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RESEARCH ARTICLE
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THE POTENTIALS OF *PASTEURELLA MULTOCIDA OMPA* PROTEIN AS THE CANDIDATE FOR SUB-UNIT VACCINE AND FOR THE DEVELOPMENT OF AN ELISA KIT TO EVALUATE THE VACCINE RESPONSE IN THE ANIMALS

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Abstract:
Pasteurella multocida is the primary causative agent of Hemorrhagic Septicemia (HS) in cattle and Buffalo. It is responsible for causing huge economic loses every year. The *P. multocida* vaccines are used for livestock, but because sensitive and specific serological tests are not available, sero-conversion in these animals is unknown. As a result, the vaccine and it's the immune response cannot be evaluated. So, the present study was designed to evaluate immunogenic potential of *OmpA* gene of *P. multocida*. PCR primers were designed to have restriction sites to cut amplicon and ligate product into expression vector. Purified PCR product was subjected to cloning PCR and the product was sequenced. The product was excised from cloning vector and ligated into expression vector (pET 40b (+)). Expression vector was transformed to chemically competent *Escherichia coli* strain DH5-α by heat shock method. Plasmid was extracted and ligation was confirmed by restriction digestion. IPTG was used to trigger expression. Total cell protein and medium were SDS-PAGE was used to analyze the expression. Recombinant proteins were injected into mice to test their immunogenicity. *OmpA* was proved to be highly immunogenic in nature, making it a more suitable candidate for sub-unit vaccine preparation and development of ELISA kit for the detection of immune responses in animals.

Keywords: Animal vaccines; Immune responses: Farm animals; Respiratory infections; Pasteurellosis.

1. Introduction

Pasteurella multocida is principal cause of respiratory infections in farm animals. It causes pneumonia, genital infections, abscesses, and septicemia. Thus, leads to shortage in production and high mortality rate (Ahmad et al. 2014). Almost 30% of cattle deaths are caused by pneumonic pasteurellosis (Mohamed & Abdelsalam 2008). HS is regarded as a disease of greater economic importance in Pakistan. In Punjab, HS is responsible for more than 2.17 billion Pakistani rupees loss (Farooq et al. 2007).

HS is mainly present in Africa and South-east Asia. Various sero-groups have been assigned to *P. multocida* based on capsular antigen and these groups are further divided on the basis of somatic antigen. HS is caused mainly by two serotypes B: 2 and 6: E present in Asia and Africa respectively (De Alwis 1992).

In well-established farms, rectal temperatures of all in-contact animals are tested periodically for early detection (Al-Hasani et al. 2007). Initially, sulfonamides were recommended for the treatment of HS. Periodic vaccination is recommended to avoid the disease. HS vaccination has become a routine, but vaccine potency remains unclear. Indecisive vaccination zones and penniless vaccination analyses are major factors in the vaccine's failure. Vaccinations have been used to combat hemorrhagic septicemia for decades, but ineffectiveness and cross-protection have limited their use'(Abbas et al. 2018). Scientists now use subunit vaccines to provide immunity to different animals. Outer membrane protein (OMP) of bacteria display heterogeneity and play a key role in colonization and invasion (Gao et al. 2021).

Pasteurella multocida B:2 has outer membrane proteins that aid in cell contact (Peng et al. 2019). There is various *Omps* as *OmpA, OmpH, Omp087* and *Omp est* but major immunogenic proteins are *OmpA* and *OmpH*. *P. multocida OmpA* acts as a bridge between outer membrane protein and peptidoglycan, stabilizes the membrane envelope, and is slightly porin-like. The outer membrane protein *OmpH* acts like a porin and is 100% isolated from bovine (Al-Hasani et al. 2007).

