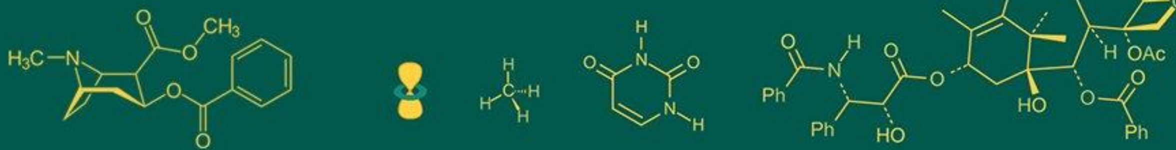

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Cloning and expression of ompH gene of type A *Pasteurella multocida* of pig origin

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Abstract
A study was undertaken to clone, express and purify the OmpH recombinant protein of *Pasteurella multocida* type A of pig origin. OmpH is a highly immunogenic porin protein found on the outer surface of *P. multocida*. For cloning and expression of ompH gene belonging to most pathogenic isolates of *P. multocida* capsular type A, pET303CT/His plasmid vectors were used. The amplified products of ompH and plasmid vector were digested with *Xba1* and *Xho1* at 37 °C for 5 hours separately. The restriction enzyme (RE) digested PCR product was ligated with the vector by using T4 ligase enzyme which was confirmed by performing T7 plasmid specific PCR. The ligated product was transformed into *E. coli* DH5α cell. Recombinant plasmid was extracted from positive clone and confirmed by T7 plasmid specific PCR, ompH gene specific PCR, double RE digestion and nucleotide sequence. Nucleotide sequence of the subjected recombinant plasmids showed 97-98% homology with ompH gene specific of porcine *P. multocida,* available in the GeneBank, NCBI. The recombinant OmpH protein was expressed in *E. coli* BL21 host in the insoluble fraction, which was purified by using His-Trap columns. The purified protein was confirmed by SDS-PAGE and western blotting by using anti His tag antibody which showed dark intense band at 37 kDa position.

Keywords: OmpH, cloning, expression, porcine, SDS-PAGE, Assam

1. Introduction
Swine pasteurellosis, stemming from *Pasteurella multocida* bacteria, stands out among various swine illnesses due to its significant mortality and morbidity rates. Although the disease is endemic in India especially in pig rearing areas of North Eastern region, there are no specific vaccines for pigs for controlling pig pasteurellosis. In India, the vaccines produced to control hemorrhagic septicaemia in cattle and buffaloes are used to control swine pasteurellosis. Hence, there is an urgent demand for the creation of an advanced vaccine system to combat swine pasteurellosis, aiming to overcome the limitations of the existing vaccine. The outer membrane proteins (OMP) may be considered as potent immunogenic candidate for controlling pasteurellosis in different species of animals including avian pasteurellosis (Zhang *et al*. 1994). Like many gram-negative bacteria, *P. multocida* also have one or more predominant OMP and these proteins have been shown to play essential role in host pathogen interaction and disease production (Lin *et al*. 2002 and Davies *et al.* 2003) [6, 1]. OmpH, a porin protein present in some Gram negative bacteria possesses both specific and cross reacting epitopes which is abundantly expressed in the bacterial surface (Tan *et al.* 2010) [12]. They are generally conserved in a bacterial species or even in a bacterial family in a way that they have high homology in primary amino acid sequence and secondary structure and are antigenically related (Jeanteur *et al.* 1991) [2]. The attributes of OmpH render it a promising candidate for a vaccine. However, research on the cloning and expression of the ompH gene from *P. multocida* in porcine isolates from the North Eastern states of India is notably deficient. Since such studies are essential prerequisites for vaccine development, their absence is noteworthy. Therefore, to establish a foundation study for using OmpH as potential vaccine candidate in future as well as a reagent for the development of a diagnostic kit in an ELISA format, this study has been aimed with the following objectives - (1) To clone and express ompH gene of a capsular type

A *P. multocida* of pig origin, (2) To purify the recombinant OmpH protein.

