was observed in the absence of adjuvant. The secondary response was not restricted by the carrier since animals primed with 141-160KLH could be boosted with uncoupled 141–160 or 141–160 coupled to tetanus toxoid. It also showed that uncoupled peptide 141–160 will prime for a neutralizing antibody response when it is incorporated into a relatively non-immunogenic carrier such as small unilamellar liposomes. These results indicate that the 141–160 peptide of FMDV, contain an important neutralizing antibody site, which can initiate its own T-helper cell response.

A chemically synthesized peptide consisting essentially of two separate regions (residues 141 to 158 and 200 to 213) of a virus coat protein (VP1) from the O1 Kaufbeuren strain of foot-and-mouth disease virus was prepared free of any carrier protein. It elicited high levels of neutralizing antibody and protected cattle against intradermolingual challenge by inoculation with infectious virus. Comparative evaluation of this peptide with a single-site peptide (residues 141 to 158) in guinea pigs suggests the importance of the VP1 carboxyl terminal residues in enhancing the protective response (DiMarchi *et al* 1986).

Synthetic peptides representing regions of the VP1 protein of foot-and- mouth disease virus strain 01 Kaufbeuren were screened for their ability to stimulate proliferation of peripheral blood mononuclear cells from vaccinated cattle. Sites were identified at residue 21-40 (peptide FMDV32) and in the region C-terminal to residue 161. Cells responding to FMDV32 were MHC class II-restricted, CD4+ and secreted IL-2. Thus, this region is defined as a Th site. Of 19 virus vaccinated Friesian cattle,

89% (17/19) responded to purified virus while 37% (7/19; 41% of virus responders) also responded to FMDV32 suggesting that this site is immunodominant for the cattle used. Furthermore, immunisation of FMDV32 responder and non-responder cattle with a related peptide, FMDV5 (FMDV32 co-linearly synthesized with the 141-160 VP1 B cell site), induced neutralizing antibody and a virus-specific T cell population in in the FMDV32-responder but not the non-responder animals (Collen *et al* 1991).

A new peptide construct Palm135-158–GGA-170–188(Acm) has been synthesized and investigated in a number of in vitro and in vivo test systems. The construct contains a virus specific T-helper epitope within the 170–188 sequence of VP_1, in addition to the main antigenic 135–158 region of the foot-and-mouth disease viral VP_1 protein (strain A_{22}). The construct has higher protective, antigenic, immunogenic and T-cell proliferative activity then the previously described shorter peptide $Palm_2$135–159. The 170–188 part of the construct serves as a virus specific T-epitope, responsible for the enhanced immunogenic and protective activity of the construct (Volpina *et al* 1999).

2.6 Antigenic variation

Foot and mouth disease is endemic in India since many centuries. It is prevalent almost in all parts of the country and occurs round the year. The disease exhibits complex epidemiology, existing in seven immunologically distinct serotypes and numerous divergent strains within the serotypes which can manifest continuous genomic and antigenic evolution. Out of the possible seven (O, A, C, Asia1, SAT1, SAT2, & SAT3) only four serotypes O, A, C and Asia 1 were ever recorded in India. Serotype ‘C’ too has not been recorded in the country since 1995.

Rowlands *et al* (1983) reported the separation of three natural antigenic variants, distinguishable in cross-neutralization tests from an isolate of foot-and-mouth disease virus. The serological differences were also demonstrated by antisera elicited by synthetic peptides corresponding to residues 141-160 of the capsid polypeptide VP1 showing that this region contains a major immunogenic site of the virus.

The primary structure of VP3, VP2 and VP4 capsid protein genes was determined by Sobrino *et al* (1989) for six epizootiologically-related foot-and-mouth disease virus isolates of serotype C1, two of which presented immunogenic differences as determined by a cross-protection assay. The results obtained were compared with those previously reported for the corresponding VP1 genes. High rates of fixation of mutations were estimated for the four capsid protein genes that ranged from 3.9 X 10(-4) to 4.5 X 10(-3) substitutions per nucleotide per year, with the highest values corresponding to VP1. Despite this genetic heterogeneity most of the amino acid exchanges were within the VP1 protein. Of the fourteen amino acid substitutions one was located in VP2 and two in VP3. Five out of the eleven amino acid exchanges that affected VP1 were located within residues 138-149, part of a main immunogenic site in FMDV. These results showed that in the course of a foot-and-mouth disease outbreak, immunologically relevant amino acid substitutions occur mainly in viral capsid protein VP1.

To investigate the mechanism of antigenic variation in foot-and-mouth disease virus, variants that escape neutralization by a monoclonal antibody were compared crystallographically and serologically with parental virus by Parry *et al* (1990). G–H loop, which is a major antigenic site of FMDV, forms a prominent, highly accessible protrusion, a feature not seen in other picornaviruses. It was this

loop that was perturbed in the variant viruses that were studied. The amino acid mutations characterizing the variants were not at positions directly involved in antibody binding, but resulted in far-reaching perturbations of the surface structure of the virus. Thus, this virus seemed to use a novel escape mechanism whereby an induced confor-mational change in a major antigenic loop destroyed the integrity of the epitope.

Foot and mouth disease virus by nature of its RNA genome, possesses a high rate of mutations during replication. This results in extensive genetic polymorphism of virus population in nature and thus emergence of FMDV variants during replication (Meyer *et al* 1994).

Antigenic variation in a major discontinuous site (site D) of foot-and-mouth disease virus of serotype C was evaluated by Mateau *et al* (1994) with neutralizing monoclonal antibodies. Isolates representing the major evolutionary sublines previously defined for serotype C were compared. Extensive variation, comparable to that of continuous epitopes within the hypervariable immunodominant site A (the VP1 G-H loop), was found. The amino acid sequences of the complete capsids of three antigenically highly divergent FMD viruses (C, Haute Loire-Fr/69, C. Argentina/69, and C3 Argentina/85) were determined and compared with the corresponding sequences previously determined for seven additional type C viruses. Differences in antigenicity were due to a very limited number of substitutions of surface amino acids accessible to antibodies and located within antigenic sites previously identified on FMDV. The results suggested that within a serotype of FMDV, antigenically highly divergent viruses can arise in the field by very limited sequence variation at exposed key residues of each of several antigenic sites.

Mohanty *et al* (1994) serologically determined the relationship of 29 field isolates from different outbreaks in the country to A22 vaccine strain currently in use in India. They reported that two of the field isolates had broader antigenic spectrum compared to the A22 vaccine strain based on serological relationship.

The antigenic relationship of sixty type A foot-and-mouth disease viruses isolated between 1968 and 1993 was determined with reference to a post-vaccinal bovine serum produced against type A IND 17/82. A micro-neutralization test and ELISA were used to compare isolates. It indicated that there was a positive correlation between the data from the two methods. The study indicated that type A IND 17/82 had a broad immunogenic spectrum and could be considered as a candidate vaccine strain for incorporation in FMD vaccines in India (Azad *et al* 1995)

PCR assays were carried out by Stram *et al* (1995) to identify the virus and its serotype in suspect animals from 2 outbreaks of FMD type O virus. Sequence analysis of the amplified VP1 cDNA showed 78% homology with O1K and over 95% homology between the samples. These findings suggest that the 2 outbreaks were due to infection with the same virus serosubtype.

The sequences of the antigenically relevant capsid proteins VP1-3 of 10 isolates obtained during an epizootic of serotype A foot-and-mouth disease virus in Iran, and collected within two and a half years, were found to be highly similar. However, each isolate differed by at least one amino acid from all others. This prompted Marquardt *et al* (2000), to analyse the immunological reactivity of the isolates. Monoclonal antibodies (mAbs) against one isolate were generated and characterized with regard to neutralizing activity and reactivity with trypsinized virus. These mAbs as well as others raised against A22 virus were used for antigen

profiling. This distinguished four antigenic conditions among the isolates and 16 reactivities among the mAbs. These findings, together with the observed sequence differences indicated the location of several epitopes. Many mAbs recognized the minor antigenic sites on VP2 and 3 and some the major site, the GH-loop of VP1. One epitope was composed of residues of the capsid proteins VP1 and 2.

Tulasiram *et al* (2006) studied FMDV isolates from vaccinated and unvaccinated animals from different parts of the country and compared their relationship with Asia 1 vaccine virus. The immunogenic, hypervariable region of viral protein 1 (VP1) gene was amplified by RT-PCR and sequenced. Analysis of sequence data showed that the viruses from two field outbreaks of Southern India were closely related to each other when compared to the isolate from the North and all the three isolates are away from the vaccine virus.

2.7 Diagnosis

The accurate diagnosis of infection with FMDV is of prime most importance for both control and eradication campaigns in FMD endemic areas and as a supportive measure to the stamping out policy in FMD free areas.

Earlier typing of FMDV was done by cross-immunity test in guineapigs (Waldmann and Trautwein 1926).

Bachrach in 1968 performed typing of FMDV cross immunity test in cattle. As this test was time consuming, expensive and imprecise, different serological tests like complement fixation test (CFT), virus neutralization test (VNT) and enzyme linked immunosorbent assay (ELISA) were developed and the most recent is the development of molecular techniques, the polymerase chain reaction (PCR) method making diagnosis more rapid and precise.

Longjam et al (2011) demonstrated that multiplex PCR could detect FMD virus in the highest number of samples (65.47%) followed by sandwich ELISA (53.57%) and virus isolation (42.85%).

2.7.1 Virus isolation

Primary cell culture of bovine (Sellers 1955, Snowden 1966 and House and House 1989) origin have exhibited susceptibility to FMDV from infected tissues.

However, the most sensitive culture system for virus isolation is primary bovine thyroid cells (Snowden 1966). Some stable cell lines, like IBRS-2 (Nair 1987), MVPK-1 clone 7 (Dinka *et al* 1977 and Nair 1987) and BHK-21 (House *et al* 1988) are also susceptible to FMDV and so are most desirable for diagnostic system but these are less sensitive than primary cells for detecting low amount of infectivity (Clarke and Spier 1980). Revenson and Segura (1963) reported that FMDV grew well on BHK-21 cell line enabling large scale production of antigen with good complement fixing properties. It has also been reported that with subsequent passage in BHK-21 clone 13 cell line, the titre of FMDV increased significantly (Sellers 1955). Mishra et al (1995) also adapted FMDV field isolates to BHK-21 clone 13 cells in 3-7 serial passage. Goel and Rai (1985) reported that the field isolates of FMDV could be passaged in BHK- 21 clone 13 monolayer cell culture, which showed characteristic CPE and were readily adapted between 3rd and 5th passage. The CPE usually develops within 48 hours, if no CPE is detected the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours.

2.7.2 Enzyme Linked Immunosorbent Assay (ELISA)

The first report of use of an Indirect ELISA to screen cattle for antibodies against FMDV was by Abu Elzein and Crowther (1978). Subsequently, a sandwich

ELISA using convalescent bovine immunoglobulin (Igs) as capture and anti-146S guinea pig sera as tracing sera was found suitable for detection and quantification of FMD virus in infected tissue culture fluid and epithelial tissue samples (Crowther and Abu Elzein 1979). The use of anti-146S rabbit immunoglobulin in place of convalescent bovine immunoglobulin as capture antibody increased the sensitivity of sandwich ELISA (Ouldridge et al 1982). Later ELISA and its various modifications were applied for detection, typing and strain differentiation of FMDV isolates with better sensitivity than CFT and, the results were comparable to that obtained with MNT (Abu Elzein and Crowther 1978, Rai and Lahiri 1981, Hambling *et al* 1984, Ouldridge *et al* 1984, Roeder and Smith 1987 and Pattnaik and Venkataramanan 1989 a & b). Liquid phase blocking ELISA using bovine convalescent sera for characterization of field isolates was done and result tallied with conventional VNT (Kitching *et al* 1988, Pattnaik *et al* 1991 and Lunt *et al* 1994). ELISA results were much more reproducible than those obtained with VNT and are not influenced by variations in tissue culture susceptibility. At the FAO/WRL for FMD, the preferred procedure for the detection of FMDV antigen and identification of viral serotypes is ELISA (Ferris and Dawson 1988). Indirect ELISA was initially used for detection of FMDV antigen in infected cell culture fluid, mice carcass and cattle tongue as well as antibodies in sera samples (Rai and Lahiri 1981, Pattnaik and Venkataramanan 1989a). Later a sandwich ELISA was used for subtype analysis of FMDV isolates (Pattnaik and Venkataramanan 1989b). Subsequently, a Sandwich ELISA was developed for detection and typing of FMDV directly from field materials (Bhattacharya *et al* 1996). The 3AB protein of FMDV was expressed in *E. coli* (Silberstein *et al* 1997, Suryanarayana *et al* 1999 and Nanni *et al* 2005) or in *P. pastoris* (Latha *et al* 2007) has been used for the diagnosis of FMD infection in

cattle. Similarly, 3ABC proteins expressed in heterologous systems were used in ELISA (3ABC ELISA) for sero-diagnosis of FMD (Bruderer et al 2004 and Lu et al 2007). Further, four serotypes of FMDV structural proteins expressed in P. pastoris and its potential utility either as immunogen or antigen has been successfully assessed in animal model (Balamurugan *et al* 2003 and Renji *et al* 2003). A recombinant FMDV polyprotein (P1) with 3C expressed in insect cells was evaluated for detecting antibodies to FMDV serotype Asia 1 in ELISA and has the potential to replace the liquid phase blocking (LPB)-ELISA using an inactivated FMDV antigen as a simple and robust serological tool for screening antibodies to FMDV serotype Asia 1 (Ko *et al* 2009).

