RESULTS

FMD is endemic in India, which with all its ethical issues leaves vaccination as the only option in controlling the disease. While the existent vaccine does provide protection against the disease, the associated disadvantages necessities to search for a alternative vaccine. DNA vaccine is one of such alternative approach. The results presented here is an develop and evaluate the Interleukin 18 adjuvanted DNA vaccine against FMDV delivered through cationic PLG micro particles.

4.1. Cloning of bovine interleukin-18

4.1.1. Amplification of precursor bovine interleukin 18 gene

Bovine macrophages were isolated from whole blood and cultured in RPMI 1640 medium (10% FCS) were stimulated with PHA at a concentration of (10 µg/ml) for 17 hours. Total cellular RNA was extracted from the stimulated Macrophages using the Trizol reagent and used in cDNA synthesis as described under materials and methods. cDNA specific for bovine Interleukin 18 was synthesized using oligo dT specific primer, purified and used as a template for the amplification IL18 sequences PCR. Gene specific forward (bIL18L) and reverse (bIL18R) primers were used for amplification and the amplicons analyzed by agarose gel electrophoresis (fig. 6). An intensely amplified DNA band was seen in lane 1 is of 0.582 kb in size, corresponds to the amplified IL18 PCR product where no amplification
could be seen lane 2 which is negative control. The size of the amplified bIL18 PCR product is similar to the reported size of the bIL18 gene.
**Fig. 6. Agarose gel electrophoresis of amplified Bovine interleukin 18 gene**

Bovine interleukin (b IL18) was amplified from cDNA using bIL18(L) and bIL18(R) primers by standard PCR reaction. The PCR product was analyzed by 1% agarose gel electrophoresis using DNA molecular weight marker (Fermentas) and documented. The gel shows the DNA bands from

Lane M – 1Kbp Ladder

Lane 1 – PCR amplified B IL18 from cDNA

Lane 2 – Negative control
4.1.2. Cloning of bovine precursor IL18 gene into pBSK+

bIL18 amplicon was purified, digested with EcoRI and NotI and ligated with similarly digested PBSK+ plasmid. Three µl of the ligated DNA was transferred into competent DH5α cells and plated on X-gal-IPTG-Ampicillin agar plate. There were several white colonies along with a few blue colonies. The blue colonies represent the presence of vector alone that provides intact α-complementation peptide. The white colonies may represent recombinant clones carrying bIL18 insert in the plasmid. The white colonies were screened for the presence of vector with insert. The transformants that resulted were screened for the presence of recombinant plasmid by colony PCR by using vector specific primer. Amplification of PCR product of 0.852 kb in size seen in lane 1, 2, 3 (Fig-7) these colonies are could be the recombinants contains the insert. Recombinant plasmids isolated from the two positive clones were subjected to digestion with EcoRI and NotI and the products analyzed by 1% agarose gel electrophoresis using a 1Kbp DNA Molecular Weight Marker (Fig.8). Release of DNA fragment of 0.582kb size seen in lane 1 could be the IL18 insert. The size of the insert was deduced from the standard curve drawn from the log molecular sizes of the marker against their mobility and found to be 0.58 Kbp, which is the size of the bIL18 gene. The specificity of the insert was confirmed by sequencing.
Fig. 7. Agarose gel electrophoresis of PCR amplified 852 bp DNA from colonies.

Individual colonies obtained after transformation with pBSK1+ ligation mixture were screened for the presence of b IL18 using vector specific T3 and T7 primers. The PCR products were analyzed by electrophoresis alongside DNA molecular weight marker (Fermentas) in 1.2% agarose gel and the result documented.

Lane M – 1kb Ladder

Lanes 1 to 3 – PCR amplified b IL18 from different transformants / colonies.

Lane 4- Negative control
Fig. 8. Agarose gel electrophoresis of EcoR I and NotI digested recombinant pBSK B IL18 plasmid DNA.

Purified plasmid DNAs (0.5µg, each) were digested with EcoR I and NotI I. The digested DNAs were subjected to electrophoresis in 1% agarose gel and analyzed alongside DNA molecular weight marker (Fermentas) and vector control. The ethidium bromide stained gel showing DNA fragments
Lane M – 1Kbp Ladder
Lanes 1 – EcoR I and Not I digested vector pBSK b IL18 gene.
Lane 2 – Vector without insert control digestion.
4.1.3. **Sequence Analysis of the BIL18 Gene**

bIL18 gene of Hallikar breed of cattle in pBSK+ vector was subjected to nucleotide sequence analysis from both the directions as described under materials and methods. The gene represented by the total coding sequence 582 bases (192aa). The predicted molecular weight from the translated product of 166aa would be 18kda including a signal sequence at n-terminal end of the protein. When compared with the published nucleic acid sequences of Interleukin 18 of other species (fig. 9), it showed a homology of 99% with Bos taurus, 97% with buffalo and 96% with sheep and goat indicating similarities with other species. Sequence was submitted to the genebank (Acc noFJ985771).

![Phylogenetic analysis of bovine IL 18](image)

**Fig. 9a. Phylogenetic analysis of bovine IL 18**
Bos indicus IL18 protein mRNA, complete cds

GenBank: FJ985771.1

FASTA Graphics

Go to:

locus 582 bp mRNA linear May 01-Mar-2010
codon 582 mRNA complete cds.