2. Material and Methods

A total of 10 nos. of isolate from *P. multocida* capsular type A of pig origin were obtained from Department of Microbiology, College of Veterinary Science, AAU, Khanapara which were further subjected for molecular confirmation by PM-PCR and CAP-PCR as per the methods described by Townsend *et al.* (1998) [14] and Townsend *et al.* (2001) [13], respectively. The 8 nos. of *P. multocida* capsular type A isolates were further subjected for detection of ompH gene (Luo *et al.* 1997) [7]. The highly pathogenic strain of *P. multocida* type A was selected for cloning and expression of ompH gene on the basis of mice pathogenicity test.

2.1 Amplification of ompH gene

The ompH gene of *P. multocida* type A was amplified in PCR using gene specific designed primers, details of which are mentioned below-

Forward primer: ATGCTCTAGAATGAAAAAAACACTTATTGC.

Reverse primer: GCATCTCGAGGAAGAACACGCGTAAACCTAC.

For that 4 µl of DNA template was added to 25 µl (2X) master mix (Thermo scientific) which contained 0.05 U/µl Taq DNA polymerase, 4 mM $MgCl_2$ and 0.4 mM of each dNTPs and onto which added 0.5 µl of ompH gene specific forward and reverse primers (10 picomol/ µl). The final volume of reaction mixture was made up to 25 µl with sufficient volume of nuclease free water. The amplification was for 35 cycles where each cycle had a denaturation at 94 °C for 15 sec, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. PCR amplified product was analyzed on 1% agarose gel along with DNA molecular weight marker.

2.2 Cloning of ompH gene

For cloning and expression of ompH gene pET303/CT-His (5369 bp) expression vector was used. The plasmid DNA extraction was carried out as per manufacturer's protocol using plasmid extraction kit (Qiagen, Hilden, Germany) and visualized on 1% agarose gel by electrophoresis. The restriction enzyme (RE) digested PCR product of ompH gene was ligated with pET303/CT-His vector using T4 Ligase enzyme which was confirmed by T7 plasmid specific PCR. The recombinant plasmid was transformed into calcium treated *E. coli* DH5α competent cells which was confirmed by T7 plasmid specific PCR, ompH gene specific PCR, RE digestion and nucleotide sequence (Kanaiyalal, 2010 and Sambrook *et al.* 2001) [3, 8]. The sequences of recombinant plasmid were analyzed by using NCBI web portal to identify the sequence specificity.

2.3 Expression of the recombinant OmpH protein

The expression host *E. coli* BL21 was transformed with recombinant plasmid which was inoculated into 5 ml of LB medium containing ampicillin antibiotics (50 µg /ml). Cultures were grown overnight at 37 °C with constant agitation at 250 rpm in shaker incubator. Three ml of overnight grown cultures were inoculated into 10 ml of LB broth with ampicillin antibiotics and incubated at 37 °C with constant shaking until the OD600 is 0.5-0.7. The culture was induced with 1 mM Isopropyl - β-D-1- thio galactopyranoside (IPTG) and incubated at different time (2 hours, 4 hours and 6 hours) with the same conditions. Then 1 ml culture from each induced sample was centrifuged at 11,000 g for 3 min and resuspended in 30 µl of 2X sample buffer (Amresco). Then samples were loaded to 12% SDS-polyacrylamide gel for analysis of OmpH protein using a standard protein molecular weight marker (Laemmli, 1970).

2.4 Purification of Recombinant OmpH Protein

The recombinant OmpH with 6 histidine residues at the C-terminal end of the protein was purified using Ni-NTA affinity chromatography (Qiagen, Hilden, Germany) as per manufacture's protocol. For this, the cell pellet of transformed BL21 was resuspended in lysis buffer and lysozyme (1 mg/ml). The cell suspension was stirred for 1 hour at room temperature and then sonicated on ice at 20-30 W with six 10 sec bursts. The lysate was centrifuged at 11,000 rpm for 30 min at 4 °C to pellet the cellular debris. For processing of denaturing protein, this cell pellet was resuspended in lysis buffer containing 8 M urea which was incubated at 4 °C for 60 min and then sonicated on ice. The cellular debris was centrifuged at 11,000 rpm for 30 min at 4 °C. The denatured protein supernatant was mixed well with pre-equilibrated Ni-NTA agarose (Genetix) for 1 hour at room temperature, and then allowed to pass through Ni-NTA column. The recombinant OmpH protein bound to Ni-NTA agarose was eluted with 4 ml of elution buffer. The purified recombinant OmpH proteins were analyzed by SDS-PAGE.

