

## Original Article

# Molecular heterogeneity of *plpE* gene in Indian isolates of *Pasteurella multocida* and expression of recombinant PlpE in vaccine strain of *P. multocida* serotype B: 2

Ajay Pratap Singh<sup>1</sup>, Satparkash Singh<sup>1</sup>, Rajeev Ranjan<sup>3,\*</sup>, Santosh Kumar Gupta<sup>1</sup>, Vijendra Pal Singh<sup>1</sup>, Bhaskar Sharma<sup>2</sup>

<sup>1</sup>Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, Bareilly- 243122, UP, India

<sup>2</sup>Division of Animal Biochemistry, Indian Veterinary Research Institute, Izatnagar, Bareilly- 243122, UP, India

<sup>3</sup>Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar- 751003, Orissa, India

Outer membrane proteins of *Pasteurella (P.) multocida* have been known to be protective immunogens. *Pasteurella* lipoprotein E (PlpE) has been reported to be an important cross reactive outer membrane protein in *P. multocida*. The gene encoding the PlpE of *P. multocida* serotypes A: 3, B: 2 and D: 1 was amplified from the genomic DNA. The amplified products were cloned and the nucleotide sequence was determined. Sequence analysis of the recombinant clones revealed a single open reading frame of 1,011 bp, 1,008 bp and 1,017 bp encoding a protein with a calculated molecular mass of 37.829 kDa, 37.389 kDa and 37.965 kDa for serotypes A: 3, B: 2 and D: 1 respectively. The comparison of the *plpE* sequence in different capsular types revealed a high degree (>90%) of homology. Furthermore, the *plpE* gene of Haemorrhagic septicaemia causing serotype (B: 2) was expressed in *E. coli* and recombinant PlpE was strongly immunostained by antiserum against whole cell antigen, indicating that the protein is expressed *in vivo*.

**Keywords:** Haemorrhagic septicaemia, *Pasteurella multocida*, PlpE

## Introduction

The population of cattle and buffaloes in India is the largest in the world and represents a large portion of the Indian livestock industry. Hemorrhagic septicaemia (HS) is an economically important disease responsible for a high morbidity and mortality in cattle and buffaloes in India and other parts of Asia and Africa [8]. In India alone, it is estimated to cause economic losses of more than 10 million

rupees annually [23]. HS is primarily caused by serotypes B: 2 and E: 2 of *Pasteurella (P.) multocida*. Besides B: 2, several other serotypes are associated with sporadic outbreaks of disease, particularly in feral ruminants [12]. Apart from HS causing serotype B: 2, serotype A has also been associated with HS like conditions in ruminants [14]. The organism is also responsible for bovine pneumonia, porcine pneumonia, fowl cholera, and snuffles in livestock and poultry [22].

Considering its significance as an important animal pathogen, research has been focused mainly on identifying the immunogens of *P. multocida* that can elicit cross-protective immunity and thus be used in vaccines against Pasteurellosis. Membrane antigens have been identified as potential immunogens and shown to elicit protective immune responses. It has been recognized that *in vivo*-grown *P. multocida* and membrane-associated proteins of *in vivo* grown cells can induce cross-protective immunity [10]. Partially purified membrane proteins, prepared from *in vivo* grown cells with apparent molecular weight of 39 kDa and 59 to 65 kDa have been reported to be associated with cross-protective immunity [20].

Outer membrane protein (OMP) profiling and western blot analysis have shown several immunogenic proteins in the outer membrane fractions of *P. multocida* [3,7,13,15]. Among these, a 39 kDa protein has been reported to be highly immunogenic and cross protective, when challenged with heterologous strains [11,21]. The monoclonal antibodies to this protein were found to be protective in mice against lethal challenge with virulent strains [2]. Later, Wu *et al.* [25] recognized 39 kDa cross protective immunogen as *Pasteurella* lipoprotein E (PlpE). They also reported the protective nature of recombinant PlpE (r-PlpE) against a challenge infection with virulent *P. multocida* strain X-73 serotype A: 1, in mice and chickens. PlpE has also been

\*Corresponding author

Tel: +91-9359097099; Fax: +91-5812301865

E-mail: drrajraj@gmail.com

reported in other bacterial species [19].

