Arch Virol (2005) 150: 967–979
DOI 10.1007/s00705-004-0460-2

Archives of Virology
Printed in Austria

# Protective immune response against foot-and-mouth disease virus challenge in guinea pigs vaccinated with recombinant P1 polyprotein expressed in *Pichia pastoris*

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Received January 20, 2004; accepted October 29, 2004
Published online January 19, 2005 

**Summary.** Vaccination of the susceptible livestock with potent, safe and cost effective vaccine is the primary requirement to control foot-and-mouth disease (FMD) in an endemic country. In this study, an alternative approach was used in which structural protein genes of all the four serotypes of FMDV (O, Asia 1, $A_{22}$ and C) were expressed separately in methylotrophic yeast *Pichia pastoris.* The recombinant polyproteins (P1) were characterized by SDS-PAGE and in Western Blot analysis. Partially purified protein was used for immunization in guinea pigs with different adjuvant formulations and immune response studied. Ninety micrograms of the recombinant protein per monovalent dose was used for immunization. A single injection of a monovalent or polyvalent vaccine was given to guinea pigs with various adjuvant combinations viz., Monovalent recombinant protein either adjuvanted with Montanide-ISA50V or Indigenous oil, Monovalent recombinant protein mixed with $1/10^{th}$ dose of inactivated oil-adjuvanted virus vaccine and Polyvalent recombinant protein with Montanide ISA50V. FMDV specific humoral immune response was observed at about $28^{th}$ day post vaccination. The immune response as assessed by indirect ELISA and Serum neutralization test titres was found to be 320–640 and 16–32, respectively. When challenged with virulent homologous type 'O' virus, the guinea pigs showed protective C index of 2.01,1.81, 2.56 and 2.48, respectively, with above said adjuvant combinations. The study has shown that yeast-expressed FMDV P1 polyprotein in a single dose could elicit a protective immune response in guinea pigs, and this could be a possible future vaccine candidate in homologous host.

This article is dedicated in memory of late Dr. S. M. Lal.

## Introduction

Foot-and-mouth disease (FMD) is an acute, febrile and highly contagious vesicular disease affecting cloven-hoofed animals having potential to cause explosive epidemics and heavy economic losses to the livestock industry worldwide. This disease is enzootic in several countries in Africa, Asia and South America as well as the Indian subcontinent. FMD is threatening even in non-endemic countries, since it has devastating effect on the economy. Outbreak in UK during the year 2001 is the best example of such an effect. FMD is caused by a virus of the species *Foot-and-mouth disease virus* (FMDV), belonging to the genus *Aphthovirus* of the family *Picornaviridae*. The virion consists of a positive sense 8.5 kb ssRNA, enclosed in an icosahedral capsid comprising 60 copies each of four structural polypeptides VP1-4 [3] which are secondary cleavage products of P1 polyprotein, a primary cleavage product of a 250 kDa polyprotein coded by the genome [41].

FMDV shows high antigenic variability, which is reflected in the existence of seven serotypes (O, A, C Asia 1, and SAT 1–3) and several strains [17], of which O, Asia 1, $A_{22}$ and C are common serotypes prevalent in India. Frequent emergence of antigenically variant viruses is increasingly recognized as one of the major problems for designing an effective vaccine [27]. The strategy used for the control of a FMD outbreak depends upon the countries, which have been affected by the epizootic. In countries with no previous history of FMD, which are classified as FMD free country, the disease is controlled by strict movement controls and slaughter of infected and exposed animals, whereas regular prophylactic vaccination is the only method of choice in endemic countries such as India. Vaccination with one serotype does not protect animals from infection with other serotypes and therefore vaccination programmes often utilize multivalent inoculation schedule. Currently, conventional binary ethyleneimine (BEI) inactivated vaccines emulsified with adjuvant have been widely used in effective control and eradication programmes in South America, Africa, and Asia. However, production methodology of conventional vaccine is associated with the risk of virus escape from containment laboratories [9]. In addition, the presence of uninactivated virus in the vaccine is leading to vaccination related outbreaks [22]. Moreover cell culture based vaccine produced in highly sophisticated laboratory is cost intensive. These limitations have led to the search for an alternative safe vaccine, which avoids the handling of live virus.