Bos indicus IL18 protein mRNA, complete cds.

ncDNA  .

Bos indicus (Bos taurus indicus)

Ontology
code�n

nucleotide

1 (base 1 to 582)

authcode 1 (base 1 to 582)

authname 1 (base 1 to 582)

rtcode

direct Submission

journal Submitted (01-Mar-2010) molecular virology lab, indian veterinary research institute, mehbal, bangalore, karnataka 560024, india

character

location/qualifiers

codon

1...582

product "mRNA"

code...582

1...582

/organism "Bos indicus"

mRNA_type "mRNA"

codons...582


Fig. 9 (b) sequence submitted to the gene bank
4.1.4. Subcloning of BIL18 into pcDNA3.1+

BIL18 gene fragment was released from the pBSK—bIL18 by digesting the latter with NotI and EoRI, purified and ligated into similarly digested and purified pcDNA3.1+. The ligated DNA (2µl) was transferred into competent DH5α cells. The transformants that resulted were screened for the presence of recombinant plasmid by colony PCR by using insert specific primers and analysis by agarose gel electrophoresis. Amplification of PCR product of 0.58 kb in size seen in lane 1, 2, 3, 4, 5, 7 (Fig-10) are putative recombinants. The two suspected clones carrying possible recombinant plasmids were inoculated in LB broth and plasmids extracted from them. RE digestion of these plasmids with Not I and E.coR I showed the presence of two fragments of size 0.582kb and 5.4 Kbp in case of positive clones Lane 1 (Fig.11). The 0.582 kb fragment is the expected size of the insert.
Fig. 10. Agarose gel electrophoresis of PCR amplified 582 bp DNA from colonies.

Individual colonies obtained after transformation with pcDNA+ ligation mixture were screened for the presence of b IL18 using gene specific IL18-L and IL18-Rprimers. The PCR products were analyzed by electrophoresis alongside DNA molecular weight marker (Fermentas) in 1.2% agarose gel and the result documented.

Lane 1M – 1Kbp Ladder

Lanes 1, 2,3,4,7,8,9 – PCR amplified b IL18 from different transformants /colonies.
Fig. 11. Agarose gel electrophoresis of EcoR I and NotI digested recombinant pcDNA b IL18 plasmid DNA.

Purified plasmid DNAs (0.5µg, each) were digested with EcoR I and NotI I. The digested DNAs were subjected to electrophoresis in 1% agarose gel and analyzed alongside DNA molecular weight marker (Fermentas) and vector control. The ethidium bromide stained gel showing DNA fragments

Lane M – 1Kbp Ladder

Lanes 1 – EcoR I and NotI digested vector pcDNA b IL18 gene.
4.1.5. Sub cloning of BIL18 gene into pET32a

The bIL18 gene was released from the pBSKbIL18 plasmid by digesting the latter with NotI and EcoRI and ligated into pET32a, which was digested similarly. The ligated DNA was transferred into DH5α competent cells and plated on ampicillin plates. Several colonies were observed of which few colonies were picked up and screened for the presence of inserted bIL18 gene. Colony PCR showed 5 of these colonies carry plasmid with inserted DNA (FIG -12) Lanes 1, 2, 3, 5 & 6. Two suspected positive clones were inoculated into LB broth and plasmids extracted from them. The DNAs were digested with and the products NotI and EcoRI analyzed by 1% analytical agarose gel electrophoresis using DNA molecular size markers (Fig.13). Two well separated DNA bands of 0.582 Kbp and 5.9 Kbp could be seen in the gel (lane 1,2). These confirmed recombinants have been designated PET32A-IL18.
Fig. 12. Agarose gel electrophoresis of PCR amplified 582 bp DNA from colonies.

Individual colonies obtained after transformation with p ET32+ ligation mixture were screened for the presence of b IL18 using gene specific IL18-L and IL18-R primers. The PCR products were analyzed by electrophoresis alongside DNA molecular weight marker (Fermentas) in 1.2% agarose gel and the result documented.

Lane M – 100bP Ladder

Lanes 1, 2,3,5,6 – PCR amplified b IL18 from different transformants/colonies.
Purified plasmid DNAs (0.5µg, each) were digested with EcoR I and NotI I. The digested DNAs were subjected to electrophoresis in 1% agarose gel and analyzed alongside DNA molecular weight marker (Fermntas) and vector control. The ethidium bromide stained gel showing DNA fragments

Lane M – 1Kbp Ladder

Lanes 1 – EcoRI and NotI digested vector pET b IL18 gene.

Lane 2 – Vector without insert control digestion
4.1.6. Expression of cloned genes

4.1.6.1. Expression of Bovine Interleukin 18 in *E. coli*

pETbIL18 was transferred into *E. coli* (BL21DE3PlysS) competent cells. Four of these transformants was grown overnight at 37°C in LB broth. The cells were pelleted and induced in fresh LB broth contains ampicillin. This was further inoculated in fresh LB broth without ampicillin till reach OD 1.2-1.4. The cells were pelleted and resuspended in fresh LB broth induced with IPTG (1mM) at 30°C for 4hrs as described under Materials and Methods. The cells were lysed by a single freeze thaw step and the chromosomal DNA was digested using Benzonase. Crude protein was analyzed by SDS PAGE. Expressed protein seen in lane 3,4 (42kDa)(fig 14)
Fig. 14 SDS-PAGE of the bIL18 expressed in E.coli