Confer *et al.* [5] vaccinated cattle with recombinant PlpE of *Mannheimia haemolytica* which resulted in reduced severity of lung lesions in experimental study with virulent *M. haemolytica*. The *plpE* gene of *P. multocida* Pm70 encodes a lipoprotein of 335 amino acids and has 24.3% sequence homology with the *plpE* gene of *M. haemolytica* [17]. Nevertheless the major epitopes of the PlpEs of *M. haemolytica* and *P. multocida* Pm70 share no homology.

In this study, we described the molecular cloning and characterization of the *plpE* gene of *P. multocida* capsular type A: 3, B: 2 and D: 1 in order to determine the heterogeneity among these. Additionally, prokaryotic expression and purification of PlpE was carried out for immunological study.

## Materials and Methods

### Bacterial strain

Three isolates viz. *P. multocida* serotypes A: 3, B: 2 (vaccine strain P52) and D: 1 maintained in the Division of Bacteriology and Mycology, Indian Veterinary Research Institute, were employed as sources of the *plpE* gene. *Escherichia coli* (strain JM109) used as host for molecular cloning and expression of *plpE* gene, was grown in either Luria Bertani broth (Himedia Laboratories, India) at 37°C or Luria Bertani agar containing ampicillin at a final concentration of 50 µg/mL.

### Preparation of OMPs from *P. multocida* P52

The OMPs were extracted by the method described by Choi-Kim *et al.* [4]. Briefly the *P. multocida* serotype B: 2 (P-52) cells were grown in BHI broth (Himedia Laboratories, India), harvested and washed twice using sterile PBS (pH 7.2). The cells were then suspended in 10 mM HEPES (pH 7.4) and disrupted by sonication, five jerks at 10 micron for 2 min each, at 30 sec intervals. The cell debris was removed by centrifugation at 1,700 × g for 20 min. The sonicated supernatant was then centrifuged at 100,000 × g at 4°C for 60 min. The resultant Pellet was resuspended in 2 mL of 2% (W/V) sodium lauryl sarcosinate (Sigma, USA) in 10 mM HEPES (pH 7.4) and incubated at 22°C for 60 min. The insoluble OMPs were sedimented by centrifugation at 100,000 × g at 4°C for 60 min and the pellet was dissolved in PBS.

### Preparation of polyclonal antibody to whole cell antigen

The whole cell method was employed to raise the hyper immune sera against *P. multocida* B: 2 [24]. The *P. multocida* B: 2 organisms were grown in casein sucrose yeast extract agar plate and the growth was harvested into PBS containing 0.3% formalin and turbidity was adjusted by Mac Farland's tube number 4. Rabbits were inoculated intravenously at 3 ~ 4 day intervals with 0.2, 0.5, 0.75, 1.0,

1.5 and finally 2.0 mL. One week after the final injection, a booster dose (0.5 mL of live culture) was given intravenously. The animals were bled after 10 days and the serum separated.

### Amplification of *plpE* gene by PCR

Genomic DNA of *P. multocida* serotypes A: 3, B: 2 and D: 1 were isolated using DNA isolation kit (Qiagen, USA). The *plpE* genes from all three serotypes were amplified by PCR using specific primers 5'-CCATGGGCATGAAACA AATCGTTTTAAA-3' (sense primer) and 5'-CTCGAGT TGTGCTTGGTGACTTTTTTC-3' (antisense primer). The PCR mixture consisting of 50 ng of template, 25 pmol of each of the primers, 200 µM of dNTPs mix, × 1 PCR buffer, 1.5 mM MgCl<sub>2</sub> and 1 U of *Taq* DNA polymerase (MBI Fermentas, USA) were diluted to a 25 µL volume with milliQ water. PCR was performed with the initial denaturation at 94°C for 10 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 53°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplified product was visualized by electrophoresis through 2% agarose gel (Bangalore Genei, India) prepared in × 1 Tris Acetate EDTA buffer and photographed.