Investigations using liquid phase blocking sandwich enzyme linked immunosorbent assay ELISA for the measurement of antibodies against FMDVin sera from sheep and cattle were reported by Hambling *et al* (1987) and results were compared with virus neutralization (VN) test. The ELISA was considered more reliable than VN for evaluating immunological response of animals following infection and vaccination.

Blacksell *et al* (1994) produced antisera at a central laboratory in Thailand against the endemic serotypes (O, A and Asia 1) of foot and mouth disease (FMD) virus. At a regional veterinary laboratory, these antisera were used in an indirect sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and serotyping of FMD virus (FMDV) antigen. Of a total of 93 samples submitted for antigen typing, 80 (86%) tested positive by ELISA and 13 (14%) were negative. No FMDV was detected in ELISA-negative samples following attempted tissue-culture virus isolation.

2.7.3 DIVA based companion diagnostic approach

2.7.3.1 Non structural protein (NSP) serology

Although vaccination offers many advantages in control of FMD, still there is opposition to its use in many part of the world because international trade regulation put a heavy penalty on the use of vaccines against FMD in the form of import/export restriction of animals and their products. After 2001 outbreak of FMD, OIE has amended its Animal Health Code regarding time duration of regaining FMD free status after an outbreak; it has been reduced to 3 months for those countries which were free of FMD without vaccination (stamping out policy) and 6 months for those countries which were free with vaccination. These trade regulations are not based on risk assessment, but rather on notion that vaccination might perpetuate carriers in population and that those carriers may pose a risk of FMD free countries that do not practice vaccination. Also the assumption is made that there is no accurate method to detect the presence of carriers in vaccinated population. Recently after 2001 outbreak of FMD in UK, EU member states have also made provision for emergency vaccination in case of a future outbreak. So DIVA strategy is equally important both for countries which are endemic in FMD, where there is large amount of carriers exists and where vaccination is practiced and for those countries which are supposed to use vaccination in face of an outbreak.

The current FMD vaccine which is being used worldwide is an inactivated whole virus particle mixed with adjuvant. When animals are immunised against such a vaccine, they mount antibody response only against coat (structural) proteins of the virus. When an animal becomes infected, antibodies also develop against non-structural proteins (viral polymerases and proteases) of the virus because virus actually replicates inside host in such a situation. The conventional diagnostic system

(e.g. ELISA) detects antibodies only against structural proteins and so is unsuitable for differentiation of FMD vaccinated and infected animals (DIVA strategy). Detection of antibodies against non-structural proteins might be a tool for DIVA strategy. Immunised animals that are infected and subsequently become carriers will also develop antibodies against NSPs, allowing carriers to be identified in vaccinated stock.

The agar-gel immuno-diffusion (AGID) test using virus infection associated antigen (VIAA), isolated from virus cultures, was the first test to be developed (Cowan and Graves 1966 and McVicar and Sutmoller 1976). VIAA was subsequently identified as the viral RNA polymerase (3D poly) (Polatnik and Arlinghaus 1967 and Newman *et al* 1979). However, in later studies, investigator found that sera from multiply vaccinated animals (Rowland *et al* 1974 and Pinto and Garland 1979) and from some animals which have even received a single dose of vaccine, had antibodies to VIAA. Such kind of immune response elicited by vaccination usually disappear 60-90 days post vaccination as detected by AGID or 90-180 days post-vaccination when 3D-ELISA is used (O´Donnel *et al* 1997). The reason behind antibodies against NSPs in vaccinated animals is that the FMD vaccines are not purified enough and depending upon the manufacturer, contain various amounts of contaminating NSPs (Luborth *et al* 1996)

Liquid phase ELISA which also detected VIAA was developed by Alonso *et al* (1990) and showed superior sensitivity to the AGID. Berger *et al* (1990) suggested that simultaneous detection of at least two NSPs antibodies (excluding 3D) would be sufficient to demonstrate viral replication.

Later on, more sensitive tests were developed. One of these tests, the enzyme-linked immuno-electrotransfer blot (EITB) assay uses a set of purified recombinant

DNA derived NSP antigens as serological probes, instead of the traditional VIAA (Bergmann *et al* 1993, 1996). These authors compared the VIAA antibody tests, the EITB test, and the virus isolation assay (from OP fluid) using sequentially collected samples from experimentally infected cattle (Bergmann *et al* 1993, 1996). The EITB test was found highly sensitive and specific for known positive and negative anti-sera. A set of anti-sera against a number of other, non-FMD viruses were negative. For the detection of past FMD infection it was clearly superior to the other (VIAA) tests.

Antibody response to NSPs is variable; the response to 3A, 3B, 3D and 3ABC could be detected in cattle as early as 7-10 days post-infection (Bergmann *et al* 1993 and Sorensen *et al* 1998) and up to 560-742 days post infection (Silberstein *et al* 1997).

Although 3D protein is not the best choice to differentiate vaccinated from infected animals, it was shown to be the most antigenic NSP (Mackay *et al* 1998) and the 3D ELISA is more sensitive than others but less specific. Mackay *et al* (1998) discovered that 3ABC is most reliable single indicator of infection (examining with bovine and ovine sera), immune response to 3ABC appeared early after infection and antibodies to 3ABC could be detected for longer than antibodies to any other NSP, 3A generally induces a similar response, some animals fail to react against 3B, whilst 3C alone is very week immunogenic. Detection of antibodies to one or more of the NSPs 2C, 3A and 3AB in addition to those against 3ABC, provides further confirmation to infection.

The 2C is usually absent in sera from multiple vaccinated animals which is explained by the association of this viral protein with cellular debris that can be separated from the virus harvest prior to inactivation of the supernatant for vaccine

production (Luborth *et al* 1996). Antibodies to 2C could be detected in cattle up to 365 days after infection (Luborth and Brown 1995 and Meyer *et al* 1997).

FMD vaccines also contain adjuvant and they may probably enhance the immune response against NSPs but because of low concentration of NSPs in vaccine, detectable (positive) level of NSP antibody consequent to vaccination may be of shorter duration than those produced following infection with live virus.

Recently, ELISA based assays with various NS proteins produced by recombinant baculovirus (Kweon *et al* 2003), in *E.coli* (Bergmann *et al* 2000), insect larva (Lopez *et al* 2004) or synthetically produced peptides to NSPs (Oem *et al* 2005) have been developed.

The advantage of using synthetic peptides is that they are very specific while the disadvantages of synthetic peptides are with their folding *in vitro* system; the epitopes are not properly folded and hence they may loose sensitivity. Currently all these assays are being validated and still there in no gold standard NSP based test.

There are also 3 commercial tests to detect antibodies against NSP (3ABC); United Biomedical, Inc., New York, Cedi-Diagnostics B.V., The Netherland and SYANOVA Biotech AB, Sweden. These tests vary with each other for their sensitivity and specificity (Bruderer *et al* 2004, Lee *et al* 2004 and Moonen *et al* 2004). These tests are also not completely validated and OIE is not in a position for prescribing these as a standard tool. Furthermore, all these tests are very expensive to be afforded by developing countries.

2.7.4 Pen side diagnostic approach

Routine diagnosis of FMD is made at several laboratories by the combined use of enzyme-linked immunosorbent assay (ELISA) ,virus isolation techniques, supplemented by reverse transcriptase PCR (RT-PCR). However, most of these

diagnostic methods require the availability of a dedicated laboratory facility, highly trained laboratory personnel, stable reagents, multistep sample handling or preparation and management of the logistical considerations associated with sample collection and transport is also required (Oem *et al* 2009). A rapid and easy-to-perform test, which would allow for on-site diagnosis to be made in the case of a suspected disease outbreak would circumvent problems associated with the transportation of samples to the laboratory and would be especially useful for a faster diagnosis in areas where the disease is endemic. Availability of 'Point of care' or 'Pen-side' diagnostic tests would have the advantage of rapid, user friendly, correct identification of a particular strain and economically feasible diagnosis of FMD in field condition. Development of a rapid chromatographic strip test , lateral flow device (LFD) for pen-side diagnosis based on a monoclonal antibodies that reacts against FMDV of all seven serotypes (Reid *et al* 2001). The Lateral Flow Assay technique permits rapid diagnosis, allowing time for the early implementation of control measures to reduce the possibility of spread of FMD. A rapid lateral-flow assay (LFA) based on FMDV antigen detection, which is easy to use and can be utilized on the farm to reduce the time required for transport and laboratory diagnosis.

Oem *et al* (2009) showed that the diagnostic sensitivity of the LFA for FMDV types O, A, C, and Asia 1 was similar, at approximately 87.3%, to that of 87.7% obtained with antigen enzyme-linked immunosorbent assay (Ag-ELISA). But the specificity of the LFA was 98.8%, compared to 100% for the Ag-ELISA (Oem et al., 2009). Recently a lateral flow device (LFD) for the detection of foot and mouth disease virus (FMDV) of the SAT 2 serotype was developed using a monoclonal antibody (Mab 2H6) for providing rapid and objective support to veterinarians in

their clinical judgment of the disease and for specific confirmation of a FMDV type SAT 2 infection (Ferris *et al* 2010). A simple , rapid, colloidal gold based immuno chromatographic strip test were developed for easy clinical testing of serotype A of FMDV in field sites was developed with sensitivity and specificity 88.7% and 98.7% respectively (Jiang *et al* 2011).

2.7.5 Serotype specific synthetic peptides for diagnosis of FMD

Petrov *et al* (1996) synthesised peptides according to theoretical analysis of antigenic structure of protein VP1 of FMDV types O, A, Asia-1 which corresponded to potential immunodominant protein sites. Activities of the peptides were studied by solid phase indirect radioimmunoassay on polyethylene film with purified immunoglobulins against intact FMDV. Virtually no cross reaction were observed. Blood sera of the cattle convalescent after FMD were tested with FMDV and peptides containing VP1 fragments 141-160 (A22), 140-160 (O1), and 140-153 (Asia-1). The specificity of interactions between the sera and the peptides and the virus was uniform, this permitting the identification of the virus type which caused the disease.

2.7.6 Latex Agglutination Test

Latex agglutination tests have been in use since 1956 to detect a wide range of analytes in the clinical laboratory. When spectrophotometers and nephelometers are used in place of the human eye to detect agglutination, it is possible to measure quantitatively and to develop sensitive particle immunoassays. Latex particles may be build from different organic materials to a desired diameter, and may be functionalized with chemical groups to facilitate attachment of molecules. Proteins and other molecules may be passively adsorbed to the latex particles or covalently coupled to functional groups. Some described automated latex agglutination tests have sensitivities of a few picograms of analyte (Gella *et al* 1991).

Yap (1994) conducted a study to optimize the conditions for the passive adsorption of polyclonal antibody onto plain surface polystyrene latex particles and tested its performance in a slide latex agglutination test for rotavirus antigen detection. Cleaning of latex particles by washing through repetitive centrifuging, decanting and resuspending in distilled water was adequate in removing surfactants from the particles' surfaces to enable coating. A study of antibody concentration, incubation temperature and buffer pH revealed that optimum coating was achieved with a 3-fold excess of antibody to the calculated total particle surface capacity for the antibody in a glycine-saline buffer of pH 9.2 at 40°C for 4 hours. The ionic strength and pH of the latex suspending buffer and the sample buffer were critical factors determining the sensitivity of the test and the appearance of non-specific agglutination. Ultrasonication, addition of glycerol and Tween 20, either individually or in combination, were able to suppress non-specific agglutination in some batches of latex reagents. Polyethylene glycol 6000 enhanced the quality of agglutination as well as reduced the time of its appearance, especially in reagents that produced poor agglutination.

With the specific immunodominant epitope (ESAT6-p) of *M. bovis*, Koo *et al* (2004) developed a Latex bead agglutination assay (LBAA) and enzyme immunoassay (EIA) and compared them with the "gold standard" culture method and skin test for their efficacy in detecting bovine tuberculosis. The data suggesed that rapid, sensitive, and specific assays can be developed with peptides containing immunodominant epitopes present in proteins uniquely expressed in *M. bovis* or *M. avium* subsp. *paratuberculosis* for differential diagnosis of cattle infected with *M. bovis* or *M. avium* subsp. *paratuberculosis*.