Chemically inactivated viral particle contains non-infectious genome while retaining the antigenic epitopes present in infectious particles against which immune response is elicited. Similarly, empty capsids, which are naturally produced during virus replication in cell culture lack viral nucleic acid and have been shown to produce neutralizing antibody response similar to that of native virus particle [16, 39]. Therefore production of structural proteins in heterologous host systems using recombinant DNA technology and using them as vaccines could be an ideal approach.

Several approaches have been made to develop alternative FMD vaccines, which include biosynthetic proteins such as recombinant capsid precursor protein, synthetic peptides, naked DNA vectors, and recombinant viruses. Capsid precursor

polyprotein (P1-2A) may fold in such a way that it maintain discontinuous epitopes involved in virus neutralization, which opens the possibility of using unprocessed capsid precursor as novel antiviral immunogen [40]. Production of capsid (P1 protein) in various heterologous host systems such as, bacteria [25], and insect cells [37] have been tried with limited success. Desired levels of expression and stability of the capsid could not be achieved in these systems, possibly due to the low pH of the medium and/or the nature of the construct. In view of this constraint, mammalian cells have been tried as expression system [1]. However production of stable transformants is not possible in mammalian cells and hence, further studies have not been pursued. Alternatively, yeast based expression system is unique, because it combines the advantages of both prokaryotic (high expression levels, easy to scale-up and inexpensive growth media) and eukaryotic (capacity to carry out most of the post-translational modifications) expression systems. In recent years, the methylotrophic yeast, *Pichia pastoris* has emerged as a powerful heterologous expression system for the production of high levels of functionally active recombinant proteins [14]. This presumption and other success reports on yeast expressed Polio [36] and Dengue virus [42] protein as vaccines have prompted us to use *Pichia pastoris* as a host to express the FMDV proteins. In this paper we report the production of structural proteins of FMDV serotypes (O, A, C, Asia 1) in *Pichia pastoris* and their application as vaccine candidate to protect the animal against FMD.

## Materials and methods

### *Vaccine viruses*

FMDV vaccine strains serotype "O" [Ind 75Madras], "Asia 1" (Ind 63/72), "A 22" [Ind 17/77] and "C" [Ind] maintained at the FMD centre, Indian Veterinary Research Institute, Bangalore grown in BHK-21 (Baby Hamster Kidney cell line) $Clone_{13}$ cells were used in this study.

### *Sera samples and conjugate*

Rabbit hyper immune serum raised against either the whole virus (146S particles) or the immunodominant peptide (C – terminal half of VP1) [43], anti-guinea pig and anti-rabbit IgG-HRPO conjugates were procured from Sigma, USA and Bangalore Genei, India, respectively and were used in this study.

### *Host strain and plasmid vector*

The $His^-$ *Pichia pastoris* host strain GS115 (his4) and the yeast transfer vector; pPIC-9K, were from Invitrogen. The vector contains the *P. pastoris* AOX1 (Alcohol Oxidase) promoter and transcription termination sequences, which are separated by MCS for insertion of the foreign genes of interest. Transformants, which become $His^+$ are selected on $His^-$ plates.

### *Amplification and cloning of P1 gene*

Viral RNA (from all the four serotypes) was extracted directly from BHK-21 infected cell culture supernatant by using TRIZOL reagent (Gibco BRL) following the protocol of the manufacturer. The cDNA was synthesized by reverse transcription (RT) reaction using downstream 2Br primer {5′d GCCGTTCTTGAGAATGGC-OH3′} and amplification of P1-2A was carried

out using upstream P2 {5′dGGGAATTCCACACAACCAACACTCAAAACAAA-OH3′} and nested downstream 2AR{5′dGCTAGTGCGGCCGCGAAGGGCCCAGGGTTGGACTC-OH3′} primers as described elsewhere [7].

The amplified PCR product in each case was purified from gel using wizard PCR preps DNA purification system (Promega) and ligated into the pPIC-9K at EcoRI and NotI sites and transferred into *E. coli* DH5α. The recombinant colonies were screened by colony PCR, using 5′ VP1L (5′dGGGGAATCTGCAGATCCAGTCAC-OH3′) and 3′ 2AR primers, followed by insert release. The P1 insert of each type was sequenced (at the 5′end) using the ABI 377 Perkin-Elmer automated DNA Sequencer to confirm the frame of the insert in pPIC-9K vector.