### Cloning, sequencing and analysis

The agarose gel containing DNA fragments was excised and the gel extraction of DNA fragments was carried out using Min Elute Gel extraction kit (Qiagen, USA). The *Taq* DNA polymerase-generated PCR products from each serotype were separately ligated to pDrive TA cloning vector (Qiagen, USA) and transformed into *E. coli* JM109 by rapid DNA ligation and a transformation kit (MBI Fermentas, USA). Preliminary identification of recombinant clones was done by blue/white screening. Plasmid isolation was carried out using Plasmid miniprep kit (Quiagen, USA) and the clones were confirmed by restriction digestion using *Nco*I and *Xho*I restriction endonuclease (MBI Fermentas, USA). The confirmed clones were then used for sequencing of the inserted gene. The obtained sequences were compared with the reported *plpE* gene of other *P. multocida* serotypes viz. X-73 (A: 1), P-470 (A: 3), P-1059 (A: 3), P-1662 (A: 4), P-61 (D: 3) and ATCC 12948 (D: 11), using DNASTar software (DNASTAR, USA). Secondary structure predictions and physiochemical properties of PlpE in the form of the algorithm were generated with the Protean program (DNASTar, USA).

### Prokaryotic expression of *plpE* gene of *P. multocida* B: 2

The truncated form of *plpE* gene without the sequence encoding the putative signal peptide was amplified using genomic DNA of *P. multocida* serotype B: 2. The oligonucleotide sequence for the forward primer was 5'-CATGGGCATGTAGCGGTGGTGCC-3' starting 58 nucleotides downstream of the 5' end and 5'-CTCGAGTT

ATTGTGCTTGGTGAC-3' for reverse primer. The 960 bp amplicon thus obtained was digested with restriction enzymes *Nco*I and *Xho*I before cloning into expression vector pPRO.EX.HTb, which was double digested with the same enzyme pair. The digested fragments were ligated followed by transformation into competent *E. coli* JM109. Subsequently the transformed cells were plated on LB agar containing ampicillin (50 µg/mL). The recombinant plasmids were identified by restriction endonuclease analysis. For the synthesis of recombinant protein, a single colony of *E. coli* JM109 harboring the *plpE* gene in pPRO.EX.HTb was inoculated into 100 mL of LB broth supplemented with ampicillin and incubated with shaking at 37°C until the culture reached an OD of 0.4 to 0.6 at A<sub>600</sub> nm (~4 h incubation). Mid log phase culture was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, USA) and 1 mM MgCl<sub>2</sub>. The induced culture was incubated at 30°C for 6 h with vigorous shaking (300 rpm).

#### Purification of recombinant protein

The culture was harvested and lysed by using lysis buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris, 8 M urea, pH 8.0). The bacterial lysate was centrifuged at 10,000 × g for 30 min at 4°C to remove insoluble cellular debris. The supernatant containing the recombinant protein was loaded onto affinity column packed with nickel chelating resin (Qiagen, USA) that selectively binds recombinant proteins with 6 histidine residues (His-Tag). The lysate was washed with 10 ml of wash buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris, 8 M urea, pH 6.3) for removing the non-specific proteins. The recombinant protein bound to resin was then eluted with a low pH buffer.

