REVIEW OF LITERATURE

Even after a century of its discovery of the virus, FMD is still persisting as a global menace. Worldwide eradication should be undertaken because the existence of the disease anywhere in the world puts any country at risk. Many international regulations have been designed which impose embargo on import of animals; their meat and milk products from endemic countries to minimize the risk of disease introduction into FMD free countries (Brown, 1999). FMD in India is endemic but slaughter is not feasible, so, the only practical alternative is to go in for regular vaccination. Due to the existence of several serotypes, polyvalent vaccine containing antigens of all circulating serotypes is used to confer protection. However, the existence of carrier animals poses a constant threat of fresh outbreaks. Consequently, elimination of these carriers constitutes an important element in any endeavour to eradicate the disease.

2.1. DISEASE

Foot and mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed wild and domesticated animals (Bachrach, 1968). High morbidity and low mortality characterize the disease with clinical signs in typical cases being vesicular lesions in the mouth, especially on tongue, occasionally on the nose (in pigs), muzzle, interdigital space, on the coronary band of the foot and on the teats in female animals. Among the domesticated species, cattle and swine are the most important target species susceptible to FMD (Pereira, 1981).
In addition, many species of cloven-hoofed wildlife such as deer, antelope, wild pigs and African buffalo may become infected. It causes mortality in young animals due to myocarditis and in adult animals the infection results in loss of milk production due to the involvement of mammary epithelial cells, reduction in draught power due to lameness, repeat breeding and abortions in pregnant animals due to pituitary involvement and acute infection. The productivity losses of affected animals may be as high as 25% in terms of milk yield, meat production and drought power.
Fig. 1. Foot and mouth disease virus genome and its proteolytic processing events.
2.2. ETIOLOGICAL AGENT

It was the first animal disease demonstrated to be caused by a filterable agent in 1897 by Loeffler and Frosch. The agent later has been identified as an RNA virus (FMDV), belonging to the genus *apthovirus* of the family *picornaviridae* (Franki, *et al.*, 1991). The virion is a non-enveloped icosahedron of 25-30 nm in diameter with a molecular weight of $8.6 \times 10^6$ dalton, and its buoyant density in CsCl is 1.43-1.45 g/cm$^3$. The purified whole virus particle has sedimentation co-efficient of 146S. It has been found that viral particle could be disintegrated, with loss of infectivity, by aging, mild heat treatment or exposure to acidic pH of 6.5 or lower.

2.3. GENOME ORGANIZATION AND ASSEMBLY

FMDV genome consists of a linear, single stranded, positive sense RNA of 8.5kb. The RNA is uncapped, however, virus coded protein VPg (3 kDa) is covalently bound to it at 5' end and 3' end has a poly A tail. The genome (Fig. 1) has a single open reading frame (ORF) flanked on either ends by untranslated regions (UTRs). The 5' UTR of 1300 nts comprises of an S fragment (400 nts) followed by poly C tract (80 - 200 nts) (Harris and Brown 1977, De la Torre *et al.*, 1988), a L segment of 700 bases which form a number of highly conserved secondary structures including tandemly repeated pseudoknots (PKs), a *cis-*acting element involved in RNA replication (the *cis*-acting replication element, *cre*) and a 450bp structure involved in initiation of translation ( Internal Ribosomal Entry Site, IRES). It has a major role in initiation of viral translation in a cap independent fashion.
(Belsham, 1993). The cre consists of a highly conserved loop containing the consensus AAACA and nestles between the PKs and the IRES. Preliminary data have shown that alteration of the conserved AAACA motif in this loop abrogates genome replication, but does not significantly affect RNA translation, suggesting that this stem loop is the FMDV cre. The single viral ORF is of 6996 nts, has two initiation sites both in frame with strong initiation from the downstream start codon and codes for a polyprotein (Fross, et al., 1984). Following the ORF is a short 3’ UTR (190 nts) and a poly A tract (10 - 100 nts) (Chatarjee, et al., 1976). The 3’ UTR is believed to contain major cis-acting specific sequence signal required for negative-strand RNA synthesis, essential to complete a full replication cycle (Saiz, et al., 2001). The single ORF consists of 3 polyprotein-coding regions (Fig. 1), from the 5’ end to the 3’ end, P1, P2, and P3. The P1 region consists of genes coding for 1A, 1B, 1C, 1D viral structural protein (Rueckert and Wimmer, 1985). P2 and P3 regions encode non-structural proteins 2A, 2B, 2C and 3A, 3B, 3C, 3D, respectively. 2C is probably involved in virion RNA synthesis. 3B encodes VPg (Fross and Schaller, 1982) and protein 3D functions as the RNA dependent RNA polymerase, also known as the virus infection associated antigen (VIAA) (Polatnick, 1980).

The mature virion is an icosahedran consisting of a capsid in which RNA is packed. The capsid is composed of 60 copies each of the four structural proteins [VP1 (213aa), VP2 (218aa), VP3 (220aa) and VP4 [85aa], which are derived from the processing of the polyprotein
VP1, VP2 and VP3 proteins are partly exposed on the capsid surface whereas VP4 is internal and is myristylated at the n-terminus (Madshus, et al., 1984; Chow, et al., 1987).