### *Expression of cloned P1 genes in Pichia pastoris*

Recombinant plasmid (all the four types) was linearized with SalI and transferred into GS115 strain of *Pichia* by electroporation as described elsewhere [7]. Transformants harbouring the plasmid-borne His 4 marker were selected on minimal agar plates lacking Histidine. The presence of the insert in the genome of the positive colonies was confirmed by PCR amplification using 5′ AOX1 (Invitrogen) and 2AR (3′end), the vector and insert specific primers, respectively. Induction of protein expression was carried out by the procedures described by Cregg et al. [15]. Briefly, 25 ml each of buffered glycerol-complex medium (BMGY) in two 250 ml flasks was inoculated with single colony of $His^{+}Mut^{+}$ positive clone and the vector transformant of *Pichia.* Both the flasks were incubated at 28–30 °C in a shaking incubator (250–300 rpm) to reach an $A_{600}$ of 2–6 (≈16–18-hrs). The cells were harvested and resuspended in Buffered Methanol-complex Medium (BMMY) to an $A_{600}$ of 1.0 (about 100–200 ml mediums) in one-litre flasks to induce expression [7]. After 96 hrs of induction, the entire culture supernatant was harvested and the secreted proteins were precipitated with 50% saturation of ammonium sulphate, dialyzed against 1 X PBS and used for further studies. Protein content was estimated by dye binding assay [13]. The expressed proteins were analyzed by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions as per the standard protocol [24] and the specificity of the proteins was confirmed by immunoblot assay [7].

### *Vaccine preparation*

The concentrated recombinant proteins were filtered through 0.2 μm membrane, mixed with formalin (formaldehyde 37.87%) at a final concentration of 0.06% (v/v) and incubated for one hour at 4 °C to stabilize the protein and was used as an immunogen in vaccine preparation.

The recombinant protein of type ‘O’ was mixed with Montanide – ISA50V (Seppic, Paris, France) water in oil adjuvant, or the indigenous oil adjuvant (developed at IVRI, Bangalore, applied for Patent) at an 1:1 ratio and emulsified by mixing several times through glass syringe with hobbled needle until a clear stable emulsion was formed. The stability of the emulsions on storage at 4 °C, room temperature and at 37 °C was tested by drop test. Similarly, 250 μl vector transformed yeast proteins (90 μg/dose) was mixed with the Montanide adjuvant as described above.

### *Vaccination of guinea pigs*

Albino guinea pigs of either sex weighing 400 to 500 g were obtained from National Tuberculosis Institute, Bangalore, India and were used for vaccination studies. Two groups of guinea pigs (20 each) were vaccinated separately with 0.5 ml/dose vaccine containing 90 μg of type ‘O’ antigen after mixing with either Montanide ISA 50V (group I) or Indigenous oil

(group II). Third group of 20 animals were vaccinated with polyvalent vaccine after mixing with 90 μg each of the four serotype specific proteins/dose with Montanide – ISA 50V. Fourth group of 20 animals were vaccinated with recombinant P1 protein (90 μg) of type 'O' mixed with 1/10$^{th}$ guinea pig dose of inactivated cell culture adjuvanted vaccine as an adjuvant as well as for boosting the conventional vaccine. Fifth group of 15 animals were vaccinated with *Pichia pastoris* native secretory proteins (90 μg)/dose after mixing with Montanide – ISA 50V as a control.

Each vaccine in 0.5 ml volume was administered intramuscularly in a single dose at the gluteal region [5]. The animals were bled at 28$^{th}$ day after post vaccination (d.p.v.) and the collected sera were tested for the presence of antibodies against the virus by ELISA and Serum neutralization test.

*Analysis of immune response to recombinant protein vaccine*

A. Indirect enzyme linked immunosorbent assay (ELISA)

Immune response to the recombinant protein vaccine was determined by indirect ELISA as described by earlier [2]. Briefly, tissue culture FMDV ($10^4$ Plaque forming units) was coated in flat bottomed 96 wells plate (Nunc, Denmark) and the left over sites were saturated with 3% BSA in PBS-T. The test sera from the vaccinated animals were two fold diluted starting from 1:40 to 1:640 in PBS-T containing 1% BSA. The antigen-antibody reaction was followed by further incubated with anti-guinea pig HRPO conjugate (1:3000 dilution) and detected by colour development with chromogen, ortho diamine dihydrochloride (ODD) as substrate. The highest dilution of the sera showing difference of 0.2 O.D. at an absorbance of 492 nm than the control at the same dilution (healthy serum/pre-vaccinated serum) was taken as ELISA titre.

