Cloning and expression of FMDV-VP1 immunoreactive peptide in trivalent form and its application as immunogen

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A search for alternatives to conventional inactivated virus vaccine for FMD with an aim to control and eradicate the disease globally, is a continuous process till a promising one is identified. Development of such vaccines underlines necessity of avoiding the use of active virus, and to have broader antigenic coverage so as to make them suitable even for disease free countries. Subunit or peptide vaccines have been shown to elicit neutralizing antibody response. However, the titres are low as compared to sera from animals vaccinated with conventional vaccine and fail to protect animals against virus challenge. This is probably due to the inclusion of only limited epitopes. Under such conditions, mixing heterologous epitopes from the various serotypes may be a better approach for elicitation of high titred antibody response. Keeping this in view, we have linked C-terminal half of VP1 carrying two B cell and one T cell epitope of three FMDV serotypes (O, A and Asia 1), which are presently in use as vaccine strains in India. The linked polyvalent gene was expressed in Escherichia coli and the 59 kDa fusion protein was studied for its immunogenicity in guinea pigs in comparison with the specific epitopes of type ‘O’ produced as a similar fusion protein of 30 kDa. The trivalent protein showed better neutralizing antibody response, even with single booster injection, as compared to monovalent protein as observed in ELISA and SNT. These studies show future scope for the development of protein/DNA-based vaccine for FMD.

Keywords: Trivalent immunogen; FMDV; immunoreactive; proteins; vaccine

Introduction

Foot-and-mouth disease (FMD) is one of the most contagious viral diseases of cloven footed animals having high economic impact and is caused by a ssRNA virus belonging to the genus Aphthovirus, family Picornaviridae. Epidemics of FMD pose constant threat to domestic livestock throughout the world. Vaccination combined with stamping out campaigns in western world has resulted in the eradication of this disease but is still a constant threat. On the other hand, due to prevailing socio-economic conditions and geographical situations, continuous disease outbreaks are reported in endemic countries. Most of the disease outbreaks in FMD endemic countries like India are due to the serotype O followed by Asia 1 and A. Currently, vaccination in India is carried out using trivalent inactivated viral vaccines. There are several limitations associated with inactivated vaccines, major of which is the release of live virus either while handling or through the vaccine. Presence of active virus may have negative effect in the disease free country, if used even in emergency situation. Under this situation, production and utilization of non-virus based vaccine may be safe and ideal.

Foot and mouth disease virus (FMDV) has 60 copies each of four structural proteins viz., VP1, VP2, VP3 and VP4, respectively¹. Five sites of B-cell epitopes were defined on these proteins by studies with monoclonal antibody escape mutant². Site 1 is the predominant site, which comprises two VP1 epitopes (residues 141-160 and 200-213 of VP1), that has been shown to protect animals from the disease³. Site 1 is linear whereas, all the other identified sites are conformational or less predominantly B-cell dependent⁴. The structural VP1₁₄₁-₁₆₀ and VP1₂₀₀-₂₁₃ peptide sequences are the most serologically dominant epitopes in FMDV infection⁵ and indeed the synthetic peptide FMDV15, comprising these sequences of VP1 (AA 141-160 and AA 200-213), has stimulated synthesis of neutralizing antibodies and protection in guinea pigs, but not in cattle⁶,⁷.

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VP1 isolated from the virus\(^8\) or recombinant VP1 derived from *Escherichia coli*\(^9,10\) and plant\(^11\) has been shown to induce neutralizing antibodies and providing partial protection to animals from FMDV infection. Although, there is an immune response, the efficacy of protection in those immunized animals does not correlate well with the level of antibodies against peptides (or) VP1\(^12\). Nevertheless, VP1 region is the most relevant to the immunogenicity of FMDV\(^13\).

