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Cloning and Expression Studies of the Major Outer Membrane Protein (*OmpH*) Gene of *Pasteurella multocida* P52 in Prokaryotic Vector

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ABSTRACT

Background: The present study was aimed at cloning and expression of the major outer membrane porin (*ompH*) gene of *P. multocida* serotype B:2 P52, the strain used in vaccines against HS in India. The Porin H (*OmpH*) is major immunodominant outer membrane protein in the envelope of *Pasteurella multocida.*

Methods: The gene encoding *OmpH* was amplified by PCR, cloned and expressed in prokaryotic expression vector. The recombinant *OmpH* protein was expressed as a protein with *E. coli* BL21 (DE3) cells transformed with recombinant plasmid pQE30-Xa-*ompH*.

Result: The expressed protein was purified from *E. coli* and characterized by SDS-PAGE and western blot analysis. The fusion recombinant protein eluted had a molecular mass of about 34 kDa. The expressed recombinant protein was confirmed with western blot analysis using RGS-His antibody and anti-*P. multocida* serum raised against whole cell lysate.

Key words: Cloning and expression, Haemorrhagic septicaemia, Outer membrane protein, *Pasteurella multocida*, Recombinant protein.

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute fatal septicaemic disease of cattle and buffaloes caused by *Pasteurella multocida* type B:2. Prophylaxis plays a major role in controlling the disease and whole-cell bacterin vaccines in use has certain limitations and outbreaks of HS have been reported to occur despite vaccinations. In order to generate a vaccine of superior quality, antigenic components that include lipopolysaccharides (LPS), outer membrane proteins (OMPs) and capsules of *P. multocida* have been analysed for their immunogenic properties. Although capsules and LPS possess antigenic properties, none has been accepted as a candidate vaccine for cattle either due to toxicity or poor immunogenicity. Studies utilizing OMPs of gram negative bacteria indicated OMPs as protective immunogens that could play an important role in bacterial adherence and invasion. Several studies have been reported to identify the potentially important OMPs of *P. multocida*, but only a few clarify the basic characteristics of the OMPs of *P. multocida* (Al-Hasani *et al.,* 2007; Wheeler, 2009), Outer membrane protein H (OmpH) is one such major protein in the envelope of *P. multocida* has been purified and characterized as a porin. Both native and recombinant OmpH proteins have been analysed for their protective ability in *P. multocida i*solates of serotype A and D associated with fowl cholera and atrophic rhinitis, respectively (Lee *et al.,* 2007) vaccine using synthetic peptide derived from the nucleotide sequence mimicking the conformational epitopes of native protein *OmpH* was also found to be protective in experimental studies (Lu *et al.,* 1999). In the present study, we have cloned and expressed the major outer membrane porin gene (*ompH*) of *P. multocida* P52 (vaccine strain for HS) and purified the major outer membrane protein *OmpH*.

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MATERIALS AND METHODS

Location and place of work

Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh.

Study period

The work was carried out from August 2017 to February 2018.

Bacterial strain

Vaccine strain P52 of *P. multocida* (prototype B:2) was obtained from Division of Biological Standardization, Mhow, M.P., India. The culture was maintained on blood agar medium.

Extraction of outer membrane proteins

OMP rich extracts were prepared as per the standard protocol (Choi Kim *et al*., 1991). Briefly, the P52 cells were grown with slow shaking in 1 liter of BHI broth for 18-20 h at 37°C. The cells were harvested by centrifugation at 10,000 ×g for 30 min, washed twice in phosphate-buffered saline (PBS). The cells were disrupted by sonication at 10 micron for a total of 5 min (5 cycles of 1 min each). Intact cells and cell debris were removed by centrifugation at 1700 × g for 20 min. The supernatant was centrifuged at 1,00,000 × g for 1 h at 4°C in an ultracentrifuge. The pellet was suspended in 10 mmol/L HEPES buffer (pH 7.4) and incubated at 22°C for 1 h. The suspension was again centrifuged at 1,00,000 × g for 1 h at 4°C to sediment detergent insoluble outer membrane enriched fraction. The pellet containing the outer membrane proteins was then washed and suspended in 5 ml sterile PBS and stored at -20°C. Protein concentration was determined using bovine serum albumin as standard (Lowry *et al*., 1951).