B. Micro Serum Neutralization Test (SNT)

Micro serum neutralization test was carried out using BHK-21 clone 13 cells according to Rweyemamu et al. [38]. The anti sera were diluted in Eagle's medium in a two-fold dilution starting from a 1:4 to 1:64 in 96-well flat-bottomed tissue culture plates (Nunc, Denmark). Virus suspension having a titre of 100 $TCID_{50}$ in 50 μl was added to each well and the mixture was incubated for 1 hour at 37 °C in a humidified $CO_2$ incubator. 50 μl of BHK-21 cell suspension ($\sim 1.5 \times 10^6$/ml) was added to each well and further incubated for 48 hrs. Appropriate serum, virus and cell controls were included in the test. The plates were observed under microscope for cytopathic effect.

C. Challenge study for protective response

All the challenge experiments were carried out in a highly contained environment at Animal Experimental Station of the Institute. The challenge virus was prepared from guinea pig adapted homologous FMDV type 'O' virus (at 6$^{th}$ passage). Virus was prepared from footpad as 10% (w/v) suspension, clarified, passaged freshly in two guinea pigs footpads by intradermoplantar tunnelling route and the pads from the secondary lesions were collected for subsequent preparation for the challenge study. Challenge experiment in guinea pigs was carried out according to Lucam et al. [26]. Both the vaccinated and control animals were challenged with virulent virus (at 7$^{th}$ passage) at different dilutions ($10^{-2}$ to $10^{-5}$ fold) on 28$^{th}$ day of post vaccination (DPV). The animals were observed after 2–3 days of the challenge for the development of primary and secondary lesions. Appearance of vesicles on the un-inoculated footpad was considered as a positive reaction. The observations were recorded and the virus titre ($GPI_{50}$-Fifty percent Guinea pig infective dose) was calculated

both in vaccinated and control animals [34]. The protective C index was calculated from the difference of virus titres between control and vaccinated animals. The titre difference of 10 log 2.0 was considered to be protective.

## Results

### *Expression of P1 polyprotein in Pichia*

The sequences coding for P1-2A of four serotypes (O, A22, C and Asia 1) were amplified by RT PCR separately. The amplified 2.1 Kbp fragments which includes VP1-3, partial VP4 and 2A in each case (data not shown) was cloned into pPIC-9K. The positive colonies in each case were confirmed by the insert release followed by nucleotide sequence analysis. The recombinant plasmid of each serotype was transferred into GS115 strain of *Pichia* by electroporation. The $His^+$ *Pichia* transformants were analyzed for the presence of the insert in the yeast genome by PCR (fig. not shown). PCR positive *Pichia* clones of each type were grown separately and induced for expression with 0.5% methanol. Since the inserted gene is in the down stream of the secretary signal sequences, the expressed gene product was expected to be secreted out in to the medium. Therefore, culture supernatants were collected at 96 hrs and the proteins secreted out were analyzed along with supernatant from the control vector transformants.

Proteins secreted after 96 hrs inductions were analyzed by SDS-PAGE (Fig. 1[A]). A single intense band of 78 kDa (in lane, 1) protein, in addition to minor *Pichia* native proteins were observed in case of clone with P1-2A insert corresponding to the truncated P1-2A protein expressed after induction.

