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Cloning, *In silico* analysis, Expression and testing of Immune-potential of outer membrane protein (Omp 87) of *Pasteurella multocida* serotype B: 2

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ABSTRACT

Haemorrhagic septicemia (HS) caused by Pasteurella multocida (serotype B: 2) is an important disease of cattle and buffaloes in Asian sub-continent. Presently available vaccines for HS have their own limitations. Efforts have been made to develop better vaccine targeting outer membrane proteins and few of them have shown potential . Omp 87 protein has shown a protective potential against fowl cholera which is caused by Pasteurella multocida serotype A:1, so in present study gene encoding for Omp 87 protein of P. multocida serotype B:2 was cloned, expressed and tested for its immune-potential. An amplicon of 2300 bp was amplified by PCR and cloned in pGEMT vector and sequenced. Sequence was analyzed for open reading frame, structural analysis and epitope mapping. clones containing complete reading frame were sub-cloned in pQE30 expression vector. Recombinant plasmid was transformed in E. coli strain M $_{15}$ for over expression. Purified recombinant protein was tested for its antigencity by Western blot analysis. Clones containing Omp 87 Gene were confirmed by colony PCR and enzymatic digestion. Nuleotides sequence of serotype B:2 was compared with Omp87 of serotype A:1 and shown 94.8% similarity. In silico analysis revealed that Omp 87 is a stable trans-membrane protein having nine major B cell epitopes and several T cell epitopes which indicates immunogenic potential of Omp 87 . On expression recombinant protein of 80 kDa was obtained which produced strong signal on western blot. Our findings indicate that Omp 87 may be a potential candidate for r-DNA vaccine against Pasteurella multocida serotype B:2, causative agent of Haemorrhagic septicemia in Asia .

Key Words: Haemorragic septicemia, *Pasteurella multocida* , Epitope, Omp87, r-DNA vaccine

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INTRODUCTION

Hemorrhagic Septicemia (HS), is an important acute disease responsible for high morbidity and mortality rate in cattle and buffaloes in India and other parts of Asia and Africa [1,2]. Currently, oil emulsion vaccines are used in India to control the HS which have several limitations [3] like tissue reaction and short term immunity [4] . Molecular epidemiological studies conducted in last decades have shown high genetic variation among the field isolates of *Pasteurella multocida* [2,5,6],which resulted into vaccination failure [2,6] .Therefore, there is a need for

better vaccine candidate having high immunogenic potential and conserved among field isolates to avoid vaccination failure.

Outer membrane proteins (Omps) of *P. multocida* have been reported to be immunogenic [7,8,9]. Number of Omps have been identified in *P.multocida*, however, limited studies have been done to identify the genes encoding these proteins [10,11]. Few Omp(s) genes have been cloned, expressed and tested for their immunogenic potential such as Omp A [9], Omp H [11] and Omp 87 [10]mong these recombinant proteins , Omp 87 have shown protection against fowl cholera in birds [10] which is caused by *Pasteurella multocida* serotype A:1 . Present study comprises for molecular cloning and *In silico* characterization of the gene coading for Omp 87 of *P. multocida* serotype B:2 followed by expression, purification and testing of immune-potential of recombinant protein.

MATERIALS AND METHODS

Bacterial Strains and vectors:

Vaccine strain (P 52) of *P. multocida* (serotype B: 2), *Escherichia coli* (Strain DH5α and M15) were procured from Indian Veterinary Research Institute, Izatnagar (India) . Cloning vectors pGEMT and pQE 30 were procured from Promega and Qiagen, USA, respectively. Antibiotics Ampicillin and Kanamycin used for the selection of recombinants were procured from Hi-media, India.

Preparation of antiserum against to whole cell antigen of *Pasteurella multocida*

The whole cell antigen was used to raise hyper-immune sera against *P.multocida* B:2 in New Zealand rabbit. Single colony was inoculated in broth and overnight grown culter was palleted by centrifugation at 10000 x g . Pallet was washed thrice with PBS and re-suspended in HEPES buffer and sonicated on ice for ten minutes, debris was removed by centrifugation at 10000 x g . Supernatant was filtered through 0 .45 µm filter paper and was quantified by Lowry's method . For the initial dose 500 µg of sonicated crude antigen in 500 µl of PBS was emulsified with equal volume of Freuend's complete adjuvant and injected subcutaneously in rabbit. Booster doses of antigen (250 µg of sonicated crude antigen in 250 µl of PBS) were given along with Freund's incomplete adjuvant on 10^{th} day and repeated thrice at 10 days intervals . After 7 days of last booster, rabbits were bled and serum was separated and tested with total cell protein as antigen by double immune-diffusion test .Serum was stored at-20 ^{0}C till further use.