### 2.3.1. Sub-Viral Particles

The intact virus particle has mostly matured VP1- VP4 and a small amount of unprocessed VP0 (VP4 + VP2) with the VPg attached to RNA sediments at 146S. Natural empty particles have no RNA and have more VP0 and sediment at 75S. The 12S pentamer units are derived from intact virus particle below pH 7.0 consist of VP2, VP3 and VP1. The protomer has a single copy of each of VP0, VP3 and VP1 and sediments as 5S (Rowlands, et al., 1975).

### 2.4. VIRUS REPLICATION AND PROTEIN PROCESSING

FMDV receptor belongs to an integrin family of proteins to which, a conserved Arg-Gly-Asp (R-G-D) motif present in the major antigenic domain (145 to 147 aa) of VP1 binds (Fox, et al., 1989). Following adsorption, penetration and intracellular uncoating, the positive sense viral RNA, is translated into a polyprotein, which on cleavage by viral protease yields four primary products namely L (20kDa), P1-2A (88kDa), P2 (56kDa) and P3 (100kDa) (Sanger, et al., 1977). Since, the RNA has two initiation codons in the same reading frame, two proteins differing in their amino terminal sequence with a common carboxy terminal are produced (Belsham, 1992, Beck et al., 1983). The first cleavage occurs at L/P1 junction resulting in the production of either smaller L protein (Lb) or larger protein (Lab) both having similar functions (Medina, et al., 1993). The leader protease, L\textsuperscript{pro}, is an active
protease and cleaves itself at its carboxy terminus from amino terminus of the P1 precursor (Strebel et al., 1986) and is responsible for cleavage of eIF4G resulting in shut-off of host protein synthesis. The second cleavage between P1 and P2 occurs at 2A/2B junction, which is mediated by 2A 'cleaving' at its own C terminus in a process of putative ribosomal 'skip' (Donnelly, et al., 2001a, b). This does not involve L or 3C (Clarke and Sanger, 1988, Ryan, et al., 1989). All the other cleavages including the cleavage between the structural proteins are performed by 3C protease. The final cleavage, which occurs after encapsidation, at 1A/1B (VP4-VP2 also called as VP0) junction is still an enigma (Belsham, 1993). Cleavage (autocatalytic) of L protein is considered responsible for the exposure of N-terminus of VP4 for myristoylation that is needed for the proper assembly of capsomers into the icosahedral capsid. The published sequences of FMDV carry the myristoylation site at the N-terminal end of VP4 (Gly-x-x-x-Ser/Thr where x is any aa). The empty capsids have 1AB, 1C and 1D and 1A/1B cleavage occurs after encapsidation.

2.5. ANTIGENICITY OF FMDV

Four virus-specified antigens are found in the harvests of virus grown in tissue culture: (a) the infectious virus particle, (b) the so-called empty particle, (c) the 12S protein sub-unit, and (d) the virus infection-associated (VIA) antigen. The four antigens can readily be separated and the fractionation studies have shown that most of the neutralising activity is elicited by the intact virus particles. However, the empty particles also elicit neutralising antibody, provided they are
stabilised by the cross-linking agent (Rowlands, et al., 1975). In contrast, the 12S protein sub-unit elicits very low levels of neutralising antibody and the VIA antigen is devoid of this activity (Brown, 1992). The capsid protein VP1 is the most exposed polypeptide located on the capsid surface. It is highly polymorphic and contains several of the major immunogenic sites important for effective antibody neutralisation and subsequent viral clearance by the immune system. Immunological studies with FMDV serotype ‘C’ led to the discovery of topologically independent antigenic sites viz., site a, located within the G-H loop of VP1; site c, located at the C-terminal end of VP1 and site d, which involves segments of VP1, VP2 and VP3 (Mateu, et al., 1989; 1990; Lea, et al., 1994). Residues 138 - 158 of VP1 (G-H loop) has been identified as the major immunodominant region and the peptides incorporating these sequences were able to induce high level of neutralising antibodies. Other epitopes on the capsid proteins VP1, VP2 and VP3 were also identified. The core of each protein (VP1-3) is an 8-stranded β barrel, similar in tertiary structure (Acharya, et al., 1989). A tandem peptide containing the T-cell peptide 3A [21aa-38aa] and the B cell antigenic site VP1 [137aa-156aa] efficiently stimulated lymphocytes from infected animals in vitro (Blanco et al., 2001).