The ability of inactivated vaccine containing heterogenous virus population in eliciting protective response shows that a mixture of antigenic epitopes may be necessary for successful immunization. Therefore, we propose to make complex antigen by linking the site 1 epitopes present in C-terminal half of VP1 of the three circulating serotypes and study the immune response in guinea pig model. The work presented here consists of linking the C-terminal half of VP1 (AA 120 to 213) of FMDV serotype A, Asia 1 and O, production of trivalent protein in *E. coli* and study the immune response of the protein in comparison with monovalent type ‘O’ protein.

**Materials and Methods**

**Virus, Sera and Host Systems**

FMDV types A (Ind 17/77), Asia 1 (Ind 63/72) and O (Ind R2/75) vaccine strains grown in BHK21 clone 13 cells, sera against the purified *E. coli* expressed C-terminal half of VP1 of type A\(^{22}\) and ‘O’ raised in rabbits, as well as, *E. coli* DH5\(^{\alpha}\) and BL21 (DE3) pLysS for gene cloning and expression studies, respectively, were used.

**Cloning of Trivalent Gene Cassette in pET32(a)**

Standard protocols were followed for the construction of trivalent gene cassette. Immunoreactive C-terminal half of VP1 genes of all the three FMDV vaccine strains (serotypes A, Asia 1 and O) were amplified separately using the forward SV1(BamHI)(5′GCCGGATCCGCCACACCGTCT GTTGGCC3′) primer, which corresponds to amino acid 120-227\(^{th}\) of VP1 and reverse 2AR(EcoR I)(5′GCCGGAAATTCGGAGGGCCCGAGGGTTTG GACTC3′) primer corresponding to 2A region. Amplicons of the size about 330 bp, corresponding to immunoreactive protein region of FMDV A and Asia 1 were cloned in pBSKS+ vector separately to get pBA330, pBAs330 (Stratagene, USA) while corresponding product of FMDV type O was cloned in pETBlue2 (Novagen) to get pETO330. Immunoreactive protein genes of all the serotypes were sub cloned separately into pET32a vector to get pETA330, pETAs330 and pETO330. The 330 bp DNA of Asia 1 was linked at 3′ end to the pBSKS+recombinant plasmid carrying 330 bp ‘A’ without altering the frame to get pBA+Asia (660). Recombinant plasmid pBA+Asia+O (990) was constructed by linking the 330 bp of type ‘O’ at 3′ end of pBA+Asia (660). The order of the inserted genes was A-Asia1-O in 5′-3′ direction. The gene 660 or 990 bp constructs were sub cloned in pET32a vector separately for the expression after releasing from pBA+Asia (660) or pBA-Asia-O (990) by Not I-Xho I digestion. The frame and the intactness of the linked genes were confirmed by sequence analysis from both the directions using T7 and T3 primers in an automated DNA sequencer.

**Expression of Cloned Genes in E. coli and Affinity Purification of E. coli Expressed Proteins using HIS-Select nickel Affinity Gel**

The recombinant plasmids, pETA, pETA-Asia and pETA-Asia-O were separately transferred into BL21 (DE3) pLysS cells. Single colony in each case was grown in 10 mL LB broth with ampicillin (50 µg/mL) for overnight at 37°C with shaking. One hundred microlitre of the overnight culture was inoculated into fresh 10 mL LB broth without ampicillin for 3 h at 37°C. The protein expression was induced by adding IPTG (isopropyl-beta-D-thiogalactopyranoside) to a final concentration of 1 mM at 30°C for 6 h and the cells were pelleted and resuspended in 1 mL of TE-PMSF (pH 8.0) (1 mM phenyl methyl sulfonyl fluoride) and stored at –80°C. The cell lysate was analyzed by 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) as per the published protocol\(^14\). Lysate supernatants from induced bacterial cells containing the monovalent, bivalent or trivalent proteins (C-terminal VP1 of A, Asia 1 and O) were analysed along with the vector transformed cells and uninduced recombinant clone as control. The specificities of the expressed proteins were confirmed by western blotting as per the protocol\(^15\) with modification. The rabbit antiserum raised against the VP1 of type A\(^{22}\) as primary antibody, goat anti rabbit IgG horse radish peroxidase conjugate as secondary antibody and O-Dianisidine Dihydrochloride as substrate were used for the detection of the antigen-antibody reaction.