Amplification of Omp 87 gene by PCR:

Genomic DNA of *P.multocida* B:2 was isolated using C-TAB method . Primers used for amplification of Omp 87 gene were as followed. To ensure correct orientation in multiple cloning site of (expression vector) in the forward primer *Bam* HI and in reverse primer *Hind* III sites were incorporated.

5'CCG GAT CCA TGA AAA AAC TTT TAA TTGC3'(Forward Primer)
5'CCA AGC TTT TAG AAC GTC CCA CCA ATG CTG3'(Reverse Primer)

Reaction mixture (25µl) used in PCR contained 20 ng of genomic DNA, 20 pmol of primer, 200µM of dNTPs (each) and 2 units of Jump Start Accu *Taq* Polymerase (Sigma, USA). The PCR programme comprised : initial denaturation at 94°C for 5 minute, followed by 30 cycles of denaturation at 94°C for one minute , 55°C for 30 seconds and at 68°C for 3 min followed by final elongation at 68°C for 10 min. Size of amplicon was measured by comparing standard molecular marker by agrose gel electrophoresis.

Cloning, sequencing and bioinformatics analysis:

The agarose gel containing DNA fragments was excised and extraction of DNA fragments was carried out using Gel extraction Kit (Qiagen, USA). Extracted DNA fragment was ligated in pGEMT vector (Promega, USA) and transformed into *E.coli* DH5α cells . Plasmid from recombinant clones was isolated using plasmid miniprep kit (Qiagen, USA) and presence of clones was confirmed by restriction digestion using *Bam* HI and *Hind* III (MBI Fermentas, USA).Positive clones were sequenced at DNA sequencing facility UDSC (New Delhi, India). Sequences deduced was submitted to NCBI. Sequence was compared with the reported Omp 87 gene of other serotype ie A:1 using DNA star software (DNASTAR,USA) . Structural analysis of Omp 87 was carried out using different on line servers viz., Protparm, Pfam and PDB server. Antigenic characterization of the protein was performed by protean [12] . Protein was characterized for B cell and T cell epitopes using IEDB analysis [13]. Three dimensional structure of protein was deduced using Swiss-Prot model analysis.

Prokaryotic Expression of Omp 87 gene of *P. multocida* B:2:
Gene insert (2,300 bp) was released from the positive clones using *Bam* HI and *Hind* III and sub-cloned in pQE 30 vector and transformed into *E.coli* M15 host cells . Recombinants, containing Omp 87 gene were selected on LB agar plate supplemented with Ampicillin (100µg/ml) and Kanamycin (25µg/ml). Transformants were screened by colony PCR and positive clones were selected for testing of expression of recombinant protein. Selected clones were inoculated in LB medium (2 ml) containing above mentioned antibiotics and incubated in shaker cum incubator - shaker at 250 rpm

37^0C until the OD at 600 nm reached 0.6. At this stage, the cultures were induced with 1mM isopropyl D-1-thiogalactopyranoside (IPTG) and further for 6 hr at 37^0 C and 1 ml aliquots of each induced culture was pelleted at 12,000x g for 2 min at 4^0 C. The induced and negative control culture (M15 cells) pellets were lysed and analyzed by 12.5% SDS-PAGE.

Purification of recombinant protein:
Recombinant Omp 87 was purified using QIA express Ni-NTA fast start kit (QIAGEN, USA) . Positive clone was inoculated in 250 ml of LB broth and incubated for 8 hours in incubator shaker at 37°C. Cells were then induced by adding IPTG(1m M final Concentration) and further incubated for 18 hours and recombinant protein was purified in denatured stage.Cells were pelleted by centrifugation at 6000 x g for 15 minutes and cell pellet was re-suspended in 10 ml of denaturation lysis buffer and kept at room temperature for 60 minutes. Lysate was centrifuged at 14000 x g for 30 minutes and supernatant thus obtained was loaded on fast start column and allowed to pass through nickel agrose. After washing the column twice with washing buffer protein was eluted in elution buffer and quantified .

SDS-PAGE and Western blot Analysis:-
Recombinant protein was subjected to SDS-PAGE (12.5% gel) and transferred to nitrocellulose membrane using semi dry blotting apparatus (Atto, Japan) using transfer buffer (Tris-glycine pH 8.2 with 20% methanol). Membranes were blocked with 5% skimmed milk powder in PBS-T(Phosphate buffer saline pH 7.4 containing 0.1 % Tween-20) for 2 hrs at room temperature fallowed by washing with PBS-T. Membrane was incubated with rabbit antiserum against crude antigen of *P. multocida* (1^{st} antibody). Washing of membrane was done with PBST following anti-rabbit horseradish peroxidase conjugate at a dilution of 1;10,000 was used as secondary antibody. Colour development was carried out with the substrate hydrogen peroxide and diaminobenzidine in Tris buffer pH 9.5. After development of optimum color the reaction was stopped by washing membrane with excess of water.