#### SDS-PAGE and Western blots

Samples were analyzed on a polyacrylamide gel using gels of 12% acrylamide contents and 3.3% cross linking. The bacterial OMP preparation and purified recombinant PlpE were applied to 12% gels and electrophoresed at 20mA. Before loading, samples were boiled for 3.5 min in an equal volume of buffer containing 2% SDS. Samples run under reducing conditions were boiled in the presence of 5% 2-mercaptoethanol. The gels were stained with Coomassie blue R-250 (Bio-Rad, USA) for detection of proteins. For Western blots, the proteins were transferred to Nitrocellulose membranes at 80 V for 2 h. The membranes were placed in 5 mL blocking buffer (5% Skim milk powder in TBS) and incubated at 4°C overnight followed by washing twice with TBS-T [1 M Tris HCl (pH 8.0), 5 M NaCl, and Tween 20 (0.05%)] for 10 min each. Subsequently, the membranes were probed with hyper immune sera raised against *P. multocida* serotype B: 2 diluted in blocking buffer (1 : 500) and washed. Rabbit anti-goat peroxidase-conjugate (diluted 1 : 5,000) (Sigma, USA) was used as secondary antibody. The blots were developed by adding chromogenic substrate solution

(Bio-Rad, USA) for 5-10 min. The reaction was terminated by washing the membrane with distilled water.

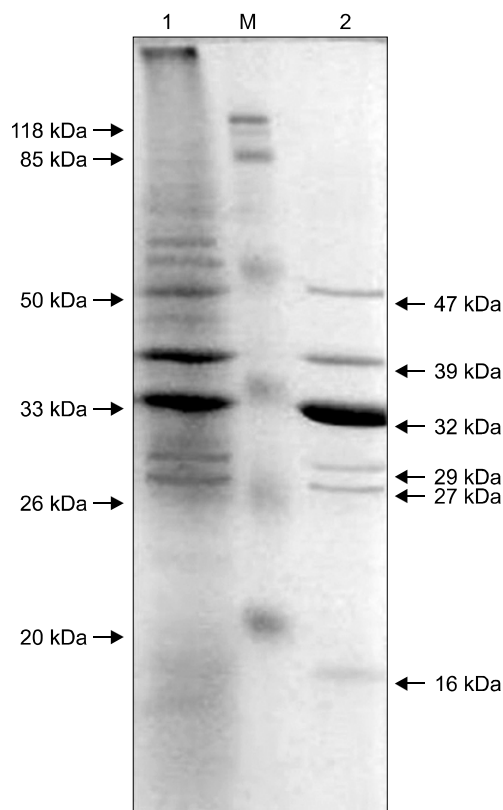
## Results

#### OMPs profiling and immunoblot analysis

OMPs of *P. multocida* B: 2 were extracted and upon sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, bands of apparent molecular weight 16, 27, 29, 32, 39 and 47 kDa proteins were observed in SDS-PAGE (Fig. 1). The polypeptides of approximate molecular weight 32 and 39 kDa were found to be major OMPs. On Western blots, 32 and 39 kDa proteins were identified as major immunodominant proteins, giving a strong immunostaining reaction with hyper-immune sera against whole cell antigen of *P. multocida* serotype B: 2 strain P52.

#### PCR, cloning and sequencing of the *plpE* gene

The *plpE* gene from *P. multocida* serotype A: 3, B: 2 and D: 1 was successfully amplified using the *plpE* specific primers. A single PCR product with a molecular size of approximately 1 kb was amplified in all of the three



**Fig. 1.** SDS-PAGE of outer membrane preparation. Lane M represents protein molecular weight marker. The most prominent band corresponding to molecular weights of 39 kDa and 32 kDa is indicated.

serotypes of *P. multocida* (Fig. 2). The amplified products were separately cloned into pDrive TA cloning vector (Qiagen, USA). The restriction digestion of each of the recombinant clones with *NcoI* and *XhoI* enzymes confirmed the presence of an approximately 1,010 bp sequence within the recombinant plasmid.