The expressed protein was purified by nickel affinity column as per the manufacturer’s protocol (Sigma, USA). The pooled protein solution was
concentrated by polyethylene glycol, dialyzed against phosphate buffered saline (PBS) and stored at – 70°C.

Comparison of Immune Response of Monovalent (O) Protein with Trivalent Recombinant Protein for Neutralization of ‘O’ Virus

Two groups of guinea pigs, each comprising five animals of body weight about 500 g were used in the experiment. Group I animals were inoculated subcutaneously with 50 µg of purified E. coli expressed monovalent protein of type ‘O’ in sterile PBS, pH 7.2, while the Group II animals received 150 µg of purified trivalent protein (of Type A, Asia 1 and O), both were adjuvanted with Freund’s complete adjuvant (FCA). Booster injections were given 30 d post inoculation (dpi) with the same dose of antigen adjuvanted with incomplete Freund’s adjuvant. Animals were bled 10 d later and the sera were subjected to indirect ELISA using purified Pichia pastoris expressed type ‘O’ antigen and SNT using 100 TCID$_{50}$ of type ‘O’ virus, to assess the humoral immune response against recombinant proteins as per the protocol$^{16}$.

Results and Discussion

Production of safe and efficacious vaccine as an alternative to the conventional virus vaccine for FMD is the need of present time to control FMD and contain the pathogen both in endemic and disease free countries in the world. To achieve this, several alternative approaches have been tried, which include the use of subunit$^{17}$ and synthetic peptide vaccines$^{18}$ reporting partial protection. However, the animal vaccinated with inactivated virus even at low concentration elicits protective response which may be due to the presence of heterogeneous population of the virus antigens in the vaccine. Thus, revealing the possible necessity of complex mixture of epitopes for good immune response. Though newly developed recombinant vaccines are considered as alternatives in terms of safety and economics, they have yet to prove their efficacy in inducing protective response in vaccinated animals when challenged with virulent virus, which may have heterogeneous populations of virus. Based on this hypothesis, we made an attempt to produce trivalent immunoreactive protein antigen of FMDV by linking the gene segments for major epitopes of the three serotypes A, Asia-1 and O, expressed the protein and studied the immunogenicity in comparison to the protein from single serotype. In the construct, both B- and T-cell epitopes from the C-terminal region of VP1 gene of all three serotypes have been included so that the protein would display the multiple epitopes. In the case of FMD, the immune response is not only B-cell but also T-cell dependent$^{19}$. It is also observed that FMDV carrier animals often have significant levels of neutralizing antibodies, but no immunopathological changes have been found in infected tissues$^{20}$, suggesting that cell mediated immunity may be involved in the clearance of persistent FMDV.

Characterization of Cloned Trivalent Gene Construct

The genes corresponding to mono (type O), bi (A+Asia 1) and trivalent (A+Asia-1+O) immunoreactive proteins in pET32a vector were confirmed by restriction enzyme digestion (Fig. 1). Frame and junctions of linked genes were confirmed by nucleotide sequencing and sequence analysis (Fig. 2). As shown in Fig. 3, all the three linked genes were in frame. The junctions have 8 and 9 amino acids between A and Asia, and Asia and O, respectively. The sequence was in frame with the ATG codon of pET32a vector thereby resulting in the production of recombinant protein carrying N-terminal fusion that has all the tags including His tag. By inserting the gene the His tag at C-terminus of the vector also was in frame, after which a termination codon was present. Therefore, the total coding region excluding tag protein comprises 1089 nucleotides i.e. 363 amino acids corresponding to 40 kDa of protein. The total size of the protein including N and C terminal tags is therefore 40+19 i.e. 59 kDa, which is the observed size.