2.6. MOLECULAR BASIS OF PATHOGENICITY

FMDV is an explosive cytolytic virus, which has a short replication cycle of about 2 to 3 hrs from the stage of virus entry to release. The host machinery is paralyzed in several ways; many of these
mechanisms are still unknown. FMDV coded proteases are involved in most of the changes in the host cell (Porter, 1993; Dougherty and Semler, 1993 and Salas and Domingo, 1995). Predominant among the known activities are the rapid inhibition of host cell protein synthesis by cleavage of eIF4g, a component of cap binding complex eIF4F by L protease activity (Devaney, et al., 1988). Recent studies indicated that eIF4g is also cleaved by 3C protease at a site that is different from that of L protease. eIF4G binds mainly to the large y-shaped stem loop 4 (2 sub domains) of RNA structure in the 3' region of internal ribosome entry site (IRES) element (Saleh, et al., 2001). 3C was also shown to cleave at the N-terminus of eIF4A of the eIF4F complex (Belsham, et al., 2000). The consensus sequences of the cleavage sites indicated the least conserved nature of these sites for 3C suggesting the involvement of extensive processing activity on the host system by this protease. 3C has been shown to affect transcription and histone H3 processing (Tesar and Marquardt, 1990; Falk, et al., 1990). The amino acid substitution of q [44]→r in 3a, either alone or in combination with the replacement I [248]→T in 2c, / l [147]→P in G-H loop of vp1 capsid protein, was sufficient to give FMDV the ability to produce lesions in guinea pigs (Nunez, et al., 2001).

2.7. CAPSID STABILITY

2.7.1. Effect of pH

FMDV is relatively unstable particularly with respect to deviations in pH and temperature from the optimal. Various reports have shown that the stability of 146S particle is adversely affected by sub-optimal
temperatures and pH, the extent of which varies with the serotype. Strain C, noville, is more acid labile than A or O. reports for type C indicated that the infectivity was reduced at pH 8.5; drastic reduction at pH 7.5 and loss of integrity of capsid at pH 7.0. Efforts were made to produce acid resistant strains of FMDV to increase shelf life of capsids produced in insect cells, which were grown at acidic conditions. Twomey et al., (1995) produced mutant acid resistant viruses, which were found to be 100 fold less infectious in suckling mice compared to BHK 21 cells. The sequence showed three effective amino acid substitutions; one in VP1 and two in VP2, producing virions of 24.7± 2 nm in diameter. This finding has significant impact in basic studies as well as applied aspect in vaccine production.

2.7.2. Effect of temperature

Thermal stability of the virus with respect to particle integrity varies with serotype. Type A and Asia 1 are most stable, O and C have intermediate stability and SAT serotypes are least stable. However, the effect of temperature on viability is different among different viruses. The Asia 1 virus genome is less stable at 37°C compared to type O (Razdan, et al., 1996). Immunogenicity and thermal stability seem to be well correlated. Natural 75S particles appear to be less thermo-stable compared to 146s particles, and the immunogenicity of the former is also comparatively lower (Doel and Baccarini, 1981, Doel and Chong, 1982). Glutaraldehyde or formaldehyde fixation enhanced thermal stability in some strains (Doel and Baccarini, 1981) but it drastically reduced the 146S content of five out of six SAT serotypes
and their stability on storage at 4°C (Rweyemamu, et al., 1989) when compared with aziridine fixatives. Though formaldehyde or glutaraldehyde is simpler, the chemistry of these solutions is complex and variable. Therefore a better approach will be to use modern protein cross linking agents that would cross-link at specific sites at precise inter-atomic distances in the light of x-ray crystallographic studies on FMDV (Logan, et al., 1993).

2.8. CHALLENGES IN FMD ERADICATION

2.8.1. Antigenic variation in FMDV

FMDV was the first virus for which antigenic diversity was recognized, with seven different serotypes namely A, O, C, Asia 1, SAT 1, SAT 2 and SAT3, more than 65 subtypes and many antigenic variants. India harboured four of the seven serotypes A, O, C and Asia 1. Vallee and Carre, (1922), in France, established experimentally, for the first time, the existence of difference in serotypes between strains. The serotypes from this experiment were named ‘O’ (from the department of Oise) and ‘A’ (Allemagne). Shortly afterwards, serotype ‘C’ (Waldmann and Trautwein, 1926) and later SAT-1, SAT-2, SAT-3 and Asia 1 were identified (Galloway et al., 1948, Brooksby and Rogers, 1957). Within each serotype there is extensive intratypic variation.

Based on homology between the RNA s, the viruses were divided into two groups, viz.: group a the three European serotypes A, O and C and single Asian type Asia 1 serotypes and group b] the south african
territory types sat 1, 2 and 3. Homology between the RNAs of two groups is only 25% to 40% but it is 65% between viruses of the same group (Robson, et al., 1977). Frequent emergence of antigenically variant viruses is increasingly recognized as one of the main problems for the design of an effective vaccine (Mateu, et al., 1994). As a consequence of this antigenic complexity, control of the disease by vaccination has become difficult (Kitching, et al., 1988). FMDV is prone to unpredictable shifts/drifts of the genomic distribution contributory to the multitudes of antigenic variants that often co-circulate in a given geographical area (Mateu, et al., 1988). This has led to extreme genetic heterogeneity or the quasi-species concept (Domingo, et al., 1980), leading to a persistence status in the animals, the mechanism of which is yet to be understood.

