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# Cloning, expression and protective capacity of 37 kDa outer membrane protein gene (*ompH*) of *Pasteurella multocida* serotype B:2

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**Abstract.** The major outer membrane protein (OmpH) of 4 local Malaysian strains of *Pasteurella multocida* serotype B:2 were characterized in comparison to ATCC strains. Three major peptide bands of MW 26, 32 and 37 kDa were characterized using SDS-PAGE. Two of these fragments, the 32 kDa and 37 kDa were observed to be more reactive with a mouse polyclonal antiserum in all of the local isolates as well as the ATCC strains in a Western blot. However, the 32 kDa fragment was found to cross react with other Gram negative bacteria. Therefore, the 37 kDa OmpH was selected as vaccine candidate. The 37 kDa *ompH* gene of the isolated strain 1710 was cloned into an *Escherichia coli* expression vector to produce large amounts of recombinant OmpH (rOmpH). The 37 kDa *ompH* gene of strain 1710 was sequenced. In comparison to a reference strain X-73 of the *ompH* of *P. multocida*, 39bp was found deleted in the 37 kDa *ompH* gene. However, the deletion did not shift the reading frame or change the amino acid sequence. The rOmpH was used in a mice protection study. Mice immunized and challenged intraperitoneally resulted 100% protection against *P. multocida* whilst mice immunized subcutaneously and challenged intraperitoneally only resulted 80% protection. The rOmpH is therefore a suitable candidate for vaccination field studies. The same rOmpH was also used to develop a potential diagnostic kit in an ELISA format.

## INTRODUCTION

*Pasteurella multocida*, the gram-negative, non-motile, rod shaped, facultative anaerobic bacterium has been isolated from a wide range of host throughout the world. This bacterium is the etiologic agent of a variety of economically significant diseases in both wild and domestic animals, including fowl cholera in poultry, haemorrhagic septicemia in cattle and buffalo, atrophic rhinitis in swine, and snuffles in rabbits (Morishita *et al.*, 1996). Many *P. multocida* strains express a polysaccharide capsule on their surface outside of the outer membrane. These isolates can be differentiated based on the antigenicity of their capsular type A, B, D, E and F and somatic serotypes 1 to 16 based on lipopolysaccharide (LPS) antigens (Boyce & Alder, 2002). The serogroup is generally related to disease predilection, with haemorrhagic septicemia strains belonging to serogroup B or E. The majority of fowl cholera strains belong to serogroup A (Christensen & Bisgaard, 1997; Boyce & Alder, 2002); and progressive atrophic rhinitis (PAR) cause by serogroup D (Davies *et al.*, 2003).

Haemorrhagic septicemia (HS) is endemic in most tropical countries such as South East Asia, India and Africa causing high mortality in livestock (Verma & Jaiswal, 1998). Cattle and buffalo are the

most common hosts, but pigs, sheep, goats, deer and camel are also susceptible to this infection (Blackall *et al.*, 2000).

The vaccines currently in use are either inactivated bacteria or live attenuated bacteria (Boyce & Alder**,** 2002). The inactivated bacteria vaccines, provides serotype-specific protection whereas the live vaccines composed of attenuated strains, confers protection against both homologous and heterologous serotypes (Boyce & Alder**,** 2002). Although considerable reduction in deaths due to this disease had been achieved by immunization with these vaccines, problems still remain in providing complete protection. Some of these problems are quality of vaccine, breakdown in immunity, duration of immunity, post-vaccination shock. Bain *et al*. (1982) found the oil adjuvant vaccines (OAV) is generally credited with reducing the incidence of HS. However, this vaccine also raised some major disadvantage apart from the difficulty in injecting the OVA due to its high viscosity, formation of abscesses at the site of injection and post-vaccination shock reactions have often been reported. Breakdown in immunity where a reversion to virulence in seen in flocks vaccinated with attenuated live vaccines and causing outbreaks of fowl cholera have been reported.

Many gram-negative bacteria have one or more predominant outer membrane proteins and these proteins have been shown to play essential role in host-pathogen interaction and disease processes (Lin *et al.*, 2002; Davies *et al.*, 2003;). *Pasteurella multocida* expresses both heat-modifiable protein (OmpA) and porin (OmpH). OmpH possessed both specific and cross-reacting epitopes which are abundantly expressed on the bacterial surface, whereas OmpA possessed cross-reacting epitopes which are not exposed on the cell surface, as shown by immunoelectron microscopy (Vasfi Marandi *et. al.*, 1995b, 1996).