RESULTS

Amplification, Cloning and Sequencing of Omp 87 gene:
Amplification of Omp 87 gene from *P. multocida* serotype B:2 using the Omp 87 specific primers resulted into an amplicon 2.3 kb. The amplified product was cloned into pGEMT cloning vector. Restriction digestion of each of the recombinant clones with *Bam* HI and *Hind* III enzymes confirmed the presence of an insert of 2.3 kb sequence with in the recombinant plasmid (Fig 1).The sequence data of Omp 87 gene of *P. multocida* serotype B:2 was been submitted to NCBI assigned the accession number EU 570212.

***In silico* analysis of Omp 87 gene:**
The nucleotide sequence of Omp 87 revealed a putative open reading frame with an ATG initation codon which remained open to the end of the sequence. The sequence analysis of coding region of Omp 87 gene showed GC content in the range of 40-46% .Nucleotide sequence of Omp 87gene of serotype B:2 (EU 570212) on comparison with that of Omp 87 of serotype A:1(U 60439) revealed 94.8% similarity . Primary structural analysis of protein revealed that it is composed of 790 amino acids and had isoelectric point at pH 5.94 and negatively charged (-5.46) at physiological pH .The protein showed six conserved domain (Table 1). Protein exhibited two trans-membrane helix one from inside to outside (4 to 23 residues,644 to 664 residues) and another outside to inside 640-656 and 675-695(Table 2).The instability index of the protein was 13.93 which indicated that Omp 87 is a stable protein.

Secondary structural analysis of the protein showed that the protein belonged to Pfam family and had six conserved domains . Omp 87 had shown high antigenic index which indicates that it may be an immunogenic protein (Fig 2 I).On epitope analysis by IEDB tools, total 18 B cell epitopes were observed among which nine were major

epitopes (Table III).On T cell epitope mapping, a sum of 19 MHC Class I epitopes were observed among which three with high affinity, seven with intermediate and eight had low affinity(Fig 2 II).In MHC Class II several epitopes of high affinity were exhibited (Fig 2 III).

Expression and purification of Omp 87:
The 2.3 Kb gene amplicon without signal peptide sequence was sub-cloned in an expression vector pQE 30. The expression construct was used to transform *E. coli* strain M15 cells . Transformants were selected on ampicillin (50 µg/µl) and kanamycin (25µg/µl). Clones were induced with IPTG as described previously . Un-induced cultures were used as control. Total lysates (prepared by directly boiling the cell pellets in SDS-PAGE sample buffer) were analyzed by SDS-PAGE. The polypeptide profiles obtained in the absence and presence of IPTG indicated that induction of the 2.3kb gene resulted in the expression of 80 kDa protein, which is consistent with the predicted size of the cloned gene product (Fig 3). Ni-NTA affinity chromatography was used for purification of expressed protein. The yield of purified protein was found to be 850 µg/ml (Fig 4 I).

Western blot analysis To ensure the antigenicity of recombinant Omp 87 western blot was performed using hyper-immune serum against whole cell antigen. Sharp signal of 80 kDa was observed in western blot which confirmed the antigencity of the recombinant Omp 87 (Fig 4 II).

DISCUSSION

Hemorrhagic Septicemia (HS) is an important disease of cattle and buffaloes in many countries including India .For effective control of the disease, an efficacious vaccine providing longer duration immunity is required. The identification and functional characterization of immunogenic proteins are prerequisite for the development of an effective vaccine. Omps of gram negative bacteria including *Pasteurella multocida* have been demonstrated to play in immuno-protections [7,8] . Some of Omps had been expressed and tested for their immune-potential like Omp 87 [10], Omp A [9] and Omp H [11]. Among these Omp 87 has been reported to be immunogenic against serotype A:1 of *Pasteurella multocida* , is a surface exposed outer membrane protein which has significant similarity D15 protective surface antigen of *Haemophilus influnzae* [10] . They further reported that specific Omp87 antiserum protected mice against homologous lethal *Pasteurella multocida* (serotype A:1)challenge. On basis of their findings they suggested that Omp87 is a protective outer membrane antigen of *Pasteurella multocida* (serotype A:1) which can provide protection against fowl cholera. Omp 87 of *Pasteurella multocida* (serotype B:2) exhibited its antigencity [14] because this protein reacted with serum vaccinated animals. They further reported that the gene was found to be localized in 9.0 Kb *Hind* III fragment of *Pasteurella multocida* genome. In serotype D Omp 87 gene was cloned and four fragments of Omp 87 gene were expressed with GST fusion protein [15] among which GST-F1 fusion protein which contains amino acid 18 through 130 of Omp87 was reacted on western blot with convalescent chicken serum and confirmed the immune-reactivity of protein.