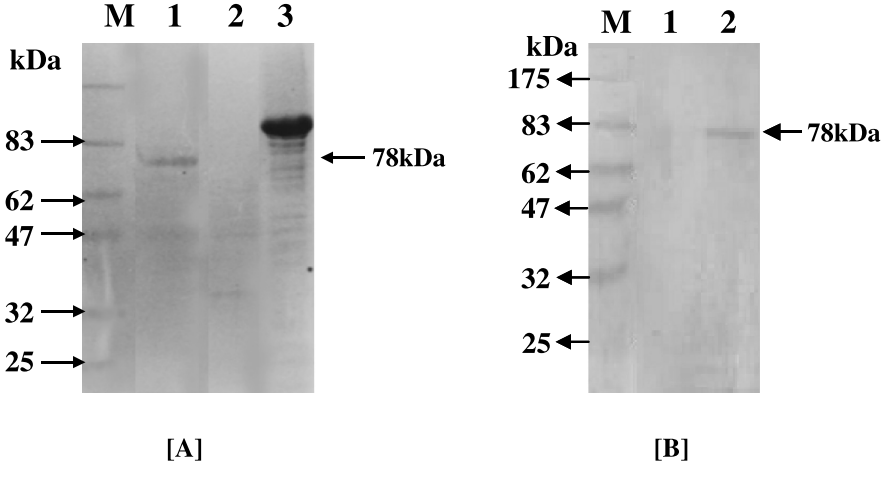


**Fig. 1.** Characterization of expressed FMDV protein. **A** Protein profile by SDS-PAGE: *M*: Standard protein molecular Weight Marker (New England Biolab). *1*, *2*: Proteins from induced and uninduced culture supernatant of yeast cells carrying P1-2A insert of type O, respectively. *3*: Yeast native proteins from culture supernatant of yeast cells carrying without insert. **B** Western blot: *1*, *2*: Culture supernatant of induced yeast cell culture carrying vector without insert and with insert, respectively

The same intense band was not seen in case of clone without insert (lane 3) but has more number of *Pichia* native proteins. This indicates that the intense band of 78 kDa protein may be the expressed protein by induced clone containing the insert. The specificity of the expressed protein was confirmed in immunoblotting. The protein was transferred on to the nitrocellulose membrane and immunodetected with antiserum specific to either the whole virus or immunodominant VP1 region (Fig. 1[B]). An intense colour reaction was observed with the protein band corresponding to 78 kDa (lane 2) indicating that 78 kDa protein is FMDV specific and no colour reaction was observed with the proteins from uninduced culture and vector-transformed clones.

### *Immune response of recombinant protein vaccine*

The immunological responses to polyvalent as well as type 'O' Monovalent P1 protein vaccines were studied using guinea pig (G.pig) model as host system. Since the type 'O' is a poor immunogen, monovalent was tried with various adjuvant combinations. The antibody response was measured by ELISA and micro neutralization assay as described earlier (Table 1). Though, FMDV specific humoral response was observed in all the G.pigs after the single injection of recombinant protein, the response was higher in the case of group III and group IV. Control animals vaccinated with yeast secretary protein did not show any increase in neutralizing antibody titres in the serum, where as increase of neutralizing antibody titre measured by micro SNT was maximum in the case of vaccinated group III and IV. Sera collected from polyvalent group (group III) were tested for the presence of antibodies against FMDV serotypes by SNT.

**Table 1.** Immune response of recombinant P1 polyprotein with various adjuvant formulations after 28$^{th}$ day of immunization

| Test | Group | | | | |
|---|---|---|---|---|---|
| | I | II | III | IV | V |
| ELISATitre[a] | 320–640 | <320 | 640 | ≥640 | <40 |
| SNT titre[b] | 1:16 | 1:8 | 1:16, 1:32<br>1:16, 1:16–32[c] | 1:16–32 | 1:2 |

[a]The highest serum dilution at which A 492 nm reading was double that of the control

[b]The highest serum dilution at which cytopathic effect could be seen in 50% of the monolayer cells

[c]SNT titre of FMDV Type O, Asia 1, A22 & C, respectively

Group I: Monovalent recombinant protein (type O) adjuvanted with Montanide ISA 50V
II: Monovalent recombinant protein (type O) adjuvanted with Indigenous oil
III: Polyvalent recombinant protein adjuvanted with Montanide ISA 50V
IV: Monovalent recombinant protein (type O) with 1/10$^{th}$ dose of cell culture vaccine in indigenous oil
V: Control group vaccinated with yeast's native protein

**Table 2.** Results of the guinea pig challenge studies[a]

| Groups | Challenge virus dilutions[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $10^{-2}$ | | $10^{-3}$ | | $10^{-4}$ | | $10^{-5}$ | | Protective index |
| | Infected | Protected | Infected | Protected | Infected | Protected | Infected | Protected | |
| Group I | 3 | 2 | 1 | 4 | 0 | 5 | 0 | 5 | 2.01 |
| Group II | 3 | 2 | 2 | 3 | 0 | 5 | 0 | 5 | 1.81 |
| Group III | 2 | 3 | 0 | 5 | 0 | 5 | 0 | 5 | 2.48 |
| Group IV | 1 | 4 | 1 | 4 | 0 | 5 | 0 | 5 | 2.56 |
| Group V | – | – | 5 | 0 | 3 | 2 | 1 | 4 | – |

