

Expression and purification of virus like particles (VLPs) of foot-and-mouth disease virus in Eri silkworm (*Samia cynthia ricini*) larvae

Manoj Kumar¹ · P. Saravanan¹ · S. K. Jalali²

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Abstract Foot-and-mouth disease (FMD) is a highly contagious viral disease, which causes severe economic loss to livestock. Virus like particles (VLPs) produced by recombinant DNA technology are gaining importance because of their immunogenic properties and safety in developing a new vaccine for FMD. In the present study, a practical and economically feasible approach of expression, purification and characterization of VLPs of FMDV in Eri silkworm (*Samia cynthia ricini*) larvae was described. Although three lepidopteran insect larvae (*Helicoverpa armigera*, *Spodoptera litura* and *Samia cynthia ricini*) were tested for production of VLPs, expression was obtained only in Eri silkworm larvae. High titred recombinant baculovirus encoding the polyprotein P1-2A-3C of FMDV was prepared in Sf9 cells. Injection of recombinant baculovirus into hemocoel of Eri silkworm larvae resulted in increasing levels of expression of VLPs in the hemolymph from 3 to 7 days post infection (dpi) compared to low level expression by oral feeding. The VLPs reacted in Sandwich ELISA with serum raised against whole virus particles of FMDV type O/IND/R2/75 and protein banding pattern of 26, 37 and 47 kDa in Western blotting demonstrated their antigenic resemblance to native virus. Sucrose density gradient purified VLPs were used for immunization of rabbits and guinea pigs for assessing immunogenicity.

Further, the reactivity of serum samples of rabbits and guinea pigs in Indirect-ELISA with titres (1.30–2.81 Log₁₀) indicated that the VLPs were antigenic and immunogenic in nature. We demonstrate that Eri silkworm larvae could be used for production of VLPs of FMDV type O/IND/R2/75 for the first time. This approach could be useful for large scale production of recombinant VLPs for vaccine or diagnostic use in FMD control programme.

Keywords FMDV · Virus like particles (VLPs) · Eri silkworm larva · Hemolymph · Baculovirus expression · Purification of VLPs

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of livestock that causes severe economic loss as a result of restrictions on international trade of animals and animal products. In India, the annual estimated economic loss due to FMD ranges from 3.0 to 4.5 billion dollars [17]. FMD is a transboundary disease, caused by foot-and-mouth disease virus (FMDV), a positive-sense single-stranded RNA virus of the genus *Aphthovirus* within the *Picornaviridae* family [16]. The viral RNA is translated from a single open reading frame into a polyprotein P1, which is cleaved by 3C viral protease to produce structural and non-structural proteins. These proteins self assemble to form mature virus after encapsidating viral RNA [6]. Vaccination of livestock with oil adjuvanted binary ethyleneimine (BEI) inactivated FMD viral antigens is the commonly practiced method in several countries to control and eradicate FMD [15]. The disadvantages associated with oil adjuvanted vaccine include short duration of immunity, risk of incomplete inactivation and live virus escape from

✉ P. Saravanan
drsaravana72@dr.com; drsaravana72@yahoo.com

¹ Foot-and-Mouth Disease Vaccine Centre, Indian Veterinary Research Institute, Bangalore Campus, Hebbal, Bangalore, Karnataka 560024, India

² National Bureau of Agricultural Insect Resources, H A Farm Post, Bellary Road, Hebbal, Bangalore, Karnataka 560024, India

production facility, requirement of high containment biosecurity to handle the virus and inability to distinguish vaccinated from infected animals (DIVA). The concerns related to usage of inactivated FMD vaccine coupled with requirement of regular booster to sustain herd immunity [19] warrants continuous search for a better FMD vaccine. Further, use of BEI inactivated cell culture based vaccine in mass vaccination programme does not provide opportunity in employing differentiation of infected from vaccinated animals (DIVA) [3]. With the advent of genetic engineering and recombinant DNA technology, scientists are searching for alternative approach to develop safe vaccine using expression systems like yeasts [5], *Escherichia coli* [10] and insect cells [1].