The cell lysate of E.coli (BL21DE3PlysS) carrying pETbIL18 (after induction) was alongwith the cell lysate before purification and a prestained standard protein molecular weight marker (Fermentas) was separated by SDS-PAGE in 12% gel,

Lane M - Standard Protein Prestained Mol.Wt. Marker
Lane 1 – Cell lysare (negative control)
Lane 2- before induction with IPTG
Lane 3and 4 – IPTG induced Cell lysate
4.1.6.2. Affinity purification of bIL18

Expressed IL18 protein was purified from column by using HIS-Select Nickel Affinity gel under denaturing conditions in the presence of 8M urea as per the protocol described in Materials and Methods. After all the non-specific proteins were washed out, the His-tagged bIL18 was eluted with elution buffer containing 200mM imidazole. The eluted protein was analyzed by SDS PAGE (Fig 15). The eluted protein seen in fraction 3,4 as a single band of 42kDa size which corresponds to IL18 fusion protein.
Fig. 15 SDS-PAGE of the bIL18 expressed in *E. coli* after purification through Ni-NTA affinity purification agarose column

The cell lysate of *E. coli* (BL21DE3PlysS) carrying pETbIL18 (after induction) was passed through Ni-NTA agarose column and the eluate and a prestained standard protein molecular weight marker (Fermentas) was separated by SDS-PAGE in 12% gel.

Lane 1 – Cell lysate before purification of the protein through Ni-NTA column

Lane 1,2,3,4 – Eluate obtained after passing the cell lysate through Ni-NTA column

Lane M - Standard Protein Prestained Mol.Wt. Marker
4.1.6.3 Caspase Treatment

Precursor IL18 undergoes proteolytic cleavage by ICE (Interleukin 1 beta converting enzyme) to generate a mature bioactive 18kDa molecule. The fusion protein was Purified IL18 was renatured by dialysed. 42kDa was treated with caspase1 to get the mature 18kDa protein."

4.2. Characterization of proteins

4.2.1. SDS PAGE Analysis of p ET32 expressed BIL18

Subsequent to induction for 4 hours, the protein expressed in E.coli were analyzed in 10% SDS PAGE gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 (CBB) stain, destained and examined under transmitted white light and the result documented (Fig.14). Several proteins bands of low concentration were seen in at the lane 1,2 including control, un induced and induced. However protein band of 42kDa is seen in induced cells (lane 3, 4) whereas this band was seen at lower concentration in uninduced cells and not present in control cells.

4.2.2. Western blot analysis of expressed proteins

The specificity of the expressed IL18 was analyzed by western blotting by using human IL18 monoclonol antibodies (fig16) protein band of 42kDa reacted with IL18 antibody seen as a thick band (lane2) whereas no reaction could be seen in case of control(lane1). Expressed protein was treated with caspase-1 for getting the mature form and then checked by western blot 18 kDa
reacted with IL18 antibody seen as thick band(lane2) where as no reaction could be seen in case of control(lane1) (fig17)

**Fig 16. Immunoblotting of recombinant Bovine precursor IL-18.**
Immunoblotting of recombinant IL-18 using anti human IL-18 Mab. Prestained molecular weight marker(Lane 1), Purified IL-18(lane-2), BL-21 cell control(lane-3)
Fig. 17 Immunoblotting of recombinant Bovine mature IL18 after treatment with caspase I. Immunoblotting of recombinant IL18 using anti human IL18 Mab. BL-21, cell control (lane1), mature IL18 (lane2), Pre stained molecular weight marker (Lane 3).
4.2.3. Biological activity of expressed BIL18

Biological activity of IL18 as evaluated as per followed above mentioned in materials and methods by measuring IFNγ production stimulated PBMC’s which was quantified by q PCR, ELISA.

(A) Real time PCR

Induction of IFN measured by quantification of m RNA coding IFN was seen in fig (19a). In PBMC treated with IL18 m RNA levels increased by 30 folds. Since IL18 is a potent inducer of IFNγ, we studied the level of IFNγ specific mRNA production in the presence of recombinant IL18 protein by qPCR to assay the biological activity of IL18. Quantitation of IFNγ has shown an increase of 30 folds in the copy number IL 18 treated cells compared to basal level.
Fig 18. Biologic activity of recombinant Bovine IL18. Briefly, 2x10⁶ bovine PBMC were incubated with purified recombinant protein 200ng/ml 24 hr. The copies of bovine IFN-γ mRNA in cells were measured by real time RT-PCR. 1: PBMC 2: PBMC with mature Bovine IL18
(B) Gamma interferon ELISA

Quantification IFN $\gamma$ of in the secreted by the stimulated cells (whole blood) was carried out by ELISA has shown an increase of 35 folds compared to basal levels (Fig 19b). The quantities of IFN$\gamma$ were 120 pgs/ml and 2400 pg/ml in the unstimulated and IL18 stimulated cells respectively. The stimulation of IFN$\gamma$ by IL 18 measured by qPCR is more or less similar to values observed in ELISA. These studies have shown the IL18 expressed in *E.Coli* is biologically active.