CHAPTER III

MATERIALS AND METHODS

3.1 Collection of sera

A total of 200 blood samples were collected from cattle (141) and buffaloes (59), of different ages and sex from both Foot and Mouth Disease (FMD) vaccinated and unvaccinated animals. These samples were obtained from places in and around Ludhiana during the period from December 2010 to April 2011. Serum was collected from the clotted blood in serum collection vials which were later stored at -20°C.

3.2 Selection of serotype specific sequences

The subsequence 140-160 amino acid residues of VP1 of FMD virus (FMDV) has been clearly identified as a major site for induction of virus neutralizing antibodies (VNA). Keeping this in view, the following peptide sequences were chosen based on published sequences of VP1 proteins of A_{24} Cruzeiro and O_1 Kaufbeuren strains of FMDV used by Doel *et al* (1990) in the peptide vaccines.

Peptide A: G-S-G-R-R-G-D-M-G-S-L-A-A-R-V-V-K-Q

Peptide O: V-P-N-L-R-G-D-L-Q-V-L-A-Q-K-V-A-R-T

Strohmaier *et al* in 1982 recognised 200-213 amino acid residues on VP1 protein of FMDV as minor neutralizing site. Hence amino acid residues 140-160 and 200-213 were picked from VP1 sequence of FMDV type Asia 1 Ind 63/72 as per Reddy *et al* (1999). The two sequences were linked by P-P-S (proline-proline-serine) as per Doel *et al* (1990).

Peptide Asia 1:

R-R-K-Q-E-I-I-A-P-E-K-Q-V-L-P-P-S-Q-P-T-R-R-G-D-L-A-V-L-A-Q-R-V-S-N-R

Non structural peptide is important to differentiate between vaccinated and infected animals. Hence non structural peptide (NSP) 2B of FMDV serotype O1K was selected as per Inoue *et al* (2006).

Peptide NSP 2B: R-S-T-P-E-D-L-E-R-A-E-K-Q

3.3 Bioinformatics

Various bioinformatics tools were used to compare and analyse viral protein sequences. BLAST is a collection of programs which looks for matches of short words. It was used for sequence similarity search using the protein sequence database GENBANK.

The NCBI BLAST web server was used to compare the selected viral peptide sequences with the protein sequences available in GenBank. This was done to identify serotype-specific sequences to prevent any cross reactions or non specific reactions between serotypes during diagnosis.

3.4 Synthesis and conjugation of peptides with colored latex beads

The latex beads of different colors were commercially procured from Sigma Aldrich Ltd. Three serotype specific peptides of the FMD virus i.e. O, A, Asia 1 and a non structural peptide (NSP) 2B were commercially synthesized and their coating on to varied colored latex beads was done by Bioconcept Labs, Pvt ltd ; Gurgaon, Haryana. The peptides O, A, Asia 1 and NSP 2B, were coated with deep blue, white , dark red and fluorescent orange coloured latex beads, respectively as shown in Table 1.

3.5 Latex agglutination test using synthetic peptides

All the serum samples were tested against all the four differently coated latex beads individually and in multiplex agglutination assay. Equal quantity (5µl) of serum sample and antigen coated on to latex beads was put on to a clean grease free glass slide. For type A, peptide was diluted to 1:8 to achieve visible agglutination. The serum sample and latex beads were mixed thoroughly using a clean wooden stick and observed for the clump formation within one minute indicative of agglutination. The samples were classified as negative, slight positive, positive and highly positive depending on the intensity of the agglutination. Fetal calf serum was used as negative control.

Table 1: Latex beads conjugated to FMDV type O, A, Asia 1 specific peptides and NSP 2B peptide.

S. No.	Colored particles used	Peptides conjugated to beads	Peptide sequence
1.	Latex beads, carboxylate –modified polystyrene latex beads, carboxylate –modified polystyrene. (Aqueous suspension, 0.9µm, mean particle size)	Peptide A	G-S-G-R-R-G-D-M-G-S-L-A-A-R-V-V-K-Q
2.	Latex beads, deep blue dyed (Aqueous suspension, 0.80µm, Average diameter)	Peptide O	V-P-N-L-R-G-D-L-Q-V-L-A-Q-K-V-A-R-T
3.	Microparticles based on polystyrene, dark red, size: 1.0 µm	Peptide Asia-1 Ind 63/72	R-R-K-Q-E-I-I-A-P-E-K-Q-V-L-P-P-S-Q-P-T-R-R-G-D-L-A-V-L-A-Q-R-V-S-N-R
4..	Flourescent Orange (Aqueous suspension, 0.5µm, mean particle size)	Non structural peptide 2B	R-S-T-P-E-D-L-E-R-A-E-K-Q

3.6 Multiplex latex agglutination test

Five microlitre of serum sample, 5µl each of FMDV type O, A, Asia1 and NSP 2B peptides conjugated to latex beads were put on a clean grease free glass slide. The serum sample and latex beads were mixed thoroughly using a clean wooden stick and observed for the clump formation within one minute. Clumps of different types were indicative of positive reaction.

To enhance the agglutination, anti bovine immunoglobulin was added.

3.7 Differentiate Infected and Vaccinated Animals - Enzyme Linked Immunosorbent Assay (DIVA-ELISA)

The SVANOVIR Foot and Mouth Disease Virus (FMDV) 3ABC-Ab ELISA Kit was used to detect antibodies to the non structural protein 3ABC of the FMDV and hence differentiate naturally infected animals from those vaccinated against FMDV. The kit is based on Solid Phase Indirect ELISA.

3.7.1 Preparation of reagents

PBS-Tween Buffer: 20 x concentrate of PBS-Tween solution was diluted 1/20 in distilled water. Prepared 500 ml per plate by adding 25 ml PBST solution to 475 ml distilled water and mixed thoroughly.

Anti-bovine IgG conjugate: Reconstituted the lyophilized HRP conjugate with 6 ml PBS-Tween buffer. Added the buffer carefully to the bottle. The solution was left for one minute and mixed thoroughly. The solution was prepared immediately before use.

Pre-dilution of controls and samples: For testing, the serum controls and serum samples were pre diluted 1/40 in sample dilution buffer.

3.7.2 Procedure

1. All reagents were equilibrated to room temperature (18-25°C) before use.
2. In duplicates added 50µl of pre-diluted positive control and negative control serum respectively, into selected wells.
3. Added 50µl of pre-diluted samples to the selected wells.
4. The plate was sealed and incubated at 37°C for 30 minutes.
5. The plates were rinsed 3 times with PBS-Tween buffer. At each rinse cycle the wells were filled and the plate emptied and tapped hard to remove all the remains of fluid.
6. Added 50µl of HRP conjugate to each well. Sealed the plate and incubated at 37°C for 30 minutes.
7. Repeated the step 5.
8. Added 50µl of substrate solution to each well and incubated for 30 minutes at room temperature in dark.
9. The reaction was stopped by adding 50µl of stop solution to each well and mixed thoroughly. Added the stop solution in the same order as the substrate solution in step 8.
10. Measured the optical density (OD) of the controls and samples at 405nm in a microplate photometer with air as blank. Measured the OD within 15 minutes after the addition of stop solution in order to prevent fluctuation in OD values.

3.7.3 Calculations

1. Corrected OD values (OD_{corr})

The optical density (OD) values in wells coated with NSP 3ABC were corrected by subtracting the OD values of the corresponding wells containing the control antigen.

$$OD_{NSP\ 3ABC} - OD_{Control} = OD_{Corr}$$

2. Percent Positivity Values

All corrected OD values for the test samples as well as negative control (Neg C) were related to the corrected OD value of the positive control as follows:

$$PP = \frac{\text{Test sample or Neg C } (OD_{corr})}{\text{Positive control } (OD_{corr})} \times 100$$

3.7.4 Test validity

The positive controls had corrected OD values greater than 0.8 and negative controls had corrected OD values of less than 0.3.

3.8 Liquid Phase Blocking (LPB) ELISA

Out of 138 samples, only 35 samples with a PP value of ≥ 48 were sent to Regional Disease Diagnostic Laboratory, Jallandhar, Punjab for LPB ELISA.

CHAPTER-IV

RESULTS AND DISCUSSION

4.1 Collection of samples

The present study was conducted on serum samples from 200 animals (cattle and buffaloes) collected from places in and around Ludhiana during the period from December 2010 to April 2011. The aim was to detect the prevalence of antibodies to different serotypes (O, A, Asia-1) of Foot and Mouth Disease Virus (FMDV) and to differentiate vaccinated and infected animals. For this, blood samples from these animals were collected and analysed for the presence of antibodies in sera against A, O, and Asia-1 serotypes of FMDV using Latex Agglutination Test (LAT) and Multiplex Latex Agglutination Test (MLAT) as described in section 3.5 and 3.6.

Out of the 200 serum samples collected, 141 samples were from cattle (70.5%) and 59 were from buffaloes (29.5%). Out of 141 cattle, 122 were vaccinated (86.52%) while 19 (13.4%) were not vaccinated. Out of 59 buffaloes, 51 were vaccinated (86.4%) while 8 (13.5%) were not. Out of 141 cattle, 7 were under 6 months of age (4.9%), 12 were of 6-12 months of age (8.5%), 15 of age between 1-2 years (10.6%) and 107 were of age more than 2 years (75.88%) (Table 2). All the 59 buffaloes were above 6 months of age. Among these 3 were of 6-12 months of age (5%), 11 of 1-2 years (18.64%) and 45 were of age more than 2 years (76.27%) (Table 3).

4.2 Selection of serotype specific and non structural amino acid sequences

Serotype specific sequences reported in the published literature were used for construction of serotype specific peptides. Sequences were confirmed to be serotype specific using the bioinformatic tool Basic Local Alignment Search Tool (BLAST).

Table 2: Age groups of cattle included in the study

Age	No. of cattle
0-6 months	7
6-12 months	12
1-2 years	15
>2 years	107

Table 3: Age groups of buffaloes included in the study

Age	No. of buffaloes
0-6 months	0
6-12 months	3
1-2 years	11
>2 years	45

FMDV type A and O specific amino acid sequences 140-160 were picked up from the protein sequences used by Doel *et al* (1990) in the peptide vaccines of general sequence cys-cys-(200-213)-pro-pro-ser-(141-158)-pro-cys-gly, where the numbered residues refer to VP1 sequences of two different serotypes (A and O).

Selected FMDV type O sequence was compared with the protein sequences available in the GenBank employing the NCBI BLAST.

VP1 protein **[**Foot and mouth disease virus - type O (O_1 Kaufbeuren strain)]

The FASTA sequence of the VP1 protein of FMDV type O_1 Kaufbeuren strain available from the GenBank is given below

TTSXGEXADPXTATVENYGGETQIQRRQHTDVSFIMDRFVKVTPQNQINILD

LMQIPSHTLVGALLRASTYYFSDLEIAVKHEGDLTWVPNGAPEKXLDNTTNP

TAYHKAPLTRLALPYTAPHRVLATVYNGECRYSRNAVPNVRGDLQVLAQK
VARTLPTSFNYGAIKATRVTELLYRMKRAXTYCPRPLLAIHPTEARDKQKIV
APVKQTL

Genbank accession number GenBank: CAC48181.1

The sequence selected from the total protein sequence was from amino acid residues 142 to 159 which corresponds to the immunogenic epitope of VP1 protein of FMDV as per the published literature.

Selected sequence

142-159-VPNVRGDLQVLAQKVART

When this sequence was compared with other sequences of the protein sequence database using NCBI BLAST, it was found to have 100% identity only with the VP1 protein FMDV type O.

VP1 protein **[**Foot-and-mouth disease virus - type A (A_{24} Cruzeiro strain)]

Specificity of the selected peptides for FMDV type A was checked using NCBI BLAST. FASTA sequence of VP1 protein of FMDV type A_{24} Cruzeiro strain as obtained from GenBank is given below:

TTTVENYGGETQIQRRHHTDIGFIMDRFVKIQSLSPTHVIDLMQTHQHGLVG
ALLRATTYYFSDLEIVVRHEGNLTWVPNGAPESALLNTSNPTAYNKAPFTRL
ALPYTAPHRVPATVYNGTSKYAVGGSGRRGDMGSLAARVVKQLPASFNYG
AIKADAIHELLVRMKRAELYCPRPLLAIEVSSQDRHKQKIIAPAKQLLNFDLL
KLAGDVESN

Accession number Swiss-Prot: P03309.1.

From this sequence, the subsequence corresponding to amino acid residues 129-146 was selected as per Doel *et al* (1990).