[a]Observations were taken 96 hr after challenge. Appearance of the secondary lesion was taken as infected
[b]Five numbers of animals in each dilution were included
Groups: As described under Table 1

*Protective response of the P1 polyprotein*

Since the vaccinated animals showed humoral antibody response, they were challenged with virulent FMDV type 'O' G.pig adopted virus (at 7$^{th}$ passage) at 28$^{th}$ DPV. Results of the challenge study (Table 2) shows that yeast expressed P1-2A protein adjuvanted with Montanide ISA 50V elicited virus specific immune response in guinea pigs and the magnitude was sufficient enough to protect the animals against infection. The challenge virus titre as expressed in 10 log dilution at which 50% of the guinea pigs were affected in the control group was 4.32. While the virus titre in vaccinated (Montanide ISA-50V adjuvanted) group was 2.31. Four animals exhibited primary lesions in this group with a G.pig protective index (C-index) of 2.01. Surprisingly, the Guinea pigs vaccinated with recombinant protein mixed with 1/10$^{th}$ dose of cell culture inactivated oil-adjuvanted vaccine and polyvalent recombinant vaccine adjuvanted with Montanide ISA 50V showed C indices of 2.56 & 2.48 with only one & two animals showing primary lesions, respectively. Vaccine with indigenous oil gave lower titre, with a protective C index of 1.81 with 8 animal showing primary lesions. Guinea pigs vaccinated either with a mixture of 1/10$^{th}$ dose of conventional vaccine and monovalent recombinant protein (90 μg) or polyvalent recombinant protein vaccine (90 μg of each protein) gave much higher guinea pig protective C indices compared to monovalent protein vaccine alone.

## Discussion

Production of safe and potent vaccine for FMD is always a priority task to FMD endemic countries. If the vaccine production strategy by any means avoids the handling of live virus it could even be much better to all the countries. Recombinant P1 protein derived from the vaccine strains (representative strains of the serotypes) of the specific region could be better approach for the production

of such vaccines. To expedite such an approach, capsid protein precursor (P1-2A) was produced in yeast and studied as vaccine. The method circumvents several problems encountered in expressing the P1 as, reported earlier. P1-2A sequence of vaccine strains of the four serotypes (O, A22, C and Asia 1) were expressed as secretary proteins using *Pichia pastoris* and evaluated the ability of the recombinant proteins to induce neutralizing antibodies in guinea pigs.

P1-2A sequence of each serotype was cloned downstream of the highly inducible AOX1 promoter along with signal peptide sequence at 5′end for high-level expression and secretion. The calculated size of the truncated P1 lacking 50 aa (150 nts) at N-terminal and carrying 16 aa 2A as a fusion at C-terminal end along with the vector-coded sequences of 18 aa (7 and 11 at N and C termini, respectively) comes to 78 kDa, which is in agreement with mobility of protein observed in SDS-PAGE which is the reported size, indicating that the 78 kDa protein is the product from the cloned P1-2A insert [18]. The major band in Fig. 1A, lane 3 could be the proteins from the lysed yeast cells. Western blot analysis of the proteins with anti serum raised in guinea pigs against the yeast cell protein reacted with the major band (lane 3) but not with the 78 kDa protein band confirming that, 78 kDa proteins is FMDV specific. Immuno-blot analysis with serum against whole virus as well as VP1, confirmed that the epitopes on 78 kDa protein are available for reaction with antibodies. Bayry et al. [10] has reported that the Asia 1 specific serum raised against ISCOM preparation of immunoreactive protein (C-terminal half) reacted more efficiently with the native virus in ELISA, than denatured. Western blot analysis of 78 kDa protein gave positive reaction with VP1 specific serum as well as serum against 146S virus particles indicating that the immuno-reactive sites are available for reaction. The quantity of protein produced by the recombinant yeast clone of each type varied between 120–150 mg/L culture which is in the reported range of 6.3 mg to 12 g/L culture by various groups depends on the nature of the protein [35, 45].