**Nucleotide sequence accession number**

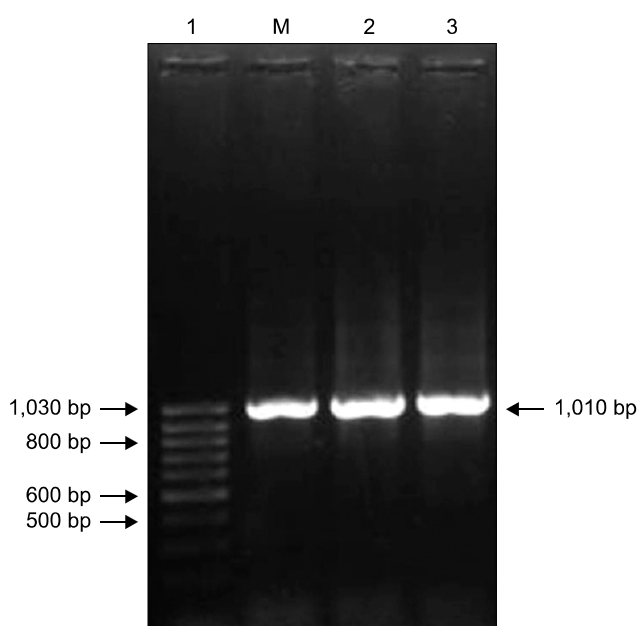
The sequence data for the *plpE* gene of *P. multocida* serotypes A: 3, B: 2 and D: 1 have been submitted to the GenBank database and assigned the accession numbers

GQ202239, GQ202240 and GQ202241.

**Sequence analysis of *P. multocida plpE* gene**

The nucleotide sequence of *plpE* revealed a putative open reading frame, beginning with an ATG initiation codon which remained open to the end of the sequence obtained. The 1kb functional *plpE* gene was found to be 1,011 bp, 1,008 bp and 1,017 bp in length for the *P. multocida* serotypes A: 3, B: 2 and D: 1, respectively.

The *plpE* gene of Indian isolates of *P. multocida* serotypes A: 3, B: 2 and D: 1 were aligned for homology with the reported sequence of *P. multocida* strains X-73 (A: 1), P-470 (A: 3), P-1059 (A: 3), P-1662 (A: 4), P-61 (D: 3), ATCC 12948 (D: 11) and the percentage of identity and divergence among nine strains of *P. multocida* has been depicted in Fig. 3. The result of phylogenetic tree analysis showed close relationship between Indian isolate of *P. multocida* serotype A: 3 to the reported strain X-73, while serotype D: 1 was close to strain ATCC 12948. However, serotype B: 2 of *P. multocida* was found to be distantly related to the X-73 and ATCC 12948 strains of *P. multocida*. The detailed description of the isolates used in this study for *plpE* gene sequence comparison has been depicted in Table 1.



**Fig. 2.** The *plpE* gene amplified by PCR using genomic DNA of *P. multocida*. Lane M represents DNA ladder and Lane 1-3 shows an amplified *plpE* gene in *P. multocida* serotypes A: 3, B: 2 and D: 3 respectively.

**Analysis of amino acid sequence and secondary structure predictions**

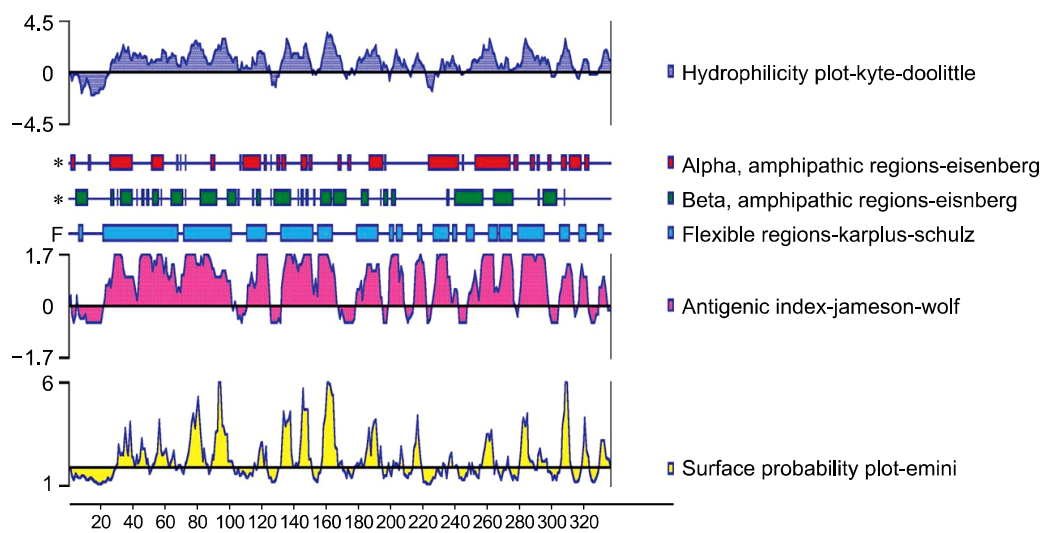
The deduced sequences of PlpEs of *P. multocida* serotypes A: 3, B: 2, and D: 1 were found to contain 336, 335 and 338 amino acids, respectively, having predicted molecular masses of 37.829, 37.389 and 37.965 kDa, respectively. When the amino acid sequences were compared it was observed that two amino acids (both serine) were found missing at the 26th and 27th position in the *P. multocida* serotype A: 3 when compared to serotype D: 1. Additionally, methionine was found missing at the 59th position in serotype B: 2.