Fig 19. Biologic activity of Bovine IL18. IFN $\gamma$ measured by whole blood ELISA. 1: Blood with out IL18 protein treatment 2: Blood treated with mature Bovine IL18
4.3. Amplification and cloning of P1-2A-3C gene into pc DNA

Polyprotein gene (P1) of FMDV type ‘Asia I’ was cloned into PBSK+ by Reddy et al., 1999 and the same was made use of in this work. However, the sites required for subcloning the P1 gene into mammalian expression vector were different. Hence the need for amplifying the gene from the above mentioned recombinant DNA as template using VP4L BAC (Asia) & 2AR primers both of which have BamHI, NotI sites for cloning and for bringing the gene into proper reading frame to ensure expression. The amplified product was confirmed in 0.8% analytical agarose gel alongside a 1 kbp DNA ladder (Fig.20). A single DNA band of high intensity (LANE 1) which was absent in the case of control (Lane 2) was in the gel. The size of the band was deduced from the standard curve drawn between the molecular sizes of the markers against their mobilities and found to be 2.9 kbp the size corresponded to the size of the cloned P1-2A-3C of type ‘Asia I’ indicating that the amplified product is P1-2A-3C gene. The amplicon was purified and digested with BamHI, NotI and ligated into similarly digested and dephosphorylated pcDNA as described.
**Fig. 20. Agarose gel electrophoresis of FMDV P1-2A-3C PCR product**

FMDV P1-2A-3C PCR was amplified from clone using VP4-L (BamHI) and 3CR(Not) primers by standard PCR reaction. The PCR product was analyzed by 1.0% agarose gel electrophoresis along with DNA molecular weight marker (Fermenta) and documented. The ethidium bromide stained gel shows the DNA bands from

Lane M – 1kb Ladder

Lane 1 – PCR amplified P1-2A-3C DNA from Clone

Lane 2 – Negative control.
Three µl of the ligated DNA was transferred into competent DH5α cells and plated on to Ampicillin agar plate. The colonies were screened for the presence of vector with insert. The transformants that resulted were screened for the presence of recombinant plasmid by colony PCR by using VP1 insert specific primer (fig21). Recombinant plasmids from the two positive clones were subjected to digestion with and BamHI and Not I products analyzed by 1% agarose gel electrophoresis using a 1kbp DNA Molecular Weight Marker (Fig.22). The size of the insert was deduced from the standard curve drawn from the log molecular sizes of the marker against their mobility and found to be 2.9 kbp, which is the size of the P1-2A-3C gene.
Fig. 21. Agarose gel electrophoresis of PCR amplified 680 bp DNA from colonies.

Individual colonies obtained after transformation with p CDNA+ ligation mixture were screened for the presence of using ID gene specific VP1-L and Vector specific T7 primers. The PCR products were analyzed by electrophoresis alongside DNA molecular weight marker (Fermentas) in 1.2% agarose gel and the result documented.

Lane 1 – 100bb Ladder

Lanes 1, 2, 3, 4, 6, 7, 8, 10, 11 – PCR amplified b IL18 from different transformants /colonies.

Lane 9- Negative control.
**Fig. 22. Agarose gel electrophoresis of BamH I and NotI digested recombinant p CDNA P1-2A-3C plasmid DNA.**

Purified plasmid DNAs (0.5µg, each) were digested with BamHI and NotI I. The digested DNAs were subjected to electrophoresis in 1% agarose gel and analyzed alongside DNA molecular weight marker (Fermentas) and vector control. The ethidium bromide stained gel showing DNA fragments

Lane M – 1Kbp Ladder

Lanes 1-BamHI and Not I digested vector p CDNAP1-2A-3C plasmid DNA.

Lane 2 – Vector without insert control digestion.
4.3.1. Genes expressed under CMV promoter in mammalian cells

Genes cloned under CMV promoter within the mammalian expression vector were checked for expression of the corresponding proteins in BHK 21 cells after transfection with lipid based Lipofectamine (Invitrogen) as described in Materials and Methods. Subsequently, the proteins were western transferred onto NC membrane and the proteins immunodetected using anti FMDV serum from experimentally infected cattle (Fig.23). Cell lysates from pCDNA P1-2A transfected BHK 21 cells (lanes 1 respectively) showed intensely reacted bands of 83 kDa corresponding to unprocessed P1-2A. However, cell lysate from pcDNA P1-2A-3C transfected BHK21 cells (lane 3,) showed no band corresponding to the 83 kDa. This is probably due to the processing of the P1-2A into the various structural viral proteins, VP0, VP3 and VP1, all of them about 24-28 kDa each and hence the reaction seen corresponding to about 24-30 kDa (lane 3). This processing of P1-2A was probably due to the expression of FMDV 3C protease, the gene for which was included in the DNA construct, P1-2A-3C. The molecular size of 3C protease is reported to be 24 kDa and hence the colored band corresponding to 3C must have also merged with those of structural proteins BHK21 control cell lysate showed bands corresponding to any of those described above indicating the specificity of expression in plasmid DNA transfected cells.
**Fig. 23. Western blot analysis of cell lysates of BHK21 cells transfected with DNA vaccine constructs along with cell lysate of untransfected BHK21 cells. (P1-2A-3C p CDNA)**

BHK21 cells of 80% confluence in monolayer cultures were transfected with the different DNA vaccine constructs using Lipofectamine (Invitrogen) besides a cell control without transfection. The cells were lysed and the proteins from the cell lysates were separated by SDS-PAGE in 10% gel along with prestained standard protein molecular weight marker (MBI Fermentas), electro transferred onto NCM and immunodetected using serum from convalescing cattle after infection with FMD serotype ‘Asia I’ virus and result documented after color development. The color reaction of the proteins.