Selected sequence

129-146- GSGRRGDMGSLAARVVKQ

Comparison of this sequence with others in the Swiss Prot using BLAST showed that it had identity only with FMDV type A.

Polypeptide [Foot-and-mouth disease virus - type Asia 1**]**

Amino acid sequence 141-160 and 200-213 were picked from VP1 sequence of FMDV type Asia 1 Ind 63/72 as per Reddy et al (1999).

VP1 sequence reported by Reddy *et al* which was deduced from the polypeptide sequence of FMDV type Asia-1 of accession number GenBank: CAA71080.1 was employed.

FASTA sequence of the polypeptide obtained from GenBank is given below:

DPSDVLVYVPYDQEPLNGEWKAKVQKRLKGAGQSPATGSQNQSGNTGSIIN

NYYMQQYQNSMDTQLGDNAISGGSNEGSTDTTSTHTNNTQNNDWFSRLAS

SAFTGLFGALLADKKTEETTLLEDRILTTRNGHTTSTTQSSVGVTYGYAVAE

DAVSGPNTSGLETRVTQAERFFKKHLFDWTPNLSFGHCHYLELPSEHKGVFG

SLMDSYAYMRNGWDIEVTAVGNQFNGGCLLVALVPELKELDTRQKYQLTL

FPHQFINPRTNMTAHINVPFVGVNRYDQYKLHKPWTLVVVVVAPLTVKTGG

SEQIKVYMNAAPTHVHVAGELPSKEGIVPVACADGYGNMVTTDPKTADPVY

GKVFNPPRTNLPGRFTYFLDVAEACPTFLRFGEVPFVKTVNSGDRLLAKFDV

SLAAGHMSNTYLAGLAQYYTQYSGTMNNHIMFTGPTDAKARYMVAYVPPG

MEPPTEPERAAHCIHSEWDTGLNSKFTFSIPYLSAADYAYTASDVAETTSVQ

GWVCIYQITHGKAEGDALVVSVSAGKDFEFRLPVDARRETTTAGESADPVTT

TVENYGGETQSARRLHTDVAFVLDRFVKLTPKNTQILDLMQIPSHTLVGALL

RSATYYFSDLEVALVHTGSVTWVPNGAPKDALDNHTNPTAYQKQPITRLAL

PYTAPHRVLATVYNGKTTYGTQPTRRGDLAVLAQRVSNRLPTSFNYGAVKA

DTITELLIRMKRAETYCPRPLLALDTTHDRRKQEIIAPEKQVLNFDLLKLAGD

V

The sequence selected corresponds to amino acid residues 140-160 of VP1 and 200-213 amino acid residues of VP1 of FMDV type Asia -1.

Selected sequence

140-160 QPTRRGDLAVLAQRVSNR

200-213 RRKQEIIAPEKQVL

BLAST analysis of the peptide with amino acid residues 140-160 and 200-213 showed homology with FMDV type Asia-1. However, residues 140-160 showed identitiy of 93% with polyprotein of FMDV type SAT 3. Residues 4-15 of the selected peptide were identical with residues 868-879 of polyprotein of FMDV type SAT3 as shown below.

Selected sequence 4 RRGDLAVLAQRVSN 17

RRGDLAVLAQRV N

FMDV SAT3 868 RRGDLAVLAQRVEN 881

Non Structural Peptide 2B

Non structural peptide 2B of FMDV serotype O1K sequence reported and evaluated by Inoue *et al* (2006) was selected.

Selected sequence

R-S-T-P-E-D-L-E-R-A-E-K-Q

4.3 Latex agglutination test using beads conjugated with serotype specific peptides and non structural peptide

The serum samples were tested using a Latex Agglutination Test with serotype specific peptides of FMDV type O, A, Asia-1 and non structural peptide (NSP 2B) conjugated to latex beads of different colors.

No agglutination could be seen with control serum (fetal calf serum) with both naked eyes and under the microscope (Fig.1).

Agglutination was visible with naked eye in case of FMDV type O, Asia1 and NSP 2B peptides. However specific agglutination with FMDV type A specific peptide could not be clearly differentiated from non specific clumps by naked eye. So to enhance the visibility and differentiation, equal quantity of serum and amido black dye (2X) diluted to 1:2 was mixed and then 5µl of 1:8 diluted bead tagged peptide suspension was added. Non specific clumps could thus be differentiated from specific agglutination.

However, under the microscope, positive serum samples showed agglutination with all the serotype specific peptides and NSP 2B peptide of FMDV conjugated with beads (Fig. 2, 3, 4, 5).

Out of 200 serum samples, 14 samples were highly positive, 60 were positive, 2 were slightly positive and 124 were negative for antibodies to FMDV type O; 36 were positive, 6 were slightly positive and 158 were negative for antibodies to FMDV type A; 22 were highly positive for type Asia 1, 63 were positive, 19 were slightly positive and 96 were negative for antibodies to FMDV type Asia 1; 4 were highly positive, 45 were positive, 20 were slight positive and 131 were negative for antibodies to NSP 2B (Table 4).

Thus out of total 200 serum samples , 22 samples were positive for FMDV type O, 14 for type A, 14 for type Asia 1, 12 for both type O and A, 26 for type O and Asia 1, 4 for A and Asia 1, 14 were positive for all the three serotypes (O, A and Asia 1) and 94 were negative for all the three serotypes (Table 5).

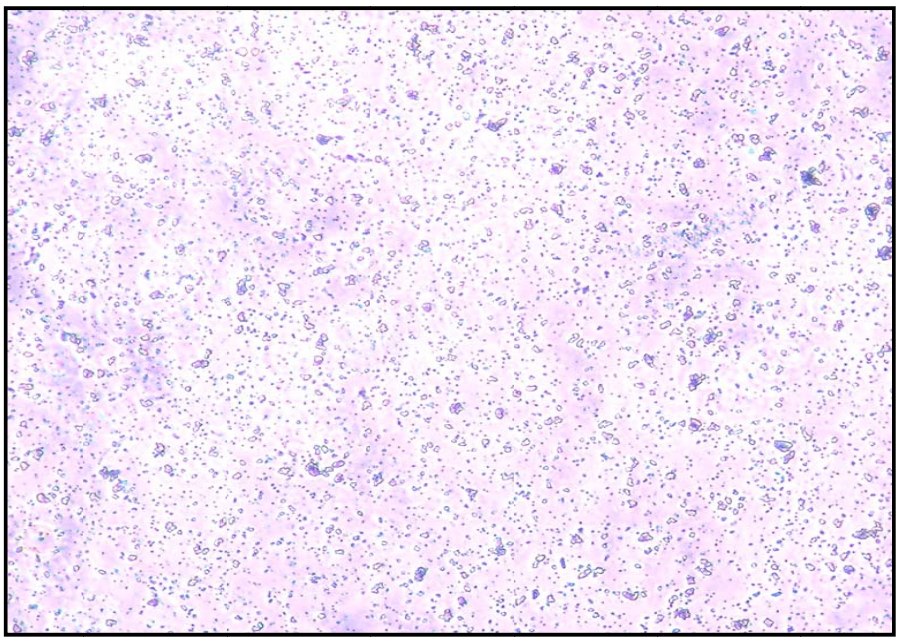

Fig. 1: Absence of agglutination of peptide conjugated beads in FMD negative serum sample (control serum)

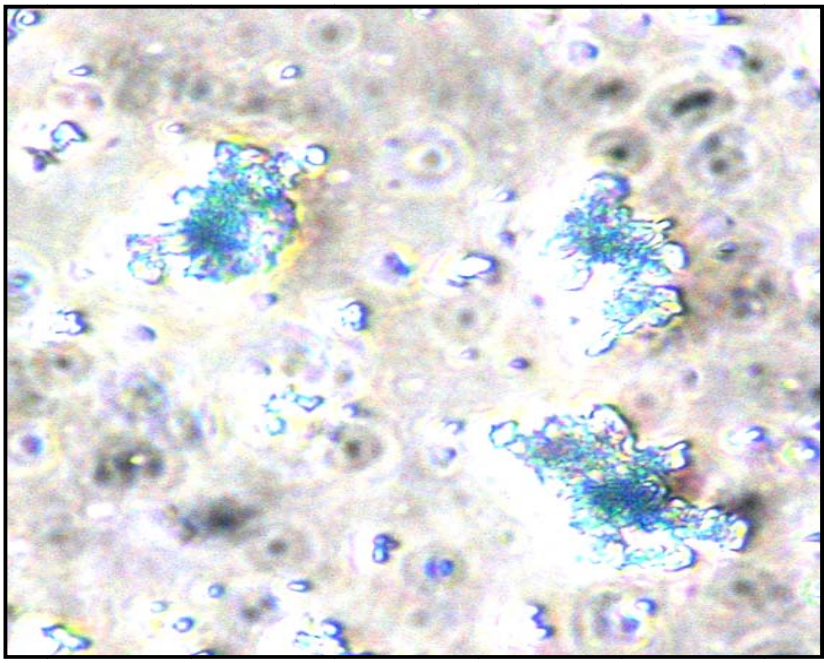

Fig. 2: Agglutination of FMDV type O specific peptide conjugated to blue beads by serum

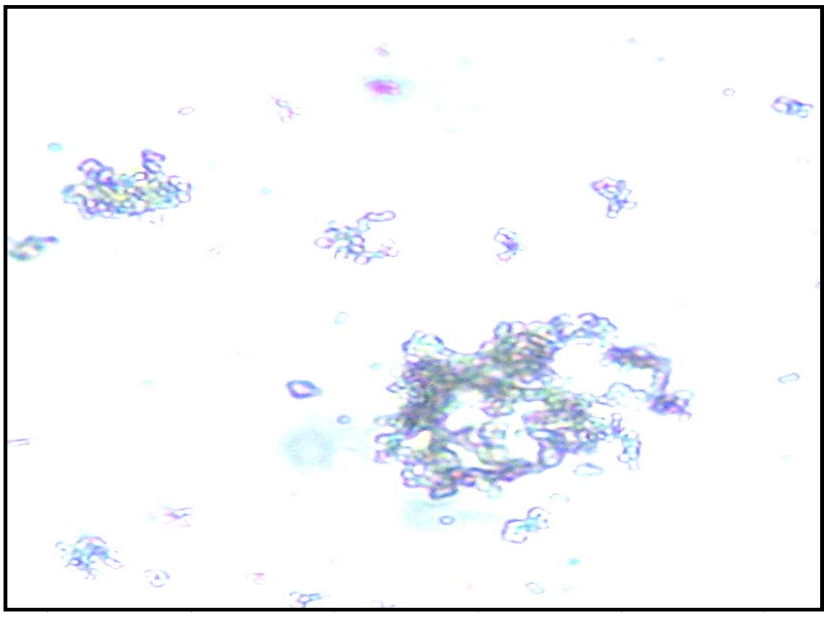

Fig. 3: Agglutination of FMDV type A specific peptide conjugated to white beads by serum

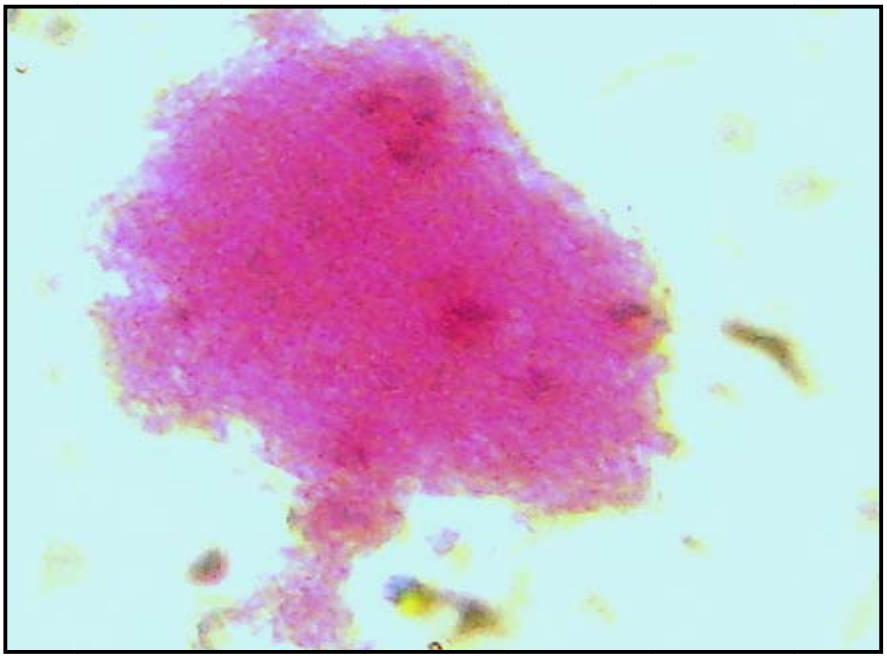

Fig. 4: Agglutination of FMDV type Asia 1 specific peptide conjugated to dark red by serum