2.5 Western Blot analysis of His tagged recombinant OmpH proteins

The expressed recombinant protein was confirmed by western blot analysis as per the standard protocol (Towbin *et al*. 1979 and Singh *et al.* 2009) [18, 10] using semi-dry blot system (Bio Rad). The purified protein was separated in SDS-PAGE and transferred to nitrocellulose membrane. Then, the membrane was incubated at 37 °C with anti-rabbit HRPO conjugate (Sigma-Aldrich, USA) (1:7000 dilution) for 1 hour.

3. Results and Discussion

All the 10 isolates had showed an amplified product of 460 base pair (bp) (Fig. 1) which is similar with the findings of Miflin and Blackall (2011) [16]. Eight out of the 10 number of *P. multocida* isolates were identified as capsular type A with a corresponding product size of 1044 bp as depicted in Fig. 2 (Ragavendhar *et al*. 2015) [17]. During the study, all eight isolates of capsular type A *P. multocida* were found to possess the ompH gene, resulting in the amplification of a 1000 bp PCR product (Fig. 3), (Bethe *et al.* 2009) [19].

3.1 Cloning of ompH gene of *P. multocida* of serotype A

The PCR amplification of ompH gene by using designed primers showed an amplicon size of 1100 bp in 1 % agarose gel electrophoresis (Fig. 4). The RE digestion of plasmid of pET303 vector was confirmed by detection of a single 5.3 kb band in 1 % agarose gel (Fig. 5). The ligation was confirmed by performing PCR with T7 plasmid specific primers showed expected band 1237 bp in 1% agarose which indicated successful ligation reaction (Fig. 6).

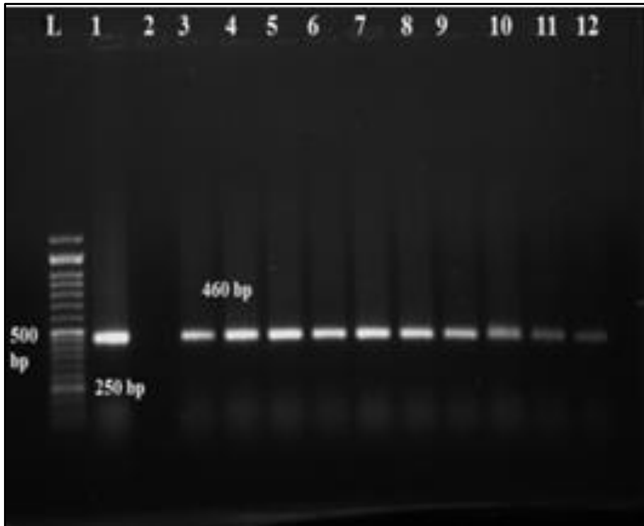

Fig 1: *P. multocida* species specific PM-PCR showing an amplified product of 460 bp in agarose gel electrophoresis. L = 100 bp ladder, 1= Positive control, 2 = Non-Template control, 3-12= Repository *P. multocida* isolates.

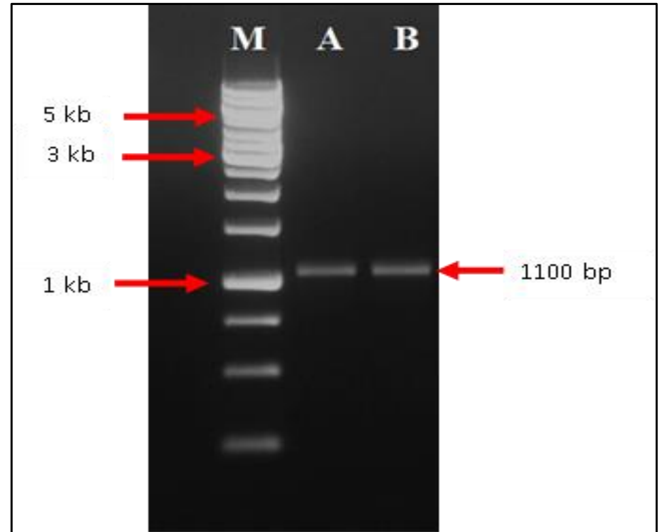

Fig 4: ompH gene of *P. multocida* with a product size of 1100 bp. M= 1kbp ladder, A, B=Amplified product of ompH gene.