However, very little is known about the precise roles of these protein in pathogenesis, but previous studies had shown that OmpA and OmpH based vaccine can induce protective immunity against bacteria infection. In one study, antibodies to OmpA were observed present in sera from rabbits naturally infected with *P. multocida* suggesting that OmpA protein may potentially be an important immunogen (Vasfi Marandi *et al.*, 1996). However, more studies involving OmpH have been demonstrated to induce protective immunity in animal models against *P. multocida*. Tomer *et al.*(2002) applied anti-*P. multocida* vaccine strain P-52 whole-cell hyperimmune serum raised in rabbits and buffalo to the immunoblotting studies, identified that the polypeptide 37kDa was the most antigenic Omp in the profile of all the serotype B:2 field isolates(19). Luo *et al*. (1997) found that OmpH of bacterial strain X-73 was able to induce homologous protection in chickens against *P. multocida*. This makes OmpH peptide an attractive vaccine candidate. Later, Luo *et al*. (1999) found that the heterogeneity of the OmpH protein in somatic serotype strains of *P. multocida* is due to the presence of a hypervariable surface-exposed loop region. Thus, a cyclic synthetic peptide (Cyclic-L2) that mimicked the conformational epitopes of the native protein was synthesized. This peptide was able to provide 70% protection in chickens against *P. multocida* infection (Luo *et al.*, 1999).

In this study, we have cloned the predominant 37 kDa *ompH* gene of *P. multocida* strain which was isolated from infected buffalo during local outbreaks. The expressed and purified OmpH was evaluated as a potential vaccine candidate using BALB/C mice. In addition, an ELISA assay was also developed to evaluate the protection levels of the 37 kDa OmpH on mice. The usage of this antigen-specific assay diagnostically was also investigated.

## MATERIALS AND METHODS

### Bacterial strains

*Pasteurella multocida* serotype B-2 (strain no: 513, 1710, 4412, and 4996) were isolated

by the Veterinary Research Institute (VRI) Ipoh, Malaysia, from a buffalo with HS and was cultured on 5% sheep blood agar at 37°C. At the same time, three ATCC strains of *P. multocida* were also provided by VRI. These include ATCC 366, ATCC 367, and ATCC 368.

**Analysis of Omps**

**Preparation of Omps**

In order to compare the Omps profile of *P. multocida* serotype B-2 and three ATCC strains obtained from VRI, Omps was purified according to the method described by Snipes *et al.* (1990). Briefly, the bacteria were cultured in brain heart infusion broth at 37ºC overnight, harvested by centrifugation at 4ºC, 10 000 g for 30 min and washed twice in cool PBS, pH 7.4. The washed pellet was resuspended in cool 10mM HEPES, pH7.4 and was sonicated for 4-5 min with duty cycle of 50%. The supernatant was collected by centrifugation at 4ºC, 5000 g for 20 min and was further clarified by centrifugation at 4ºC, 10 000 g for 60 min. The pellet were resuspended with 2ml of 2% (w/v) N-Lauroylsrcosine in 10 mM HEPES, pH 7.4 and were incubated at 22ºC for 60 min. The suspension was then centrifuged at 4ºC, 10 000 g for 60 min and the pellet was rinsed with distilled water three times and resuspended in distilled water. The protein concentrations were determined by the BCA™ Protein Assay kit (Pierce) and the Omps were adjusted to 1 mg/ml and store at -20ºC till use.

**SDS-PAGE and Western blots analysis**

The *P. multocida* serotype B-2 and three ATCC strains were analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli's method (1970). The SDS-PAGE was stained with Coomassie Blue. For Western blot analysis the gel was prepared and transferred onto nitrocellulose membrane at 100V for 1 hr (Bio-Rad). The membrane was blocked with 4% BSA and washed three times for 10 min each time using 0.05% Tween-20 in Tris-buffered saline (TBS) (10 mMTris, 150 mM NaCl, pH 7.5). 1: 800 mouse antiserum (See section 2.5) was then incubated for 1 hr followed by three washes. The membrane was then incubated with 1:5000 dilution of alkaline phosphotase anti-mouse IgG (Dako) for 1 hr followed by three washes again. The antigens were detected by adding 1ml of BCIP/NBT Western Blue® stabilized substrate (Promega) for 5 min in the dark, and then rinsed with 20 mM EDTA for 3 min to stop the reaction.

**Cloning *ompH* gene**

The *ompH* gene was amplified from *P. multocida* genomic DNA by PCR. PCR primers were designed based on the sequence of *P. multocida* strain X-73, obtained from GeneBank accession no. U50907. Based on this sequence, the total length of *ompH* gene is 1604bp; including promoter sequence and open reading frame (ORF). The ORF started at position 392bp to 1454bp, with length of 1062bp. Further analysis shows that this genomic sequence contains one signal peptidase I cleavage site in between position 451-452bp. Thus the mature *ompH* gene is coded from 452 to 1454bp (Figure 1).

To clone this *ompH* gene, cloning of full sequence is not needed, thus the primers were designed to flank the matured *ompH* sequence. In order to facilitate purification of the recombinant OmpH (rOmpH) using nikel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices, DNA sequence of 6-His tag was incorporated into reverse primer (Table 1).

