Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol.25 No.3 Ser. No.88 2024

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Cloning and expression of Fusion and Hemagglutinin proteins of peste des petits ruminants virus in the baculovirus system: an immunogenicity study in mice

Taheri Asl, M.[1]; Moghbeli, M.[2*]; Kargar, M.[3, 5]; Lotfi, M.[4] and Kafilzadeh, F.[5]

[1]Ph.D. Student in Microbiology, Department of Microbiology, College of Science, Jahrom Branch, Islamic Azad University, Jahrom, Iran; [2]Department of Biology, College of Science, Damghan Branch, Islamic Azad University, Damghan, Iran; [3]Department of Biology, Zand Institute of Higher Education, Shiraz, Iran (current address); [4]Department of Quality Control, Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization (AREEO), Karaj, Iran; [5]Department of Microbiology, College of Science, Jahrom Branch, Islamic Azad University, Jahrom, Iran

[*]***Correspondence:*** *M. Moghbeli, Department of Biology, College of Science, Damghan Branch, Islamic Azad University, Damghan, Iran. E-mail: moghbeli552@gmail.com*

10.22099/ijvr.2024.48368.7047

(Received 16 Sept 2023; revised version 26 Aug 2024; accepted 19 Sept 2024)

Abstract

Background: Peste des petits ruminants virus (PPRV) is one of the most economically important pathogens in sheep and goats. Fusion (F) and Hemagglutinin (H) proteins are the main immune-stimulating antigens. **Aims:** The present study aimed to clone and express F and H genes in baculovirus, and evaluate the immunogenicity of recombinant proteins produced by sf9 cells in mice. **Methods:** Amplified F and H genes (by RT-PCR using specific primers) were cloned into pFastBac Dual plasmid. The recombinant plasmid was transformed in DH10Bac host cells. SDS-PAGE and Western blotting was performed to control the recombinant protein, and a whole pure and specific recombinant protein was obtained. **Results:** The immunogenicity of 20 µg of non-adjuvant recombinant proteins in Balb-c mice showed better results compared with the attenuated PPR vaccine. **Conclusion:** The recombinant protein obtained from this study can be a suitable candidate for the production of recombinant vaccines against PPRV.

Key words: pFastBac Dual, PPRV, Recombinant vaccine, sf9 cell

Introduction

Peste des petits ruminants (PPR) is one of the most fatal and contagious diseases in small ruminants, particularly goats (Patel *et al*., 2015; Marashi *et al*., 2017). PPR is diagnosed with fever and mucosal erosion, which occur within a few days of diarrhea and pneumonia, and may increase over a long period of time, often leading to death (Cotmore *et al*., 2019). According to the World Organization for Animal Health (OIE), PPR is one of the most reported diseases due to severe financial loss in sheep and goats (Truong *et al*., 2014). PPR can cause extensive economic loss in enzootic areas so that its incidence and mortality rate may reach 90% and 80%, respectively (Woma *et al*., 2016). Vaccination against PPR is an effective way to control the disease (Kumar *et al*., 2017). Given that, the best available vaccine against this disease is the PPRV homolog, PPRV/Nigeria/75/1, which has played a vital role in the history of PPRV vaccination (Kumar *et al*., 2017). However, attenuated live PPRV vaccines are not temperature resistant. In addition, the possibility of infection with live pathogens, the persistence of virulence, and the risk of recurrence of virulence are some of the disadvantages of these vaccines (Baazizi *et al*., 2017). The causative agent of PPR belongs to the genus Morbillivirus of the Paramyxoviridae family (OIE Office International des Epizooties of the World Organization for Animal Health). PPRV is an enveloped single-stranded negative-sense RNA virus with 16 kb length that encodes 8 proteins: nucleocapsids protein (N), RNA polymerase phosphoprotein cofactor (P), two nonstructural proteins (C and V) transcribed from the phosphoprotein gene, matrix protein (M), fusion protein (F), hemagglutinin protein (H), and viral RNA polymerase (L) (Niyokwishimira *et al*., 2019). Protein F stimulates viral hemolysis, cell attachment, and the onset of infection. Proteins F and H constitute spikes and play a significant role in initiating cell division (Niyokwishimira *et al*., 2019). The virus attaches to the host cell membrane by protein H using protein F, which leads to nucleocapsid import (Tounkara *et al*., 2019). Proteins F and H of morbilliviruses are capable of stimulating autophagy (Tounkara *et al*., 2019). Therefore, these surface proteins are immunogenic and provide immunity against this virus. Therefore, the

present study aimed to clone and express F and H proteins of Morbillivirus in the baculovirus system and evaluate its immunogenicity in an animal model by making subunit vaccines.