Characterization of outer membrane proteins

Purified outer membrane proteins of *P. multocida* was analysed in SDS-PAGE using the discontinuous buffer system (Laemmeli 1970). The proteins were subjected to electrophoretic separation in 12% resolving and 5% stacking polyacrylamide gels. Sample containing about 25 µg of protein was loaded into each lane and electrophoresis was then performed at 60V for 10-12 h and visualized by staining with Coomassie brilliant blue. The molecular weight of the OMP bands was determined using standard protein molecular weight marker. The polypeptides from the gels were transferred on to 0.45 µm nitrocellulose membrane (NCM) using a semi-dry electroblotting apparatus for western blotting (Towbin *et al*., 1979). After blocking non-specific sites by 5% dry skimmed-milk the immunoblots were treated with anti-*P. multocida* (polyclonal hyper immune rabbit serum as the primary antibody and goat anti-rabbit IgG horseradish peroxidase (HRPO) conjugate as the secondary antibody. Immunoblot was developed with freshly prepared substrate solution (10 mg diaminobenzidine tetrahydrocholride in 50 mL 50 nmol/L Tris hydrochloride, pH 7.6, with the addition of 30 µL H_2O_2).

Genomic DNA extraction

P. multocida genomic DNA was isolated by alkaline lysis method as described by Sambrook and Russell, (2001). Purity and concentration of DNA was determined by UV// VIS spectrophotometry and it was run in a 0.8% agarose gel.

Amplification of ompH gene by polymerase chain reaction (PCR)

The gene for OmpH was amplified in PCR using gene specific oligonucleotide primers (F- TCAGGATCCCAG CA ACAGTTTACAATCAAGA and R- CTACCCGGGTTAGAAG TGTACGCGTAAACCA) as per Joshi *et al.* (2013).

PCR was performed using genomic DNA (150 ng) along with forward and reverse primers (20 pmol each), 4 µl of 2.5 mM dNTPs, 5 µl of 10× buffer with $MgSO_4$ and 0.2 µl of *Pfu* DNA polymerase and 38 µl of NFW in 50 µl reaction buffer. The amplification cycle was Initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 90s, final extension at 72°C for 5 min. The PCR amplified product was analysed on 1% agarose gel along with DNA molecular weight marker.

Cloning of *ompH* gene into prokaryotic expression vector pQE30-Xa

The amplified *ompH* gene fragment was gel purified using the QIA quick gel extraction kit (Qiagen) following manufacturer's instructions. Restriction enzyme (RE) digestion of insert DNA was carried out using two restriction endonucleases, *Bam*HI and *Sma*I. This gel purified *ompH* gene fragment was ligated to pQE-30 × a plasmid (Qiagen, USA) with T4 DNA ligase utilizing cloning. The ligated plasmid was transformed into *E. coli* DH5α competent cells. The positive clones were isolated by miniprep method of plasmid isolation using the methods described by Sambrook and Russell, (2001).

Expression and purification of recombinant *OmpH* in *E. coli* BL21 (DE3) host cells

The pQE30-×a-*ompH* plasmid with *ompH* in correct orientation was transformed in *E. coli* BL21 (DE3) host cells following the instructions from the manufacturer on LB agar containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml). For the induction, the pQE30-Xa-*ompH* plasmid carrying *E. coli* BL21 (DE3) colony was grown overnight at 37°C in LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. Two ml freshly grown culture was inoculated into 50 ml LB broth containing the same antibiotics and incubated at 37°C with vigorous shaking until the broth reached an OD_{600} of 0.6 was recorded. The cells were induced by adding 1 mM IPTG and were allowed to grow further for 4-6 hrs. The induced *E. coli* cells were then harvested by centrifuging the broth at 6,000 rpm for 10 min. The recombinant OmpH with 6 histidine residues at the N-terminal end of the protein was purified under denaturing conditions using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. For this, a Ni-NTA Fast Start Kit (Qiagen) was used as per the manufacturer's instructions. Briefly, the bacterial pellet was resuspended in lysis buffer (8M urea, 0.1 M NaH_2PO_4, pH 8.0). Incubation was carried out at room temperature for 60 min by gently swirling the cell suspension. Lysis was complete when the suspension was translucent. Lysate was centrifuged at 1,100 g for 10 min at room temperature to pellet the cellular debris. Cell lysate supernatant containing the recombinant protein was applied to the column. The recombinant protein bound to Ni-NTA agarose was washed twice with wash buffer

(8M urea, 0.1 M NaH_2PO_4, pH 6.3) and eluted with elution buffer (8M urea, 0.1 M NaH_2PO_4, pH 4.5).

Characterization of recombinant OmpH

The recombinant protein was analysed in SDS-PAGE and western blot analysis. The eluted protein was separated on 12% SDSPAGE along with induced and non induced bacterial cells and stained with Coomassie brilliant blue. For western blot analysis, the recombinant protein was separated in SDS-PAGE, electroblotted to nitrocellulose and probed with anti-Pasteurella serum raised against whole cell lysate of *P. multocida* and anti-His antibodies. Blots were also probed with anti-His antibodies for the detection of 6xHis tagged recombinant protein followed by secondary antibody and chromogenic detection with goat anti-mouse-IgG HRPO conjugate.