**Extraction of total genomic DNA of *P. multocida* B-2**

The total genomic DNA of *P. multocida* B-2 was extracted by standard DNAzol® protocol. Briefly, the bacteria were cultured in brain heart infusion broth at 37ºC overnight. 1.5ml of the culture was transferred into a 1.5ml tube and was spun at 14 000 rpm for 5 min. Supernatant was discarded and the bacteria pellet was resuspended in 1.0 ml of DNAzol. After 15 min of incubation at room temperature, the mixture was spun down at 8000 rpm for 5 min. the total genomic DNA containing

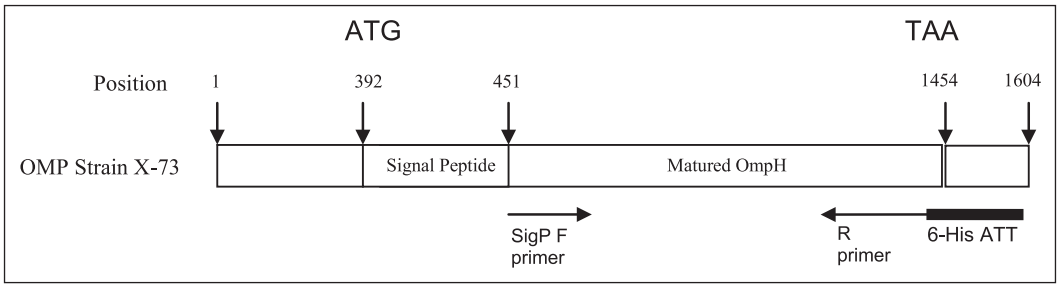


Figure 1. Genomic analysis of *ompH* gene from *P. multocida* strain X-73 (U50907). The primers were designed franking the matured *ompH* sequence with 6-His tag sequence incorporated at reverse primer, followed by stop codon

Table 1. Primers use to amplify matured *ompH* gene. SigP F is the forward primer start right after signal peptidase I cleavage site. 6His R is the reverse primer with 6-His tag DNA sequence and stop codon incorporated. Sequence in bold represent H-his tag DNA sequence; *sequence in italic* represent stop codon

| Primer | Sequence (5'—) | Length (bp) | Tm (°C) |
|---|---|---|---|
| SigP F | CAGCAACAGTTTACAATCAAGACGGTACCAAAGTTGATGTA | 41 | 64.3 |
| 6His R | *TTA* **GTGATGGTGATGGTGATG** GAAGTGTACGCGTAAACCTGT | 42 | 37.6 |

supernatant was transfer to a fresh tube, and the cell debris containing pellet was discarded.

0.5ml of 100% ethanol was than added to the supernatant and mix well by brief vortex. The mixture was then spun at 14000 rpm for 15 min to pellet down the total genomic DNA. The pellet was then washed twice with 75% ethanol and finally resuspended in 50ul DNase/RNase free water.

**PCR of *ompH* gene**

Amplification of *ompH* gene was done using both SigP F and 6His R primer in a 50ul PCR reaction. The 50 µl PCR mixture consisted of 5µl of 100ng *P. multocida* genomic DNA, 1.5µl of 10µM primers, 1.25µl of 10µM deoxynucleotide triphosphate (Genecraft), 1.5mM of 10mM $MgCl_2$, 1.25u of *Taq* DNA polymerase (Genecraft), 5µl of 10X PCR buffer, and 35.75 µl of distilled water. The thermal cycling conditions were: 1 cycle at 95°C for 5 min; 40 cycles at 94°C for 30 sc, 50°C for 1 min, and 72°C for 1.5 min; 1 cycle at 72°C for 10 min. This PCR profile was repeated for another three times to confirm it is a specific PCR product. The PCR product was analyzed by electrophoresis in 1.5% agarose gel and sequencing.

**Cloning, transformation and selection of *ompH* positive clone**

Cloning was performed according to the protocol QIAexpressionist™ (Qiagen). The expression vector pQE30-UA is a linear vector which readily to be ligated with PCR product. The PCR product of *ompH* gene was ligated with expression vector and was incubated at 16°C for overnight. The ligated vector was transformed into the competent *Escherichia coli* M15. The vector competent cell mixture was incubated at 4°C for 20 minutes followed by incubation at 42°C for 90 seconds for plasmid absorption. Later the transformed cells were cultured at 37°C for 90 min. *Escherichia coli* M15 harboring a plasmid [pREP4] will confer kanamycin resistance, whereas the expression plasmid vector, pQE-30 UA confers ampicillin resistance.

Hence a successful clone should be able to grow in agar plates containing both antibiotics.

**Plasmids extraction and amplification of *ompH* gene**

The plasmid of pREP4, non-insert pQE-30 UA and *ompH* gene inserted pQE-30 UA were extracted by Bioneer® Plasmid extraction kit. In order to linearlize the plasmid, pREP4 was digested by *Hind*III, insert and non-insert pQE-30 UA were digested by *BamH*I according to the restriction map on QIAexpressionist™ (Qiagen). The DNA plasmids of positive clones were confirmed again by PCR. The results are analyzed by gel electrophoresis and λ DNA/Eco130I (StyI) marker (Fermentas) was used to size the fragments.

**Expression of *ompH* gene**

**Expression of *ompH* gene in *E.coli* M15**

The positive clone was selected by culturing the single colony with ampicillin and kanamycin on LB agar. The expression and purification were carried out according to the Qiagen manufacturer's instructions. Briefly, positive clones were cultured in medium containing both ampicillin (100µg/ml) and kanamycin (25µg/ml) at 37ºC overnight. 100 ml of pre-warmed media (with antibiotics) was inoculated with 5 ml of the overnight cultures. The culture was grown with vigorous shaking until $OD_{600}$ of 0.6 was reached (30-60 min). A final concentration of 1 mM IPTG was added to induce expression for 4-5 hrs.