Materials and Methods

RNA extraction, cDNA synthesis and RT-PCR

PPR virus was obtained as an attenuated vaccine from Razi Vaccine and Serum Research Institute (Iran). RNA extraction was performed from the prepared strain using RNA extraction kit (Sinaclon Co., Iran) with the standard protocol of kit. Specific primers were designed for the amplification of F and H genes according to the sequence of PPRV/Nigeria/75/1 available in the NCBI gene bank. *Nco*I and *Kpn*I recognition site sequences were designed at the beginning and the end of H gene primers, respectively. Furthermore, *Bam*HI and *Xba*I sequences were designed at the beginning and the end of F gene primers, respectively (Table 1). Reverse transcriptase-polymerase chain reaction (RT-PCR) reaction was performed for cDNA synthesis using ThermoScript™ RT-PCR System (Invitrogen™, USA) and reverse primers. The temperature program cycle for PCR reactions was performed at 65°C for 5 min, followed by 42°C for 60 min for reverse transcription activity, 5 min at 37°C for completing the synthesis period, 5 min at 70 for inactivating the enzyme, and finally one step at 4°C for 15 min.

Table 1: Primers used for amplifying F and H genes. The cut site on each primer is underlined and colored

Name	Primer sequence	Tm	Size (bp)
FPPR	F: GGATCCATGACACGGGTCGCAA R: TCTACACTACAGTGATCTCACGTACG	64	1701
HPPR	F: CCATGGATGTCCGCACAAAGGGAAAG R: GGTACCTCAGACTGGATTACATGTTACCTC	66	1833

Amplification of F and H genes

The PCR mixture consisted of 12.5 µL of Mastermix (Exprime 2X), 1 µL (10 picomoles) of each of the forward and reverse primers, and 1 µL of cDNA (approximately 20 ng). The reaction volume was increased to 25 µL with distilled water.

The temperature program included 94°C for 3 min (1 cycle), 95°C for 30 s, 64°C for 30 s, 72°C for 90 s (32 cycles), and 72°C for 5 min (1 cycle). The products were run on 1% agarose gel.

Construction of transfer vector and recombinant virus

To make the construction (Fig. 1), first, the H and F genes were cloned with pFast DualTM vector, respectively. Enzymatic digestion was performed using two enzymes *BamH*I and *Xba*I for the F gene and *Nco*I and *Kpn*I for the H gene (ThermoFisher Scientific, USA) to obtain two adhesive ends for binding. The ligation reaction was performed between F, H genes and pFast DualTM vector. The reaction mixture consisted of 2 µL of pFast DualTM vector, 5 µL of F and H genes, 1 µL of T4 DNA ligase (ThermoFisher Scientific, USA), 1 µL of ligation buffer 10X, and 1 µL of PEG4000 in a total volume of 10 µL. The reaction mixture was incubated at 22°C for 60 min. Enzyme inactivation was performed at 60°C for 10 min. Then, 5 µL of the ligation product was transformed into the DH5α competent cell by standard heat shock method (Sambrook and Russell, 2006). Colonies obtained from Lorian Bertani (LB) agar medium containing ampicillin (100 µg/ml) were examined for the presence of plasmids containing F and H genes.

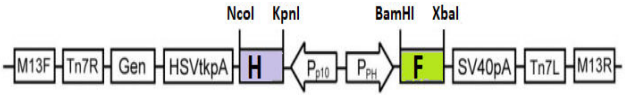

Fig. 1: Construction of H and F genes in the desired loci

Recombinant plasmid pfastHF was extracted using the purification kit (Pouya Gene Azma Ltd., Iran) according to the standard protocol of the kit. To make the recombinant Bacmid, a special strain of *E. coli* DH10 Bac was used. Recombinant pfastHF plasmids were transferred to *E. coli* DH10 Bac competent cells using the standard heat shock method (Sambrook and Russell, 2006). *E. coli* DH10 Bac cells were cultivated in the LB medium containing kanamycin (10 µg/ml), tetracycline (10 µg/ml), and gentamicin (7 µg/ml). Resistance genes for each antibiotic are present in the Bacmid, adjuvant plasmid, and pFastDualTM, respectively. Recombinant Bacmid (Bac-PPRHF) was confirmed by PCR using DreamTaq DNA polymerase enzyme. The primers used in this reaction included forward primer, 5´-CCC AGT CAC GAC GTT GTA AAA CG-3´ and reverse primer, 5´-AGC GGA TAA CAA TTT CAC ACA GG-3´. PCR reaction consisted of 2.5 µL Buffer (10X), 0.5 µL dNTP Mix (10 mM each), 1 µL of each primer, 1 unit of DreamTaq DNA polymerase, and 5 µL of the recombinant plasmid in total volume of 25 µL.