Lane 1 – Lysate from cells transfected with P1-2A

Lane 2 - Lysate from untransfected cells
Lane 3 - Lysate from cells transfected with P1-2A-3C p CDNA

Lane 4 - Lysate from cells transfected with vector control

Lane M – Standard prestained protein molecular weight marker

4.4 IL18pcDNA

Genes cloned under CMV promoter within the mammalian expression vector were checked for expression of the corresponding proteins in BHK 21 cells after transfection with lipid based Lipofectamine (Invitrogen) as described in Materials and Methods. Subsequently, the proteins were western transferred onto NC membrane and the proteins immunodetected using anti human IL18 Mab. Cell lysates from pcDNA bIL18 transfected BHK 21 cells (Fig 24) (lanes 2, respectively) showed intensely reacted bands of 18 kDa corresponding to bIL18. However, cell lysate from BHK21 cells (lane 3) showed no band corresponding to the 18kDa.
Fig. 24. Western blot analysis of cell lysates of BHK21 cells
ansfected with DNA Vaccine constructs along with cell lysate of
untransfected BHK21 cells. (b IL18 p CDNA)

BHK21 cells of 80% confluence in monolayer cultures were
transfected with the different DNA vaccine constructs using
Lipofectamine (Invitrogen) besides a cell control without transfection.
The cells were lysed and the proteins from the cell lysates were
separated by SDS-PAGE in 10% gel along with prestained standard
protein molecular weight marker (MBI Fermentas), electro transferred
onto NCM and immunodetected using anti human IL-18 Mab and
result documented after color development. The color reaction of the
proteins.

Lane 1 - Lysate from untransfected cells
Lane 2- Lysate from cells transfected with b IL18 p CDNA
Lane M – Standard prestained protein molecular weight marker
4.4.1 PLG Microparticles

PLG microparticles size and distribution was measured by electron microscope (fig25). DNA vaccine constructs was adsorbed on PLG particles and the quantity of the DNA adsorbed on the particles was quantified by spectrophotometer.

Fig. 25. PLG Microparticles  size and distribution was measured by electron microscope.

4.5. Vaccine efficacy studies in guinea pigs

4.5.1. Humural Immune response in Vaccinated Guinea Pigs

Sera samples collected from the vaccinated animals were evaluated for the ability to neutralize FMDV in vitro by serum neutralizing assay (Fig.26). Higher level of neutralizing antibody titers was observed in the group vaccinated with P1-2A-3CpcDNA+IL18pcDNA+PLG compared to inactivated vaccine and P1-2A-3CpcDNAPLG groups at 21st dpv. Booster dose given at 21st dpv has increased the neutralizing antibody titres in all the groups. However, the titre was higher in P1-2A-3CpcDNA+ IL18pcDNA (1.8) group compared to the
other two groups. These results indicated that P1-2A-3CpcDNA+IL18pcDNA could induce significantly higher humoral immune responses than P1-2A-3CpcDNA and inactivated FMD vaccine group (P < 0.05).

The sera samples collected on the 0, 14th, 21st day after primary immunization and 14th (35 the development after primary immunization) day of after booster dose were subjected to sandwich ELISA to assess IgG isotyping. IgG typing ELISA was used to characterize the isotype response in the vaccinated animals (Fig 27a) shows that IgG1 levels increased from 0 day to 35 dpv in all the vaccinated animals. However, P1-2A-3CpcDNA+ IL18 pcDNA+PLG was able to induce higher IgG1 at 14, 21 and 35 dpv of primary immunization compared to other groups. Inactivated vaccine group has shown higher IgG1 levels compared to the P1-2A-3CpcDNA+PLG group indicating higher humoral response elicited by the inactivated vaccine compared to the unadjuvant DNA vaccine. Similarly, P1-2A-3CpcDNA+IL18pc DNA+PLG construct was able to induce higher IgG2 group followed by P1-2A-3CpcDNA+ IL18 pcDNA (Fig27b). Animals vaccinated with inactivated FMD vaccine have shown lower levels of IgG2 compared to the DNA vaccine groups. These observations have shown higher CMI responses in both the DNA vaccine construct groups compared to the inactivated virus vaccine which being commercially used.
Fig. 26. Neutralizing antibody responses in immunized Guinea pigs. Serum samples (n=6) were collected at 0, 14, 21, 35 dpv and neutralizing antibodies were analyzed by serum neutralizing assay. The titers of neutralizing antibodies were expressed as the reciprocal of the last serum dilution that neutralizes 100 TCID50 of FMDV in 50% of the wells. Data were shown as mean ± standard error.
Fig. 27a & b: Levels of IgG subclass in immunized guinea pigs. Serum collected at 0, 14, 21, 35 dpv and IgG sub classes were analyzed by ELISA (n=6). Data were shown as mean ± standard error. a. IgG1 profile b. IgG2 profile.
4.5.2 Cell Mediated Immune responses in vaccinated Guinea Pigs

Proliferation responses of lymphocytes isolated at 0, 14, 21 and 35 day after primary immunization are seen in Fig 28. FMDV-specific T lymphocyte proliferation responses in P1-2A-3CpcDNA+ IL18 pcDNA+PLG vaccinated group was significantly higher than that from other groups P1-2A-3CpcDNA and inactivated vaccine groups (P < 0.05). Even naked DNA vaccine (P1-2A-3CpcDNA) was able to induce significantly higher proliferation response than the inactivated FMD Vaccine (P < 0.05).