Baculovirus expression system has been employed for producing virus like particles (VLPs) of swine vesicular disease virus (SVDV), enteroviruses, hepatitis C virus (HCV), poliovirus and FMDV type Asia 1 and type O viruses [2, 4, 7, 8, 14, 18, 20]. Although VLPs may successfully be produced using insect cells, large scale production needs standardized protocol to avoid batch to batch variation and degradation of the expressed VLPs in acidic pH of cell culture system. Production of VLPs in vitro in insect cells involves time consuming and laborious methods for scaling up of cells and downstream processing. In this context, the present study was conducted to express VLPs or capsid proteins of FMDV in Eri silkworm larvae. Eri silkworm larvae can be produced economically at reduced cost in large scale when compared to insect cells. Various species of silkworms have been used in expression and their utilization for production of vaccine in China [10–14]. This research work was envisaged with aim of expressing VLPs of FMDV type O/IND/R2/75 in insect larvae of Eri silkworm as bio-reactor and characterizes their antigenic and immunogenic nature.

Materials and methods

Eri silkworm, cotton bollworm and tobacco caterpillar larvae

Larvae of Eri silkworm, cotton bollworm and tobacco caterpillar used in the present study were procured from National Bureau of Agricultural Insect Resources (NBAIR), ICAR, Hebbal, Bangalore, India. The larvae were reared on castor leaves, *Ricinus communis*, in the laboratory. Each mated females lays about 750 eggs and larvae after hatching are provided with castor leaves to feed. The larvae are reared in groups and pupate in about 30 days. Therefore, large scale rearing is possible as no disease is encountered while rearing them.

Cells and viruses

Spodoptera frugiperda (Sf9) insect cells procured from M/S Invitrogen were maintained at 27 °C in Sf-900 II SFM medium (Invitrogen), BHK₂₁ cells and recombinant baculovirus expressing FMDV P1 polyprotein with mutated 3C protease were available at FMD Vaccine Production Laboratory, Indian Veterinary Research Institute (IVRI), Bangalore, India. The recombinant baculovirus expressing P1-2A-3C of FMDV type O was propagated in Sf9 cells and stored at 4 °C.

Recombinant baculovirus produced in Sf9 cells was confirmed for the presence of gene of interest, P1-2A-3C, by isolating DNA and subsequent PCR amplification using VP4-F: 5'TGGGATCCATGGGAGCYGGGCAATCCAG3' and 3C-R: 5'TGAAGCTTACTCGTGGTGTGGTTCGG3' primers.

Infection of Eri silkworm, cotton bollworm and tobacco caterpillar larvae

Eri silkworm larvae were infected via per oral route with stock recombinant baculovirus by feeding the starved larvae with castor leaves soaked in the recombinant baculovirus (200 µL). In case of injection route, 50 µL of recombinant baculovirus was injected into hemocoel of each larva. The larvae were daily observed for changes in the behaviour, feeding habit and mortality due to baculovirus infection. Preparation of whole larval extract and collection of hemolymph was performed on specified time intervals of 1–8 days post infection (dpi). Hemolymph and whole larval extract prepared at defined time intervals were tested in sandwich ELISA (S-ELISA) to optimize route of infection. Similarly larvae of cotton bollworm [*Helicoverpa armigera*] and tobacco caterpillar [*Spodoptera litura*] were tested for permissiveness to baculovirus by per oral and hemocoel routes of administration.

Purification and quantification

Hemolymph (2.5 mL) collected from Eri silkworm at 7 and 8 dpi was purified through 20–60 % sucrose gradient (0.5 mL each of 20, 30, 40, 50 and 60 % sucrose) by ultracentrifugation at 120000×g for 16 h at 4 °C in 5 mL polyallomer tubes. After ultracentrifugation, fractions of 0.5 mL each were collected from bottom to top and tested in S-ELISA. S-ELISA was conducted on hemolymph and whole larval extracts as per the method of Bhat et al. [2] with slight modifications. Briefly, the 96-well ELISA plate (Nunc, Maxisorp) was coated with 50 microlitre (µL) of FMDV anti-146S serum raised in rabbit at 1 in 1000 dilution in carbonate-bicarbonate buffer (pH 9.6) and incubated at 37 °C for 1 h. All reagents were added in

50 μ L volume for each well. After incubation, the plate was washed three times with PBST (Phosphate buffered saline (PBS) containing 0.05 % Tween-20). Samples were added in duplicates, with four wells each for positive (viral antigen) and negative (Sf9 cellular antigen) controls. The plate was incubated and washed as described earlier. The anti-146S guinea pig tracing antibody diluted 1:4000 in blocking buffer (PBST + 5 % adult donor bovine serum) was then added to each well. The plate was washed after incubation at 37 °C for 1 h and anti-guinea pig IgG conjugated to horse raddish peroxidase (Dako, Germany) at 1:3000 dilution in blocking buffer was added and incubated. After washing, freshly prepared orthophenylene diamine/hydrogen peroxide substrate was added and incubated at 37 °C for 15 min for colour development. Then the reaction was stopped using 1 M H₂SO₄. The plate absorbance was read at 492 nm in an ELISA reader (Tecan Infinite50) with 620 nm reference wavelength. Positive–negative cut off value was calculated as twice mean \pm standard deviation of four intermediate blank wells.