Earlier studies [6, 8, 20, 21, 30], using several oil adjuvants of the Montanide series (Seppic, Paris) have suggested that some of these adjuvants appear to be promising candidates in the formulation of new generation FMD vaccines. In this study, the guinea pigs immunized with adjuvanted recombinant P1-2A protein at a single dose, induced FMDV specific immune response at $28^{th}$ d.p.v. ELISA and SN titre was found to be 320–640 and 16–32, respectively. The neutralizing antibody response (as shown in the Table 1) indicated that, the yeast expressed P1-2A proteins have native confirmation and elicited significant neutralizing antibody response.

The serum neutralizing titres have been shown to have positive correlation with protection against FMDV challenge in cattle, sheep and pigs [12, 31, 32]. However, this correlation as per few reports is not precise. Some animals that possess antibodies lower than the threshold antibody titre can resist challenge, whereas others with higher titres do not, indicating certain non-antibody mediated immune mechanisms play a role [28, 46].

In our studies no positive correlation was observed between antibody titers and animal protection. The high ELISA titers in this experiment may be due to the

exposure of various epitopes on the tricellar surface and the background titer of 1:40 in case of control may be due to the non-specific response to yeast proteins. Similar results were reported in case of a 45 kDa fusion protein of type A12 as detected by mice protection test [23] and in case of truncated 20 kDa protein of Asia1 by SNT [44]. It has been reported by McCullough et al. [29] that, there was no positive correlation between antibody titers and protection, as immunization with recombinant proteins along with different adjuvant combinations showed relatively moderate neutralizing antibody response (1:16) but protected the animals against the challenge by the virulent virus at different degrees. Few animals, could not withstand the challenge with virus (as indicated by the appearance of secondary lesions), which may be due to low humoral response or individual variation.

In our study, guinea pigs group vaccinated with Montanide ISA-50V adjuvanted vaccine, showed better protection (C index 2.01) and local reaction was observed only in a few animals compared to that of other groups. The group vaccinated with, recombinant protein along with $1/10^{th}$ dose of cell culture inactivated oil-adjuvanted vaccine has showed highest protective C index (2.56) followed by polyvalent vaccine group adjuvanted with Montanide ISA 50V (2.48). The highest protective response with the mixed vaccine could be due to the presence of inactivated virus and other virus-induced proteins, which may have the priming effect; however, this needs further study. Low SN titres and protective response in case of indigenous oil adjuvanted vaccine may be due to improper emulsification as observed during preparation. Gomes et al. [19] found that the method used for blending does affect the quality of the vaccine in terms of the protection achieved. Peptide/recombinant protein vaccines can be administered at high concentrations in small volumes. Volume-dependent local reactions can be minimized in this case [4] by concentrating the purified proteins in the aggregated form or in the form of ISCOMs [11]. It is advantageous to use proteins in the form of emulsion rather than in aggregated form in order to get native conformation as well as adjuvant effect.

In a disease outbreak situation, high quality vaccine, which elicits rapid immune response is highly desirable as it can be used in emergency. Such vaccines should be efficacious in all susceptible species and induce a rapid and high protective response after primary vaccination [8]. Further studies are to be carried out to evaluate the efficacy in the homologous host.

In conclusion, our present results showed the potentiality of recombinant vaccine produced in *Pichia pastoris* and vaccination with a single injection of 90 μg of protein could elicit a high immune response, which is sufficient enough to protect guinea pigs from virulent virus. Though our preliminary experiments with guinea pigs are quite encouraging, further studies are needed to determine the efficiency of these proteins as vaccines in natural hosts. Subsequent validation of the vaccine in homologous host, evaluation on large number of animals at the field level and development of suitable method for scaling up the production of recombinant protein using *Pichia pastoris* will help in effectively implementing the FMD control programme.

## Acknowledgments

We wish to thank the Director, IVRI, Izatnagar, Joint Director, IVRI, Bangalore for providing all facilities, ICAR, New Delhi for providing SRF to V.B. and Dr. Vincent Ganne, Manager, Vaccine Development, Seppic, Paris, for providing us the Montanide adjuvant to carryout this work. We are indebted to late Dr. S.M. Lal, ex-Joint Director, IVRI Bangalore Campus, for his support and encouragement.

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