		Percent Identity										
		1	2	3	4	5	6	7	8	9		
Percent divergence	1		95.2	92.8	99.5	93.8	93.8	93.7	91.8	95.3	1	<i>Pasteurella multocida</i> D1
	2	4.3		94.4	95.2	95.9	95.5	95.8	93.4	99.6	2	<i>Pasteurella multocida</i> A3
	3	5.9	5.1		92.8	96.4	96.4	96.5	94.1	94.6	3	<i>Pasteurella multocida</i> strain P52
	4	0.5	4.3	5.8		93.8	93.8	93.7	91.9	95.4	4	<i>Pasteurella multocida</i> strain ATCC 12948
	5	5.1	3.7	3.5	5.0		99.8	99.7	96.9	96.1	5	<i>Pasteurella multocida</i> strain P-61
	6	5.1	3.7	3.5	5.0	0.2		99.7	96.9	96.1	6	<i>Pasteurella multocida</i> strain P-470
	7	5.2	3.8	3.4	5.1	0.3	0.3		96.8	96.0	7	<i>Pasteurella multocida</i> strain P-1059
	8	7.0	6.0	5.6	6.9	2.8	2.8	2.9		93.7	8	<i>Pasteurella multocida</i> strain P-1662
	9	4.2	0.4	4.9	4.1	3.5	3.5	3.6	5.8		9	<i>Pasteurella multocida</i> strain X-73
		1	2	3	4	5	6	7	8	9		

**Fig. 3.** DNA identity matrix based on pair wise comparison of *plpE* sequences of different strains of *Pasteurella multocida*.

**Table 1.** Details of the isolates included in the *plpE* gene sequence comparison

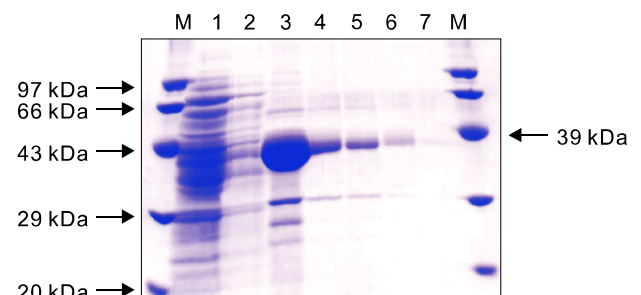
Isolates	Serotype	Host species	No. of nucleotides (bp)	Molecular mass of protein (Da)	Isoelectric point	Gene bank accession number
<i>Pasteurella multocida</i>						
X-73	A: 1	Chicken	1,011	37738.64	5.89	EF219452
P-1059	A: 3	Turkey	1,008	37413.26	6.19	EF219455
P-1662	A: 4	Turkey	1,008	37546.41	5.81	EF219456
P-470	A: 3	Pig	1,008	37441.28	6.19	EF219453
P-61	D: 3	Pig	1,008	37413.26	6.19	EF219454
ATCC	D: 11	Pig	1,017			EF219457
12948	B: 2	Cattle	1,008		5.81	GQ202239
BNM-P52	A: 3	Buffalo	1,011	37389.25	5.89	GQ202240
BNM-194	D: 1	Buffalo	1017	37829.71	6.34	GQ202241
BNM-47				37965.99		
<i>Mannheimia haemolytica</i>						
89010807N	S1	Sheep	1,310	39127.82	6.65	AF059036