2.8.2. Persistence

The asymptomatic infection in cattle, buffaloes, sheep and goats, may be the outcome of an acute infection established by pharyngeal / nasal exposure to the virus, or as a result of immunization with some live attenuated vaccine (Sutmoller and Gaggero, 1965). Established features of FMDV persistence uncovered by research include the facts that the virus can be recovered from cattle for over 2 years, the duration being influenced by the strain of the virus; virus multiplies to a low titre in the epithelial cells of the soft palate and pharynx (Brooksby, 1982). Ruminants carry the virus following exposure and the virus can persist in the pharynx for up to 2 years (Hargreaves, 1994). The virus persistence and carrier status in convalescent and
recovered animals makes them main reservoirs though it is extremely
difficult to demonstrate transmission from one animal to another
under controlled conditions, only circumstantial evidence suggests
involvement of persistently infected animals in the spread of the
disease (Salt, 1993). Sutmoller and Gaggero, (1965) stated that
carriers cannot be cured of the disease by vaccination but persistence
may be established in vaccinated cattle. Doel, et al., (1994) suggested
that the antibodies against antigenically conserved nonstructural
proteins like RNA polymerase reduce and prevent carrier status. Viral
persistence may be responsible for mixed infections observed in the
animals (Gajendragad, et al., 1999). Though mechanism of persistence
is obscure, carriers pose the gravest problem, in their ability to select
antigenically variant viral sub-populations (Gebauer, et al., 1988).
This aspect constitutes a major drawback in the control of FMD.

2.9. DIAGNOSIS

The effective control of the disease in endemic countries needs
better vaccines and the strong support of effective diagnostic tests,
which can detect both type and strain variation. Detection of FMDV
was previously done by isolation of virus either in host animals
(Skinner and Knight, 1964) or in tissue culture (Snowdon, 1966;
House and Yedloutsching, 1982), Later complement fixation test was
developed to detect viral antigens in tissues (Casey, 1965). Off late,
enzyme linked immunosorbent assay is used for typing of the virus
( Abu elzein and Crowther, 1978).
With the upsurge of recombinant DNA technology and PCR methodologies new vistas opened in diagnostic virology. The PCR-amplification of viral nucleic acid (Rodriguez and Schudel, 1993, Locher et al., 1995, Suryanarayana, et al., 1999), development of specific nucleic acid probes (Mcfarlane, et al., 1990), and RT PCR - oligonucleotide probing ELISA (Alexandersen, et al., 2000), are some of the tests, which are noteworthy.

Though nucleic acid based techniques are sensitive, their application at the field level is still limited. Subsequently recombinant proteins have found their way in diagnosis. Detection of antibodies against viral replicase or other infection associated recombinant antigens (via) have been tried (Berger, et al., 1990).

The diagnosis of FMD was carried out previously by CFT, however the trend changed and ELISA is considered as the test of choice but modern approaches like immune biosensor (Gajendragad, et al., 2001) may be attractive prospects. Similarly recombinant proteins of non-structural genes are being used to differentiate the vaccinated from the infected animals (Priyadharshini, 2001).

2.10. CONTROL

Countries, which are committed to FMD control usually, take up virus eradication from the territory as their final goal. The countries in entire Western Europe and parts of South America followed this strategy and eradicated FMD. The benefits of FMD control and eradication results in improvement of livestock health and productivity, which in turn will enhance national economy by
increasing opportunities for trade. These benefits are dependent upon the efficiency of the measures taken not only to control and eradicate the disease, but also to prevent its re-introduction. Central to this are the diagnostic methods applied, active epidemiological surveillance, and control on the movement of animals, vaccination programs and monitoring of vaccine efficiency.

Many international regulations have been designed which impose embargo on import of animals; their meat and milk products from endemic countries to minimize the risk of disease introduction into FMD free countries (Brown, 1999). FMD is endemic in India. Hence slaughter is not feasible. The only practical alternative is to go in for regular vaccination, as vaccines against FMD are effective means for controlling outbreaks of the disease (Dus santos, et al., 2000). Due to the existence of different serotypes, polyvalent vaccine containing antigens of all circulating serotypes is used to confer protection.

**2.11. VACCINES AGAINST FMD**

Attempts to develop FMD vaccines started in the early years of the 20th century when Belin (1927) described his experiments with attenuation of the virus. Later researchers also worked on attenuated FMD vaccines, but major problems were encountered such as unpredictable virulence in the field. Effectively, this undermined any belief that a safe and stable attenuated product could be realized within a reasonable time frame. Further, its use would most probably complicate the discrimination of naturally infected and vaccinated
animals with the added potential problem of attenuated vaccine virus spreading to non-vaccinated livestock.