The amount of VLPs in terms of recombinant protein content of peak fractions of the hemolymph collected from infected Eri silkworm larvae was determined by spectrophotometry. The purified VLPs were quantified by spectrophotometric reading at 260 and 280 nm.

Antigenicity

The purified fractions of Eri silkworm expressed VLPs were characterized by S-ELISA and Western blotting to check for antigenicity. The hemolymph collected from Eri silkworm on 7 and 8 dpi were separated by 13 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on polyvinylidene difluoride (PVDF) membrane. Briefly the PVDF membrane containing the bound proteins was blocked with Phosphate buffered saline (PBS) containing 5 % skimmed milk powder. Then it was allowed to react with 1 in 100 diluted rabbit serum (diluted in PBS) raised against 146 s of FMDV type O and washed with wash buffer (PBST, PBS + 0.05 % Tween 20) and then incubated for 1 h at 37 °C. After three times washing, the membrane was reacted with anti-rabbit IgG conjugated with HRPO antibody raised in guinea pig at 1 in 1000 dilution in 6 mL of PBS. The colour was developed using DAB (Diamino benzidine) and metal enhancer tablet (M/S Sigma Aldrich). The membrane was washed with distilled water to stop the colour reaction and photographed.

Immunogenicity

Immunization experiments were carried out in accordance with the guidelines of the Institute Animal Ethics Committee (IAEC). To check for immunogenicity, six guinea

pigs and six rabbits were administered with VLPs at dose rate of 2 μ g per animal in 0.5 mL volume with ISA206 adjuvant by i/m route. Booster injection was given on 28 dpv by i/m route in thigh region. Animals were bled on 14, 28, 42, and 56 dpv to collect serum. Detection of antibody levels in rabbits and guinea pigs was carried out by indirect ELISA (I-ELISA). Polystyrene 96-well microtitre plate (NUNC) was coated with 1:50 dilution of purified inactivated FMDV ‘O’ antigen (concentration of 20 μ g/mL) 50 μ L/well in coating buffer (Carbonate-bicarbonate, pH 9.6) at 37 °C for 1 h. After washing thrice with wash buffer (PBS-T; NaCl—137 mM, KCl—2.7 mM, Na₂HPO₄—10 mM, KH₂PO₄—1.8 mM, pH 7.4 containing 0.05 % Tween 20) at 2 min interval for each wash, the plates were blocked with blocking buffer (PBS-T containing 5 % adult bovine serum) at the rate of 50 μ L/well and incubated at 37 °C for 1 h. After washing, 50 μ L of serum samples (1:10 diluted in PBS solution) in two-fold series in blocking buffer were added in duplicate wells and further incubated at 37 °C for 1 h. FMDV type ‘O’ hyper immune sera raised in rabbit/guinea pig were used as positive controls. Following washing, 1:3000 dilutions of horse radish peroxidase conjugated anti species (rabbit/guinea pig) IgG was added to all the wells at the rate of 50 μ L/well. The plates were incubated at 37 °C for 1 h and washed as described earlier. Finally 50 μ L of OPD substrate solution containing 3 mg of Ortho-phenylene diamine (OPD) and 2.4 μ L of H₂O₂ in 6 mL of citrate buffer pH 5.0 was added to each well for colour development. The reaction was stopped by addition of 1 M H₂SO₄ (50 μ L/well). Absorbance was measured at 492 nm using Bio-Rad ELISA reader. Indirect ELISA was repeated by coating with purified VLPs diluted 1:200 in blocking buffer to check the antibody titre against the injected VLPs. Positive negative cutoff values were calculated as the thrice the mean of blank wells. The serum I-ELISA titre was calculated as the highest dilution of serum which showed positive in 50 % of the wells.

