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Molecular characterization and computational analysis of the major outer membrane protein (*ompH*) gene of *Pasteurella multocida* P52

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ABSTRACT

The major outer membrane protein (OmpH) of *P. multocida* P52 was identified as one of the major immunodominant antigens. The gene *ompH*, encoding OmpH, was amplified, cloned and sequenced. The coding region of OmpH is 1,002 bp long. The predicted primary protein is composed of 333 amino acids, with a 20-amino acid signal peptide. The mature protein contains 313 amino acids with a predicted molecular mass of 33,760 Da. The nucleotide sequence and the predicted amino acid sequence of the *ompH* gene of *P. multocida* P52 showed a high level of homology to the OmpH of other serotypes of *P. multocida,* confirming that the *ompH* gene is conserved among all the serotypes of *P. multocida.* Multiple sequence alignment revealed high homology among the serotypes, with major variations confined to two discrete regions (amino acids 82-102 and 223-240), which corresponded to hydrophilic domains showing high antigenicity. The sequence information, presented in this study will open new vistas in progress towards the development of suitable prophylaxis and molecular epidemiological analysis.

Key words: gene, haemorrhagic septicaemia, outer membrane protein, *Pasteurella multocida*, sequencing

Introduction

Haemorrhagic septicaemia (HS) is an acute fatal septicaemic disease of cattle and buffaloes caused by *Pasteurella multocida* serotype B:2. Prophylaxis plays a major role in

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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* (LU et al., 1991; ABDULLAHI et al., 1990; AL-HASANI et al., 2007; WHEELER, 2009).

Outer membrane protein H (OmpH) is one such major protein in the envelope of *P. multocida* that has been purified and characterized as a porin (CHEVALIER et al., 1993). Both native and recombinant OmpH proteins have been analysed for their protective ability in *P. multocida* isolates of serotype A and D associated with fowl cholera and atrophic rhinitis, respectively (LUO et al., 1997; LUO et al., 1999; 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 (LUO et al., 1999).

In the present study, we have cloned and characterized the major outer membrane porin gene (*ompH*) of *P. multocida* P52 (vaccine strain for HS) encoding major outer membrane protein OmpH.

Materials and methods

Bacterial strain. Vaccine strain P52 of *P. multocida* (serotype B:2) was obtained from Division of Standardization, Indian Veterinary Research Institute, Izatnagar, 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 ^{0}C. The cells were harvested by centrifugation at 10,000 × g for 30 min, washed twice in phosphate-buffered saline (PBS) containing 10 mmol/L phosphate buffer, pH 7.4, and 150 mmol/L sodium chloride and then suspended in 10 mmol/L HEPES (N-2- hydroxyethyl piperazine-N-2ethanesulfonic acid) buffer (pH 7.4). 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 ^{0}C in an ultracentrifuge. The pellet which contained total membrane was suspended in 2 mL of 2% (w/v) sodium lauryl sarcosine detergent in 10 mmol/L HEPES buffer (pH 7.4) and

incubated at 22 ^{0}C for 1 h. The suspension was again centrifuged at 1,00,000 × g for 1 h at 4 ^{0}C to sediment detergent insoluble outer membrane enriched fraction. The pellet containing the outer membrane proteins was then washed and finally suspended in 5 mL sterile PBS and stored at -20 ^{0}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* were analysed in SDS-PAGE using the discontinuous buffer system (LAEMMLI, 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. The proteins were 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* (P52) polyclonal hyper immune rabbit serum as the primary antibody and goat anti-rabbit IgG horseradish peroxidase (HRPO) conjugate as the secondary antibody. Colour development was done with freshly prepared substrate solution (10 mg diaminobenzidine tetrahydrocholride in 50 mL 50 mmol/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 chemical lysis method as described by SAMBROOK and RUSSELL, 2001. Purity and concentration of DNA was determined by UV/VIS spectrophotometery 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 (forward: 5'-ACTATGAAAAAGACAATCGTAG-3, reverse: 5'-GATCCATTCCTTGCAACATATT-3') based on the sequence information reported earlier (LUO et al., 1997). PCR was performed using 20 ng of genomic DNA along with forward and reverse primers (60 pmol each), 0.1 mM of dNTPs, 1.5 mM $MgCl_2$ and 3 units of Taq DNA polymerase in 1 × reaction buffer. The amplification cycle was 35 cycles of denaturation at 94 ^{0}C for 15 s, annealing at 55 ^{0}C for 1 min and amplification at 72 ^{0}C for 1 min. The PCR amplified product was analysed on 1% agarose gel along with DNA molecular weight marker.