**2.11.1. Conventional Vaccines**

The first experimental vaccine against FMD was made by Valle, Carre 1925 using formaldehyde inactivated tongue tissue from infected cattle (Brown, 1999). It was not before 1937, when Waldmann et al., 1937 produced the first practical inactivated vaccine using virus from the epithelium and vesicular fluid of tongues of deliberately infected cattle. Frenkel’s (1947) approach of using formalin killed FMDV produced in bovine tongue epithelial suspension as vaccine has marked the era of control of FMD by vaccination. This is now replaced by mass production of virus in BHK 21 suspension cells and inactivation of the virus by formalin or aziridine compounds (BEI) and adjuvantation with either aluminum hydroxide gel and saponin for cattle or with oil (Gomes, et al., 1980) for swine and cattle. The duration of protection is prolonged in formalin treated (Fish, et al, 1969) and in oil adjuvanted vaccines (Gomes, et al., 1980). Quality of immune response is poor for BEI inactivated viruses as compared to that for formalin inactivated one, probably due to stabilization of the antigenic structure by formaldehyde (Rowlands, et al., 1975). Since the inactivated viral vaccine does not provide long lasting immunity in animals, it necessitates a biannual vaccination schedule. Though plagued with many limitations, *viz.*, improper inactivation leading to vaccine related outbreaks as reported by King, *et al* (1981).
2.11.2. Subunit Vaccines


2.11.3. Synthetic Peptide Vaccines

Numerous studies carried out with respect to synthetic peptides coding for the immunogenic regions deduced by various methods including monoclonal antibodies lead to the elucidation of T and B cell epitopes which in turn provided a better understanding of humoral and cellular immune responses to FMDV. The results
predicted that VP1 residues 21 - 40 correspond to T cell epitopes and offer cross protection to serotypes; VP1 residues 141 - 156 protect homologous virus strains and residues 200 - 213 offer cross protection for homologous and heterologous viruses. Synthetic peptides corresponding to C-terminal region of VP1 (147-160 and 200-213 aa) that could elicit high neutralizing antibody responses were tried as vaccines in guinea pigs (Strohaimer, et al., 1982). A new peptide construct, consisting of a virus specific T- helper epitope within the 170-188 sequence of VP1, in addition to the main antigenic 135-158 region of FMDV A₂₂ had, reportedly, elicited a higher protective, antigenic, immunogenic and T- cell proliferative activity (Volpina et.al., 1999).

2.11.4. Empty Capsid Vaccines

It has been shown that empty capsids of FMDV retain the antigenicity (Grubman, et al., 1985) and immunogenicity (Rowlands, et al., 1975) of infectious virus particles. It was also reported that the immunogenicity was superior as the antibodies produced are not only against sequential epitopes but also against conformational epitopes and hence may represent an efficient and safe alternative vaccine candidate. Production of empty capsids from whole virus is cumbersome and not practical. However, this can be achieved by gene cloning technology. Several host-vector systems are available for the production of empty capsids. Bacterial, insect and mammalian cell systems were tried for expressing P1 region of FMDV. Bacterial system has its limitation in that myristoylation, which is needed for capsid
assembly, does not occur and hence the stability is affected. Insect cell culture is done under acidic conditions, which are not only detrimental to capsid stability (Kumar, 2000), but also requires sophisticated infrastructure adding to the cost of bulk vaccine production. The yeast expression system combines the advantages of both the eukaryotic and prokaryotic expression systems. Yeast is faster, easier and less expensive to grow besides facilitating processing, modification and expression of proteins in a secretary form, which eases purification of the desired protein (Hollenberg and Gellissen, 1997). Empty capsids have been produced through yeast expression system for FMDV serotype Asia 1 (Renji, 2001) and for serotypes O, C, A22 (Balamurugan, 2002) that were found to be immunogenic in limited experiment animal studies. However, this system has not yet been fully exploited for the production of FMD vaccines.

2.11.5. DNA Vaccines

Wolff et al., (1990) determined that plasmid DNA encoding marker genes could be expressed following intramuscular injection in mice and made preliminary observations leading to the development of the concept of genetic vaccination. Subsequently, the generation of an immune response to marker proteins encoded by plasmids was demonstrated by two groups using plasmid DNA introduced into the skin of mice by a biolistic gene delivery system (Williams, 1991 and Tang, et al., 1992). The generation of a protective immune response upon immunization with a genetic vaccine was initially demonstrated in mice that were inoculated intramuscularly with naked plasmid DNA
encoding the internal nucleoprotein of Influenza virus (Ulmer et al., 1993). The potential efficacy of DNA vaccination into post mitotic muscle cells has since been demonstrated in a variety of murine and animal models infected with bacterial, viral or parasitic pathogens. Immunization using DNA vaccines is an approach that is being widely investigated to protect against a large number of infectious diseases including FMD (Wong et al., 2002; Cedillo-Barron et al., 2001; Benvenisti et al., 2001; Wong et al., 2000; Beard et al., 1999; and Chinsangaram et al., 1998). Immunization with plasmid DNA elicits both cell-mediated and humoral immune responses (Yang et al., 2001; Nobiron et al., 2001; Kim et al., 2001; and Tighe et al., 1998). These responses include the generation of antigen-specific cytotoxic T lymphocytes (CTL) and protective neutralizing antibodies. In earlier studies, it was found that plasmid DNA constructs could elicit CTL response that was invariably lacking in immune response elicited by protein vaccines (Raz et al., 1993). Since CTLs are considered necessary for a successful defense against intracellular pathogens like viruses (Zinkernagel, 1996), this particular aspect of DNA vaccine is one of its great advantages over protein-based vaccines. In addition, it has also been found that the B-cell and T helper (Th) cell responses are also elicited in DNA vaccination as antigen genes contained in the plasmids are translated and presented for surveillance (Tighe et al., 1998).