Comparison of the sequence of Omp 87 of serotype A:1 with that of B:2 serotype revealed more than 95% homology which indicates that both the proteins had close similarity in their structure. Amino acid sequence analysis Omp 87 exhibited the characteristic features of outer membrane protein as it contain conserved hydrophobic residues at C-terminus (Phenylalanine last residue) which is essential for stability of outer membrane proteins [16]. Epitope mapping of Omp 87 protein exhibited several B cell and T cells epitopes , for effective prolonged immunity against HS it is essential that antigen should be able to provoke both cell mediated and humoral immunity [17]. High antigenic index of recombinant Omp 87 indicates it has has high immune-potential. *In silico* study findings of present study clearly indicate that Omp87 can provoke both cell mediated as well as humoral immune responses and can provide a better protection in comparison to presently available killed vaccine. Expression and purification of bacterial porins had been found to difficult as they integrate in cell membrane of host cell and may also cause osmotic destabilization of the cell [18]. Therefore, in the present study protein was expressed without signal peptide. The host cells (M13)carrying p REP4 repressor plasmid was used for stable propagations of expression construct as it has higher repressor level. On expression of Omp 87 gene recombinant protein of 80 kDa was observed which was further tested for immune-reactivity by Western blot with antisera raised against whole cell antigen of *Pasteurella multocida* and a strong signal was observed with purified recombinant protein.

On the basis of findings (*In Silico* analysis and Western blotting) of present study and findings of earlier workers [10,14,15] it may be concluded that Omp 87 may be a candidate for development of r-DNA vaccine against Hemorrhagic Septicemia.

Table 1: Details of conserved domains of Omp proteins

	Domain	Start	End
Omp 87	Surf Ag VNR	21	88
	Surf Ag VNR	89	169
	Surf Ag VNR	172	258
	Surf Ag VNR	261	340
	Surf Ag VNR	343	416
	Bac surface Ag	442	790

Table 2: Prediction of trans membrane helix

Omp87	From	To
Inside to outside	4	23
	644	664
Outside to inside	640	656
	675	695

Table 3: Major B cells Epitopes of Omp 87

No.	Start Position	End Position	Peptide	Peptide Length
1	103	112	(SKLEDQMELQ)	10
2	123	134	(KFDQTQFNKDLE0	12
3	158	163	(SDDKKE)	6
4	215	220	(VEQRIK)	6
5	238	244	(TFDEQDK)	7
6	274	284	(STLRQEMRQQE)	11
7	309	314	(VETKTE)	6
8	327	332	(KVKERN)	6
9	352	357	(ASIKQD)	6
10	382	389	(GYNEPYFT)	8
11	402	410	(EEYDSSKSN)	9
12	453	467	(EYNRDLYRQSMKYND)	15
13	510	517	(SDNKYYKL)	8
14	578	586	(AIYRTRQCP)	9