Various killed and subunit vaccines have been prepared for *Pasteurella multocida* to treat the hemorrhagic septicemia in cattle and buffaloes (Mostaan et al. 2020). To check the efficacy of vaccine, a trial in different experimental animal models such as mostly in mice and rabbit, are tested (Martin et al. 2018). Indirect ELISA kits are widely used to check the humoral immune response. Different types of antigen are used to prepare the indirect ELISA kit as capsular and lipopolysaccharide (Kharb 2015). Indirect ELISA is developed to known the antibodies titer of subunit vaccine which is prepared from the OMPs of *Pasteurella multocida* P52 (Kharb 2015). In Pakistan vaccines of *Pasteurella multocida* is used for livestock but due to unavailability of sensitive and specific serological tests, sero-conversion is not recorded in these animals (Mushtaque et al. 2022). For this reason, vaccine evaluation cannot be performed, and immune response remains undetected. An ELISA kit is required for the detection of immune response in animals. Keeping in mind the scenario, immunogenicity of recombinant outer membrane protein *rOmpA* was performed in present study to check whether these proteins can be used in the development of an ELISA kit. So, in this study, we established a system to obtain expression of outer membrane protein (*OmpA*) of *P. multocida* B:2 in *E. coli* strain BL21, purification of recombinant outer membrane Protein (*OmpA*) and evaluation of the immunogenicity of the recombinant outer membrane proteins (*OmpA*) in mice.

2. Materials and Methods

2.1. Ethical considerations

The animal study protocol was approved by the Institutional Review Board of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan (protocol code: 2013-VA-428) approved on 14th October 2020. All the methods were performed in accordance with the relevant guidelines. Furthermore, the study results were reported in accordance with ARRIVE guidelines.

2.2. Isaolation and identification of bacteria

P. multocida serotype B-2 was obtained from Veterinary Research Institute Lahore. The strain obtained was further culture on Brain Heart Infusion (BHI) broth in biosafety containment Level 2 facilities. 250 ml of BHI broth had been inoculated with 200 µl of *P. multocida* serotype B-2 culture and this was incubated at 37°C for 18 hours in a shaker incubator at 250 RPM. Overnight culture was obtained and subjected to confirmation by Molecular identification of *P. multocida* by PCR (Abbas et al. 2018).

2.3. Detection of OmpA

Primers for the amplification of Outer Membrane Protein A *(OmpA)* gene were designed using Primer3 software. Designed primers were subjected to BLAST to check the similarity with desired gene (s) and their Melting temperature were calculated using NEB T_m calculator (Borah 2011). Primers prepared are shown in table 1. Amplified product was visualized and analyzed on 1% agarose gel with DNA ladder (marker) of known length (Sarah et al. 2006).

Table 1. List of primers for the amplification of *OmpA* gene.

Gene	**Primer**	**Sequence**
Primers for the amplification of *OmpA* gene		
OmpA	Forward Primer	5'-ATGAAAAAAACTGCAATCGCATT-3'
	Reverse Primer	5'-TTATTTGTTACCTTTAACAGCGATTTC-3'
Primers with restriction sites for amplification of *OmpA* gene.		
OmpA	Forward Primer (BamH1)	5'-AAAAAAGGATCCATGAAAAAAACTGCA ATCGCATT-3'
	Reverse Primer (Sal1)	5'-AAAAAAGTCGACTTATTTGTTACCTTT AACAGCGATTTC -3'

2.4. Ligation of selected genes into vector

Ligation of the gene (*OmpA*) was obtained in three steps: Preparation of competent cells, excision of gene of interest, ligation of gene of interest into vector and transformation.

2.4.1. Competent cell preparation

To get the expression of selected genes, first the fresh culture of BL21 derivative *E. coli* was obtained. This fresh culture was processed by Calcium Chloride Method to make competent cells (Chang et al. 2017).

2.4.2. Excision of gene of interest

For the excision of gene of interest from the plasmid (vector) double restriction digestion of the eluted product was performed (Fischer et al. 2018). The reaction mixture was incubated at 37ºC for 4 hours.

2.4.3. Extraction of digested DNA fragments from the Gel

Digested DNA was run on gel by using standard gel electrophoresis conditions along with ladder. For this, DNA band of required size was cut with sterilized blade and added into the weighed microfuge tube. The DNA was eluted using Thermo scientific Gene JET Gel extraction kit (Gao et al. 2021). The eluted product contained DNA and stored at 4°C.

2.4.4. Ligation of selected genes into Expression Vector pET 40b (+)

pET 40b (+) was selected as expression vector. For ligation of Gene of interest *OmpA,* double digestion of the vector was also performed. Reaction mixture for double digestion was prepared and the product was excised from gel using Thermo Scientific Gel Extraction kit. Gene of interest was ligated into expression vector using Thermo Scientific Rapid Ligation kit (Xie et al. 2020).