The initial denaturation cycle for the PCR reaction was done at 94°C for 4 min, followed by 35 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 5 min. Furthermore, the final extension step was performed at 72°C for 7 min.

Inoculation of DNA recombinant Bac-PPRHF into insect cells

Sf9 cells were used for recombinant protein expression. Sf9 insect cells were obtained from the National Cell Bank of Pasteur Institute of Iran. In this process, 800 $\times 10^6$ young cells in the logarithmic phase were passaged on a six-cell culture plate in Grace's insect medium (1X) supplemented containing 10% FBS, 1% antibiotics penicillin, and streptomycin, then incubated at 37°C. Complex formation was performed using a combination of 8 µL of celfectin and 1 µg of DNA recombinant Bac-PPRHF, each of which was diluted separately in 100 µL of Grace cell culture medium without supplementation, antibiotics, or serum.

The mixture was incubated at room temperature for 30 min. The transfection mixture was added to the cells kept at 27°C for 5 h. The transfection mixture was then removed, followed by washing the cells. Then, 2 ml of complete culture medium containing 10% FBS was added to the cells and placed at 27°C for five days examination. The first passage supernatant was used to increase the virus titer. Thus, six consecutive passages were performed on Sf9 cells. The supernatant was examined for the presence of protein by SDS-PAGE on 12% gel in a discontinuous manner (Schägger, 2006) and western blotting (Sambrook and Russell, 2012) using serum of mice immunized with PPR vaccine (Razi Vaccine and Serum Research Institute, Iran) and anti-mouse alkaline phosphatase-conjugated secondary antibody. Bradford standard method was used to evaluate the amount of recombinant protein produced (He, 2011).

Evaluation of recombinant protein immunogenicity in laboratory animals

Twenty-five male BALB-C mice were obtained from Razi Serum Institute. The mice were divided into three groups: ten mice as the main sample (T1-T10), ten mice as the positive control samples (PC1-PC10), and five mice as the negative control (CR1-CR5). Furthermore, 100 µL (20 µg) of mixed F and H antigens without adjuvant were injected intraperitoneally into ten main mice. PPR vaccine of Razi Vaccine and Serum Research Institute was used as a positive control and physiological serum as a negative control. Mice were kept at a temperature between 22 to 25 degrees under clean conditions with sufficient food. After 21 days, the second injections were performed, the same as the first injections. Blood samples were taken from all mice 21 days after the second injection. Blood sera were collected, and a serum neutralization test was performed using the standard method (Gauger and Vincent, 2020). The complement in the sera was inactivated by placing it at 56 degrees for 30 min. Serial dilutions from 1:2 to 1:2048 were prepared using PBS buffer. In each serum dilution, a virus suspension containing TCID501000 was added in a ratio of one to one. All dilutions were incubated at 37°C and then added onto vero cells and incubated at 37°C for 12 days. Finally, CPE was checked.

Statistical analysis

Data were subjected to a two-tailed analysis of variance using the GLM procedure of SAS 9.4. Tukey multiple comparison test was used for comparing significant differences between experimental groups. Data are declared as the mean and standard error of the mean.

Results

Isolated genomic RNA concentration was 80 ng/µL the absorption ratio of 260/280 was 1.85 and the absorption ratio of 260/230 was 1.95 representing the appropriate purity of the isolated DNA.

Using specific primers from the synthesized cDNAs, F and H genes were amplified, showing the presence of specific bands for each gene (Fig. 2).