RESULTS AND DISCUSSION

HS is an important disease of cattle and buffaloes. For effective control of the disease, an efficacious and longer duration immunity vaccine is required. Identification and characterization of important immunogens of the bacteria would not only help in designing an improved vaccine but also would help in developing a test for protection status of vaccinated animals. OMPs have been identified as playing an important role in protection. This study was aimed to perform cloning, expression and purification of the major outer membrane protein (OmpH) of *P. multocida* P52 (serotype B:2), the strain used in vaccines against haemorrhagic septicaemia in India to make available the protein in pure form and the desired amount. The result of PCR amplifying a product of the expected size of 942 bp reflects the conserved nature of the *ompH* gene among the *P. multocida* serotypes.

Outer membrane proteins

The outer membrane protein preparation of *P. multocida* P52 revealed the presence of about 06 polypeptide bands on SDS-PAGE. The molecular mass of the polypeptide bands ranged from 20 kDa to 91 kDa. Based on stain intensity and band thickness, polypeptides with approximate molecular weights of 20, 34, 48, 72, 89 and 91 kDa were considered to be the major OMPs. On western blot, the 20, 34, 48, 72, 89 and 91 kDa, were identified as major immunodominant proteins. As per the study conducted by Johnson *et al.* (1991) polypeptide bands of 32, 35, 37, 46, 52, 59, 70 and 87 kDa were identified as major OMPs. 32 kDa protein band was shown to be a major band in Asian HS isolates. B: 2 reference strain was also shown to express outer membrane proteins of 32 and 36 kDa molecular weights (Choi-Kim *et al*., 1989). Further, protein bands in the range of 25-88 kDa were reported in the OMP preparations of P52 strain and polypeptides of MW 44, 37 and 30 kDa were the major immunogens determined (Pati *et al*., 1996). About 20 polypeptide bands with molecular weight ranging from 16 to 90 kDa were observed in the OMP profile of vaccine strain P52, of which 31, 33 and 37 kDa were considered to be MOMPs (Tomer *et al*., 2002). 32 kDa OMP was also found to be the major protein in 17 isolates of *P. multocida* (serotype B: 2), including vaccine strain P52 along with 25, 28, 34, 45 and 87 kDa proteins (Arora *et al*., 2007).

Potential immunogens of *P. multocida* P52 were identified by electroblot immunoassay. Immunoblotting was performed using the hyperimmune serum raised against the whole cell antigen in order to detect immunogenic proteins in outer membrane proteins. Polypeptides separated on 12% SDS-PAGE were transferred to nitrocellulose membrane by semi-dry system. Presence of three polypeptides of 34, 72 and 91 kDa size was reported showing reaction with antiserum. On western blot analysis of major immunogens all the major protein bands appeared immunogenic; however 34 kDa protein was found to be most immunodominant among them. In the present study, out of six immunodominant outer membrane proteins, three polypeptides of 34, 72 and 91 kDa molecular weights were identified. As both sonicated whole cell lysate antigen and the purified OMPs gave similar patterns on Western blots using anti-*P. multocida* serum, it appears that the OMPs are major immunogens of *P. multocida* against which antibodies are directed. On Western blotting, the 20, 34, 48, 72, 89 and 91 kDa, were identified as major immunodominant proteins. Similarly, using sera from immune animals, major bands of 32 and 37 kDa in the Katha strain were observed (Johnson *et al.,* 1989). It has also been reported that sera collected from mice vaccinated with formalin killed B:2 vaccine recognized proteins of 14.2, 32, 35, 50, 67, 80 and 94 kDa molecular weights (Dawkins *et al*., 1991). 44, 37 and 33 kDa proteins were immunodominant in P52 strain (Pati *et al*., 1996; Tomer *et al*., 2002). 32 kDa OMP was found to be major protein in 17 isolates of *P. multocida* (serotype B: 2) including vaccine strain P52 on immunoblotting (Arora *et al*., 2007).

Amplification and cloning of *ompH* gene

PCR amplification of the *ompH* gene of *P. multocida* P52 yielded the expected product of 942 bp. On amplification using *ompH*-specific primer, a PCR product of the expected size of 942 bp was obtained. Luo *et al.* (1999) also reported a single amplicon of similar molecular size from all the serotypes of *P. multocida* associated with fowl cholera. The results of PCR reflects the conserved nature of the *ompH* gene among *P. multocida* serotypes.