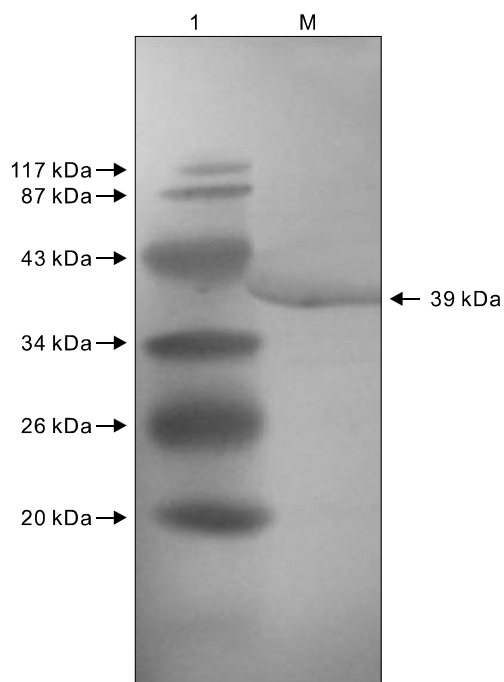
**Fig. 4.** Hydrophilicity, antigenicity and surface probability plot of deduced amino acid sequence of PlpE of *P. multocida* strain P-52 (serotype B: 2). Positive values represent hydrophilic, antigenic and surface exposed regions.

The physicochemical analysis of PlpE using Protean software showed a typical hydrophobic stretch at the N terminus, which corresponded to the putative signal peptide. The protein sequences following signal peptide had predominant hydrophilic regions, with smaller intervening hydrophobic stretches throughout the protein. The predicted protein had predominant antigenic indices throughout the length and region with probable antigenic determinant of protein was observed between 1-28 amino acids (Fig. 4).

#### Expression of *plpE* gene of *P. multocida* serotype B: 2

The ~960 bp *plpE* gene product without signal peptide sequence was amplified from *P. multocida* genome and

**Fig. 5.** Expression and purification of r-PlpE in *E. coli* JM109. Lane M represents protein molecular weight marker, Lane 1 contains lysis buffer follow through, Lane 2 contains wash buffer follow through and Lanes 3-7 contains first, second, third, fourth and fifth eluted fractions.



**Fig. 6.** Immunoblot of purified PlpE probed with hyperimmune serum against whole cell lysate of *P. multocida* serotype B: 2 and rabbit anti-goat peroxidase conjugate. 39 kDa bands corresponding to r-PlpE are indicated. Lane M contains prestained protein molecular weight marker.

cloned directionally in expression vector pPRO.EX.HTb. The recombinant *E. coli* Jm109 cells were induced by IPTG and the expressed PlpE fused with polyhistidine tag was purified by nickel chelating affinity chromatography. SDS-PAGE electrophoresis of the eluted fraction showed a very intense protein band of ~39 kDa (Fig. 5). Western blot analysis using homologous hyper immune serum against whole cell antigen further confirmed the antigenicity of 39 kDa recombinant PlpE (Fig. 6).