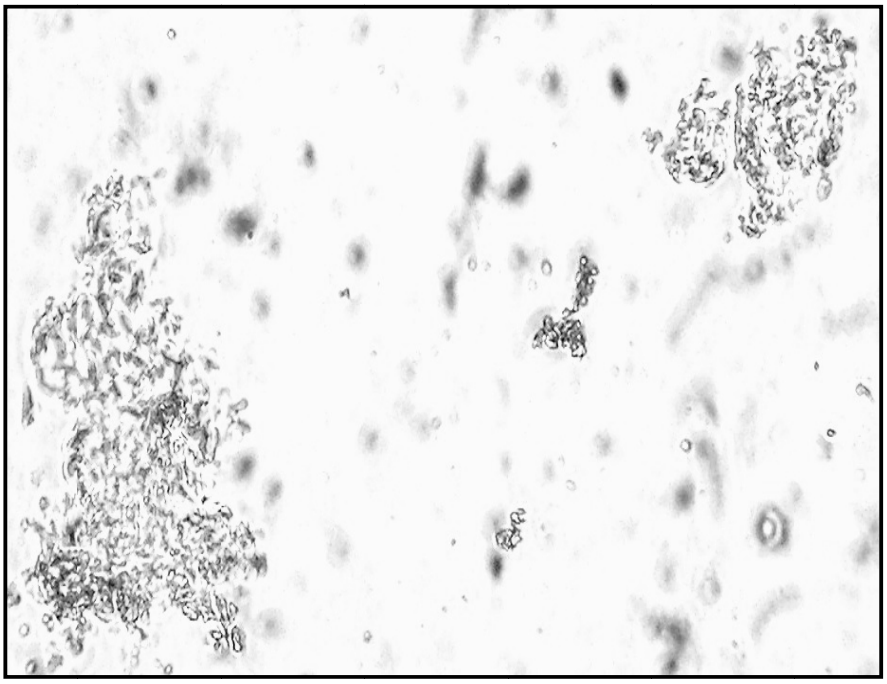

Fig. 5: Agglutination of non structural peptide (NSP 2B) conjugated to fluorescent orange beads by serum

Table 4: Detection of FMDV serotypes by latex agglutination test using synthetic peptides

Serotype	Number of Samples			
	Strong positive (++)	Positive (+)	Slight positive (SL +)	Negative (-)
O	14	60	2	124
A	0	36	6	158
Asia 1	22	63	19	96
NSP 2B	4	45	20	131

Table 5: Serum samples positive for antibodies to different serotypes of FMDV by LAT using serotype specific peptides

FMDV serotype	Number of positive samples	Percentage (%)
O	22	11%
A	14	7%
Asia 1	14	7%
O, A	12	6%
O, Asia 1	26	13%
A, Asia 1	4	2%
O, A, Asia 1	14	7%
Negative samples	94	47%
Total	200	100%

4.4 Multiplex latex agglutination test

Agglutinates with beads of different types were seen only with the aid of microscope (Fig.6). However, clumps of beads conjugated with peptide of FMDV type A were not clearly visible.

Agglutination was enhanced using anti bovine immunoglobulin.

4.5 Differentiate Infected and Vaccinated Animals - Enzyme Linked Immunosorbent Assay (DIVA-ELISA)

One hundred and thirty eight serum samples were chosen randomly irrespective of species, age and vaccination status. Reactivity of these samples was measured by DIVA- ELISA kit (Svanovir). Samples with percent positivity (PP) values of ≥48 were interpreted as positive (infected) and samples with PP values <48 were interpreted as negative, as recommended by the manufacturer. The results are summarized in Table 6.

Thirty five out of 138 samples were identified as positive with a PP value ≥ 48. Hence the prevalence of FMD in these animals as per DIVA-ELISA kit was 25.36%.

4.6 Liquid Phase Blocking (LPB) ELISA

Out of 138 samples, 35 samples with a PP value of ≥ 48 were sent to the Regional Disease Diagnostic Laboratory, Jallandhar, Punjab for LPB ELISA.

Out of the total 35 samples examined, 3 samples were positive for type O, 2 for type A, 1 for type Asia 1, 3 for both type O and A, and 10 for type O and Asia 1. None were positive for A and Asia 1, 15 were positive for all the three serotypes (O, A and Asia 1) and 1was negative for all the three serotypes (Table 7).

Table 6: Results of DIVA ELISA with commercial kit (3ABC-Ab ELISA) showing positive samples

Sample no.	OD corrected	Percent positivity (PP) value
100	0.819	94.0
148	0.876	100.6
82	1.035	118.8
140	0.894	102.6
99	0.73	83.8
176	0.57	65.4
66	0.551	63.3
172	0.877	100.7
72	0.805	92.4
156	0.599	68.8
76	1.109	127.3
93	0.652	74.9
80	0.829	95.2
169	0.971	111.5
147	0.855	98.2
146	1.194	137.1
141	0.839	96.3
175	0.991	113.8
167	0.847	97.2
26	0.364	48.0
19	0.489	64.5
24	0.553	73.0
31	0.381	50.3
96	0.478	63.1
1	0.393	51.8
5	0.679	89.6
17	0.813	107.3
15	0.626	82.6
12	0.49	64.6
74	0.714	94.2
68	0.374	49.3
95	0.403	53.2
121	1.142	75.9
139	0.775	51.5
41	0.75	49.8

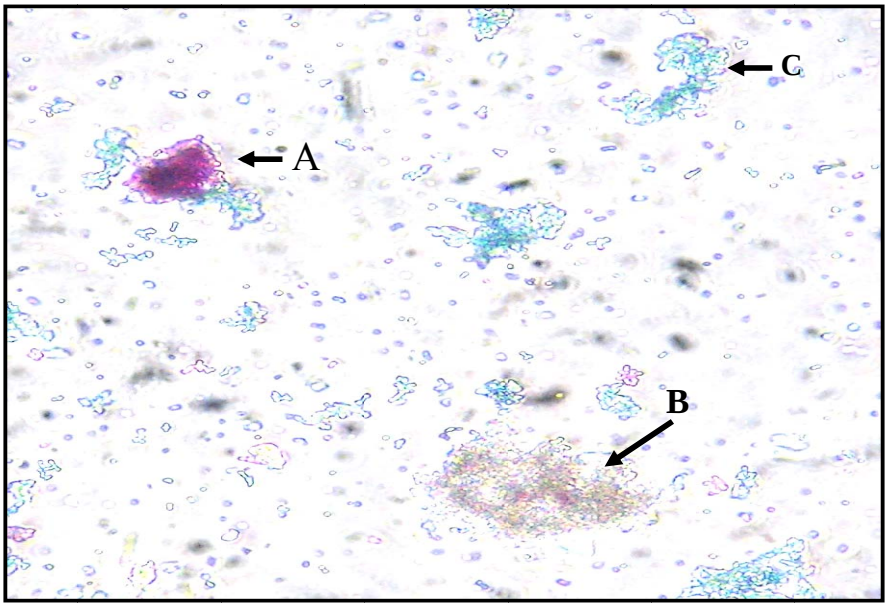

Fig. 6: Simultaneous agglutination of beads conjugated to three different serotypes of FMDV with serum from vaccinated animal

A – Type Asia 1

B – Type A

C – Type O

Table 7: Sera found to be positive for antibodies to different serotypes of FMDV by LPB ELISA

Serotype of FMDV	LPB ELISA	
	No. of positive samples	Percentage (%)
O	3	8.5%
A	2	5.7%
Asia 1	1	2.8%
O, A	3	8.5%
O, Asia 1	10	28.57%
A, Asia 1	0	0%
O, A, Asia 1	15	42.85%
Negative samples	1	2.85%
Total	35	100%

4.7 Comparison of LPB ELISA with LAT (Latex Agglutination Test) using peptides

Out of total 35 samples, 31(88.57%) samples were detected positive for FMDV type O by LPB ELISA. LAT could detect only 11 (31.42%) samples as positive. LPB ELISA detected 20 (57.41%) samples as positive for FMDV type A while 11 (31.42%) were detected as positive by LAT. Out of 35 samples tested, 26 (74.28%) were detected as positive for FMDV type Asia1 by LPB ELISA while LAT could detect only 15 samples as positive (Table 8).

4.8 Comparison of DIVA ELISA with NSP peptide based agglutination

Out of 138 samples, 54 were found to be positive by NSP agglutination while 35 were found to be positive by DIVA ELISA.

It was interesting to note that 21 samples were positive by NSP peptide agglutination but were negative by DIVA ELISA. On the other hand, 33 out of 35 samples were found to be positive by both DIVA ELISA and NSP methods. Two samples negative by NSP could be detected by DIVA ELISA (Tables 9).

These findings suggest that NSP peptide based agglutination test was superior to commercially available DIVA ELISA kit in detecting antibodies due to infection. This is rather intriguing because it is widely believed that ELISA is superior to agglutination test. However, one possible reason could be that the number of antigenic determinants inducing antibodies could be more than antigenic sites detected by ELISA.

However, this needs to be studied further to get a conclusive picture.

Table 8: A Comparison of the efficacy of LAT with that of LPB ELISA in detection of antibodies to FMDV serotypes

Serotype	LPB ELISA		LAT	
	No. of positive samples	Percentage (%)	No. of positive samples	Percentage (%)
O	31	88.57%	11	31.42%
A	20	57.41%	11	31.42%
Asia 1	26	74.28%	15	42.85%

Table 9: Comparison of DIVA ELISA with NSP peptide

Results	No. of samples
Positive by NSP agglutination	54
Positive by DIVA ELISA	35
Positive by NSP and DIVA ELISA	33
Positive by NSP and Negative by DIVA ELISA	21
Positive by DIVA ELISA and Negative by NSP.	2

CHAPTER-V

SUMMARY

Foot and mouth disease (FMD) is a highly contagious disease of cloven footed animals caused by a single stranded, positive-sense RNA virus belonging to the *Aphthovirus* genus of *Picornaviridae.* VP1 is the most frequently studied protein of FMD virus (FMDV) owing to its significant roles in virus attatchment, protective immunity, and serotype specificity. It is known that the main immunodominant region of FMD virus is present on VP1 protein of FMDV. Important antigenic sites on FMD virus are located on the sequence between amino acids 140 and 160 of VP1. Although several reliable diagnostic tests such as complement fixation test (CFT), enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) already exist; none of them are applicable in the field conditions as they are cumbersome, expensive and require skilled manpower. Hence the present study aimed at utilizing the type specific synthetic viral peptides and conjugated with coloured latex beads in a simple multiplex immunoassay based on DIVA strategy for clear cut differentiation between serotypes by the naked eye.

A total of 200 serum samples were collected from cattle (141) and buffaloes (59). The samples were categorized according to the vaccination status and age of the animal.

Serotype specific sequences and non structural sequence reported in the published literature were used for construction of serotype specific and non structural peptides. Sequences were confirmed to be serotype specific using the bioinformatic tool Basic Local Alignment Search Tool (BLAST). Sequences of the serotype specific regions are given below :

Peptide A: G-S-G-R-R-G-D-M-G-S-L-A-A-R-V-V-K-Q

Peptide O: V-P-N-L-R-G-D-L-Q-V-L-A-Q-K-V-A-R-T

Peptide Asia 1: R-R-K-Q-E-I-I-A-P-E-K-Q-V-L-P-P-S-Q-P-T-R-R-G-D-L-A-V-L-A-Q-R-V-S-N-R

Peptide NSP 2B: R-S-T-P-E-D-L-E-R-A-E-K-Q

Serotype specific peptides of the FMD virus and a non structural peptide (NSP) 2B were commercially synthesized and conjugated to varied coloured latex beads by Bioconcept Labs, pvt ltd ; Gurgaon, Haryana.

LAT was employed for all the samples using peptide FMDV type O, A, Asia 1 and non structural peptide (NSP) 2B. Out of total 200 samples , 22 samples were positive for FMDV type O, 14 for type A, 14 for type Asia 1, 12 for both type O and A, 26 for type O and Asia 1, 4 for A and Asia 1, 14 were positive for all the three serotypes (O, A and Asia 1) and 94 were negative for all the three serotypes. Sixty nine samples showed positive results with NSP 2B peptide.

With MLAT, clumps of different types were seen only with the aid of microscope. However clumps of type A were not clearly visible. Agglutination was enhanced by using anti bovine immunoglobulin.

Thirty five out of 138 samples were identified as positive with a PP value ≥ 48 by DIVA ELISA. Hence the prevalence of FMD infected animals among the tested population as per DIVA-ELISA was 25.36%.