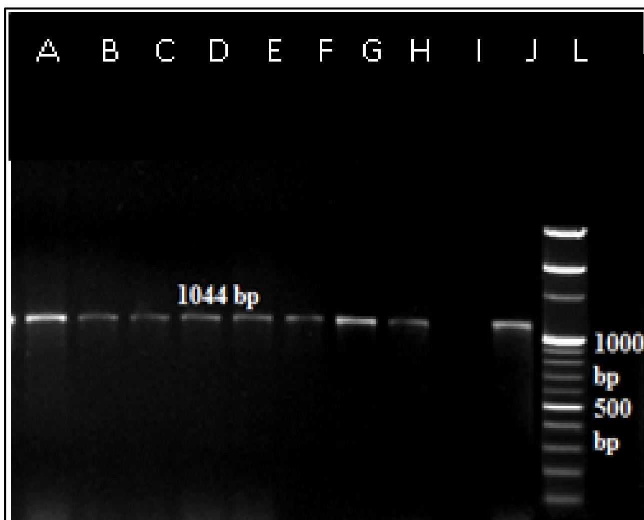

Fig 2: *P. multocida* type specific Cap A-PCR showing an amplified product of 1044 bp of capsular type A in agarose gel electrophoresis. A-H= *P. multocida* isolates, I= Non Template control, J= Positive control, L= 100 bp ladder.

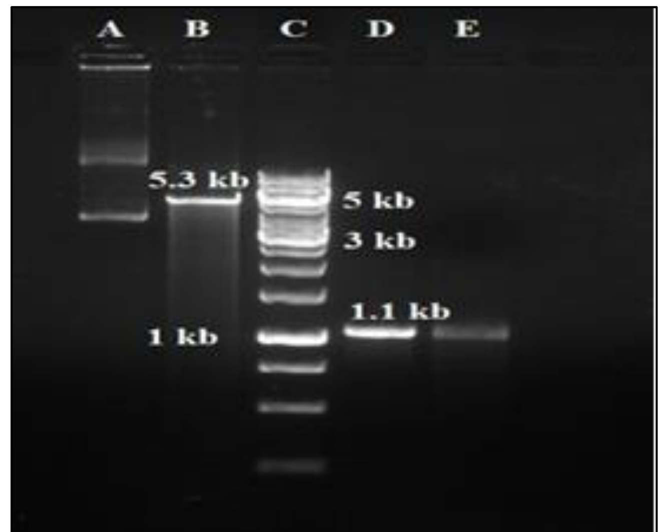

Fig 5: RE digestion of PCR product of ompH gene and pET303 plasmid vector. A=Undigested plasmid, B= Digested plasmid with RE, C=1 kb Marker, D=Undigested PCR product of ompH gene, E=Digested Insert ompH.

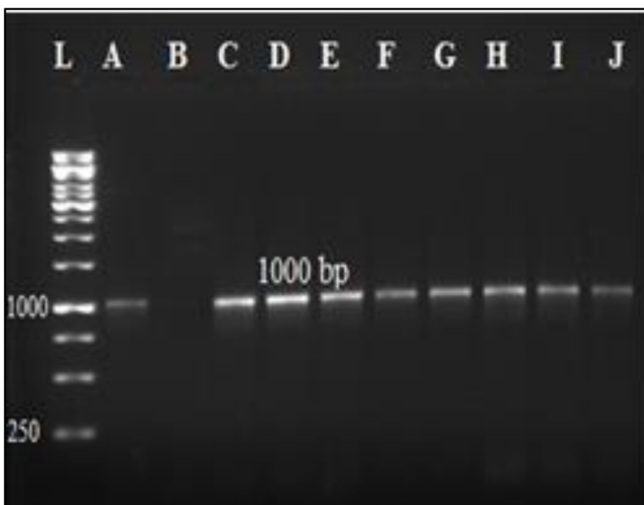

Fig 3: PCR amplified ompH gene of *P. multocida* with a product size of 1000 bp in agarose gel electrophoresis. L= 1kbp ladder, A= Positive control, B= Non template control, C-J= P. multocida isolates.