2.5. Transformation of ligated Expression vector to BL21 derivative E. coli strain

Frozen competent cells were thawed in ice for ten minutes. Prepared ligation mixture (5 µl) was added to the thawed competent cells. Transformation was performed by Heat-Shock Method. On LB agar plate containing 50 µg/ml Kanamycin, 100 µl of the cells were inoculated by spreading. Plates were incubated at 37°C for 18 to 24 hours. Isolated colonies were observed. The positively transformed cells was preserved in 50% glycerol and stored at -80°C for further use.

2.5.1. Induction of Expression of selected genes in Transformed BL21 derivative *E. coli* Glycerol preserved culture was streaked on LB agar plate followed by incubation at 37°C for 24 hrs. After incubation, single isolated colony from LB agar was shifted to LB broth followed by incubation at 37°C for 3 hrs. Sterilized 1 mL (100 mM) IPTG medium was added to it followed by overnight incubation at 37°C for 3 hours (Xing et al. 2021). The expression of the recombinant protein was evaluated by SDS polyacrylamide gel.

2.5.2. SDS polyacrylamide gel Analysis of prepared samples
Stacking and resolving gels were prepared according to protocol described in a previous study (Simpson 2006). In the first well, 5 µl of protein marker was loaded. Total cell protein, induced media, un-induced media, induced periplasmic and uninduced periplasmic samples were loaded in the well 1,2,3,4,5 and 6 respectively. Electrodes were connected to a power supply with the help of a cable. Voltage of 120V was applied to the gel for one and a half hour. Gel was removed from the tank and results were observed.

2.5.3. Purification of recombinant protein by affinity columns
Recombinant protein was purified by employing HisPur^{TM}-Ni-NTA purification kit at 4°C (Mayahi et al. 2018). Protein extract was mixed with the equilibration buffer to attain two resin equal volume. Recombinant protein was eluted by adding elution buffer to the column followed by centrifugation at 700 × g for 2 minutes.

2.6. Vaccination and animal challenge
For evaluation of protective immunity induced by recombinant outer membrane protein A (*rOmpA*), vaccination and challenge study was performed (Dabo et al. 2008). For this, specific pathogen free, 6-8 weeks old female Albino mice were divided into two groups. Each group was further divided into four groups. Each group had 20 mice per group. Each group was challenged with virulent *P. multocida strain B:2* after 14 to 21 days. Phosphate buffer saline (PBS) was used as a negative control and injected into one Sub-group from each main group. Protection was determined by mice survival within 4-day post challenge. Mortality in infected mice was monitored daily, and results were noted. Institutional animal care and use committee reviewed and approved the protocol used in the animal challenge study. None of the anaesthesia or euthanasia methods were used in the current study as none of the tested animals went under that condition to use the euthanasia method. Division of each group is given in the table below (Table 2).

Table 2. *In-vivo* Experiment for evaluation of Vaccination and animal challenge

Group	Sub-Group	Purpose/Contents	Number of mice
G1 (*rOmpA*)	A1	Negative Control Group	5
	A2	25 µg of recombinant protein	5
	A3	50 µg of recombinant protein	5
	A4	75 µg of recombinant protein	5

2.7. Statistical Analysis
The data was transferred to the Excel spreadsheet (2016). The data was then analyzed by Jamovi Software. Based on concentration of protein (*OmpA*) paired t-test (dependent) was used for evaluation of immunogenicity in mice.

3. Results
3.1. Identification of Pasturela multocida and *OmpA*
Pasturela multocida was isolated on blood agar through conventional culture technique and identification was done through PCR. Results of identification and confirmation results are given as below in figure 1. To confirm the presence of our desired gene, PCR was done. The presence of the

genes was confirmed by the appearance of band sizes of 1076 bp (for *OmpA*) and both genes were present in isolated bacterial strain. Results are shown in the figure 1.