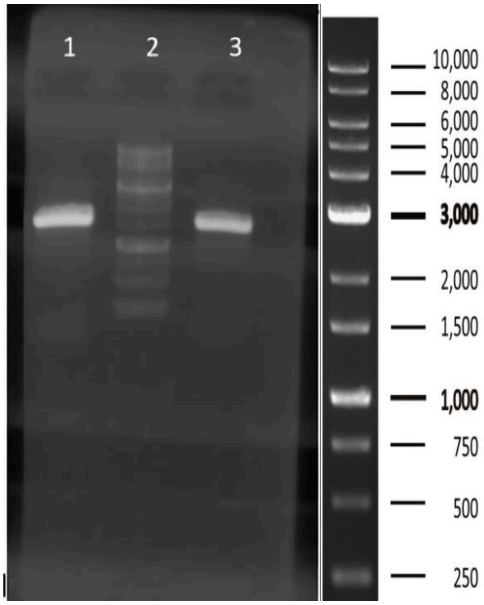

Fig. 2: Electrophoresis of PCR product on agarose gel. Line 1: H gene PCR product, Line 3: F gene PCR product, and Line 2: 1 kb ladder

H gene and pFastBacTM Dual vector ligation were confirmed using enzymatic digestion. Digestion was performed using *Nco*I and *Kpn*I enzymes and electrophoresis of the two fragments, 1833 bp, and 5000 bp, indicating the presence of the H gene in the pFastBacTM Dual vector as pFastH (Fig. 3).

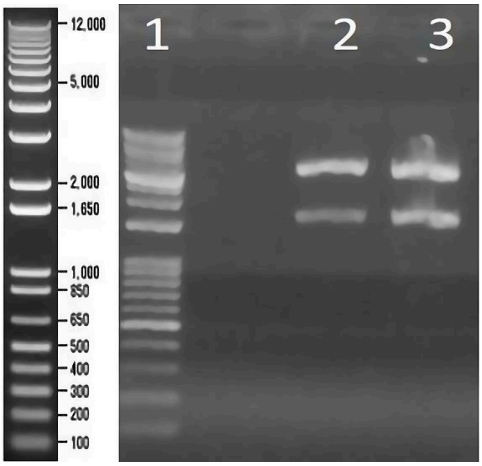

Fig. 3: Electrophoresis of pFastBacTM Dual vector carrying H gene (pFastH) double digested with *Nco*I and *Kpn*I enzymes. Line 1: 1 kb plus ladder, and lines 2 and 3: Double digested vectors

Ligation of the F gene and pFastH vector was also confirmed by enzymatic digestion. Digestion was performed using *BamH*I and *Xba*I enzymes and electrophoresis of the two fragments, 1700 bp, and 7000 bp, indicating the presence of the F gene in pFastH vector as pFast HF (Fig. 4).

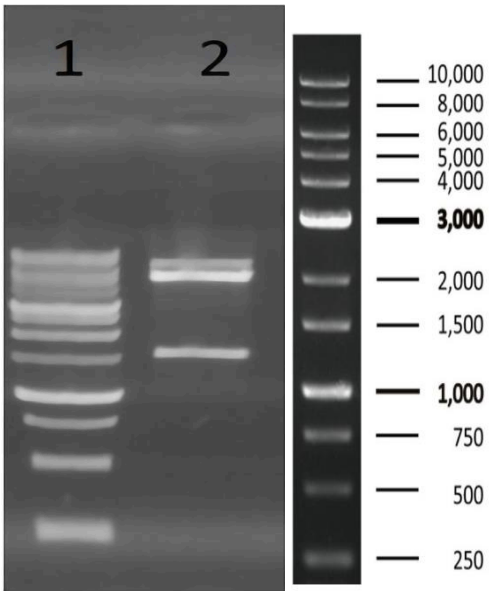

Fig. 4: Enzymatic digestion reaction electrophoresis of vector carrying H and F genes with *BamH*I and *Xba*I enzymes. Line 1: 1 kb ladder, and Line 2: Vector carrying H and F genes after enzymatic digestion

As shown, two bands are formed: the 1.7 kb band belongs to the F gene and the 7 kb band belongs to the vector carrying the F gene. PCR with general M13 primers was used to evaluate the presence of the gene on a recombinant plasmid. As shown in Fig. 5, 6200 bp band genes are obtained in the presence of both genes (Fig. 5).