Cloning of ompH gene into pGEM-T Easy Vector System. The amplified *ompH* gene fragment was gel purified using the QIA quick gel extraction kit following manufacturer's instructions. This gel purified *ompH* gene fragment was ligated to pGEM-T Easy plasmid with T4 DNA ligase utilizing TA cloning. The ligated plasmid was transformed into *E. coli* DH5α competent cells. Selection of recombinant clones was done using blue/white

screening procedure by plating on a Luria Bertani (LB) agar plate containing 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 μg/mL X-gal, supplemented with 100 μg/mL ampicillin. The recombinant clones produced white colonies. The positive clones were analyzed using standard methods (SAMBROOK and RUSSELL, 2001).

DNA sequence determination. The cloned PCR product designated as p*OMPH* was sequenced by the DNA sequencing facility at UDSC, department of Biochemistry, University of Delhi, South Campus, New Delhi.

DNA sequence analysis. Nucleic acid sequence of the *ompH* gene of *P. multocida* was derived by using the electropherograms and sequence data of both forward and reverse primers. Amino acid sequence was also deduced from the nucleotide sequence and sequence similarity searches were performed using NCBI BLAST network service and Laser gene software (DNA STAR, Madison, WI, USA). The amino acid sequence was also compared and aligned with the OmpH sequences of different *P. multocida* serotypes using the sequence data available in the GenBank (Accession numbers viz: U52200, U52201.1, U52202, U52203.1, U52204, U52205.1, U52206, U52207.1, U52208, U52209, U52209, U52210, U52211.1, U52212.1, U52213.1, AJ459785, AY603962, AY606823, AY864815) by MegAlign Clustal multiple sequence alignment in DNA STAR. Percent similarity/percent divergence and phylogenetic trees were constructed using the programme MegAlign available in DNA STAR.

Antigenicity plot. Antigenicity plot along the polypeptide chain was predicted by the algorithm (HOPP and WOODS, 1981) using the web site http://www.bioinformatics.org/JaMBW/3/1/7.

Nucleotide accession number. The DNA sequence of the *ompH* gene of *Pasteurella multocida* P52 was submitted to GenBank and assigned the accession number EU 016232.

Results

Outer membrane proteins. The OMP extract of the P52 strain contained about 20.6 mg protein. The outer membrane protein preparation of *P. multocida* P52 revealed the presence of about 15 polypeptide bands on SDS-PAGE (Fig. 1, A). The molecular mass of the polypeptide bands ranged from 25 kDa to 94 kDa. Based on stain intensity and band thickness, polypeptides with approximate molecular weights of 32, 35, 37, 46, 52, 59, 70 and 87 kDa were considered to be the major OMPs. On western blot, the 32, 35, 37, 46, 59 and 87 kDa, were identified as major immunodominant proteins (Fig. 1, B).

Amplification and cloning of ompH gene. PCR amplification of the *ompH* gene of *P. multocida* P52 yielded the expected product of 1.2 kb (Fig. 2, Lane 1). When the amplified *ompH* gene was cloned into pGEM-T Easy vector, it released an identical insert

upon single enzyme digestion of the recombinant clone with *Bst*ZI restriction nuclease (Fig. 2, Lane 2).

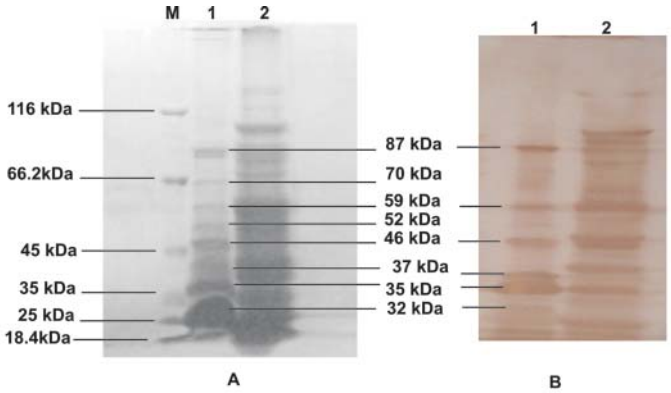

Fig. 1. SDS-PAGE and Western blot assay of the outer membrane proteins (OMPs) of *Pasteurella multocida* P52. A. SDS-PAGE of sonicated whole cell lysate and OMPs of *Pasteurella multocida* P52 on a 12% gel stained with Coomassie blue. B. Western blot of the gel probed with antiserum against whole *Pasteurella multocida* cells. Lane M: Marker; Lane 1: OMPs; Lane 2: Sonicated whole cell lysate