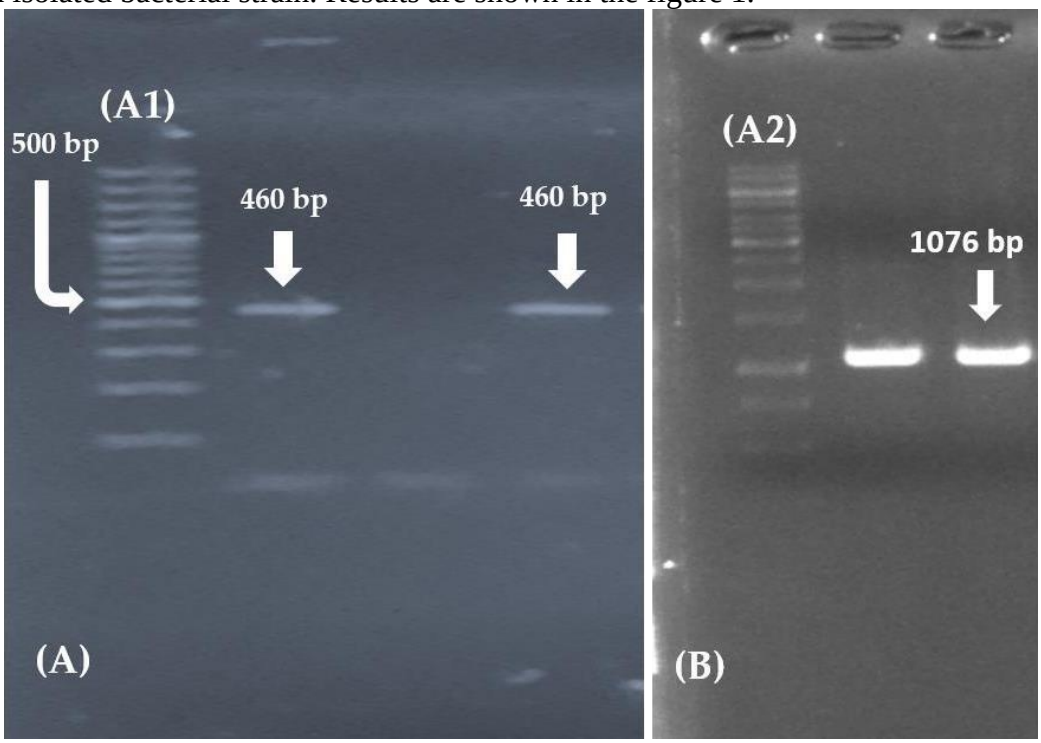

Figure 1. (A) Picture shows Gel electrophoresis results of PCR for the confirmation of *Pasturella multocida*. Band size of 460bp, confirmed the presence of *Pasturella multocida*. (B) 1076 bp of protein *rOmpA*, indicating successful detection of desired genes in samples. (A1) Ladder of 500 bp was used as marker. (A2) Ladder of 1000 bp was used as marker.

3.2. Ligation of gene of interest

Gene of interest was excised by double restriction digestion and ligated into expression vector. Selective culture media was used to grow the cells and growth of *E. coli* on culture media confirmed successful ligation of desired genes into our expression vector. The Figure 2 shows growth of transformed cells on to culture media confirming successful ligation process. *E. coli* obtained after ligation process was confirmed by colony PCR (Figure 3).

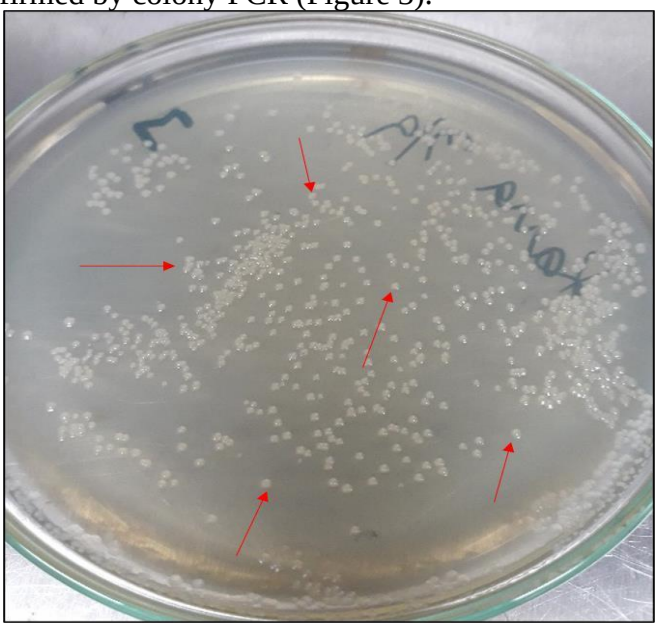

Figure 2. The growth of transformed *E. coli* DH5-α. The red arrows shows the isolated colonies of *Escherichia coli*.

Figure 3. Results of Agarose gel electrophoresis of cloning PCR showing band of our desired gene after successful ligation of it into expression vector. 1000 bp ladder was used as marker (control).