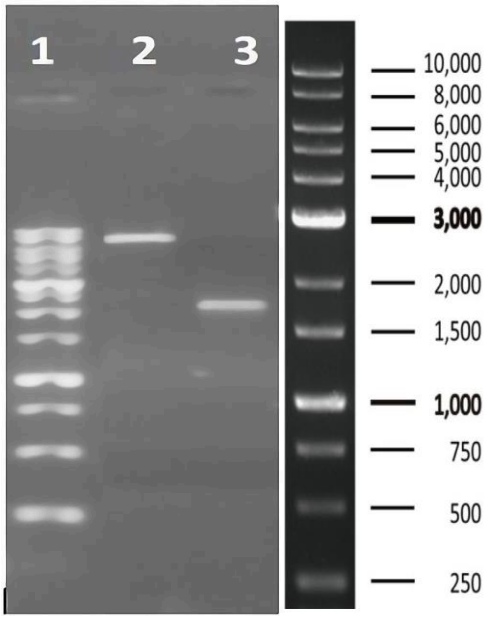

Fig. 5: Electrophoresis of PCR product of recombinant vector carrying H and F genes (Bac-PPRHF) and negative control (bacmid without H and F genes). Line 1: 1 kb ladder, Line 2: PCR product of recombinant vector using M13 general primers, and Line 3: Negative control PCR product using general primers of M13

The SDS-PAGE of sf9 cells infected with recombinant virus supernatant had a 62 kDa band. Given the molecular weight of protein F (about 61 kDa) and protein H (about 65 kDa), it is not possible to separate them on SDS-PAGE gel; thus, a band of about 60 to 65 kDa was expected. As shown in Fig. 6, only a band using Coomassie Brilliant Blue staining was observed.

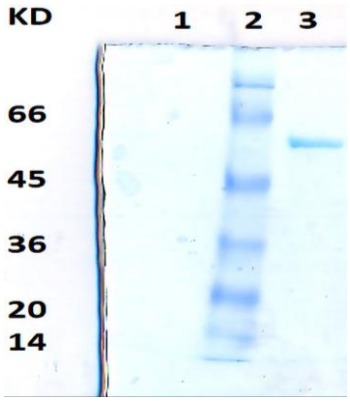

Fig. 6: Investigation of recombinant proteins on SDS-PAGE. Lane 1: Supernatant solution of transfected cells with Bacmid without gene, Lane 2: Ladder, and Lane 3: Transfected cells supernatant with recombinant Bacmid

As shown in Fig. 6, the 63 kDa band of the transfected cells supernatant with the recombinant Bacmid was completely characterized by polyclonal antibodies in Western blotting while the transfected cells supernatant contained Bacmid without genes did not detect any band (Fig. 7).

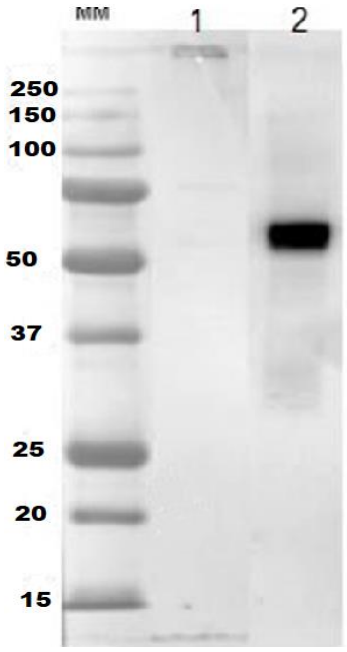

Fig. 7: Investigation of recombinant proteins with Western blotting. MM: Molecular marker. Lane 1: Supernatant solution of transfected cells with Bacmid without gene, and Lane 2: Transfected cells supernatant with recombinant Bacmid

Evaluation of recombinant protein immunogenicity in laboratory animals

Following the evaluation of recombinant protein immunogenicity in mice, the results of neutralization test (Table 2) and statistical analysis (Table 3 and Fig. 8) reveal a significant difference in the rate of neutralization ($P<0.05$).

Table 2: Comparison of immunogenicity of recombinant protein with attenuated live vaccine in mice by Serum Neutralization Test method

Mouse No.	Day 42		Mouse No.	Day 42	
	ALog	T		ALog	T
PC1	0.85	7	TE1	1.25	18
PC2	0.95	9	TE2	1.15	14
PC3	0.95	9	TE3	0.85	7
PC4	0.85	7	TE4	1.05	11
PC5	0.85	7	TE5	1.15	14
PC6	1.25	18	TE6	1.05	11
PC7	1.15	14	TE7	1.05	11
PC8	1.15	14	TE8	1.05	11
PC9	0.85	7	TE9	0.85	7
PC10	0.95	9	TE10	1.05	11

PC: Positive control, TE: Test group, and ALog: Antilog T (antibody titer)