Out of the total 35 samples, 3 samples were positive for antibodies to type O, 2 for type A, 1 for type Asia 1, 3 for both type O and A, 10 for type O and Asia 1, while none was positive for A and Asia, 15 were positive for antibodies to all the three serotypes (O, A and Asia 1) and 1was negative for all the three serotypes by LPB ELISA.

On comparison of LPB ELISA with LAT using peptide, it was found that LPB ELISA was more sensitive than LAT as it could detect more samples positive for different serotypes.

On comparison of DIVA ELISA with NSP Peptide based agglutination, the latter could detect more positive samples than DIVA ELISA.

The following conclusions could be drawn from the present study:-

1. Latex Agglutination is an inexpensive technique that does not require expertise and can be used for penside diagnosis and screening for antibodies to different serotypes of FMDV and to differentiate infected and vaccinated animals.
2. Visible clumping without the aid of microscope was observed with serotype specific and non structural peptides conjugated to latex beads of different colours.
3. On multiplexing, agglutinates of different types were seen only under the microscope. However agglutinates of type A were not clearly visible.
4. Agglutination can be enhanced using anti-bovine immunoglobulin.
5. Sensitivity of Latex agglutination Test was less than LPB ELISA.
6. NSP 2B peptide based agglutination test could detect more samples positive as compared to the commercial DIVA kit.

REFERENCES

Abu Elzein E M E and Crowther J R. 1978. Enzyme-labelled immunosorbent assay techniques in foot-and-mouth disease virus research. *Journal of Hygiene Cambridge* **80**: 391-99.

Ahuja K L, Kumar A and Prasad S and Sharma R. 1986. Incidence and distribution of foot and mouth disease virus types in North-West India (1982-84). *Indian Journal of Animal Sciences* **56**: 503-05.

Ahuja K L, Kumar K and Prasad S. 1996. Incidence and distribution of foot and mouth disease virus types in North-West India (1991-93). *Indian Journal of Virology* **12**: 125-27.

Alonso A, Gomes M P D, Martins M A and Sondahl M S. 1990. Detection of foot-and-mouth disease virus infected-associated antigen antibodies: comparison of the enzyme-linked immunosorbent assay and agar gel immunodiffusion tests.*Preventive Veterinary Medicine* **9:** 223-40.

Ansell D M, Samuel A R, Carpenter W C and Knowles N J. 1994. Genetic relationships between foot-and-mouth disease type Asia 1 viruses. *Epidemiology and Infections* **112**: 213–24.

Arlinghaus R B and Polatnick J. 1969. The isolation of two enzyme-ribonucleic acid complexes involved in the synthesis of foot-and-mouth disease virus ribonucleic acid. *Proceedings of National Academy of Sciences* USA **26:** 821-29.

Azad H M, Srinivasan V A and Crowther J R. 1995. Serological study of type A Indian foot and mouth disease virus isolates. *Acta Virologica* **39**: 193-96.

Bachrach H L. 1968. Foot-and-mouth disease virus. *Annual Review of Microbiology* **22**: 201-44.

Bachrach H L. 1977. Foot-and-mouth disease virus, properties, molecular biology and immunogenicity. Beltsville Symposia in Agricultural Research. **1 :** 3–32.

Balamurugan V, Renji R, Saha S N, Reddy GR, Gopalakrishna S and Suryanarayana V V S. 2003. Protective immune response of the capsid precursor polypeptide (P1) of foot and mouth disease virus type 'O' produced in Pichia pastoris. *Virus Research* **92**: 141–49.

Barnett P V, Ouldridge E J, Rowlands D J, Brown F and Parry N R. 1989. Neutralizing epitopes of type O foot-and-mouth disease virus. I. Identification

and characterization of three functionally independent, conformational sites. *Journal of General Virology* **70 :** 1483-91.

Baxt B and Mason P W. 1995. Foot-and-mouth disease virus undergoes restricted replication in macrophage cell cultures following FC receptor mediated adsorption. *Virology* **207**: 503- 09.

Beard C W and Mason P W. 2000. Genetic determinants of altered virulence of Taiwanese foot-and-mouth disease virus. *Journal of Virology* **71**: 987-91.

Beck E, Forss S, Strebel K, Cattaneo R and Feil G. 1983. Structure of the FMDV translation initiation site and of the structural proteins. *Nucleic Acids Research* **11**: 7873-85.

Bekkum J G, Frenkel H S, Frederiks H H J and Frenkel S. 1959. Observations on the carrier state of cattle exposed to foot-and-mouth disease virus. *Tijdschr Diergeneeskd* **84** : 1159-64.

Belsham G J and Bustock C J. 1988. Studies on the infectivity of foot-and-mouth disease virus RNA using microinjection. *Journal of General Virology* **69**: 265-74.

Belsham G J. 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. *Progress in Biophysics and Molecular Biology* **60** : 41-60.

Belsham G J. 2005. Translation and replication of FMDV RNA. *Current Topics in Microbiology and Immunology* **288**: 43-70.

Berger H G, Straub O C, Ahl R, Tesar M and Marquardt O. 1990. Identification of foot-and mouth disease virus replication in vaccinated cattle by antibodies to non-structural virus proteins. *Vaccine* **8:** 213–16.

Bergmann I E, De Mello P A, Neitzert E, Beck E and Gomes I. 1993. Diagnosis of persistent aphthovirus infection and its differentiation from vaccination response in cattle by use of enzyme-linked immunoelectrotransfer blot analysis with bioengineered non-structural viral antigens. *American Journal of Veterinary Research* **54:** 825–31.

Bergmann I E, Malirat V, Auge de Mello V and Gomes I. 1996. Detection of foot and- mouth disease viral sequences in various fluids and tissues during persistence of the virus in cattle. *American Journal of Veterinary Research* **57:** 134–37.

Bergmann I E, Malirat V, Neitzert E, Beck E, Panizzutti N, Sanchez C and Falczuk A. 2000. Improvement of a serodiagnostic strategy for foot-and-mouth

disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. *Archives Virology* **145:** 473-89.

Bhattacharya S, Pattnaik B and Venkataramanan R. 1996. Development and application of sandwich enzyme linked immunosorbent assay (ELISA) for type identification of foot-and-mouth disease (FMD) virus in direct field materials. *Indian Journal of Animal Sciences* **66**: 1201-09.

Bittle J L, Houghten R A, Alexander H, Shinnick T M, Sutcliffe J G and Lerner R A. 1982. Protection against foot-and-mouth disease virus by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* **298**: 30-33.

Blacksell S D, Lunt R A, Chamnanpood C, Linchongsubongkoch W, Nakarungkul N, Gleeson L J and Megkamol C. 1994. Establishment of a typing enzyme-linked Immunosorbent assay for foot and mouth Disease antigen, using reagents against Viruses endemic in Thailand. *Review on Scientific Technology Office International Epizooites* **13**: 701-09.

Brooksby J B. 1982. Portraits of viruses: Foot-and mouth disease virus. *Intervirology* **18**: 1-23.

Brown F, Newman J F E, Scott J, Porter A, Frisby D, Newton C, Carey N and Fellner P.1974. Poly (C) in animal virus RNAs. *Nature* **251**: 342-44.

Bruderer U, Swam H, Haas B, Visser N, Brocchi E, Grazioli S, Esterhuysen J J, Vosloo W, Forsyth M, Aggarwal N, Cox S, Armstrong R and Anderson J. 2004. Differentiating infection from vaccination in foot-and-mouth-disease: evaluation of an ELISA based on recombinant 3ABC.*Veterinary Microbiology* **14**: 187-97.

Burrows R, Mann J A, Garland A J, Greig A and Goodridge D. 1981. The pathogenesis of natural and simulated natural foot-and-mouth disease infection in cattle. *Journal of Comparative Pathology* **91**: 599- 609.

Capozzo A V, Burke D J, Fox J W, Bergmann I E, La Torre J L and Grigera P R. 2002. Expression of foot and mouth disease virus non-structural polypeptide 3ABC induces histone H3 cleavage in BHK21 cells. *Virus Research* **90**: 91-96.

Carrillo C, Tulman E R, Delhon G, Lu Z, Carreno A, Vagnozzi A, Kutish G F and Rock D L. 2005. Comparative Genomics of Foot-and-Mouth Disease Virus. *Journal of Virology* **79**: 6487–504.

Cavanagh D, Sangar D V, Rowlands D J and Brown F. 1977. Immunogenic and cell attachment sites of foot-and-mouth disease virus: further evidence for their location in a single capsid polypeptide. *Journal of General Virology* **35**: 149-58.

Clarke J B and Spier R E. 1980. Variation in the susceptibility of BHK populations and cloned cell lines to three strains of foot-and-mouth disease virus. *Archives Virology* **63**: 1-9.

Clarke B E, Brown A L, Currey K M, Newton S F, Rowlands D J and Carroll A R. 1987. Potential secondary and tertiary structure in the genomic RNA of foot-and-mouth disease virus. *Nucleic Acids Research* **15**: 7067-79.

Collen T, Dimarchi R and Doel T R. 1991. A T cell epitope in VP1 of foot-and-mouth disease virus is immunodominant for vaccinated cattle. *Journal of Immunology* **146**: 749-55.

Cowan K M and Graves J H. 1966. A third antigenic component associated with foot and- mouth disease infection. *Virology* **66:** 528–40.

Crowther J R and Abu Elzein E M E. 1979. Application of the enzyme linked immunosorbent assays to the detection and identification of foot-and-mouth disease virus. *Journal of Hygiene Cambridge* **83**: 513-19.

Devaney M A, Vakharia V N and Lloyd R E 1988. Leader protein of foot and mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. *Journal of Virology* **62**: 4407-09.

Dhanda M R, Gopalakrishnan V R and Dhillon H S. 1957. Note on the occurrence of atypical strains of foot-and-mouth diseases virus in India. *Indian Journal of Veterinary Science* **27**: 79–84.

DiMarchi R, Brooke G, Gale C, Cracknell V, Doel T and Mowat N. 1986. Protection of cattle against foot-and-mouth disease by a synthetic peptide. *Science* **232**: 639- 41.

Dinka S K, Swaney L M and Mc Vicar J W.1977. Selection of a stable clone of the MVPK-1 foetal porcine kidney cell line for assays of foot-and-mouth disease virus. *Canadian Journal of Microbiology* **23**: 295-99.

Doel T R, gale C, Do Amaral C M C F, Mulcahy G and Dimarchi R. 1990. Heterotypic Protection induced by synthetic peptides corresponding to three serotypes of Foot and Mouth disease Virus. *Journal of Virology* **64** : 2260-64.

Domingo E, Escarmis C, Baronowski E, Ruiz-Jarabo C M, Carrillo E, Sobrino F. 2003. Evolution of foot-and-mouth disease virus. *Virus* **91**: 47-63.

Donaldson A I, Gibson C F, Oliver R, Hamlin C and Kitching R F. 1987. Infection of cattle by airborne foot-and-mouth disease virus: minimal doses with O2 and SAT2 strains. *Research Veterinary Sciences* **43**: 339-46.

Dong-Sheng He, Kang-Ning Li, Xiao-Min Lin, Shao-Rong Lin, Dan-Ping Su and Ming Liao. 2011. Genomic comparison of foot-and-mouth disease virus R strain and its chick-passaged attenuated strain. *Veterinary Microbiology* **150**: 185-90.

Donnell V K, Smitsaart E, Cetra B, Duffy S, Finelli J, Boyle D, Draghi G, Fondevila N and Schudel A A. 1997. Detection of virus infection associated antigen and 3D antibodies in cattle vaccinated against foot-andmouth disease. *Revue scientifique et technique* (International Office of Epizootics), **16:** 833–40.

Donnelly M L, Gani D, Flint M, Monaghan S and Ryan M D 1997. The cleavage activities of aphthovirus and cardiovirus 2A proteins. *Journal of General Virology* **78:** 13- 21.

Ferris N P and Dawson M. 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot and- mouth and swine vesicular diseases. *Veterinary Microbiology* **16:** 201–09.

Ferris N P, Nordengrahn A, Hutchings G H, Paton D J, Kristersson T, Brocchi E, Grazioli S and Merza M. 2010. Development and laboratory validation of a lateral flow device for the detection of serotype SAT 2 foot-and-mouth disease viruses in clinical samples. *Journal of Virological Methods* **163** : 474-76.

Forss S, Strebel K, Beck E and Schaller H. 1984. Nucleotide sequence and genome organization of foot-and-mouth disease virus. *Nucleic Acids Research* **12**: 16.

Francis M J, Fry C M, Rowlands D J, Brown F, Bittle J L, Houghten R A and Lerner R A. 1985. Immunological Priming with Synthetic Peptides of Foot-and-Mouth Disease Virus. *Journal of General Virology* **6**: 2347-54.