![Graph showing lymphocyte proliferative responses in immunized guinea pigs.](image)

**Fig. 28.** Lymphocyte proliferative responses in immunized guinea pigs. Lymphocytes samples (n = 6) were collected at 0, 14, 21, 35 dpv were stimulated with recombinant homologous protein (10 µg/ml) and the proliferation responses were detected by a standard MTT method. Data were shown as mean ± standard error.
4.5.3. Th1& Th2 Cytokine profile in Vaccinated Guinea Pigs

Expression of Th1 and Th2 cytokines in the vaccinated animals was determined by real time PCR by quantifying the IFNa, IFNy, IL2, IL4 and IL25 specific mRNAs. Significantly higher levels of Th1, Th2 cytokine expression was observed in the group inoculated with P1-2A-3CpcDNA+IL18pcDNA+PLG, compared to other two groups (Fig.29a-e). However, Th1 cytokine induction was higher in animals vaccinated with DNA vaccine constructs in a (P1-2A-3CpcDNA) animals vaccinated with FMD inactivated vaccine.
Fig. 29 a&e. Th1 and Th2 cytokine mRNA profile quantitated by Real time PCR

(a) IFN-α mRNA relative expression in total PBMC cells of immunized guinea pigs Data presented as mean±SE of 2ΔΔCt

(b) IFN-γ mRNA relative expression in total PBMC cells of immunized guinea pigs Data presented as mean±SE of 2ΔΔCt

(c) IL2 mRNA relative expression in total PBMC cells of immunized guinea pigs Data presented as mean±SE of 2ΔΔCt.

(d) IL4 mRNA relative expression in total PBMC cells of immunized guinea pigs Data presented as mean±SE of 2ΔΔCt.

(d) IL25 mRNA relative expression in total PBMC cells of immunized guinea pigs Data presented as mean±SE of 2ΔΔCt.
4.5.4. CD4&CD8 Profile in Vaccinated Guinea Pigs

Flow cytometric analysis (Figs. 30a&b) showed that, in all tested groups, the percentage of both CD4+ and CD8+ peripheral blood T-cell subpopulation increased significantly in comparison to the unvaccinated animals after prime immunization. Those levels of have further increased after boost immunization, at 21 day. CD4+ and CD8+ levels in P1-2A-3CpcDNA+IL18pcDNA+PLG vaccinated animals are significantly higher than P1-2A-3CpcDNA and inactivated FMD vaccine groups. However CD4+ population is significantly higher in the group vaccinated with inactivated vaccine, compared the animals vaccinated with DNA vaccine alone without IL18. Where as both the DNA vaccine groups higher CD8+ expression was seen.
Fig-30a & b CD4+ and CD8+ levels were calculated from the number of cells labelled with the fluorescent monoclonal antibodies of anti-CD4, anti-CD8 using a Flow cytometer. N= 6 in each group.
Data presented as percentage mean+SE.  a. CD4+ profile b. CD8+ profile

4.5.5. Virus Challenge experiments in Vaccinated Guinea Pigs

All guinea pigs were subcutaneously inoculated with 100 GPID50 of guinea pig adopted virus 0.2 ml on left rear foot 15 days after the second vaccination. In P1-2A-3C pcDNA+IL18pcDNA+PLG group all the six animals (100%) were protected where as four out of six animals (100%) were protected in case of P1-2A-3CpcDNA. Inactivated FMD vaccine has protected five out of six animals (83%). All the unvaccinated animals have shown the symptoms of FMD after the challenge.

4.5.6. Non Structure Protein specific ELISA

Antibodies against the non-structural proteins (NSPs) were used to differentiate the vaccinated from the infected animals. We measured the level of FMDV NSP 3AB antibodies in sera of all the guinea pigs at 21 days after the challenge. Antibodies against 3AB proteins were negative in all the animals vaccinated with P1-2A-3C pcDNA+IL18 pcDNA+PLG which are completely protected from FMDV challenge. One animal in inactivated FMD vaccine group and two animals in P12A3CpcDNA group were positive to 3AB, which had shown FMD lesions in the challenge experiment.
4.6 VACCINE EFFICACY STUDIES IN CATTLE

4.6.1. Screening of calves for FMDV neutralizing antibodies

All sera samples collected from cattle were heat inactivated. Sera samples at serial dilutions (1:8 to 1:256) incubated with virus (100 TCID50 of FMDV Asia1) for one hr at 37°C, transferred to preformed mono layers of BHK-21 cells and incubated at 37°C (with 5% CO₂) for 48 hrs. Neutralization of CPE was used to determine the end-point, and titers that were calculated as the reciprocal of serum dilution to neutralize 100 TCID50 of FMDV. The animals which are showing low titer of FMD antibodies (1:8 or Less) were used for vaccinating that particular type vaccine.