In continuation with PCR, cloning and expression of *ompH* gene encoding outer membrane protein of 34 kDa was also carried out. PCR techniques have already been employed for cloning of *ompH* gene of *P. multocida* A:1 by Luo *et al.* (1997), Singh *et al.* (2009) and Joshi *et al.* (2013). The distribution of OMP genes among different serotypes of *P. multocida* has been reported. A 16 kDa Omp gene was found to be present in all the serotypes of *P. multocida* (Goswami *et al.,* 2004). The presence of *ompH* gene and its orientation in the pQE30-Xa-*ompH* plasmid was checked by restriction digestion with BamHI restriction endonuclease

followed by analysis of digested products on 1% agarose gel. Single enzyme digestion study using *BamHI* restriction endonuclease on recombinant clone resulted in release of product size of 942 bp, respectively. Digestion with BamHI resulted in the production of linear plasmid. Recently, characterization and diversity of pathogenic *P. multocida* has been investigated on the basis of not only capsular type but also *OmpH* type by molecular methods (Davies *et al.,* 2003; Jabbari and Esmaelizadeh, 2005).

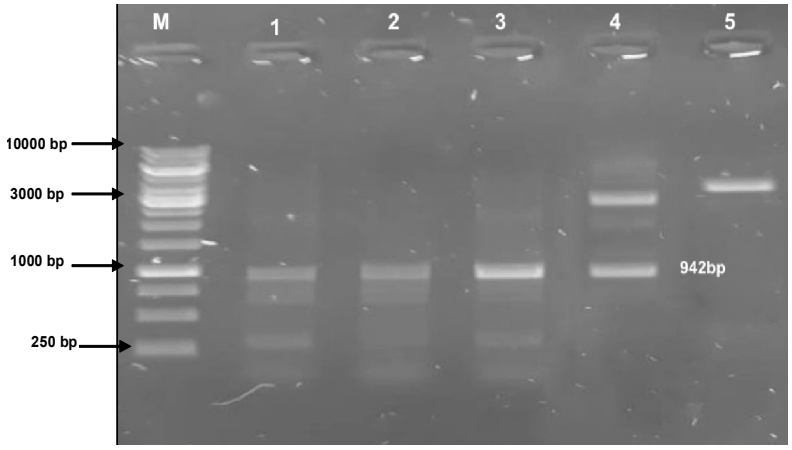
Cloning for expression was performed using pQE vector for N-terminal 6x His tag constructs. RE digestion of PCR product and pQE30-Xa vector was done using *Bam*HI and *Sma*I, the restriction sites for which are present in the multiple cloning site of vector and at the terminal ends of the PCR product. The amplified fragment was cloned into pQE30-Xa expression vector between these *Bam*HI and *Sma*I restriction sites (Fig 1).

Expression and purification of recombinant ompH protein

After characterization, the recombinant plasmid pQE30-Xa-*ompH* was transformed into the *E. coli* BL21 (DE3) expression host and induced with IPTG for recombinant protein expression. The expressed protein was purified under denaturing conditions using Ni-affinity chromatography and the eluted proteins were analysed on 12% SDS-PAGE. The poly histidine tag associated eluted recombinant protein had an approximate molecular weight of 34 kDa. In uninduced and control cultures, no additional band of protein was observed.

The expression profile of the desired 34 kDa protein was obtained highest upto 12 hrs. of induction with 1 mM IPTG. Bhat and Jain (2010) studied the induction kinetics of 49 kDa recombinant OMP protein of *Salmonella* Typhi and found that maximum induction was obtained at 4 hr. after addition of 1.0 mM IPTG to the medium. Singh *et al.* (2009) studied transformation of *E. coli* with recombinant pQE32-pEOMPH which contained the *ompH* gene for the mature protein without a signal peptide. Priyadarshini (2014) noted that the positive recombinant clones were induced by adding IPTG to the media for expression of recombinant proteins. SDS- PAGE analysis was carried out of 5 and 16 hr after induction and the intensity of ~64 kDa (rPfhB2), ~50 kDa (rHsf1), ~62 kDa (rTbpA) and ~45 kDa (rHp2) were gradually increased by 16 hr of post induction, respectively. It was indicative of satisfactory level of expression of recombinant protein in *in-vitro* grown conditions.