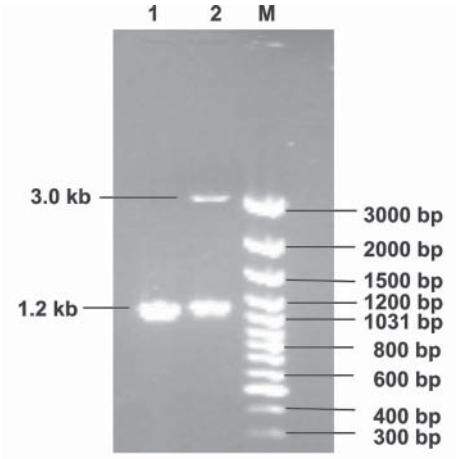

Fig. 2. Agarose gel electrophoresis of the cloned ompH gene. Lane 1: PCR amplified ompH gene; Lane 2: Released ompH insert and pGEMT vector upon digestion with BstZI.; Lane M: 100 bp DNA ladder

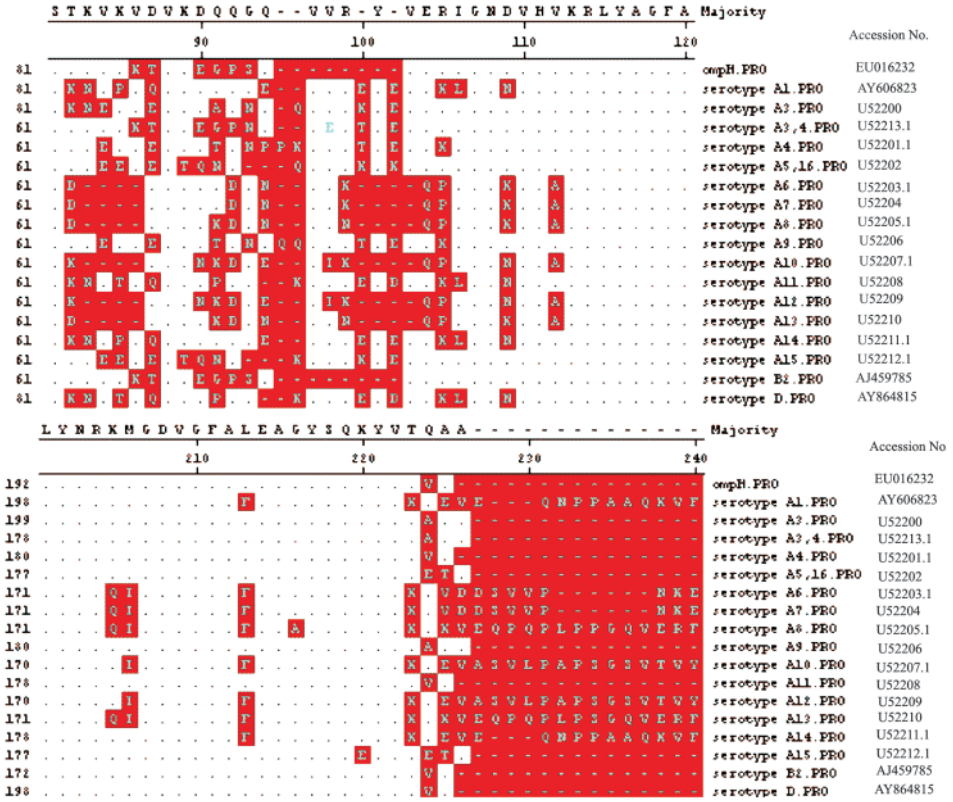

Fig. 3. Alignment and comparison of amino acid sequences of OmpH of *Pasteurella multocida* P52 and different serotypes of *Pasteurella multocida*

DNA sequence analysis. Consensus nucleic acid sequences (1156 bp) of the *ompH* gene of *P. multocida* P52 was obtained and amino acid sequences were deduced. The coding region of OmpH is 1,002 bp long. The predicted primary protein is composed of 333 amino acids, with a 20-amino acid signal peptide. The mature protein contains 313 amino acids with a predicted molecular mass of 33,760 Da. On sequence similarity search in NCBI database the *ompH* gene showed similarity to other bacterial porins, especially to *Haemophilus influenzae* porin P2. The nucleotide sequence and the predicted amino acid sequence of the *ompH* gene of *P. multocida* P52 (serotype B: 2) showed high level of homology to the OmpH of other serotypes of *P. multocida*. Multiple sequence alignment of OmpH amino acid sequences of different serotypes of *P. multocida* revealed

high homology, with variations of amino acid composition and sequence length in some regions. Major variations were confined to two discrete regions (amino acids 82-102 and 223-240) (Fig. 3).