4.6.2. SNT of the vaccinated animals

Sera samples collected from the vaccinated animals were evaluated for the ability to neutralize FMDV in vitro by serum neutralizing assay. Neutralizing antibody titers in the individual animals was shown in the and group wise average mean values in the fig 31 which describes the antibody titers in P1-2A-3CpcDNA IL18+PLG, P1-2A-3C+IL18 constructs vaccinated groups significantly higher than compared with the controls, conventional vaccine and naked vaccinated groups at 21st dpv as well as on 28th day (after the booster dose at 21dpv).
Fig 31 Neutralizing antibody responses in immunized calves. Serum samples (n=6) were collected at 0, 14, 21, 28 dpv and neutralizing antibodies were analyzed by serum neutralizing assay. The titers of neutralizing antibodies were expressed as the reciprocal of the last serum dilution that neutralizes 100 TCID50 of FMDV in 50% of the wells. Data were shown as mean ± standard error.
4.6.3. ELISA

Sera samples collected on 0, 7, 14, 21, 28 dpv were heat inactivated and subjected to Sandwich ELISA to assess humoral immune response. Sera samples were diluted two fold (1:8 to 1:512) and sandwich ELISA was carried out as described in materials and methods. Absorbance values obtained were corrected with the background absorbance and the corrected values were used to determine the positive samples Mean absorbance was calculated for the negative samples and Standard error was calculated. O.D values above mean+ 3xS.E is taken as The log values of the ELISA titers are shown in bar diagram (Fig. 32) Control animals and the pre vaccinated sera have shown the titer of 0.903. The significantly higher antibodies titers are observed in P12A-3C p CDNA +IL18+PLG and P12A-3C-IL18 (p<0.05) compared with naked vaccinated group and control group.
Fig 32 Analysis of immune response to vaccine construct in calves by sandwich ELISA

Total anti-FMDV total Igs responses in immunized calves. Serum samples (n=6) were collected at 0, 14, 21, 28 dpv.
4.6.4. CMI responses by MTT assay:

Proliferation responses of lymphocytes isolated at 14, 21 and 28\textsuperscript{th} day of primary immunization are seen in Fig 32. FMDV-specific T lymphocyte proliferation responses from the groups of different construct was shown in fig 33. The proliferative response was significantly higher in P1-2A-3C-pcDNA-IL18-pcDNA-PLG, P1-2A-3C-pcDNA-IL18-pcDNA group than that from other groups (P1-2A-3C pcDNA, inactivated vaccine groups) (P < 0.05). DNA vaccine groups have shown higher proliferative responses than the inactivated viral vaccine.
Fig. 33 Lymphocyte proliferative responses in immunized calves.

Lymphocytes samples (n = 6) were collected at 0, 14, 21, 28 dpv were stimulated with recombinant homologous protein (10 µg/ml) and the proliferation responses were detected by a standard MTT method. Data were shown as mean + standard error.
4.6.5. IgG Isotype ELISA

The sera samples collected on the 0, 14th, 21st, day after primary immunization and 7th (28th dpv after primary immunization) day of after booster dose were subjected to sandwich ELISA to assess IgG isotyping. IgG typing ELISA was used to characterize the isotype response in the vaccinated animals (Fig 34a) shows that IgG1 levels increased from 0 day to 28 dpv in all the vaccinated animals. However, P1-2A-3CpcDNA+IL18 pcDNA+PLG was able to induce higher IgG1 at 14, 21 and 28 dpv of primary immunization compared to other groups. Inactivated vaccine group has shown higher IgG1 levels compared to the P1-2A-3CpcDNA+PLG+IL18 group indicating higher humoral response elicited by the inactivated vaccine compared to the naked DNA vaccine. Similarly, P1-2A-3CpcDNA+IL18 pcDNA+PLG construct was able to induce higher IgG2 group followed by P1-2A-3CpcDNA+ IL18 pcDNA (Fig34b). Animals vaccinated with inactivated FMD vaccine have shown lower levels of IgG2 compared to the DNA vaccine groups. These observations have shown higher CMI responses in both the DNA vaccine construct groups compared to the inactivated virus vaccine which being commercially used.
Fig. 34a&b: Levels of IgG subclass in immunized calves. Serum collected at 0, 14, 21, 28 dpv and IgG sub classes were analyzed by ELISA (n=6). Data were shown as mean ± standard error. a. IgG1 profile b. IgG2 profile
4.6.6. Real Time PCR

To monitor the expression of cytokines, they were quantifying by Real Time PCR by the (35 a-e) IL2, IL4, IFNα, IFNγ and IL25 specific mRNAs in which GAPDH a house keeping gene was used as a normalizing control. After vaccination of calves, the mRNAs of Th1 and Th2 cytokines were evaluated and compared with unvaccinated group. Expression of Th1 and Th2 cytokines in the vaccinated animals was determined by real time PCR. Significantly higher levels of Th1, Th2 cytokine expression was observed in the group inoculated with P1-2A-3CpcDNA+IL18pcDNA+PLG, P1-2A-3CpcDNA + IL18pcDNA compared to other two groups. However, Th1 cytokine induction was higher in animals vaccinated with DNA vaccine constructs in a (P1-2A-3CpcDNA) animals vaccinated compared with FMD inactivated vaccine. Th2 cytokine markers IL4 and IL25 levels were significantly higher in inactivated vaccinated group than naked DNA vaccinated group.
Fig. 35 a & e. Th1 and Th2 cytokine mRNA profile quantitated by Real time PCR

(a) IFN-α mRNA relative expression in total PBMC cells of immunized calves Data presented as mean±SE of 2ΔΔCt

(b) IFN-γ mRNA relative expression in total PBMC cells of immunized calves Data presented as mean±SE of 2ΔΔCt.