Francis M J, Hastings G Z, Syred A D, McGinn B, Brown F and Rowlands D J. 1987. Nonresponsiveness to a foot-and-mouth disease virus peptide overcome by addition of foreign helper T-cell determinants. *Nature* **330**: 168-70

Gebauer F, Torre J C, Gomes I, Mateu M G, Barahona H, Tiraboschi B, Bergmann I, Auge de Mello P and Domingo E. 1988. Rapid selection of genetic and antigenic variants of foot-and- mouth disease virus during persistence in cattle. *Journal of Virology* **62**: 2041-49.

Gella F J, Serraa J and Generx J. 1991. Latex agglutination procedures in immunodiagnosis *Pure and Applied Chemistry* **63**: 1131-34.

Goel A C and Rai A. 1985. Growth curve, plaque assay and inactivation studies of FMD virus subtypes O5, O1 and O6 of Indian origin. *Indian Journal of Comparative Microbiology Immunology and Infectious Diseases* **6**: 16-28.

Goel Y P. 1989. A note on some epizootiological observations on foot and mouth disease outbreak in an organised herd. *Indian Veterinary Medicine Journal* **13**: 127-29.

Grubman M J, Zellner M and Wagner J. 1987. Antigenic comparison of the polypeptides of foot and mouth disease virus serotypes and other picornaviruses. *Virology* **158**: 133-40.

Hamblin C, Armstrong R M and Hedger R S. 1984. Rapid enzyme linked immunosorbent assay for the detection of foot and mouth disease in epithelial tissues. *Veterinary Microbiology* **9**: 435-43.

Hamblin C, Kitching R P, Donaldson A I, Crowther J R and Barnett L T R. 1987. Enzyme linked immunosorbent assay (ELISA) for detection of antibodies against foot and mouth disease virus (FMDV). *Epidemiology and Infections* **99**: 733-44.

Hedger R S and Condy J B. 1985. Transmission of footand-mouth disease from African buffalo virus carriers to bovine. *Veterinary Research* **17**: 205.

House C and House J A. 1989. Evaluation of techniques to demonstrate Foot-and mouth disease virus in bovine tongue epithelium: comparison of the sensitivity of cattle, mice, primary cell cultures, cryopreserved cell cultures and established cell lines. *Veterinary Microbiology* **20**: 99-109.

House J A, House C and Llewellyn M E. 1988. Characteristics of the porcine kidney cell line IB-RS-2 clone D10 (IR-RS-2 D10) which is free of hog cholera virus. *Canadian Journal of Comparative Medicine* **24**: 677-82.

Inoue T, Parida S, Paton D J, Linchongsubongkoch W, Mackay D, Anupomma D, Gubbins S and Saikil T. 2006. Development and evaluation of an indirect enzyme linked immunosorbent assay for detection of foot and mouth disease virus non structural protein antibody using chemically synthesized 2B peptide as antigen. *Journal of Veterinary Diagnostic Investigation* **18** : 545-52.

Islam M A, Rahman M M, Adam K H and Marquardt O. 2001. Epidemiological implications of the molecular characterization of foot-and-mouth disease virus isolated between 1996 and 2000 in Bangladesh. *Virus Genes* **23**: 203–10.

Jackson A L, Neill H, Maree F, Blignaut B, Carrillo C, Rodriguez L and Haydon D T. 2007. Mosaic structure of foot-and-mouth disease virus genomes. *Journal of General Virology* **88** : 487-92

Jiang T, Liang Z, Ren W-w, Chen J, Zhi X-y, Qi G-y, Liu X-t and Cai X-p. 2011. A simple and rapid colloidal gold-based immunochromatogarpic strip test for detection of FMDV serotype A .*Virologica Sinica* **26** : 30-39.

King A M Q, Underwood B O, Mccahon D, Newman J W and Brown F. 1981. Biochemical identification of viruses causing the 1981 outbreak of foot-and-mouth disease in the UK. *Nature* **293**: 479-80.

Kitching R P, Rendle R and Ferris N P. 1988. Rapid correlation between field isolates and vaccine strains of FMD virus. *Vaccine* **6**: 403-08.

Kitching R P. 1999. Foot-and-mouth disease: current world situation. *Vaccine* **17**: 1772- 74.

Klein J**,** Hussain M**,** Ahmad M**,** Normann P**,** Afzal M and Alexandersen S .2007**.** Genetic characterisation of the recent foot-and-mouth disease virus subtype A/IRN/2005. *Virology Journal* **4** : 122.

Knowles N J and Samuel A R. 2003. Molecular epidemiology of foot-and-mouth disease virus. *Virus* **91**: 65-80

Ko Y J, Jeoung H Y and Lee H S *et al*. 2009. A recombinant protein-based ELISA for detecting antibodies to foot-and-mouth disease virus serotype Asia1. *Journal of Virological Methods* **159**(1): 112–18.

Koo C H ,Park Y H, Ahn J, Waters W R, Hamilton M J, Barrington G, Mosaad A A, Palmer M V , Shin S, and Davis W C. 2004. New Latex Bead Agglutination Assay for Differential Diagnosis of Cattle Infected with *Mycobacterium bovis* and *Mycobacterium avium subsp. Paratuberculosis. Clinical and Diagnostic Laboratory Immunology* **11**: 1070-74.

Kurz C, Forss S, Kupper H, Strohmaier K and Schaller H. 1981. Nucleotide sequence and corresponding amino acid sequence of the gene for the major antigen of foot and mouth disease virus. *Nucleic Acids Research* **9**: 1919-23

Kweon C H, Ko Y J, Kim W I, Lee S Y, Nah J J, Lee K N, Sohn H J, Choi K S, Hyun B H, Kang S W, Joo Y S and Lubroth J. 2003. Development of a foot - and-mouth disease NSP ELISA and its comparison with differential diagnostic methods. *Vaccine* **23**: 1409-14.

Laporte J and Lenoir G. 1973. Structural proteins of foot-and-mouth disease virus. *Journal of General Virology* **20**: 161-68.

Latha Priyadharshini M, Balamurugan V, Prabhudas K, Suryanarayana V V S, Reddy G R 2007. Expression of 3AB protein of foot and mouth disease virus in Pichia pastoris. *Indian Journal of Biotechnology* **6**: 329–35.

Lea S, Hernandez J, Blakemore W, Brocchi E, Curry S, Domingo E, Fry E, Abu-Ghazaleh R, King A, Newman J, Stuart D and Mateu M G. 1994. The structure and antigenicity of a type C foot-and-mouth disease virus. *Structure* **2**: 123-39.

Lee F, Lin Y L and Jong M H. 2004. Comparison of ELISA for the detection of porcine serum antibodies to non-structural proteins of foot-and-mouth disease virus. *Journal of Virological Methods* **116**: 155-59.

Loeffler F and Frosch P. 1897. Summarischer bericht uber die ergebnisse der untersuchungen der kommoission zur erforchung der maul-und-klamenseuche, Zentralbl *Bakteriol. Parasitenk. Infektionskr* **22**: 257-59.

Longjam N, Sarmah A K, Deb R, Mathapati B, Saxena V K and Tayo T. 2011. Detection of foot and mouth disease virus serotype O by sandwich ELISA, viral isolation and multiplex PCR. *Online Journal of Veterinary Research* **15**: 76-92.

Lopez M G, Peralta A, Berinstein A, Fondevila N, Carrillo E and Taboga O. 2004. High-level expression of recombinant 3AB1 non-structural protein from FMDV in insect larvae. *Journal of Virological Methods* **124:** 221-24.

Lu Z, Cao Y and Guo J. 2007. Development and validation of a 3ABC indirect ELISA for differentiation of foot-and-mouth disease virus infected from vaccinated animals. *Veterinary Microbiology* **125** : 157–69.

Lubroth J and Brown F. 1995. Identification of native foot-and-mouth disease virus non- structural protein 2C as a serological indicator to differentiate infected from vaccinated livestock. *Research in Veterinary science* **59**: 70-78.

Lubroth J, Grubman M J, Burrage T G, Newma, J F E and Brown F. 1996. Absence of protein 2C from clarified foot-and-mouth disease virus vaccines provides the basis from distinguishing convalescent from vaccinated animals. *Vaccine* **15**: 419–26.

Lunt R A, Linchongsubongkoch W and Gleeson L J. 1994. A modified liquid phase (LP) blocking ELISA used to assess type O foot-and-mouth disease virus antigenic variation in Thailand. *Veterinary Microbiology* **42**: 79-90.

Maan S, Kumar A, Sharma R and Ahuja K L. 1998. Prevalance of foot and mouth disease virus types in North-West India (1994-96). *Indian Journal of Virology* **14**: 55-57.

MacKay D K J, Forsyth M A, Davies, P R and Salt, J S. 1998. Antibody to the non-structural proteins of foot-and-mouth disease virus in vaccinated animals exposed to infection. *Vet Q* **20** : 1-9.

Marquardt O and Freiberg B. 2000. Antigenic variation among foot-and-mouth disease virus type A field isolates of 1997-1999 from Iran. *Veterinary Microbiology* **74**: 377-861.

Mason P W, Grubman M J and Baxt B. 2003. Molecular basis of pathogenesis of FMDV. *Virus Research*. **91**: 9-32.

Mateu M G, Hernandez J, Martinez M A, Feigelstock D, Lea S, Perez J J, Giralt E, Stuart D, Palma E L and Domingo E. 1994. Antigenic heterogeneity of a Foot and Mouth Disease Virus serptype in field is mediated by very limited sequence variation at several antigenic sites. *Journal of Virology* **68**: 1407-17.

Mateu M G , Martínez M A, Capucci L, Andreu D, Giralt E, Sobrino F, Brocchi E and Domingo E. 1990. A single amino acid substitution affects multiple overlapping epitopes in the major antigenic site of foot-and-mouth disease virus of serotype C. *Journal of General Virology* **71** : 629-37.

McCahon D. 1986. The genetics of foot-and-mouth disease virus. *Review on Scientific Technology Office International Epizooites* **5** : 279-97.

McVicar, J W and Sutmoller P. 1976. Growth of foot-and- mouth disease virus in the upper respiratory tract of non-immunized, vaccinated, and recovered cattle after intranasal inoculation. *Journal of Hygiene* **76:** 467–81.

Meloen R H, Rowlands D J and Brown F. 1979. The Individual Structural Polypeptides of Foot-and-Mouth Disease and Polio Viruses. *General Virology* **45**: 761-63.

Meyer R F, Pacciarini M, Hilyard E J, Ferrari S, Vakharia V N, Donini G, Brocchi E and Molitor T W. 1994. Genetic variation of foot-and-mouth disease virus from field outbreaks to laboratory isolation. *Virus Research* **32**: 299-312.

Meyer R F, Babcock G D, Newman J F, Burrage T G, Toohey K, Lubroth J and Brown F. 1997. Baculovirus expressed 2C of foot-and-mouth disease virus has the potential for differentiating convalescent from vaccinated animals. *Journal of Virological Methods* **65**: 33-43.

Mishra N, Mishra S and Bhagwan P S K. 1997. Occurence of an outbreak of FMD in an organised goat farm. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* **18**: 2.

Mishra N, Rai D V and Pattnaik B. 1995. Strain differentiation of foot-and-mouth disease virus type 'Asia1'. *Indian Journal of Animal Science* **65**: 368-75.

Mohanty J, Rai D V, Venkataraman R, Pattnaik B and Mukhopaddhyay A K. 1994. Antigenic spectrum of foot and mouth disease type A vaccine virus n India. *Indian Journal of Animal Sciences* **64**: 1-8.

Moonen P, van der Linde E, Chenard G and Dekker A. 2004. Comparable sensitivity and specificity in three commercially available ELISAs to differentiate between cattle infected with or vaccinated against foot-andmouth disease virus. *Veterinary Microbiology* **99** : 93-101.

Nair S P. 1987. Studies on the susceptibility and growth pattern of foot-and-mouth disease virus vaccine strains in two pig kidney cell lines. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* **8**: 76-81.

Nanni M, Alegre M, Compaired D, Taboga O and Fondevila N. 2005. Novel purification method for recombinant 3AB1 nonstructural protein of foot-and mouth disease virus for use in differentiation between infected and vaccinated animals. *Journal of Veterinary Diagnostic Investigation* **17**: 248–51.

Newman J F, Cartwright B, Doel T R and Brown F. 1979. Purification and identification of the RNA-dependent RNA polymerase of foot-and-mouth disease virus. *Journal of General Virology* **45:** 497-507.

Newman J F, Piatti P G, Ryan M D and Brown F. 1994. Function of minor polypeptides in foot-and-mouth disease virus and poliovirus. *Trends in Microbiology* **2:** 494-97.

Newton S E, Carroll A R, Campbell R O, Clarke B E and Rowlands D J. 1985. The sequence of Foot and Mouth Disease Virus RNA to the 5' side of poly (c) tract. *Gene* **40** : 331-36.