3.3. Expression and purification of protein SDS polyacrylamide

Growth of transformed cells were then processed to evalute expression of desired proteins (outer membrane protein A) by SDS-PAGE. The presence of 34 kDa band showed the presence of desired proteins.

3.5. In vivo studies for evaluation of immunogenicity of rOmpA

Results of vaccination and challenge test showed that at concentrations of 75 µg and 50 µg *rOmpA*, number of mice survived were 3 and 2 respectively. The detailed description of results is mentioned in the table 3.

Table 3. Vaccination of mice and challenge evaluation.

Groups	**Sub-groups**	**Vaccination**	**No. of mice challenged**	**No. of mice survived**	$\bar{x}_d \pm S_d$	**T-value**	***p*-value**
rOmpA	A1	PBS	5	0	-3.625±1.5059	-6.8084	<.001
	A2	75µg	5	4			
	A3	50µg	5	3			
	A4	25µg	5	1			

Results of the paired-t test indicated that there is a significant large difference between Before (M = 5, SD = 0) and After (M = 1.4, SD = 1.5), t (7) = 6.8, p < .001.

4. Discussion

Greater economic losses in farm animals, sheep, goats and poultry are caused by respiratory infections (Snyder & Credille 2020). Similar infections are provoked by inimical physiological stress or co-infection of virus and bacteria. *Pasteurella multocida* is a principal cause of respiratory infections in farm animals (Yaman et al. 2018). It causes pneumonia, genital infections, abscesses and septicemia. Thus, leads to shortage in production and high mortality rate (Su et al. 2020). The current study was designed to evaluate the expression and immunogenicity of recombinant outer membrane protein A (*OmpA*) of *P. multocida.*

Pasteurella multocida strains B and E are the main causative agent of HS in cattle and Buffalo (Shivachandra et al. 2011). They cause an acute type of disease in the animals. Pathogenesis mainly involve the swollen head and neck with edema and hemorrhagic lymph nodes are also observed (Harper et al. 2006). *P. multocida* is prevalent worldwide as most abundant commensal of the buffalo and cattle and may act as opportunistic pathogen depending upon surroundings and animal husbandry

practices (Wilson & Ho 2013). Outer membrane profile of the *P. multocida* was demonstrated. 15 to 20 bands of outer protein bands was observed ranging from 16 to 90 kDa (Tomer et al. 2002).

OmpA of *P. multocida* is a two domain protein which acts as bridge to make a physically link of outer membrane and peptidoglycans of the cell (Townsend et al. 1998). One is N-terminal made of 8-stranded beta barrel having 4 external loops and a C-terminal that binds with peptidoglycan (Viale & Evans 2020). It may also act as a porin and regarded as slow porin, since slow diffusion is regulated by *OmpA* (Hatfaludi et al. 2010). Almost 12 immunogenic outer membrane proteins have been identified by immunoblot that may act for the development of a recombinant vaccine. *OmpA* is demonstrated as immunogenic protein in the same study (Al-Hasani et al. 2007).

In the present study, vaccine strain of *P. multocida* strain B:2 was isolated and revived in mice. It was confirmed by already reported specific primers. Gene for outer membrane Protein A (*OmpA*) was amplified using self-constructed primers. Restriction sites for *BamH1* and *Sal1* along with six adenine nucleotides is added on 5' end of the forward and reverse primer respectively. Both of the enzymes were analyzed first on web cutter for their digestion activity for *OmpA* gene. The PCR amplified product is then cloned into a cloning vector provided with ThermoScientific GeneJet PCR cloning kit. The cloned gene was then sequenced using forward and reverse primers of the gene. After confirmation of the sequence, the gene was excised from the vector and ligated into pET 40b (+). OmpA::pET 40b (+) vector was then transformed into competent modified *E. coli* strain DH5-α. After confirmation of the plasmid through restriction digestion, the OmpA::pET 40b (+) was transformed into *E. coli* derivative BL21. Expression was induced using pET manual standard protocol (Novagen 2002). Total cell protein and media protein was then analyzed directly and by trichloroacetic acid precipitation method respectively by SDS-PAGE electrophoresis. Recombinant outer membrane protein A *rOmpA* was then purified using ThermoScientific HisPur^{TM} Ni-NTA purification kit and analyzed by SDS-PAGE electrophoresis. The *rOmpA* was then inoculated in 6 to 8 weeks old albino mice in 3 groups in 25µg, 50µg and 75µg concentrations. Challenge was given to the mice on 14th day of inoculation and mortality was observed for 4 to 5 days. Previously, many bioinformatics tools also used to propose the potent target for vaccines against different organism e.g., Epstein-Barr virus (Ahmed et al. 2022; Ahmed et al. 2023), herpesviruses (Naveed et al. 2022d), COVID-19 (Naveed et al. 2022a), and multi drug resistant bacteria (Naveed et al. 2022b; Naveed et al. 2022c) etc.