The initial experiments by Ward et al (1997) revealed that DNA vaccine candidate against FMDV was not as effective as the
inactivated whole virus vaccine. The observed neutralizing antibody responses elicited fell short of those required for protection against the disease in swine. Chinsangaram et al., (1998) evaluated candidate FMD DNA vaccines designed to produce viral capsids lacking infectious viral nucleic acid. Plasmid DNAs containing a portion of the FMDV genome coding for the capsid precursor protein (P1-2A) and wild type or mutant viral proteinase 3C were administered to mice for evaluating the immune responses. They reported anti-FMDV antibodies in both instances. However, only the plasmid DNA containing the wild type 3C elicited a neutralizing antibody response. These results suggest that capsid formation \textit{in situ} is required for effective immunization. Further, their efforts could only partially protect against virus challenge in mice. Subsequently, Beard \textit{et al.} (1999), demonstrated that their construct consisting of P1 and 3D sequences with wild type 3C could protect swine against FMDV while a similar construct carrying mutated 3C lacking post translation processing ability failed to give protection. The immunogenicity of two recombinant viruses (Adenovirus and Vaccinia virus) expressing P1-FMDV administered either individually or sequentially was analyzed by Sanz-Parra \textit{et al.}, (1999). Results showed that double immunization with recombinant adeno-P1 virus expressing the P1 polypeptide of FMDV elicited an antiviral immune response and partially protected pigs against viral challenge. Further, they concluded that the protection elicited was the result of the FMDV-specific T cell responses but not detectable antibodies. It has been established that introduction of VP1 immunogenic domain alone could not induce
protection even though anti FMDV neutralizing antibodies were elicited (Huang et al., 1999). Benvenisti et al., (2001) tested a gene gun mediated DNA vaccine against FMDV. Their construct, which consisted of encephalomyo-carditis virus internal ribosomal entry site, the entire P1 and 2A together with 3CD sequences afforded partial protection to pigs from FMDV challenge. This partial protection was attributed to the cellular immune participation rather than humoral responses. Cedillo-Barron et al., (2001) however, have reported a marginally improved humoral response affording partial protection against challenge in swine after three sequential vaccinations with DNA (P1-2A-3C-3D). Further, co-administration of a plasmid encoding granulocyte-macrophage colony-stimulating factor had elicited improved antibody response to FMDV proteins.

Zhang et al., (2003) reported to have tested plasmid DNAs containing multiple epitopes of foot and mouth disease virus in mice. A series of plasmids encoding different combinations of B cell epitopes and a T cell epitope were constructed and characterized by inoculating BALB/c mice. Specific antibodies were detectable only in the mice inoculated with plasmids encoding the T cell epitope and B cell epitopes from sites 5 and 1, within which site 5 includes residues 135-167 of VP1 and site 1 includes 141-160 region (G-H loop) and carboxyl terminus of VP1. Stronger cellular immune responses were also observed in these mice using T cell proliferation assay.
2.11.6. Genetic Adjuvants

Wong \textit{et al.}, (2002) reported complete protection in swine construct, consisting of two FMDV VP1 epitopes (amino acid residues 141-160 and 200-213), and swine interleukin (IL2 has been demonstrated to have the ability to increase both FMDV-specific humoral and cell mediated immune response against FMDV \textit{in vivo}.

Wu \textit{et al.}, (2003) explored the possibility of using type I interferon (IFN-alpha/beta) as a novel anti-FMD agent and concluded that in vivo expression of porcine INF-alpha could partially protect cattle from FMD. Because porcine INF-alpha (PoIFN-alpha) inhibits FMDV replication in bovine cells, its potential as an anti-FMD agent was evaluated in cattle. Consequently, replication defective human adenovirus type 5 (Ad5) vector expressing PoIFN-alpha (Ad5-PoIFN-alpha) has reduced the severity of FMD less severe than in control animals. One animal was reported to have been completely protected. Similarly, although all the Ad5-PoIFN-alpha-inoculated animals developed viremia, it was delayed by one day as compared with the control group. Moraes \textit{et al.}, (2003) extended the above work with a combination of adenovirus expressing interferon alpha and a foot and mouth disease virus subunit vaccine. They examined the duration of protection afforded in swine by a recombinant, replication-defective human adenovirus type 5 containing porcine interferon alpha (Ad5-pIFNalpha) and the ability of a combination of Ad5-pIFNalpha and a FMDV subunit vaccine delivered by Ad5-A24 (Ad5 vector containing the capsid coding region of FMDV serotype A24 Cruzeiro and the 3C
proteinase coding region of FMDV serotype A12) to induce immediate as well as long-lasting protection against homologous FMDV challenge 1, 3, 5 and 7 days post inoculation (dpi). All animals challenged 1 and 3 dpi were reportedly completely protected from disease while animals in the remaining groups had either no clinical signs of disease or clinical signs were delayed and less severe compared to the control group. Further, swine inoculated with a combination of Ad5-pIFNalpha and Ad5-A24 and challenged 5dpi were all completely protected from disease and developed a significant FMDV-specific neutralizing antibody response.