Results

Confirmation of OP1-2A-3C by PCR amplification and agarose gel electrophoresis

The polyprotein P1-2A-3C gene of FMDV type O/IND/R2/75 present in the recombinant baculovirus stock produced in Sf9 cells was amplified using forward primer VP4-F and reverse primers 3C-R. The PCR amplified product was checked in 1 % agarose gel and recombinant baculovirus DNA had shown a bright band of 2.93 Kbp corresponding to the size of P1-2A-3C gene of FMDV as shown in Fig. 1.

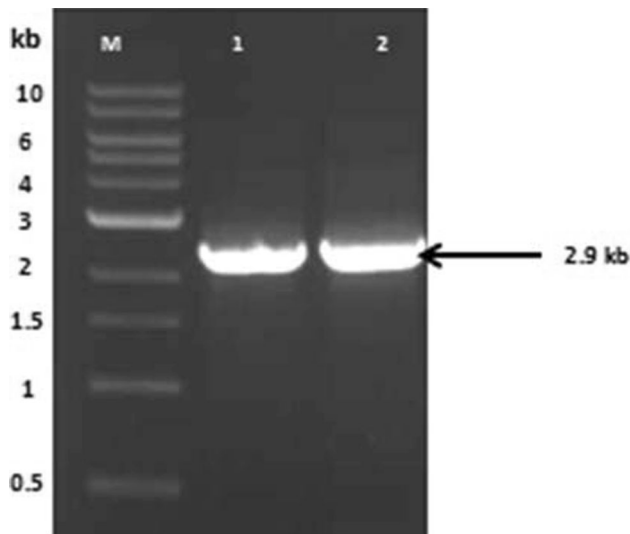


Fig. 1 Agarose gel electrophoresis of PCR amplicon (2.9 Kb) of P1-2A-3C of FMDV type O/IND/R2/75 in recombinant baculovirus stock of P5 and P6. Lane M represents 1 Kb DNA ladder (M/S Invitrogen), while lanes 2 and 3 represent passage 5 and 6 of the recombinant baculovirus stock

However no amplicon was observed in the negative control Sf9 cells or empty Bacmid DNA.

Optimization of methods for infecting Eri silkworm with recombinant baculovirus

Eri silkworm larvae were infected via per oral and injection routes with stock recombinant baculovirus of titre 10^7 pfu/mL as determined by plaque assay. The whole larval extract and hemolymph collected on day 1–8 for each species of larvae were tested for the presence of FMDV structural proteins in S-ELISA as shown in Fig. 2. Mean absorbance values are increasing as soon as 3 dpi which reached maximum on 7 dpi (0.94) and then started declining in case of injection route based on the results of S-ELISA (Fig. 2b), while increasing mean absorbance values from 4 (0.09) to 7 dpi (0.39) was observed in per oral route of infecting Eri silkworm (Fig. 2a). However, the reactivity is at basal level or comparatively less in case of larvae of *Helicoverpa armigera* and *Spodoptera litura* both in oral and injection methods as shown in Fig. 2a, b. Based on these results, the study is then only continued in Eri silkworm larvae.

Antigenic characterization of VLPs by Western blotting

The Eri silkworm expressed FMDV type O VLPs found positive in S-ELISA were further characterized by Western blotting. Western blot analysis of VLPs had shown bands