The phylogenetic construct shows that the P52 is clustered with serotype A 3:4 and is closely related to it. Most distantly related to P52 are serotypes A: 6, 7, 8 and 13 that are present in separate clusters (Fig. 4).

Overall identity among the sequences was found to be 85.67%, showing high homology. The *P. multocida* P52 strain showed maximum identity to serotype D of *P. multocida* associated with atrophic rhinitis in pigs (93.7%) and serotype A3 (93.7%) and A3:4 (93.2%) of *P. multocida* causing fowl cholera. The group of serotypes A8 and A13 of *P. multocida* of fowl cholera were less closely related to P52 (approximately 73% identity)

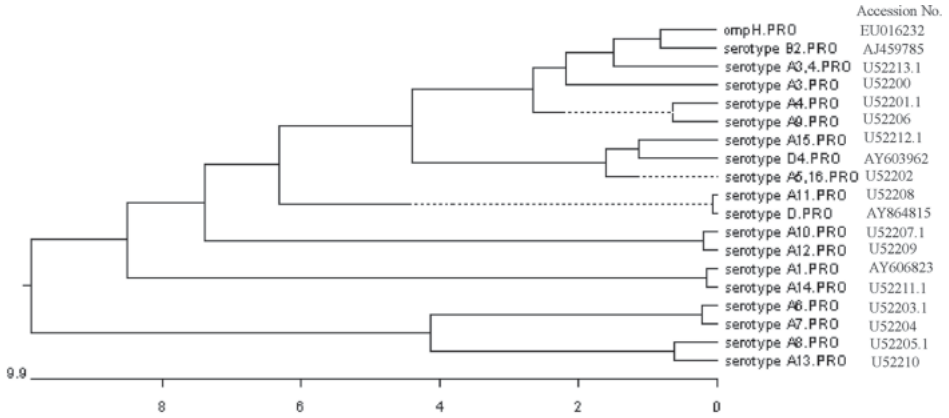

Fig. 4. Phylogenetic tree displaying relationship between amino acid sequences of ompH gene of *Pasteurella multocida* P52 and different serotypes of *Pasteurella multocida*

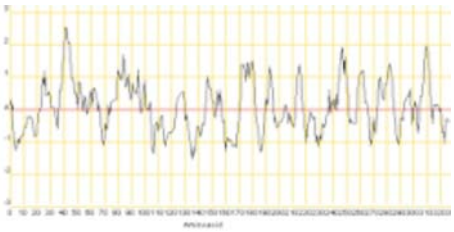

Fig. 5. Antigenicity plot of OmpH protein of *Pasteurella multocida* P52

Antigenicity plot. In silico study of the deduced amino acid sequence predicted the antigenicity plot (Fig. 5). The chart displays the variation of the antigenic index as function of amino acid position. Major variations confined to two discrete regions at amino acids 82-102 and 223-240 were seen as the hydrophilic domains.

Discussion

In the present study, 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 (JOHNSON et al., 1991). 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).

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 32, 35, 37, 46, 59 and 87 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).

On amplification using *ompH*-specific primer, a PCR product of the expected size of 1.2 kb 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 reflect the conserved nature of the *ompH* gene among *P. multocida* serotypes.

Consensus nucleic acid sequences of the *ompH* gene of *P. multocida* P52 obtained on analysis was 1156 bp. The ORF of *ompH* is 1,002 bp long. The predicted primary protein is composed of 333 amino acids, with a 20-amino acid signal peptide. The deduced mature protein of OmpH is 313 amino acids in length. Similar findings were reported by LUO et al. (1997) when they obtained the coding region of *ompH* to be 1,059 bp long in avian isolate and the predicted primary protein was composed of 353 amino acids, with a

20-amino-acid signal peptide. Also, amplification of *ompH* genes from all serotypes of *P. multocida* (type A) revealed the coding regions between 954-999 bp in length, while the deduced mature proteins were found to be 318-333 amino acids long (LUO et al., 1999). In *P. multocida* isolated from a case of atrophic rhinitis in pig, ORF of *ompH* was found to be 1,023 bp encoding 341 amino acids with a signal peptide of 20 amino acid (LEE et al., 2007).