Over the past few years, new formulations, new molecular adjuvants and more sophisticated constructs have been developed extending the scope and purview of DNA vaccines far beyond just preventing diseases. Generated DNA vaccines encoding *Schistosoma mansoni* large subunit of calpain (Sm-p80) and either mouse GM-CSF or IL-4 to determine their adjuvant effect in mice. GM-CSF may work as adjuvant through its activating effect on dendritic cells and macrophages. Intramuscular vaccination with Sm-p80 alone provided 39% protection and this protection was significantly increased to 44% with GM-CSF co-administration and 42% with IL-4. The addition of GM-CSF led to an increase in total IgG and IgG1 while Th1 type IgG2a antibody titers remained high in protected animals (Walter R. Weiss 1998). Since protection was associated with Th1 type antibodies, the Sm-p80 DNA vaccine was further enhanced with co-delivery of plasmids encoding mouse IL-2 or IL-12 (Afzal A. Siddiqui 2003).
Greater protection was observed with IL-2 and modest but significantly higher protection was provided by IL-12 co-delivery. Both IL-2 and IL-12 are key cytokines in Th1 cell differentiation. The co-delivery of these cytokines increased IgG2a antibody levels and decreased IgG1 levels, indicating that these genetic adjuvants were successful as Th1 enhancers. Other studies reported no enhancement of protection or immune responses when IL-12 was co-injected, but these differences may be attributed to the nature of the vaccine antigen. Granulocyte / macrophage colony-stimulatory factor (GM-CSF) is an good adjuvant for a DNA vaccine mainly on account of its ability to recruit antigen synthesis at antigen-presenting cells (APCs) and ability to stimulate the maturation of dendritic cells (DCs). This study evaluated the utility of GM-CSF cDNA as a DNA vaccine adjuvant for glycoprotein B (gB) of pseudorabies virus (PrV) in a murine model it has enhanced the protective immunity against a PrV infection. This immunity was caused by the induction of increased humoral and cellular immunity in response to PrV antigen.

It has been demonstrated several studies shown that intramuscular or intranasal delivery of various cytokine genes promotes antigen-specific immunity triggered by a genetic vaccine for a infections. pDNAs encoding interferon (IFN)-α, IL-2, IL-12, IL-15, IL-18 and granulocyte–macrophage colony-stimulating factor (GM-CSF) have been shown to enhance cell mediated immune response (Th1).
immune responses triggered by a pDNA vaccine against human immunodeficiency (HIV) virus, hepatitis B virus, mycobacterium and HSV, although the humoral response was elevated or down modulated, depending on the cytokines and antigens employed. In some reports, the use of cytokine genes brought significant elevation in CTL activities against the antigens. The results strongly suggest the potential usefulness of the cytokine gene delivery as molecular adjuvants.

2.11.7. Interleukin -18

Interleukin-18 belongs to the IL-1 cytokine family, it is main function as very important regulator molecule of both innate and acquired immune responses. It is earlier known as interferon (IFNγ)-inducing factor. IL18 sequencing contains 192 amino acids precursor polypeptide. It is lacking a conventional signal peptide and a mature protein of 157 amino acids. IL18 gene contains of seven exons, out of one and two are noncoding.

2.11.8 IL-18 Expression and Synthesis

IL-18 expression has been reported in macrophages, dendritic cells (DC), Kupffer cells, keratinocytes, osteoblasts, synovial fibroblasts (Nakanishi, Dinarello 2001, Gracie, J. A 2003). Pro-IL-18 is cleaved after Asp35 by the endoprotease IL-1 converting enzyme (ICE; caspase-1) to generate a biologically active, mature 18-kD moiety. In contrast, cleavage of pro-IL-18 or mature IL-18 at Asp71-Ser72 and Asp76-Asn77 by caspase-3 results in the generation of biologically inactive peptides.
2.11.9. Functional effects of IL-18

It is initially identified IFN inducing factor, later it is expanding in several functions like T and natular killer cells maturation, cytokines production and cytotoxicity (Okamura et.al 1995, Ushio et.al 1996). IL18 when it is combine with IL2 it is coinduces IL13 in murine. Stimulation of bone marrow-derived macrophages or splenic DC with IL-12 and IL-18 can induce IFN- production (Di Marzio, et.al 1994, Yoshimoto, T et.al 1997). Similarly, IL-18 promotes neutrophil activation, reactive oxygen intermediate synthesis, cytokine release, and degranulation [Gracie, J. A et.al 1999, Leung, B. P et.al 2001]. Recent studies suggest that IL-18 up-regulates intracellular adhesion molecule-1 (ICAM-1) and VCAM-1 expression on endothelial cells and synovial fibroblasts (Jacques et.al 2000).