It was proved in the current study that *rOmpA* is immunogenic in nature. This protein have potential to be used for the development of an ELISA kit to evaluate the vaccine response in the animals (Dabo et al. 2008; Liu et al. September 3 - 6, 2012). Moreover, it can also be used as candidate protein to be used in the development of the recombinant protein as it induced protective immune response in the mice. These findings are in accordance with our findings in which *rOmpA* showed greater immune response and as a result more mice survived which were vaccinated with *rOmpA* protein.

A similar study was conducted in Australia research council center of excellence in Structural and Functional Microbial Genomic by Keith-Al-Hasani, 2007 revealed the immunogenicity of *OmpA* and *Omp87* from *P. multocida* (AAT57679). *P. multocida* Pm70 gene sequence and previously published data was analyzed to discover the candidate proteins to be used as potential vaccine candidate. Immunogenic proteins was identified by the immunoblot assay and 14 of them was recognized as immunogenic including Outer membrane Protein A and protein 87 (Al-Hasani et al. 2007).

In contrast S. Mady Dabo (2008), evaluated the immunogenic potential of recombinant *rOmpA* in mice to be used as a candidate protein for the development of an ELISA kit. Although *rOmpA* was found to be antigenic in-vitro and induce production of Th-2 cells but in-vivo, *rOmpA* showed non-immunogenic characteristics thus suggesting that it cannot be used as candidate for the development of recombinant vaccine (Dabo et al. 2008).

According to another research, the outer membrane protein of Oma87 from *Pasteurella multocida* A:1 has significant similarity to the D15 protective antigen of *Haemophilus influenza* (Ruffolo & Adler 1996). A *P. multocida* serotype D strain of *Oma87* was used to clone four *Oma87* pieces into a pGEX expression vector, which was then transformed into *E. coli*. Convalescent chicken sera only responded with the GST-F1 fusion protein, which had the amino acids 18 through 130 of *Oma87*

linked to the GST protein, according to a Western blot study. The GST-F1 protein vaccine did not protect hens from a virulent *P. multocida* serotype A challenge (Ruffolo & Adler 1996).

The *rOmpA* protein act as antigenic portion of the kit and can be used to produce hyper immune sera in mice. That hyper immune sera will be used to produce anti-antibodies conjugates to be used in the development of an ELISA kit. But the titer of the antibodies produced in response to the *rOmpA* was not identifiable. It can be evaluated by using gold standard techniques i.e., Indirect Haemagglutination Assay (IHA) or already developed ELISA kit. Moreover, the *rOmpA* can also serve as a candidate for the development of a recombinant vaccine (Ayalew et al. 2011). It was proved in the current study that *rOmpA* is immunogenic in nature and have potential to be used for the development of an ELISA kit to evaluate the vaccine response in the animals. Moreover, it can also be used as candidate protein to be used in the development of the recombinant protein as it induced protective immune response in the mice.

5. Conclusions

The findings of current study suggest that *rOmpA* plays an immunoprotective function and have a high potential to be the candidate of an ELISA kit. Although *rOmpA* showed better results in small animal model (mice) *in–vitro* but more tests like IHA can be done to check the immune responses more precisely. Further studies are needed to determine if this immunogenic function may extend the protective immunity after initial immunisation when given as a booster dose. Through various immunisation techniques, the immunogenic protein may potentially be examined as a candidate for vaccine and also for ELISA kit. Moreover, further studies are required to evaluate their efficacy as recombinant vaccine and as a tool for the detection and evaluation of immune response when tested in animals such as cattle and buffaloes.

Institutional Review Board Statement: All methods were performed in accordance with the relevant guidelines and regulations. The animal study protocol was approved by the Institutional Review Board of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan (protocol code: 2013-VA-428) approved on 14th October 2020.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data related to this research work could be accessed upon a reasonable request to the corresponding authors.

Conflicts of Interest: The authors declare no conflict of interest.

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