Table 3: Statistical analysis of mice serum neutralization

Experimental group	SN	SE
Positive control	8.00^{b}	1.39
H&F protein	11.77^{a}	0.92
Negative control	0.00^{c}	0

[a, b, c] Positive control: Confirmation of commercial PPR vaccine, H and F protein: Confirmation of recombinant H and F protein, Negative control: Negative, SE: Standard error of the mean, and SN: Serum neutralization

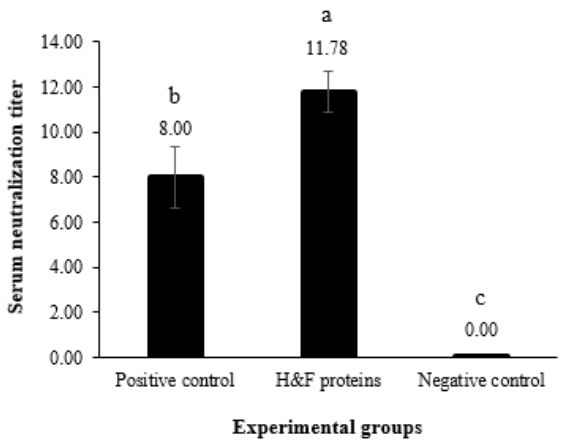

Fig. 8: Immunized mice seroconversion proportion

Discussion

Following the successful eradication of bovine plague in 2011, the eradication of PPR is on the global agenda (Zhou *et al*., 2018). Given the economic, social, and health effects of PPR on humans, the Food and Agriculture Organization (FAO) and the World Organization of Animal Health (WOAH) jointly published a PPR strategy for large ruminants in 2015 to eradicate PPR by 2030 (Pruvot *et al*., 2020). To do so, a four-phase program has been developed through which this disease can be eradicated in Africa and the Middle East and Asia (Legnardi *et al*., 2022). Iran and many countries in the Middle East are in the second phase, and in order to enter the third phase, DIVA vaccines are needed to differentiate infected animals, from vaccinated animals (DIVA). In this regard, a lot of research has been done, none of which has been commercialized so far (Liu *et al*., 2014; Fakri *et al*., 2015; Mokhtari *et al*., 2017; Fakri *et al*., 2018). The protein expression system in insect cells using baculoviruses was first described by Luckow and Summers in 1988. A number of morbillivirus proteins (mainly F and H) have been expressed in this system. In 2003, Rahman *et al.* expressed the F protein of PPR virus and H protein of bovine Rinderpest virus in the mentioned system and proved their immunity in a mouse model (Rahman *et al*., 2018).

In this study, for the first time, both immunodominant proteins of PPR virus, namely F and H, were expressed in the baculovirus system and their immunogenicity was investigated in mice. The Bac-to-Bac expression system was used as an invitrogen product. The F gene with *BamH*I and *Xba*I sites was cloned into the multiple cloning site (MCS) of the pFASTBAC DUAL polyhedrin promoter and the H gene with the *Nco*I and *Kpn*I sites was cloned into MCS of the pFASTBAC DUAL P10 promoter. The polyhedrin promoter has shown superior regulation of gene expression, leading to higher levels of expression compared with other promoters. The F protein has been extensively studied for its role in immunogenicity; hence, it was cloned next to the polyhedrin promoter to ensure enhanced expression levels when compared with the H gene. After confirmation of both genes by PCR and enzymatic digestion, the recombinant vector was transferred to *E. coli* strain DH10, which carries the modified genome of baculovirus. After expression in Sf9 cells, the purity of the recombinant proteins was confirmed by examining the cell supernatant on SDS-PAGE and observing a strong band of about 63 KD. Antibody levels in the serum of immunized mice were evaluated 2 weeks after the second dose using a viral neutralization test. It was found that the rate of neutralization was higher than that in mice immunized with live attenuated vaccine. Given that only 20 µg of recombinant protein without adjuvant was used in the sample prepared in this study, the difference in immunogenicity was significant.

The expression of F and H proteins in the baculovirus system and immunogenicity study of these recombinant proteins showed promising results. The findings suggest that the recombinant protein obtained from this study has the potential to be a suitable candidate for producing recombinant vaccines against PPRV.

Acknowledgements

We wish to express our thanks and appreciation to

the Islamic Azad University, Jahrom Branch, and Pouya Gene Azma Ltd. for their financial and executive support.

Conflict of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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