2.12. VACCINE DELIVERY

As explained in the previous section, in order to induce immune response administered antigen, the antigen must enter to the mucosal epithelial cells. The vaccine delivery adjuvants are developed based on the understanding that the improved immune response by promoting the uptake of antigen by the antigen presenting cells. One approach that to develop vaccine delivery adjuvant is to create particulate adjuvants. The antigen is encapsulated inside the particle or coated on the surface of the particle. Particles are transported through a involving the Monocytes.
2.12.1. Poly (lactide co-glycolides) or PLG microparticles

PLG microparticle is derived from aliphatic polyesters, the poly (lactide co-glycolides). The micro particles are incorporated into the antigen by using encapsulation. PLGs are biodegradable and biocompatible and have been approved for use as controlled release vehicle. PLGs biodegraded mainly involves hydrolysis of polymer to produce common metabolites, lactic and glycolic acids, which are eliminated through the Krebs cycle. The rate of biodegradation depends on the polymer composition, molecular weight and manufacture procedure.

The adjuvant effect of PLG microparticle is achieved through

1. The microparticle is design protects the entrapped antigen/coated plasmid against degradation.

2. Microparticles delivers the entrapped antigen/ coated plasmid to the antigen presenting cells.

Particulate carriers are the several attributes for use as vaccine delivery systems. Microparticles have a similar size of the pathogens that the immune system recognizes and, consequently, they are efficiently internalized by antigen presenting cells (APC). The uptake of microparticle (5 µm) by phagocytic cells has been well documented and uptake into APC is likely to be important in the ability of particles to perform as vaccine adjuvants. It has been reported that macrophages that carry micro particles to lymph nodes can mature into dendritic cells (DCs). In addition, uptake of biodegradable micro
particles directly into DCs has been demonstrated both *in vitro* and *in vivo*. The ideal size for micro particles appears to be in the range of 1–3 µm (Manmohan Singh *et al.* 2003), and it appears that cationic micro particles are particularly effective for uptake into macrophages and DCs (*Derek T O’Hagan et al.* 2003). A second useful property of micro particles is that they can present multiple copies of antigens on their surface, which has been shown to be optimal for B cell activation. Recent studies have shown that, together with the activation of innate immunity, the duration of antigen persistence is important in triggering protective T cell responses. Such persistence of antigens can be enhanced by association with microparticles which protect antigens from degradation.

### 2.12.2. Microparticles as delivery systems for DNA vaccines

It has been established that DNA vaccines over significant potential for the induction of potent CTL responses. Nevertheless, the potency of DNA vaccines in humans has so far been disappointing, particularly in relation to their ability to induce antibody responses (*Derek T O’Hagan et al.* 2003). This has prompted investigators to evaluate adjuvants and delivery systems for DNA vaccines and also to use DNA in a prime/boost regimen with alternative vaccine modalities [Derek T. O’Hagan *et al.* 2004]. An early study suggested that microparticles with entrapped DNA have the potential to improve the potency of DNA vaccines. However, encapsulating DNA into microparticles has several limitations, which include damage to DNA during microencapsulation and release, low encapsulation efficiency
and minimal initial release of entrapped DNA (Walter E., et.al 1993). Because of the limitations of PLG microparticle with entrapped DNA, other polymers have been investigated with improved characteristics for release of entrapped DNA. As an alternative approach to avoid the problems associated with microencapsulation and release of DNA, a novel cationic PLG microparticle formulation that adsorbs DNA onto the surface was developed. Importantly, cationic micro particles with adsorbed DNA induced enhanced immune responses in comparison to naked DNA and this enhancement was apparent in all species evaluated, including nonhuman primates (Derek T. O'Hagan et.al 2006). In addition, the cationic PLG micro particles efficiently adsorb DNA and could deliver several plasmids simultaneously (Derek T. O'Hagan et.al 2001). Cationic PLG micro particles with adsorbed DNA showed protective efficacy in a rodent colon cancer model [Luo.Y, D et.al 2003], increased antibody and cellular immune responses to HBsAg [Jorge E Osorio et.al 2003, and enhanced the protective efficacy of a DNA-based tuberculosis vaccine (Mollenkopf, et.al 2004). Cationic PLG micro particles with adsorbed DNA are currently being evaluated in a human clinical trial as a delivery system for a HIV vaccine and have also shown significant potential in a non-human primate study as a delivery system for a HCV vaccine (Derek T. O'Hagan et.al 2004). Cationic PLG micro particles appear to be effective predominantly as a consequence of the efficient delivery of the adsorbed DNA into DC. Following administration, the micro particles are also very effective at recruiting DC to the injection site,
and the micro particles also protect adsorbed DNA against degradation in vivo. Cationic emulsions have also been described, which are able to adsorb DNA onto their surface and to enhanced immune responses (Manmohan Singh et al 2004). In addition, the preparation of microparticles for DNA delivery involving alternative polymers was recently described (Jones, D. H et al 1997).