**Purification of rOmpH by nikel-nitrilotriacetic acid (Ni-NTA)**

The cells were harvested by centrifugation at 4000 g for 20 min. The supernatant was discarded and the *E. coli* lysate was prepared by native conditions. The cell pellet was resuspended in lysis buffer and sonicated six times on ice with 10s bursts at 250W. Then lysate was collected after centrifugation at 10 000 g for 25 min at 4ºC. 50% Ni-NTA slurry was added to the lysate and mixed gently by shaking at 200rpm on a rotary shaker for 60 min at 4ºC. The lysate-resin mixture was then loaded on to a column and eluted fraction was collected as recombinant OmpH (rOmpH). The extracted rOmpH was analyzed by SDS-PAGE followed by Western Blot.

**Production of mouse antiserum**

100 ìg of crude Omps were emulsified in complete Freund adjuvant and injected subcutaneously to balb/cJ mice (6-8 weeks of age). After 14 days, the same amount of Omps were emulsified in incomplete Freund adjuvant and injected sub-cutaneously to BALB/c mice. Blood was collected 7 days after the second injection. The blood of control mice was also collected. These sera were ready to be used in the development of ELISA assay.

**The use of rOmpH peptide in Enzyme-linked immunosorbent assay (ELISA)**

The whole cell lysate, purified Omps of *P. multocida* serotype B-2, and rOmpH were diluted to 1 µg/ml with coating buffer and 100 µl were coated on immunoplates at 4ºC overnight. The plates were washed five times with PBS containing 0.05% Tween-20, followed by 100 µl of blocking buffer (0.5% bovine serum albumin in PBS), and incubated at 37°C for 1 hr. The plates were washed five times and incubated with 100 µl of 1:100 dilution mouse polyclonal antiserum at 37ºC for 1 hr. Then, the plates were washed again and incubated with 100 µl of 1:5000 dilution horse-radish peroxidase anti-mouse IgG (Dako) at 37ºC for 1 hr. After five washes, 100 µl of OPD substrate (Abbott Diagnostics) was added and incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 50 µl of 4N sulfuric acid and absorbance was read at the wavelengths of 492/620 nm in an ELISA reader (Biotrak® II, Amerrsham).

**Determination of lethal dosage**

*Pasteurella multocida* was cultured overnight in LB medium at 37ºC with shaking. A serial of tenfold dilution were prepared in phosphate-buffered saline

(PBS) (pH 7.4) and plated on agar plates for quantification. The diluted culture with 10, $10^2$, $10^3$, and $10^4$ CFU were used to inject subcutaneously into 6- to 8-week-old BALB/c mice to obtain lethal dosage 50% ($LD_{50}$). The infected mice were observed daily, and their mortality was recorded.

**Protection studies in BALB/c mice**

Three groups of mice (6-8 weeks old) were prepared: Group1 served as the control, Group 2 were immunized intraperitoneally (IP) and Group 3 were immunized subcutaneously (SC) with 100µg of rOmpH peptide mixed with Freund's adjuvant (1:1). 100µl of PBS was given to the control group. Each group was consisted of four mice. The mice were vaccinated twice (every 2 weeks) and 14 days after the second vaccination, all three groups of mice were challenged with 10 CFU of *P. multocida*. The mice were observed for 5 days after challenged. Serum was harvested before and after immunization and after challenge for determination of antibody levels using the ELISA assay.

## RESULTS

**Extraction and analysis of Omps**

The extracted Omps of 3 ATCC strains and four outbreak strains of *P. multocida* serotype B:2 were analyzed by 12% of SDS-PAGE gel and by Western Blot. From the SDS-PAGE, the protein band with molecular weight (M.W.) of 26, 32, 37, 45, 54, and 100 Kilodaltons (kDa) were observed (Fig. 2a). In the Western Blot analysis, it was noted that OMPs with molecular weight of 26, 32, 37, and 100 reacted with greater intensity with the antisera as compared to the 45 and 54 kDa Omps (Fig. 2b).

**PCR amplification, DNA sequencing and expression construct**

To amplify the *ompH* gene fragments, we used the SigP F and 6His R (Figure 1) as forward and reverse primers. As shown in Figure 3a, the size of PCR products is in accordance with the sequencing results. As shown in Figure 4, the coding region of *ompH* gene of the *P. multocida* strain-1710 is 980bp long. The predicted primary protein is composed of 346 amino acids (Figure 5). The first 20 amino acid encoded by this gene is a signal peptide (N-terminal). Thus, the mature protein contains 325 amino acids with a predicted molecular mass of ~37 kDa. As shown in Figure 3b, using the QIAexpressionist™ system (See Section 2.3.3) the size of the pREP4 plasmid is 3740bp and the non-insert pQE-30 UA plasmid is 3500bp. The pQE-30 UA plasmid which was successfully cloned with the *ompH* gene was 4478bp. The [pREP4] plasmid was linearized with *Hind*III. However the *ompH* gene fragment and pQE-30UA both contain a *Hind*III restriction enzyme site and as such the *Bam*HI was used for this clone. Successful amplification of the *ompH* gene from the DNA plasmid of positive clone resulted in a PCR fragment of the expected size (980bp) (Figure 3b- lane 4).