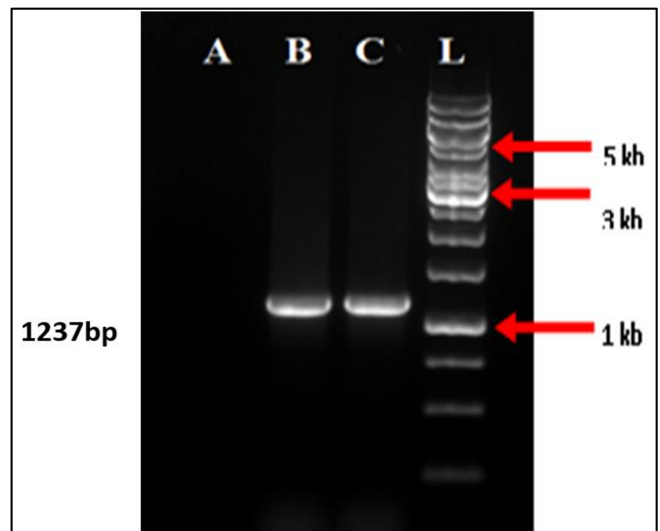

Fig 6: PCR amplified T7 promoter gene of pET303 vector with a product size of 1237 bp in agarose gel electrophoresis. A = Negative control, B & C = Ligated product, L= 1kbp ladder.

Six colonies of DH5α were examined for the presence of the correct insert (ompH) through colony PCR, utilizing T7 and ompH gene-specific primers. All six colonies tested positive in the colony PCR for the ompH gene with bands of 1100 bp, and for T7-specific PCR with bands of 1237 bp, respectively (Fig. 7 & 8). The orientation of the insert fragment was checked by digestion of recombinant plasmids with *Xba1 and Xho1* restriction enzyme. The result showed that double digestion of recombinant plasmid released an ompH fragment of approximately 1100 bp size whereas plasmid was linearized with a product size of 5369 bp (Fig. 9).

Nucleotide sequence of the subjected recombinant plasmids showed 97-98% homology with ompH gene specific of porcine *P. multocida,* published in the GeneBank, NCBI. In a detail study on *P. multocida* B: 2, Tan *et al.* (2010) [12] observed that out of 3 peptide fragments of ompH, the 37.0 kDa fraction was more reactive with a mouse polyclonal antiserum. In a similar study, Kanaiyalal (2010) [3] characterized the ompH gene of bovine *P. multocida* by cloning, sequencing and sequence analysis by bioinformatics tools. The purified PCR product, in his study was ligated in pTZ57R/T vector followed by transformation into competent *E. coli* cells. Contrary to this observation on detection of amplified recombinant clone with 1237 bp size, Kanaiyalal (2010) [3] demonstrated the size of the recombinant clones carrying desired insert of ompH gene as 1158 bp DNA fragment (1000 bp + 158 bp). This might be due to the use of different cloning vectors in two different studies. A study on nucleotide sequence of recombinant ompH of the vaccine strain of *P. multocida* (P52) revealed a high level of homology to the OmpH of other serotypes of *P. multocida* (Singh *et al.* 2011) [11]. On the basis of this observation, it was concluded that ompH gene is conserved among all the serotypes of *P. multocida.*

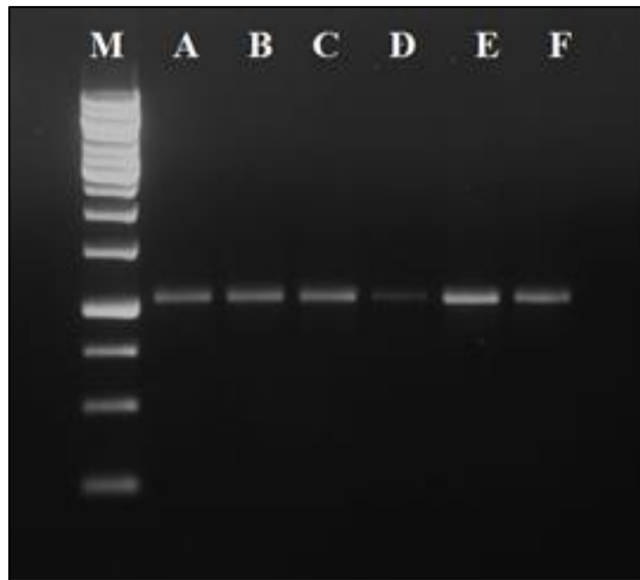

Fig 7: PCR amplified ompH gene with a product size of 1100 bp in agarose gel electrophoresis. M= 1kbp DNA ladder, A-F= Amplified product of ompH *gene* of recombinant plasmid isolated from DH5α.