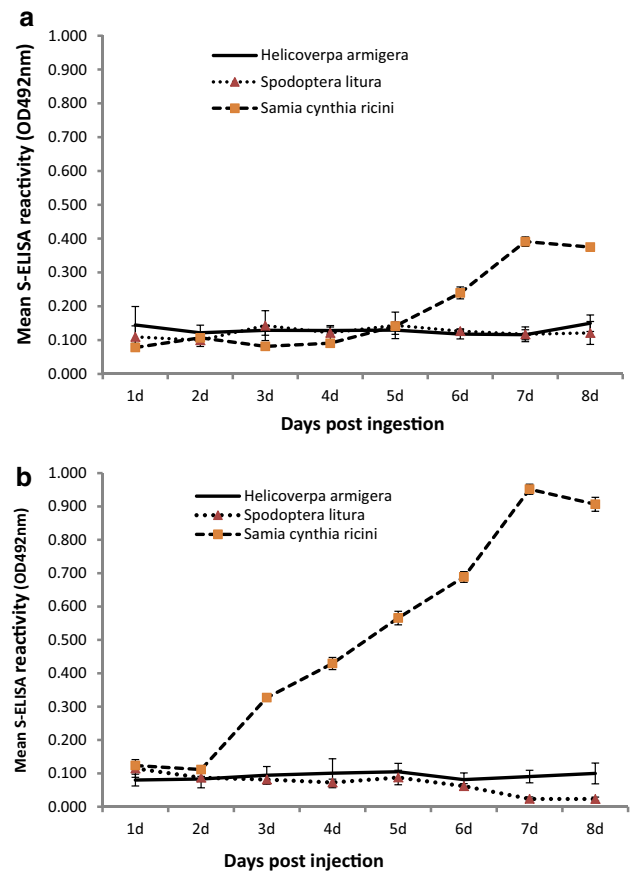


Fig. 2 S-ELISA reactivity of whole larval extract and hemolymph of three lepidopteran insect larvae collected from day 1 to 8 after infecting by **a** oral feeding and **b** hemocoel injection methods. The values are represented as the mean ($n = 5$) \pm standard deviation

of 26 (VP1/VP3), 37 (VP0), and 47 kDa (VP1 + VP3) similar to native viral antigen as depicted in (Fig. 3) indicating the antigenic nature of VLPs expressed in Eri silkworm larvae.

Purification of Eri silkworm expressed VLPs of FMDV and antigenic properties

The purified fraction of hemolymph collected from sucrose density gradient, presumed to contain 75 s empty VLPs, was selected for quantification of protein using spectrophotometry and was also tested in S-ELISA. The amount of recombinant FMDV proteins in terms of total protein content was 2.12 mg/mL as estimated by spectrophotometry. The fractions showed one peak of 0.82 corresponding to fraction no. 4 when tested in S-ELISA at OD₄₉₂ (Fig. 4). The high reactivity of peak fractions in S-ELISA indicated antigenic nature of larvae expressed VLPs.

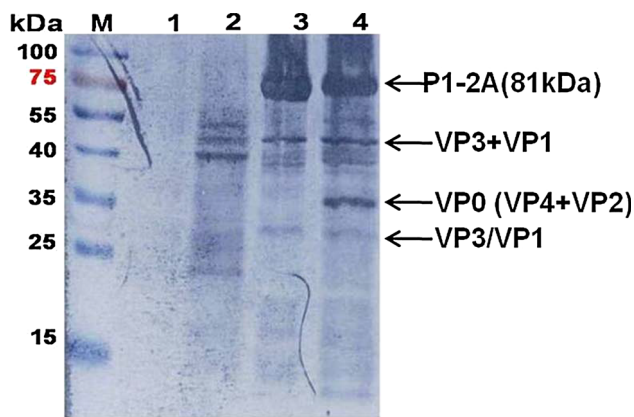


Fig. 3 Immunoblot of FMDV type O capsid proteins detected in the hemolymph of infected Eri silkworm larvae. *Note:* Lane M precision prestained molecular weight marker (BioRad), 1 hemolymph of control Eri silkworm (negative control), 2 FMDV type O cell culture grown antigen as positive control, 3 hemolymph collected on 7 dpi and 4 hemolymph collected on 8 dpi. Banding pattern of 81, 47, 35, 27 kDa corresponding to P1-2A-3C, VP3 + VP1, VP0 and VP1 or VP3, respectively as indicated by arrow in the blot

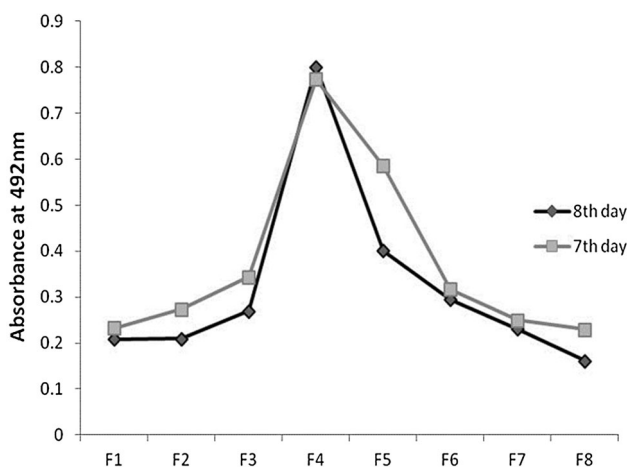


Fig. 4 S-ELISA reactivity of purified fractions of hemolymph of Eri silkworm larvae expressed VLPs. The values are represented as mean of three independent experiments. F1–F8 indicate the fraction from bottom to top of sucrose density (20–60 %) gradient