**Expression, purification, and Western-blot analysis of *ompH* gene**

The expression level of *ompH* gene in *E. coli* cells was found to be maintained at a low level of less than 1 mg/L. The construct was designed to contain six C-terminal histidine residues for ease of purification by affinity chromatography over a Ni-NTA matrix. The signal peptide region of the *ompH* gene was not being cloned. Western Blot analysis (Figure 6a, b) demonstrated the recombinant *ompH* was also able to be recognized by the mouse polyclonal antiserum in the *P. multocida* serotype B:2.

**Determination of $LD_{50}$**

From the $LD_{50}$ determination experiment, all mice in the group injected with $10^2$, $10^3$ and $10^4$ CFU died 12-24 hrs while the group injected with 10 CFU showed mortality of 50% after 24-48 hrs. Thus the $LD_{50}$ used in the experiment was 10 CFU.

**Development of ELISA**

The optical density of whole cell lysate, crude Omps of *P. multocida* and rOmpH is shown in Table 2. Omps coated wells

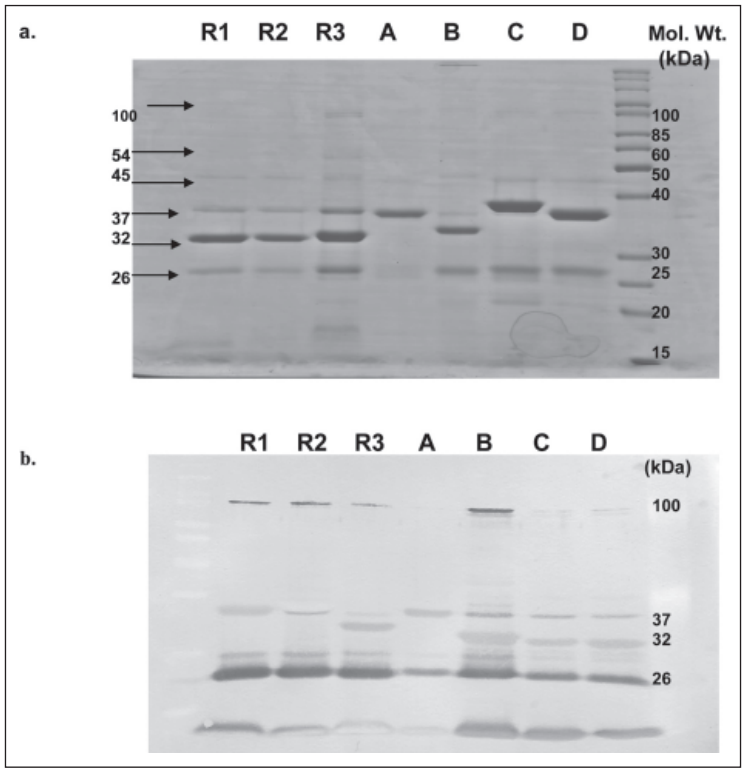


Figure 2. a. SDS-PAGE gel stained with Coomassie Blue. Lane R1, R2, and R3 are ATCC 366, 367, 368). Lane A, B, C, and D are *P. multocida* (strain no: 513, 4412, 4996 and 1710). b. The Omps transferred onto western blot membrane was detected by mouse antiserum

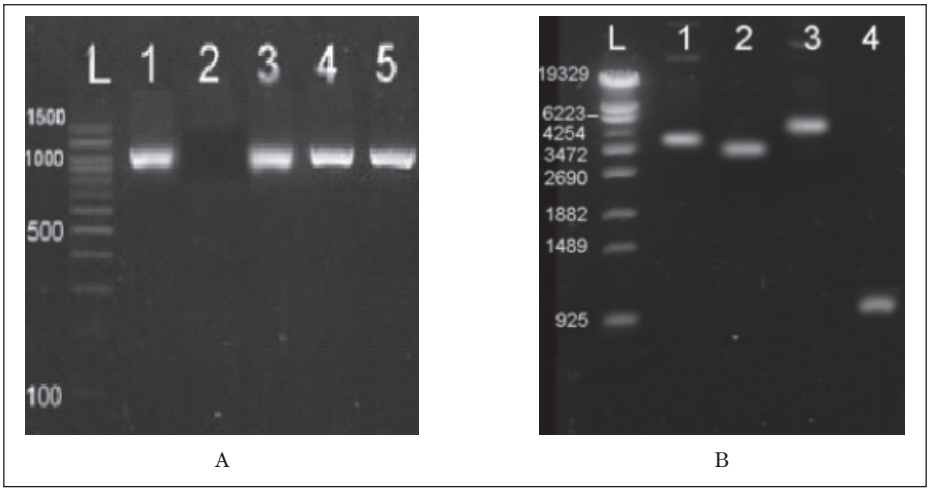


Figure 3. Generation pQE-30 UA expression vector

Gel A: Lanes: L, 100 bp DNA marker; 1, *ompH* gene PCR product from *P. multocida* genomic DNA; 2, negative control; 3-5, three PCR repeats of *ompH* gene