Bacterial porin genes are sometimes difficult to clone in *E. coli* because foreign porins are usually lethal for *E. coli*. Initial attempts to clone the entire *ompH* gene into the expression vector were unsuccessful by a number of workers (Luo *et al*., 1997; Lee *et al*., 2007). This failure could be explained due to the leaking expression of the primary protein without IPTG induction and lethality of recombinant porin protein in *E. coli*. The signal peptide in the primary protein helped to target *OmpH* to the outer membrane of *E. coli* that may cause osmotic destabilization of the cells or a change in the structural integrity of the outer membrane. In the present study, transformation of *E. coli* with recombinant pQE30-Xa, which contained the *ompH* gene for the mature protein without a signal peptide, was successful though the expression level of the recombinant gene was relatively low on induction of expression as it could not be detected in SDS-PAGE of induced cell lysate. Similar was the observation by Singh *et al.* (2009) in prokaryotic expression and purification of recombinant bovine IFN-γ from *E. coli*. There was also no leaking



Lane M: DNA ladder 100 bp.
Lane 1: Colony PCR-amplified product at 942 bp.
Lane 2: Plasmid PCR-amplified product at 942 bp.
Lane 3: Positive Recombinant clone PCR- amplified product at 942 bp.
Lane 4: Released insert of 942bp and linear vector after RE digestion with BamHI.
Lane 5: Undigested recombinant plasmid.

Fig 1: Confirmation of positive recombinant clones.

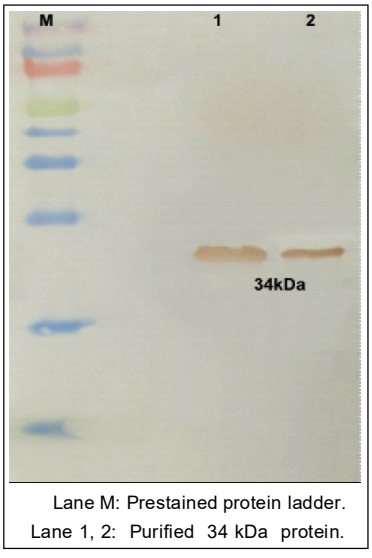
expression of the primary protein without IPTG induction as non induced lysate also did not show the presence of protein in SDS-PAGE.

After confirmation, expression of each protein was scaled up by induction of 1 litre culture of *E. coli* transformants in LB Amp+ Kanamycin+ broth. The expressed proteins fused with polyhistidine tag were purified under denaturing conditions using nickel chelating affinity chromatography. The induced cell lysate was allowed to pass through the Ni-NTA column so that expressed protein gets attached to the Ni-NTA matrix and unbound protein washes off. Then his-tagged protein was eluted out using elution buffer. The efficiency of purification was assessed by SDS-PAGE electrophoresis of the eluted fractions. The purified recombinant proteins were analyzed in SDS-PAGE and a very intense protein band of 34kDa was confirmed by western blotting (Fig 2). The poly histidine tag associated eluted recombinant proteins had an approximate molecular weight of 34 kDa in recombinant clones.

The fusion recombinant protein had a molecular mass of about 37 kDa produced by *E. coli* (Singh *et al*., 2009; Luo *et al*., 1997) found 40 kDa recombinant protein for the 37 kDa purified OmpH and it was the most abundant protein produced by *E. coli*. In native conformation, porin H is a homotrimer, stable in SDS at room temperature and is dissociated into monomers upon boiling. The molecular mass of denatured monomers range between 34 and 42 kDa depending on the serotype and the electrophoretic system used for analysis (Lugtenberg *et al*., 1986; Chevalier *et al*., 1993; Lubke *et al*., 1994). The immunoblots treated with anti-*P. multocida* serum, showed cross reactivity with *E. coli* lysate as both the induced and non induced *E. coli* harbouring pEOMPH showed coloured bands (Singh *et al*., 2009).

The isolation of pure OmpH from *Pasteurella multocida* represents a difficult challenge. Since the major outer membrane proteins and other bacterial porins are usually associated with LPS, which results in uncertainty in the immunological and functional characterization of them. Consequently, the production of recombinant OmpH in *E. coli* and its subsequent purification would greatly facilitate the characterization of *OmpH* in the absence of interference from the contaminant molecules. With the expression and purification of the ompH gene of *P. multocida* P52 in the present study, further work is needed to investigate the role of recombinant protein in protection studies and to study the antigenic properties of the recombinant OmpH as a candidate for vaccine.

Exploring the scope of this protein for molecular typing would also contribute towards conducting molecular epidemiological studies on HS causing *P. multocida* isolates and thereby understanding spread of the bacteria and thus developing suitable control strategies for preventing HS out breaks.



Lane M: Prestained protein ladder.
Lane 1, 2: Purified 34 kDa protein.

Fig 2: Western blot analysis of recombinant OMP 34kDa purified protein.

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Conflict of interest

All authors declare that they have no conflict of interest.

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