Immune response of guinea pigs and rabbits injected with VLPs obtained from Eri silkworm larvae

Foot-and-mouth disease virus (FMDV) type ‘O’ specific total IgG response for each rabbit and guinea pig immunized with VLPs was assessed by Indirect-ELISA (I-ELISA). All the rabbits and guinea pigs showed titre of less than 0.6 Log_{10} in the pre-immune sera collected before start of the experiment. The total antibody estimated by I-ELISA showed titre in the range of 1.30–2.81 Log_{10} for rabbits (Fig. 5) in the post vaccinal sera indicating a good immune response of VLPs in rabbits. Among six rabbits

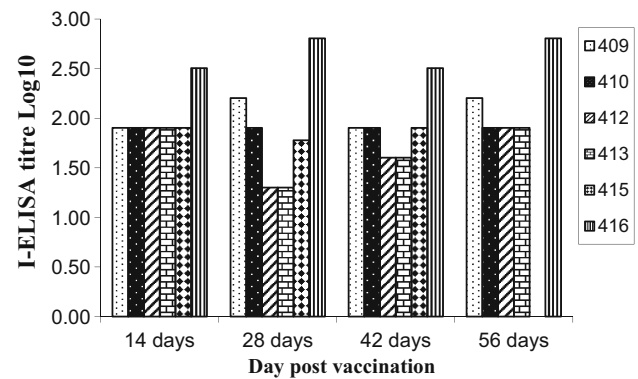


Fig. 5 I-ELISA titre of rabbits immunized with VLPs produced in the hemolymph of Eri silkworm larvae. The titres are mean of four independent experiments. Rabbit no. 415 died on 50 dpv

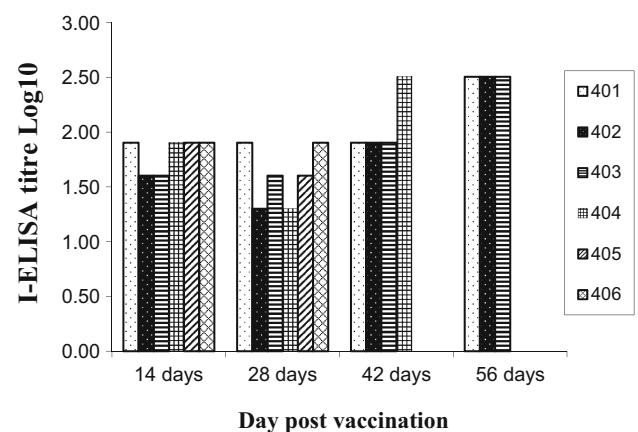


Fig. 6 I-ELISA titre of guinea pigs immunized with VLPs produced in the hemolymph of Eri silkworm larvae. The titres are mean of four independent experiments. Guinea pig no. 405 and 406 died on 45 dpv and 404 died on 55 dpv

immunized, rabbit number 416 showed highest titre of 2.51 Log_{10} on 14 and 42 dpv; and 2.81 Log_{10} on 28 and 56 dpv, while other rabbits showed moderately high titres. Similarly in guinea pigs, the total antibody measured by I-ELISA showed titre of less than 0.6 before start of the experiment, which increased to 1.30–2.51 (Log_{10}) in the post vaccination period. Majority of the guinea pigs showed peak titre on 42 and 56 dpv indicating the effect of booster in eliciting strong immune response. The immune response of guinea pigs vaccinated with VLPs of FMDV type O in terms of I-ELISA reactivity was presented in Fig. 6.

Discussion

Foot-and-mouth disease (FMD) is one of the most contagious viral diseases of cloven footed animals posing threat to livestock industry worldwide. Vaccination is the only