Gel B: Lanes: L, λ DNA marker; 1, pREP4 linearlized by *Hind*III; 2, non-insert pQE-30UA digested by *BamH*I; 3, *ompH* gene inserted pQE-30 UA digested by *BamH*I; 4, *ompH* gene fragments amplified by PCR from positive clone

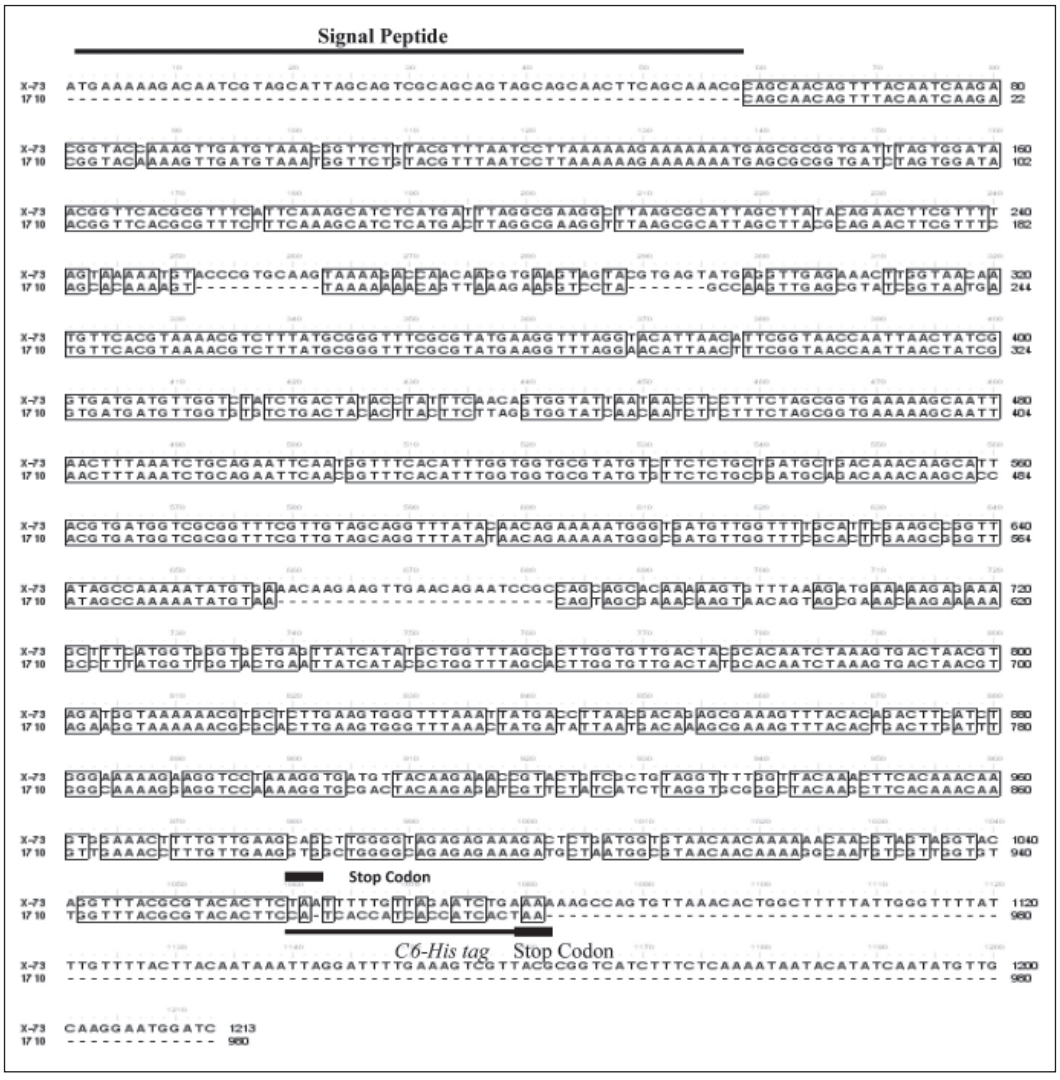


Figure 4. Comparison of nucleotide sequence of *ompH* gene for both *P. multocida* strains X-73 and strain-1710

Table 2. Development of ELISA assay. Comparisons ELISA OD of mice injected with Omps and control mice with different antigens: Omps of *P. multocida*, rOmpH and whole cell lysate

| | Mice injected with OMPs | | | | Control mice | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Min | SD | | | Min | SD |
| Omps | 3.089 | 2.828 | 2.96 | 0.18 | 0.228 | 0.317 | 0.27 | 0.06 |
| rOmpH | 2.018 | 2.211 | 2.11 | 0.14 | 0.196 | 0.203 | 0.20 | 0.00 |
| Whole cell lysate | 0.93 | 1.052 | 0.99 | 0.09 | 0.684 | 0.573 | 0.63 | 0.08 |