Fig. 1—Agarose gel electrophoresis of restriction enzyme digested recombinant pETD, pETD+Asia 1 and pETD+Asia 1+O plasmids. Lane 1-100 bp DNA ladder (New England Biolabs); Lanes 2, 3 and 4-Not I and Xho I digested pET32a vector with A, A+Asia 1, A+Asia 1+O, genes, respectively; & Lane 5-Linearized pET32a vector.
Characterization of Expressed Protein

The lysate proteins from the *E. coli* carrying insert (mono, di, trivalent) showed additional protein bands of the size 30, 45 and 59 kDa (Fig. 4, lanes 3, 4 & 5), respectively, which are not present in the case of host with pET32a plasmid without insert (Fig. 4, lane 6) or uninduced clone (Fig. 3, lane 2) indicating that they correspond to the recombinant plasmid coded gene products. High intensity protein bands revealed that the proteins are overexpressed and are present in soluble form. The proteins corresponding to mono, bi and trivalent as per the sequence were expected to have molecular weights of 12, 24 and 36 kDa, respectively. However, the observed molecular weights were 30, 45 and 59 kDa, due to 18 kDa His-tag at amino terminus of all the expressed proteins. Additional amino acids in between the linked proteins (8 AA in bivalent and 9AA in trivalent proteins) added from vector also have contributed to the respective molecular weights.
increase in the mass, at the same time, providing desired stability. It has been observed that the level of expression increased with the size (Fig. 4) contrary to the expected negative effect. However, we have not made any specific studies for understanding the molecular basis of this observation. Presence of His-tag at both carboxyl and amino terminus of expressed proteins from pET32a vector had enabled us to purify the recombinant proteins from *E. coli* using HIS-select nickel affinity gel column. The specificity of the expressed proteins was confirmed by immunoblot assay where anti serum against the VP1 of type A22 (purified *E. coli* expressed protein) raised in rabbits showed positive colour reaction. Protein bands 30, 45 and 59 kDa correspond to FMDV O, A+Asia 1 and A+Asia 1+O, respectively. No color reaction was observed with the proteins from uninduced bacteria carrying recombinant plasmid or bacteria carrying vector alone (Fig. 4).

**Immune Response of Monovalent and Trivalent Immunoreactive Proteins in Guinea Pigs**

Monovalent (type ‘O’) and trivalent recombinant proteins were purified using HIS-select nickel affinity gel column and used for immunological studies. The ELISA titres of sera of the animals which received 50 µg of monovalent recombinant protein of type ‘O’ were between 1:25 to 1:100 (Fig. 5) when compared with that of those animals which were vaccinated with 150 µg of trivalent recombinant protein showed ELISA titres of 1:200 (about 2 to 4-fold), indicating that antibody response against ‘O’ in trivalent protein is higher. On the other hand, when the serum neutralization titres of the guinea pigs vaccinated with monovalent recombinant type ‘O’ proteins and trivalent proteins were analysed using 100TCID$_{50}$ of type ‘O’ virus, the titres were between 1:4 and 1:8 in case of animals vaccinated with monovalent protein$^{21}$. However, the SN titres of the animals, which received 150 µg of trivalent recombinant protein, were 1:32 for 4 animals and 1:16 for one animal indicating that trivalent protein elicited high neutralizing antibody response against type ‘O’. The increase in ELISA titre by just 2 to 4-folds i.e. 1:100 to 1:200 in case of trivalent protein may be due to the bigger size of protein contributing better immune response or due to the presence of cross-reacting epitopes. However, very high (4 to 8-fold) increase in SN titres from 1:4 is unexpected as the molar concentration of monovalent type ‘O’ is the same in both the vaccines. The presence of higher SN antibodies against one

![Fig. 5—ELISA and SNT titres of monovalent and trivalent recombinant proteins of FMDV in guinea pigs. The ELISA and SNT titres at 40 dpi and represent the deviations from prevaccinated animal titres. The log of serum dilution at which A492 nm values was double that of prevaccinated serum in ELISA. The log of serum dilution at which 50% of BHK21 cell monolayer had shown cytopathic effect in SNT.](image)

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