strategy employed to control FMD in endemic settings. Presently available BEI inactivated FMD vaccine has disadvantages of handling live virus and could not be used as DIVA vaccine to effectively implement FMD control programme. Hence, baculovirus expression system offers a suitable method of developing safe vaccine without handling live virus and associated risks. Several VLPs based vaccines are being produced in insect cells for both veterinary and human use. VLPs mimic epitope present on the native virus (FMD) which could elicit efficient humoral and cell mediated immune responses [9]. Recombinant VLPs of FMDV type O having P1-2A-3C with mutation in 3C was previously developed, which gave protection against challenge in vaccinated guinea pigs [2]. It is tedious to produce the VLPs in insect cells which require laborious cell culture system which may hinder the large scale production and purification. In this study, larvae of three lepidopteran insect species of *Helicoverpa armigera*, *Spodoptera litura*, and *Samia cynthia ricini* were tested for producing VLPs. We could observe the VLPs production only in *Samia cynthia ricini* larvae and not in other larvae by oral and hemocoel injection methods and no literature available for comparison to the best of our knowledge. VLPs were being expressed in heterologous system of silkworm [11–13]. Expression of P1-2A-3C in vivo presumed to mimic in vivo post translational modifications and assembly of VLPs similar to production of virus in cell culture system. In this study Eri silkworm larvae and not normal silkworm larvae were chosen as in vivo system to express VLPs of FMDV proteins. Recombinant baculovirus stock prepared in Sf9 cells with high titre was used to infect the Eri silkworm larvae by oral and hemocoel injection methods. The analysis of VLPs expressed in hemolymph by SDS-PAGE and Western blot showed the banding pattern corresponding to VP1, VP0 and VP1 + VP3 proteins of FMDV which is in agreement with earlier reports of Asia 1 empty capsid [4] and FMDV type O empty capsids [2] in Sf9 cells. When two different methods of infecting of Eri silkworm larvae were compared, parenteral route of injection gave better results in producing VLPs of FMDV in terms of higher S-ELISA reactivity than the per oral route. Further optimization of parenteral route of infection was performed at specified time intervals to find out the maximum levels of expression of VLPs. It was evident that maximum expression of VLPs was observed on 7 dpi and started declining from 8 dpi in Eri silkworm by hemocoel injection method.

The amount of recombinant total protein in the peak fraction was 2.12 mg/mL. The yield of VLPs of FMDV type O produced in one 175 cm² flask in Sf9 cells by monolayer method was estimated at 100 µg for each flask, while each larva had produced 200 µg. The estimated cost

of producing 1 mg of VLPs in Sf9 cells by monolayer method was INR 8000 (on the basis of 100 µg of VLPs from 175 cm² cell culture flasks), whilst the cost of producing same amount of VLPs in the Eri silkworm larvae was INR 100 on the basis of yield of 200 µg VLPs from one larva. Further, the Eri silkworm larvae can be reared in large numbers by feeding castor leaves at cheaper rate and infected by hemocoel route to produce large quantities of VLPs in 7 days time appear to be an economical method for developing recombinant vaccine using bioreactors.

Among rabbits and guinea pigs immunized with VLPs of Eri silkworm larvae and tested for their immunogenicity by I-ELISA, significantly higher titres were observed for individual rabbits based on total antibody present in the serum samples of rabbits on 28 and 56 dpv, as evident from Fig. 5. Similarly significantly high titres were observed in guinea pigs immunized with VLPs. Guinea pigs had shown consistently higher immune response than rabbits indicating the highly immunogenic nature of VLPs expressed in Eri silkworm larvae.

Antigenicity of expressed VLPs resembling native virus was proved by the reactivity of the hyper immune sera raised in rabbits and guinea pigs in I-ELISA coated with 146S whole virus. The sera raised against VLPs retained some of the viral epitope required for eliciting considerable immune response in rabbits and guinea pigs. On screening serum samples of rabbits and guinea pigs coated with VLPs in I-ELISA showed high reactivity with titre of 2.81 indicating the highly immunogenic nature of VLPs in inducing good immunogenicity. Our results suggested that VLPs could be expressed in Eri silkworm larvae in large quantities in shortest possible time (7 days) and could be an alternative economically viable approach for large scale production of VLPs for testing antigenicity and immunogenicity. Injection of recombinant baculovirus in Eri silkworm larvae strategy could be employed for producing large quantities of recombinant VLPs without involving time consuming, expensive and laborious insect cell culture system. Further studies are needed to prove the efficacy of VLPs produced in Eri silkworm larvae in target animals like cattle by immunization and challenge experiments before contemplating its use in FMD vaccination.

Our results clearly demonstrated that the VLPs of FMD could be produced in large quantities using Eri silkworm larvae in rapid turnaround time of 1 week. FMDV type O VLPs produced by insect larvae could mimic antigenic sites present on the native virus and could elicit high titred antibody response in laboratory animals like rabbits and guinea pigs. Therefore, it is concluded that the production of VLPs in Eri silkworm larvae is highly economical and less laborious method compared to insect cell culture based production system.

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