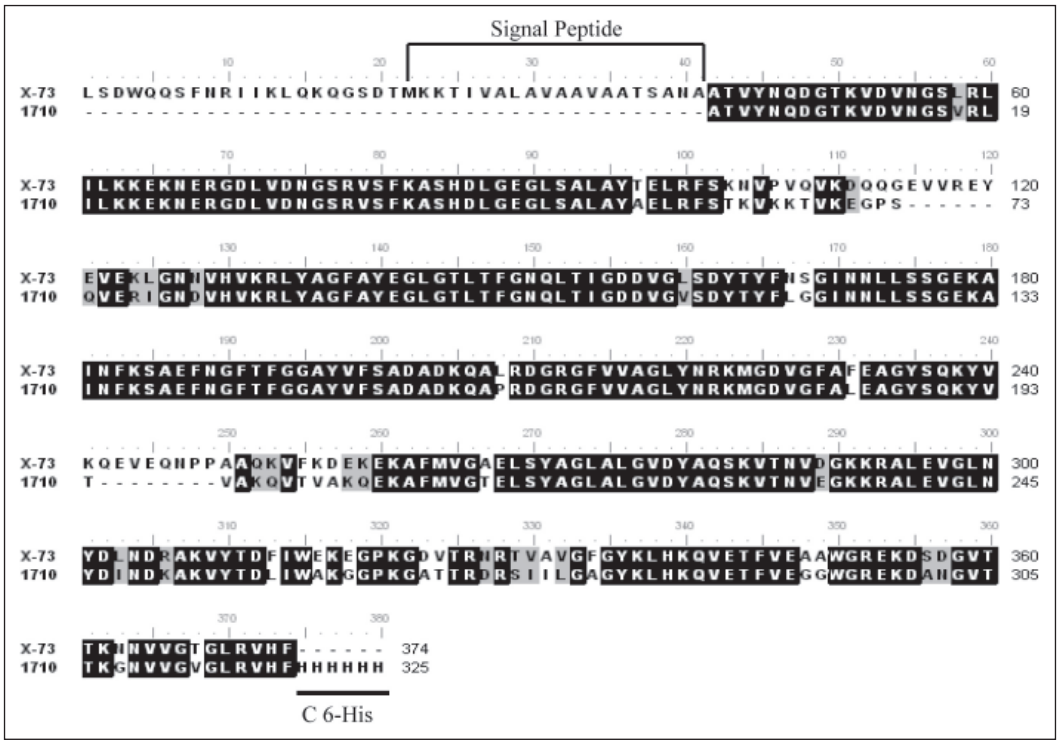


Figure 5. Comparison of deduced amino acid sequence of OmpH *P. multocida* strain X-73 and strain 1710

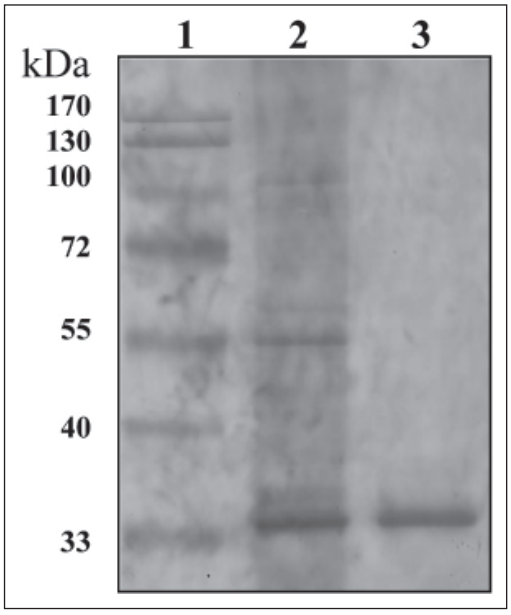


Figure 6a. SDS-PAGE gel stained with Coomassie Blue. Lanes: 1, Protein marker (kDa); 2, Whole cell lysate; 3, purified recombinant OmpH

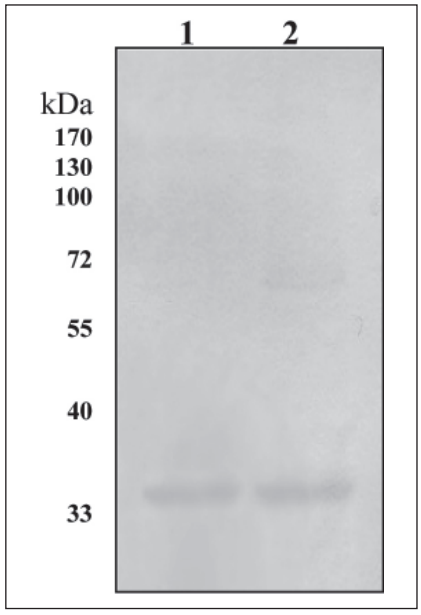


Figure 6b. Western blot analysis using immunostained with mouse polyclonal antiserum. Lanes: 1, Whole cell lysate; 2, purified recombinant OmpH

showed higher optical density readings than rOmpH coated wells, while whole cell lysate showed the lowest. Whole cell lysate gave the highest background when reacted with control blood while rOmpH showed the least background.