The signal peptide region of the OmpH has a stretch of hydrophobic amino acids and an Ala-X-Ala cleavage site. The amino acid composition of OmpH is typical of nonspecific bacterial porins in its highly negative hydropathy index, high glycine content, low proline content, and lack of cysteine. The predicted molecular mass of mature protein is 33,760 daltons. A sequence similarity search in NCBI database revealed that the *ompH* gene and the predicted amino acid sequence show similarities to other bacterial porins including *Haemophilus influenzae* porin P2 as reported earlier for serotypes associated with fowl cholera (LUO et al., 1997).

In the present study, major variations confined to two discrete regions corresponded to hydrophilic domains in antigenicity plot. Similar findings were reported in *P. multocida* isolated from a case of atrophic rhinitis in pig, where residues of amino acids from 82-103 and 219-226 constituted variable regions corresponding to hydrophilic domains of OMPs (LEE et al., 2007). LUO et al. (1999) reported that major variations were present in amino acids 60-80 and 200-220 positions for serotypes of *P. multocida* type A. The difference in amino acid number is due to the lack of 20 signal peptide amino acids. These regions were predicted as two largest exposed loops in secondary structure predictions by these workers and were relatively showing high antigenicity than other hydrophobic regions. Vaccination studies in chickens with synthetic peptides derived from these loops induced 70% protection against the challenge (LUO et al., 1999). The findings suggest that these variable regions may work as strain-specific epitopes taking an important role in serotype-specific immune response as high level of homology has been found in the amino acid sequence of P52 with other serotypes. Analysis of this gene from different field isolates of *P. multocida* B:2 for the presence of variable regions can be used for designing vaccine for HS. Higher antigenic index of variable regions indicated the more likely possibility that antibodies would "see" these group residues.

The sequence variation of OmpH may have functional consequences. It has been observed with other bacterial OMPs that sequence divergence may result in antigenic variation of surface-exposed epitopes. Examination of this possibility is important for understanding the role of OmpH in the adaptation of *Pasteurella multocida* to its host environment. It has been found that the external loops of porins function as the gate for the pores and control the molecules that pass through the pores. Therefore, there is possibility that sequence divergence in the loop structure of OmpH, may affect the

size and charge properties of the pore and consequently modulate the permeability of different molecules, thereby suggesting high degree of variability in the external loops of OmpH for adaptation under selective pressures exerted by host immune system and other environmental conditions.

High homology similar to 72.3% overall identity between OmpH amino acid sequences of different serotypes of *P. multocida* type A (LUO et al., 1999) indicated the conserved nature of the protein.

OMP preparations from *in vivo* grown *P. multocida* cells (cross-protective factors) has been shown to provide heterologous immunity. The N-terminal sequence of OmpH is almost identical to that of cross-protection factors identified as OMP 179 and OMP 153 from strain P-1059 (serotype 3), which are in high molecular mass range (42-44 kDa). The relationship between porin H and cross protection factors is unclear and needs to be studied further.

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). 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 outbreaks.

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SAŽETAK

Glavni protein (OmpH) stanične stijenke bakterije *Pasteurella multocida* P52 identificiran je kao jedan od glavnih imunodominantnih antigena. Gen ompH što kodira za OmpH bio je umnožen, kloniran i sekvencioniran. Kodirajuće područje za OmpH veličine je 1002 bp. Predviđeni primarni protein sadržava 333 aminokiseline sa signalnim peptidom od 20 aminokiselina. Zreli (konačni) protein sadržava 313 aminokiselina s predviđenom molekularnom masom od 33,760 Da. Nukleotidni slijed i predviđeni aminokiselinski slijed gena *ompH* bakterije *P. multocida* P52 pokazao je visoku razinu homolognosti s OmpH drugih serovarova bakterije *P. multocida* što potvrđuje da je gen *ompH* konzerviran u svim serovarovima bakterije *P. multocida*. Višestrukim poravnanjem sljedova dokazana je visoka homolognost među serovarovima s velikom varijabilnošću ograničenom na dva zasebna područja (aminokiseline 82-102 i 223-240), koja odgovaraju hidrofilnim područjima s jakom antigenošću. Rezultati o aminokiselinskom sljedu dobiveni u ovom radu otvorit će nove putove u razvitku prikladne profilakse i molekularne epizootiologije.

Ključne riječi: gen, hemoragijska septikemija, protein stanične stijenke, *Pasteurella multocida*, sekvencioniranje