Nunez J I, Blanco E, Hernandez T, Gomez T C, Martin M J , Dopazo J and Sobrino F. 1998. A RT-PCR assay for the differential detection of viral vesicular diseases in swine. *Journal of Virological Methods* **72**: 227-35.

Oem J K, Ferris N P, Lee K N, Joo Y S, Hyun B H and Park J H. 2009. Simple and rapid lateral-flow assay for the detection of foot-and-mouth disease virus. *Clinical and Vaccine Immunology* **16** : 1660-64.

Oem J K, Kye S J, Lee K N, Park J H, Kim Y J, Song H J and Yeh M. 2005. Development of synthetic peptide ELISA based on nonstructural protein 2C of foot and mouth disease virus. *Journal of Veterinary Science* 317-25.

Ouldridge E J, Barnett P V, Hingley P J, Head M and Rweyemamu M M. 1984. The differentiation of foot-and-mouth disease virus strains using an indirect

sandwich enzyme-linked immunosorbent assay saturation model. *Journal of Biological Standardization* **12**: 367-77.

Ouldridge E J, Barnett P and Rweyemamu M M. 1982. The relative efficiency of two ELISA techniques for the tritration of FMD antigen. *Current Topics in Veterinary Medicine and Animal Science* **22**: 142-51.

Parry N, Fox G, Rowlands D, Brown F, Fry E, Acharya R, Logan D and Stuart D. 1990. Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. *Nature* **347**: 569 – 72.

Pattnaik B, Rai, D V and Venkataramanan R. 1991. Specificity of foot-and-mouth disease virus antigen and antibody reaction in liquid phase ELISA. *Indian Journal of Animal Sciences* **61**: 235-40

Pattnaik B and Venkataramanan R. 1989a. Indirect enzyme linked immunosorbent assay for the detection of FMDV antigen. *Indian Journal of Animal Science*s **59**: 317-22.

Pattnaik B and Venkataramanan R. 1989b. A sandwich enzyme linked immunosorbent assay for subtype analysis of foot-and-mouth disease virus isolate. *Indian Journal of Animal Sciences* **59**: 1363-68.

Petrov V N, Rybakov S S, Petrova O N, Lugovskol A A and Pavlov A V. 1996. The use of synthetic peptides for detection of antibodies against foot-and-mouth disease virus in the blood from convalescent animals. *Molekuliarnaia Genetika Microbiologia Virusologia* **3**: 27-31.

Pfaff E, Thiel H J, Beck E, Strohmaier K and Schaller H. 1988. Analysis of neutralizing epitopes of foot-and-mouth disease virus. *Journal of Virology*. **62**: 2033-40.

Pinto A A and Garland A J M. 1979. Immune response to virus-infectionassociated (VIA) antigen in cattle repeatedly vaccinated with foot-andmouth disease virus inactivated by formalin or acetylethyleneimine. *Journal of Hygiene* **82**: 41-50.

Polatnick J and Arlinghaus R B. 1967. Foot-and-mouth disease virus-induced ribonucleic acid polymerase in baby hamster kidney cells. *Virology* **31**: 601-08.

Prasad S, Goel M C, Sharma V K, Tewari S C, Dogra S C, Pandey R and Ramakant. 1978. A note on the incidence and distribution of foot and mouth disease virus types in North –West region of India. *Indian Journal of Animal Science* **48**: 627-2.

Prasad S, Kumar A, Ahuja K L and Sarma R. 1992. Prevalence of foot and mouth disease virus types in north West India (1985-87). *Indian Journal of Virology* **8**: 49-52.

Rai A and Lahiri D K. 1981. A micro- enzyme linked labeled immunosorbent assay (Micro-ELISA) for the detection of foot-and-mouth disease virus antigen and antibody. *Acta Virologica* **25**: 49-52.

Reddy G R, Tratschin J D, Natarajan C and Suryanarayana V V S. 1999. Capsid protein encoding (P1) sequence of Foot and Mouth Disease Virus Type Asia 1 Ind 63/72. *Virus Genes* **18** : 161-68.

Reid S M, Ferris N P, Bruning A, Hutchings G H, Kowalska Z, and Akerbolm L. 2001. Development of a rapid chromatographic strip test for the pen-side detection of foot-and-mouth disease virus antigen. *Journal of Virological Methods* **96**: 189-202.

Renji R, Balamurugan V, Saha S N, Reddy G R, Suryanarayana V V S. 2003. Expression of the Capsid precursor polypeptide (P1) of foot and mouth disease virus type 'O' in Pichia pastoris. *Indian Journal of Biotechnology* **2**: 533–38.

Revenson S and Segura M. 1963. Multiplication of foot-and-mouth disease virus in roller tube culture BHK-21 cells of hamster kidney. *Rev. Invest. Canad* **18**: 293-99.

Rodrigo M J and Dopazo J. 1994. Evolutionary analysis of picornavirus family, *Journal of Molecular Evolution* **39** : 3662-71.

Roeder L P and Le Blanc Smith P M. 1987. Detection and typing of foot-and-mouth disease virus by enzyme linked immunosorbent assay: a sensitive,rapid and reliable technique for primary diagnosis. *Research in Veterinary Science* **43**: 225-32.

Rowlands D J, Clarke B E, Carroll A R, Brown F and Houghten R A .1983. Chemical basis of antigenic variation in foot-and-mouth disease virus. *Nature* **306**: 694-97.

Rowlands D J, Sangar D V and Brown F. 1974. A comparative chemical and serological study of the full and empty particles of foot-and-mouth disease virus. *Journal of General Virology* **26 :** 227-38.

Ryan M D, King A M and Thomas G P. 1991. Cleavage of foot-and-mouth disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. *Journal of General Virology* **72:** 2727-32.

Saini S S, Sharma J K, Kwatra M S and Singh P. 1991. Epidemiological Observations on Foot and Mouth Disease in Cattle and Buffaloes in Punjab during 1988-89. *Indian Journal of Comparative Immunology, Microbiology & Infectious Diseases* **12**: 1-4.

Salt J S. 1993. The carrier state in foot and mouth disease virus. An immunological review. *Veterinary Journal* **149** : 207-23.

Sangar D V, Rowlands D J, Harris T J R and Brown F. 1977. A protein covalently linked to foot and mouth disease virus RNA. *Nature* **268**: 648-50.

Sangar D V. 1979. The replication of picornaviruses. *Journal of General virology* **45**: 1-13.

Sanyal A, Mohapatra J K, Kumar R M, Biswas S, Hemadri D, Tosh C, Sabarinath G P, Gupta S K, Mittal M, Giridharan P and Bandyopadhyay S K. 2004. Complete nucleotide sequence analysis of a vaccine strain and a field isolate of foot and-mouth disease virus serotype asia1 with an insertion in vp1 genomic region. *Acta Virologica* **48**: 159-66.

Saravanan T, Kumar C A, Reddy G R, Dechamma H J, Nagarajan G, Ravikumar P, Srinivas G and Suryanarayana V V S. 2011. Construction of genome-length cDNA for foot and mouth disease virus serotype Asia 1 IND 63/72 vaccine strain. *International Journal for Biotechnology and Molecular Biology Research* **2**: 39-45.

Sarma D K, Barman N N, Das S K, Dutta P K and Boro B R. 1991. Foot and mouth disease in north easteren parts of india in 1988-1990. *Virus–Information-Exchange-Newsletter-For South-East-Asia ans the western-Pacific* **8**: 128.

Sarma D K and Hazarika A K. 1996. Foot and mouth disease in organised cattle farms of the North East states of India. *Journal of Assam Veterinary Council* **6**: 51-52.

Saxena R. 1995. Incidence of foot and mouth disease in India. *Indian Journal of Dairy Science* **48**: 366-73.

Schumann K R, Knowles N J, Davies P R, Midgley R J, Valarcher J F and Raoufi A Q. 2008. Genetic characterization and molecular epidemiology of foot-and-mouth disease viruses isolated from Afghanistan in 2003–2005. *Virus Genes* **36**: 401–13.

Sellers R F. 1955. Growth and titration of the viruses of foot-and-mouth disease and vesicular stomatitis in kidney monolayer tissue cultures. *Nature* **176**: 121-24.

Sellers R F. 1955. Growth and titration of the viruses of foot-and-mouth disease andvesicular stomatitis in kidney monolayer tissue cultures. *Nature* **176**: 121-24.

Sellers R F. 1971. Quantitative aspects of the spread of foot-and-mouth disease. *Veterinary Bulletin* **41:** 431-39.

Sharma S K and Singh G R. 1993. Cyclic bahaviour of foot and mouth disesse in India. *Veterinary Record* **133**: 448-50.

Silberstein, E Kaplan, G Taboga, O Duffy S and Palma E. 1997. Footand- mouth disease virus-infected but not vaccinated cattle develop antibodies against recombinant 3AB1 nonstructural protein. *Archives Virology* **142:** 795–805.

Singh K B and Singh P. 1998. Present status and recommendations for control of foot and mouth disease (Apthous Fever). *Punjab Veterinarian* **6**: 19-20.

Snowden W A. 1966. Growth of foot-and-mouth disease virus in monolayer cultures of calf thyroid cells. *Nature* **210**: 1079-80.

Snowden W A. 1968. The susceptibility of some Australian fauna to infection with foot-and- mouth disease virus. *Australian Journal of Experimental Biology & Medical Science* **46**: 667-87.

Sobrino F, Martinez MA, Carrillo C and Beck E. 1989. Antigenic variation of foot-and- mouth disease virus of serotype C during propagation in the field is mainly restricted to only one structural protein (VP1). *Virus Research* **14**: 273-80.

Sorensen K J, Madsen K G, Madsen E.S., Salt J.S., Nqindi J. and Mackay D K. 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. *Archives Virology* **143:** 1461–76.

Stram Y, Molad T, Chai D, Gelman B and Yadin H. 1995. Detection and subtyping of foot-and-mouth disease virus in infected cattle by polymerase chain reaction and amplified VP1 sequencing. *Journal of Veterinary Diagnostic Investigation* **7**: 52-55.

Strohmaier K, Franze R and Adam K H. 1982. Location and characterization of the antigenic portion of the FMDV immunizing protein. *Journal of General Virology* **59** : 295-306.

Sumption K, Rweyemamu M and Wint W. 2008. Incidence and distribution of foot-and- mouth disease in Asia, Africa and South America; combining expert

opinion, official disease information and livestock populations to assist risk assessment. *Transboundary Emerging Diseases* **55**: 5–13.

Suryanarayana V V S , Viswanathan S, Ratish G, Bist P, Prabhudas K, Gajendragad M R and Natarajan C. 1999. E. coli expressed proteins as diagnostic reagents for typing of foot-and-mouth disease virus. *Archives Virology* **144** : 1701-12.

Sutmoller P and Gaggero A. 1965. Foot-and-mouth disease carriers. *Veterinary Research* **77**: 968-69.

Tewari S C, Singh I P, Sharma R, Pandey V K and Prasad S. 1975. A note on the incidence and distribution of foot and mouth disease virus types in North-West India. *Indian Journal of Animal Sciences* **45**: 159-60

Tulasiram P, Tyagi M Srinivas K, Prabhudas K, Nataranjan C and Suryanarayanana Y. 2006. Antigenic variation in Foot and Mouth Disease Virus Type Asia 1 isolates circulated during 1993-95 in India. *Virus genes* **15**: 247-53.

Valarcher J F, Leforban Y, Rweyemamu M, Roeder P L, Gerbier G and Mackay D K J. 2008. Incursions of foot-and-mouth disease virus into Europe between 1985 and 2006. *Transboundary Emerging Diseases* **55**: 14–34.

Volpina O M , Surovoy A Y, Zhmak M N, Kuprianova M A, Koroev D O, Chepurkin A V Toloknov A S and Ivanov V T. 1999. A peptide construct containing B-cell and T-cell epitopes from the foot-and-mouth disease viral VP_1 protein induces efficient antiviral protection *Vaccine* **17** : 577-84

Waldmann O and Trautwein K. 1926. Experimentelle Untersuchungen ueber diePluralitet des Maul-und Klauenseuche Virus. *Berl Iterarztl Wschr* **42**: 569-71.

Woodbury R L. 1995. A review of the possible mechanisms for the persistence of foot-and- mouth disease virus. *Epidemiology and Infections* **114**: 1-13.

Yap K L. 1994. Development of a slide latex agglutination test for rotavirus antigen etection. *Malaysian Journal of Pathology* **16**: 49 – 56.

Yilma T. 1980. Morphogenesis of vesiculation in foot-and-mouth disease. *American Journal of Veterinary Research* **41**: 1537-42.

Zibert A, Maas G, Strebel K, Falk M M and Beck E. 1990. Infectious foot-and-mouth disease virus derived from a cloned full-length cDNA. *Journal of Virology* **64**: 2467-73.

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