**Protection studies in BALB/cJ**

All four mice in non-immunized group died after 48 hrs. 100% the IP group immunized IP challenged mice survived while 80% of the SC immunized/IP challenged survived when challenged with live bacteria at the lethal dose of 10 CFU. In the ELISA assay (Table 2), the ELISA OD readings of post immunization were elevated in the mice of both of the above two immunized groups and thus was able to protect the mice when challenged with a lethal dose of *P. multocida*.

## DISCUSSION

OmpH was selected in this study because it is a porin protein that possessed both specific and cross-reacting epitopes which are abundantly expressed on the bacterial surface. In this study, the Omps profile in the SDS-PAGE show that 32 kDa and 37 kDa OmpH are two predominant Omps found in our local isolate. Western blot analysis showed all local *P. multocida* isolates and the ATCC strains shared the common 37kDa OmpH and their reactivity with mouse polyclonal antiserum (Figure 4a). However, not all the local isolates of *P. multocida* showed their reactivity against 32kDa OmpH with mouse polyclonal antiserum. Thus, the 37kDa OmpH is a suitable candidate for vaccination. In a study conducted by Luo *et al*. (1997), the 37kDa protein serotype 1 amplified with the same primer was used as a vaccine candidate and has shown homologous protective capacity in chickens.

The sequencing results of *ompH* gene, shows this 1710 *P. multocida* strain has a 39bp deletion in the gene as compare to strain X-73. The deletion was observed at three locations, position 255 to 265 (10 bp), position 292 to 298 (6 bp) and position 662 to 685 (23 bp). However, these deletions did not shift the open reading frame of the amino acid sequence (Figure 7). A sequence similarity search in the GenBank database revealed that homology of the *ompH* gene between strain X-73 and strain 1710 is 82.6% at the nucleotide level (Figure 4) and 80.6% at the amino acid level (Figure 5). These results indicated that the *ompH* gene is highly conserved among *P. multocida* serotypes.

The *ompH* gene fragment was successfully cloned into the expression vector *E. coli* M15 by the selection of antibiotics with the confirmation by PCR and size. The rOmpH which when purified in native condition was shown to hybridize when probed with a mouse polyclonal antiserum by Western blotting. These showed that the antibodies induced by native Omps were able to react to the native conformational epitopes of rOmpH.

In the study done by Luo *et al.* (1997) the recombinant OmpH lost its antigenicity after purification. This was explained as denatured protein that was unable to fold back natively. This study overcame this problem by a few approaches. The positively cloned *E. coli* was cultured in LB broth at temperatures lower than 37°C and it was noted that the ideal temperature is 30°C. This will maintain low level of expression and the recombinant protein produced may have a higher chance of folding properly. In addition, the formations of inclusion bodies will greatly decrease. Inclusion bodies are crucial factors that decrease the solubility of the recombinant proteins in the cytoplasm. With the reduction of inclusion bodies, the native purification methods can be carried out in order to preserve the antigenicity of a recombinant protein. Thus, the rOmpH is suitable to be used as a diagnostic reagent in ELISA.

In the protection study, the expressed rOmpH showed its capability of protecting the mice from the challenge dose of 10 CFU. Mice immunized and challenged intraperitoneally shown 100% protection against *P. multocida* whilst mice

Table 3. Antibody titer and protective capacity of OmpH against lethal challenge *with P. multocida* serotype B:2

| | Antibody titer | | | |
|---|---|---|---|---|
| | Pre-immunization | Post-immunization | Post Challenged[a] | Survival %[b] |
| Non-immunized mice (control) | 0.25 ± 0.15 | 0.35 ± 0.12 | – | 0 |
| Immunized IP/Challenged IP | 0.18 ± 0.24 | 2.51 ± 0.24 | 2.24 ± 0.17 | 100% |
| ImmunizedSC/Challenged IP | 0.41 ± 0.13 | 2.59 ± 0.19 | 3.11 ± 0.30 | 80% |

[a] The 50% infective doses (ID50) used in this study was 10 CFU
[b] 4 mice for each group

immunized subcutaneously and challenged intraperitoneally only shown 80% protection (Table 3). An ELISA showed that the native rOmpH stimulated high titers of antibodies against native rOmpH (Table 3). These results indicate that the native conformation of porin is considered crucial for the induction of immunity. When rOmpH was applied as an antigen in ELISA format, the background antibody titer was greatly decreased as compare to whole cell lysate. This suggested that the rOmpH was able to enhance the specificity of the diagnostic assay.

In conclusion three major peptides of the outer membrane protein (OmpH) of 4 local Malaysian strains of *P. multocida* serotype B:2 were characterized, of which the 37 kDa OmpH was selected as vaccine candidate due to its high specificity and it being a porin protein. The expressed rOmpH showed its capability of protection in mice and was able to stimulate high titres of antbodies against native rOmpH. It was also found suitable as a reagent for the development of a diagnostic kit in an ELISA format.

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