(c) IL2- mRNA relative expression in total PBMC cells of immunized calves Data presented as mean±SE of 2ΔΔCt.

(d) IL4- mRNA relative expression in total PBMC cells of immunized calves Data presented as mean±SE of 2ΔΔCt.

(e) IL25- mRNA relative expression in total PBMC cells of immunized calves Data presented as mean±SE of 2ΔΔCt.
4.6.7 CD4&CD8 Profile in Vaccinated Cattle

Flow cytometric analysis (Figs. 36a&b) showed that, in all tested groups, the percentage of both CD4+ and CD8+ peripheral blood T-cell subpopulation increased significantly in comparison to the unvaccinated animals after prime immunization. Those levels of have further increased after boost immunization, at 21st day. CD4+ and CD8+ levels in P1-2A-3CpcDNA+IL18pcDNA+PLG, P1-2A-3CpcDNA + IL18pcDNA vaccinated animals are significantly higher than P1-2A-3CpcDNA and inactivated FMD vaccine groups. However CD4+ population is significantly higher in the group vaccinated with inactivated vaccine, compared the animals vaccinated with DNA vaccine alone without IL18. Where as all the DNA vaccine groups higher CD8+ expression was seen. All DNA vaccinated groups CD8+ levels were significantly higher when compared to the inactivated vaccinated group.
Fig-36a & b CD4+ and CD8+ levels were calculated from the number of cells labelled with the fluorescent monoclonal antibodies of anti-CD4, anti-CD8 using a Flow cytometer. N= 6 in each group. Data presented as percentage mean±SE. a. CD4+ profile b. CD8+ profile
4.6.8. Gamma Interferon assay by FACS

Gamma interferons assay was done by the flow cytometry. The percentage of cells expressing γIFN (Fig. 37) are significantly higher in the P12A3C – pcDNA + IL18pcDNA + PLG, P12A3CpcDNA + IL18pcDNA vaccinated groups compared to the other groups. But no significant differences between in these two groups. In case of the inactivated vaccinated group the percentage of gamma IFN levels were significantly higher than DNA naked vaccine group.

![Graph showing percentage of IFNγ cells over time](image)

**Fig-37** IFNγ levels were calculated from the number of cells labelled with the fluorescent monoclonal antibodies of IFNγ using a Flow cytometer. N= 6 in each group. Data presented as percentage mean±SE.
4.6.9. IL4 assay by FACS

To monitor the expression of IL4 was quantified by Flow cytometry. The percentage of lymphocytes expressing the IL4 levels were significantly higher in P1-2A-3CpcDNA + IL18pcDNA + PLG, P1-2A-3CpcDNA + IL18pcDNA groups when compared to the other groups. However in case of inactivated vaccinated group higher levels of IL4 observed compared with naked DNA vaccinated group (Fig 38).

![Graph showing IL4 levels over days of post-vaccination.](image-url)

**Fig-38** IL4 levels were calculated from the number of cells labelled with the fluorescent monoclonal antibodies of IL4 using a Flow cytometer. N = 6 in each group. Data presented as percentage mean±SE.
4.6.10. Gamma IFN ELISA

Plasma were collected 14, 21, 28 dpv blood was used to quantify the gamma IFN levels using bovine IFN monoclonal abs and recombinant IFN γ as standard. The amounts of gamma IFN γ levels were significantly higher in the P12A3CpcDNA + IL18pcDNA + PLG, P1-2A-3CpcDNA + IL18pcDNA groups when compared to the other three groups (fig 39).

Fig 39. IFNγ quantified by ELISA from the whole blood in immunized calves (n=6)
4.6.11. Challenge Studies

All animals were inoculated with 10000 CID50 of cattle adopted virus 0.5 ml on tongue 15 days after the second vaccination. The lesions were observed up to 7 days. In case of P1-2A-3CpcDNA +IL18pcDNA+ and P1-2A-3CpcDNA +IL18pcDNA groups four of six animals were fully protected one animal protected and one partially. The percentage of protection is 67% which was similar in both the groups. In case of inactivated vaccine group three out of six calves were protected one partially protected. These observations are in accordance with the observation in earlier experiments in guinea pigs that IL-18 adjuvant and PLG delivery improves the efficacy of pcDNA constructs and protects the vaccinated animals from the challenge.

4.6.12. NSP ELISA

Antibodies against the non-structural proteins (NSPs) were used to differentiate the vaccinated from the infected animals. To measured the level of FMDV NSP 3AB antibodies in sera of all the calves at 21 days after the challenge. Antibodies against 3AB proteins were negative in four animals vaccinated with P1-2A-3CpcDNA+IL18 pcDNA +PLG one animal was strong 3AB antibodies are present in another animal very weak 3AB antibodies are present. Similarly observed in case of P1-2A-3CpcDNA+IL18 pcDNA group. Two animal in inactivated FMD vaccine group and three animals in P1-2A-3CpcDNA group were positive to 3AB, which had shown FMD lesions in the challenge experiment.