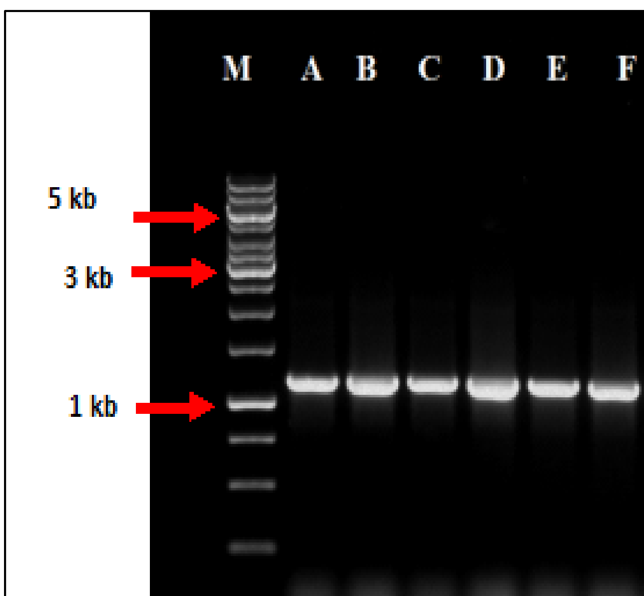

Fig. 8: PCR amplified T7 promoter gene of pET303 vector with a product size of 1237bp in agarose gel electrophoresis. M= 1kbp ladder, A-F=Amplified product of T7 gene of recombinant plasmid isolated from DH5α cell.

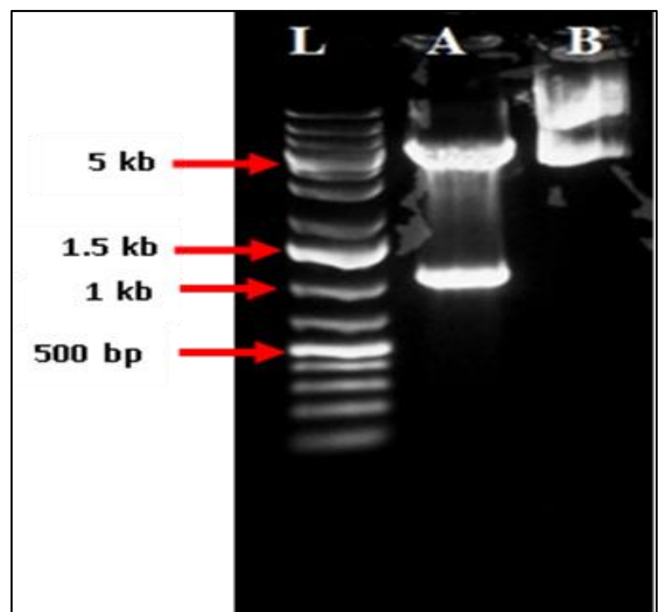

Fig 9: RE digestion of recombinant plasmid. L= 1kbp ladder, A=RE digested recombinant pET303/CT-His plasmid, B= Undigested recombinant pET303/CT-His Plasmid.

3.2 Expressions of OmpH Protein

The intensity of the SDS-PAGE pattern of 37.0 kDa OmpH in the transformed BL21 cell was found to be prominent at 6 hrs post induction with 1mM IPTG. A thick sharp distinct band of 37.0 kDa was observed which indicates the presence of expressed protein in high amount (Fig. 10). In contrary Jogi *et al*. (2022) found a prominent 34 kDa recombinant OmpH protein at 12 hrs of induction with 1 mM IPTG. Singh *et al.* (2009) [10] also successfully induced the plasmid carrying *E. coli* M15 with 1mM IPTG at 6 hrs at 37 °C in LB broth. Lee *et al.* (2004) [5] recorded repeated unsuccessful transformation of *P. multocida* of pig origin into *E. coli* BL21 (DE3). Based on their observation they opined that expression of the truncated as well as full-length forms of the recombinant OmpH could be fatal to the host *E. coli* BL21 (DE3).