## Discussion

In this study, we characterized the *plpE* gene of *P. multocida* serotypes A, B and D followed by expression and immunoblot of the PlpE of *P. multocida* P52 (the vaccine strain used in India). OMPs have been found to be potent immunogens and protective to mice, rabbits, chicken and calves [6,16,26]. A 39 kDa cross-protective antigen was identified by Rimler [20] in *P. multocida* A: 3 (strain P1059) which induced active and passive cross-protection in chickens and turkeys [11,21]. The monoclonal antibodies against 39 kDa protein of *P. multocida* P-1059 (serovar A: 3) were produced and characterized by Ali *et al.* [1,2]. Mice passively immunized with the monoclonal antibodies to 39 kDa protein were found resistant to lethal challenge with virulent strains. In

2007, Wu *et al.* [25] observed that mice immunized with purified recombinant PlpE (r-PlpE) was resistant to homologous and heterologous challenge with *P. multocida*. Also, the r-PlpE was found to confer good protection against challenge infection with *P. multocida* strain X-73 (A: 1) in chickens.

The *plpE* genes of A: 3, B: 2 and D: 1 serotypes of *P. multocida* were amplified using the primers designed on the sequence of the *plpE* gene of *P. multocida* A: 3 which was approximately 1kb in all the three isolates. Upon comparison with reported sequences of *plpE* gene of *P. multocida* strains X-73 (A: 1), P-470 (A: 3), P-1059 (A: 3), P-1662 (A: 4), P-61 (D: 3), ATCC 12948 (D: 11), more than 90% homology was observed with Indian isolates of *P. multocida* serotypes A: 3, B: 2 and D: 1. The result showed the presence of *plpE* gene in all three serotypes of Indian isolates. The nucleotide sequence of HS causing *P. multocida* serotype B: 2 had 92.8% and 94.4% identity with other Indian isolates of *P. multocida* serotypes A: 3 and D: 1 respectively. The finding indicated that Indian isolates of *P. multocida* are closely related to each other and also to other strains on the basis of the *plpE* gene sequence. Wu *et al.* [25], also observed 90.8-100% sequence homology of the *plpE* gene, shared by different strains of *P. multocida* serotypes.

The nucleotide sequences of the Indian isolates of *P. multocida* serotypes A: 3, B: 2 and D: 1 were converted to amino acids and compared for homology. The amino acid sequence showed variation in composition among serotypes A: 3, B: 2 and D: 1. The absence of one or more amino acids may attribute to the difference in immunogenicity of the PlpE among these serotypes; this can be confirmed by detailed proteomic analysis of the recombinant protein.

The predominant hydrophilic regions were found dispersed throughout the length of protein. The above finding was further supported by surface probability assay of the PlpE, by which it could be inferred that the PlpE has putative surface exposed regions randomly distributed throughout the protein. Similar findings have been reported in other PlpE of *M. haemolytica* and OmlA of *A. pleuropneumoniae* by Gerlach *et al.* [9] and Pandher *et al.* [19].

The PlpE was expressed in *E. coli* JM109 and the apparent molecular mass of the expressed PlpE was found to be ~39 kDa when analyzed by SDS-PAGE. Western Blot analysis revealed the presence of a single ~39 kDa immunogenic band, indicating the antigenic and immunodominant nature of the PlpE. This statement was further supported by the SDS-PAGE and immunoblot analysis of OMPs of *P. multocida* serotype B: 2 (P52). The OMPs were extracted and separated by SDS-PAGE, which revealed presence of major outer membrane fractions with molecular masses of approximately 39 kDa. Immunoblot of OMPs probed with hyperimmune sera against whole cell antigen

of *P. multocida* P52 revealed a deeply immunostained band corresponding to a molecular weight of 39 kDa. The results clearly indicated that PlpE is most likely to be a 39 kDa cross protective immunodominant antigen, as described by Ibrahim *et al.* [11] and Rimler [21]. It also indicates a satisfactory level of expression of PlpE grown in *in-vitro* conditions.

The findings of the present study revealed that PlpE is present in all three Indian isolates of *P. multocida* serotypes A: 3, B: 2 and D: 1. The antigenic uniformity in this protein and extent of amino acid similarity supports its cross protective nature among the three serotypes. The western blot analysis further confirmed that the PlpE is one of the major immunodominant proteins in *P. multocida*.

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