3.3 Purification of recombinant OmpH protein

The His-tagged rOmpH proteins were purified by nickel chelating affinity chromatography under denaturing conditions in the presence of 8M urea by using nickel-nitrilotriacetic acid (Ni-NTA) columns and evaluated by western blot which revealed the presence of a very intense protein band at 37.0 kDa (Fig. 11). Luo *et al.* (1997) [7] recorded the 37.0 kDa purified rOmpH in western blot analysis. Singh *et al.* (2009) [10] and Sezer *et al.* (2012) [9] also purified the rOmpH with 6 histidine residues at the N-terminal end of the protein under denaturing conditions using nickel-nitrilotriacetic acid by affinity chromatography. Contrary to this observation on detection of *P. multocida* rOmpH in denatured condition Tan *et al.* (2010) [12] reported rOmpH of *P. multocida* type B of bovine origin in native condition, which might be due to the lowering of incubation temperature from 37 °C to 30 °C. According to them, lowering of incubation temperature resulted in a higher chance of folding of recombinant protein properly.

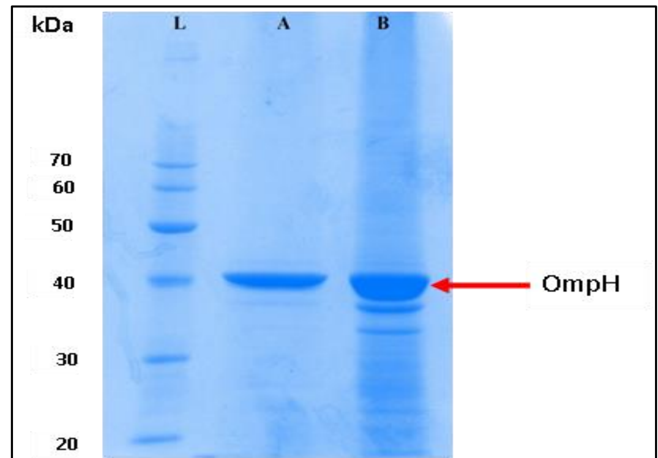

Fig 10: SDS-PAGE analysis of rOmpH after purification. L= Protein MW marker, A= Purified rOmpH protein, B= Unpurified rOmpH protein

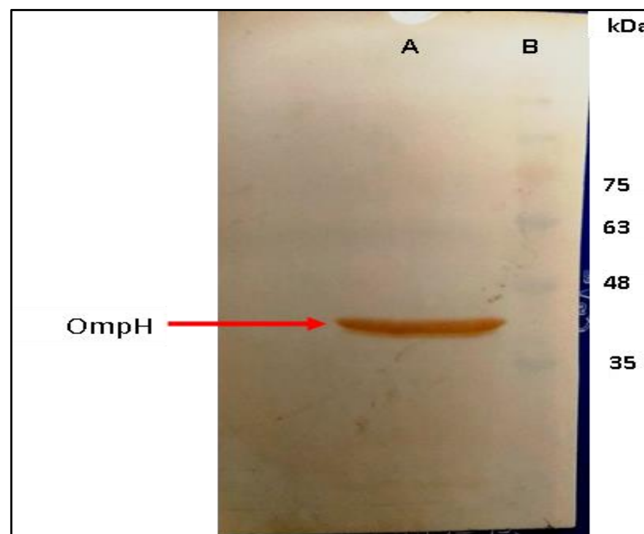

Fig 11: Western blot analysis of the purified 37.0 kDa rOmpH of *P. multocida* with anti-His antibody. Lane A = Single band of 37 kDa rOmpH, Lane B= Protein MW marker.

4. Conclusion

Form the above study it can be concluded that the recombinant protein OmpH which was cloned and expressed from a porcine isolates of *P. multocida* type A is likely to help in the development of suitable vaccine to control swine pasteurellosis. The expressed protein may also be used for development of a diagnostics kit for diagnosis of swine pasteurellosis in future.

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