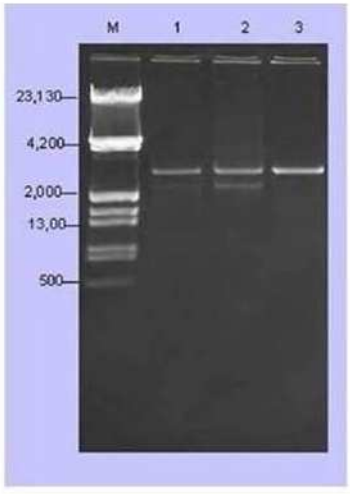

Fig 1: Release of insert of Omp 87 gene

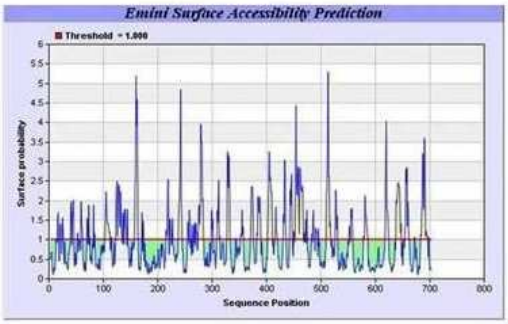

Fig 2 I: Antigenic indexing of Omp 87 Protein of *Pasteurella multocida*

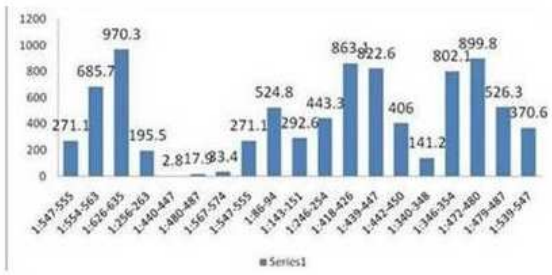

Fig 2 II: Major MHC Class I Epitope of Omp 87 gene

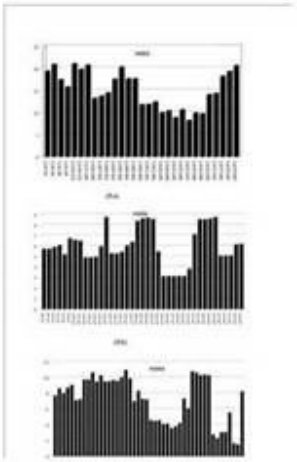

Fig 2 III: Major MHC Class II Epitope of Omp 87 gene

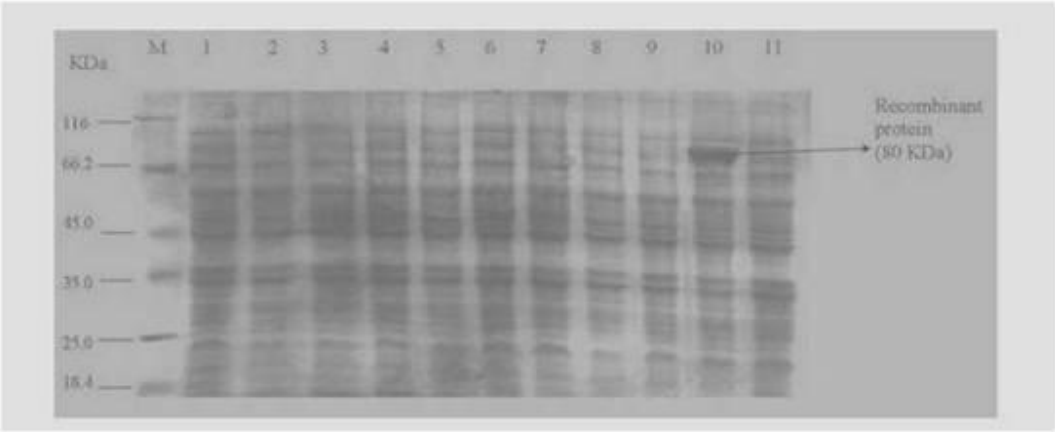

Fig 3: SDS-PAGE analysis of Omp 87 Protein Expression
M –Marker,
Lane 1 to 9: Non Expressed clones
Lane 10: Expressed Clone
Lane 11: M $_{15}$ Cells (Negative control)

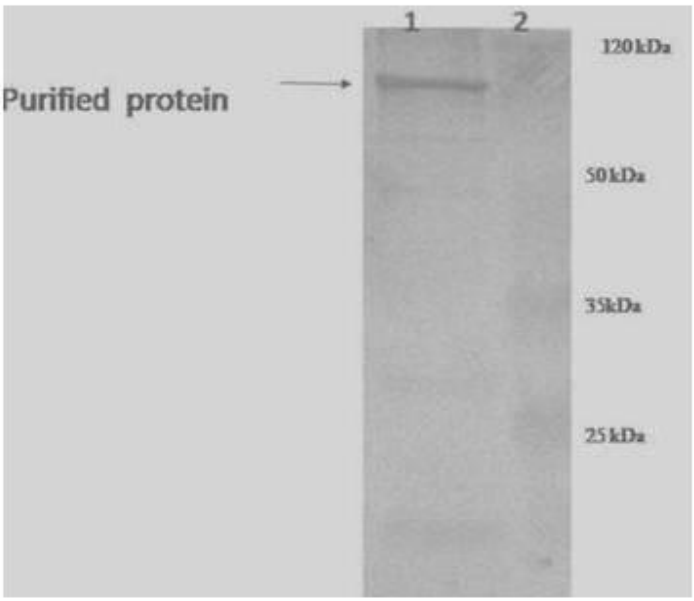

Fig 4 I: SDS-PAGE showing purified protein of Omp 87
Lane 1: Purified Protein
Lane 2: Marker

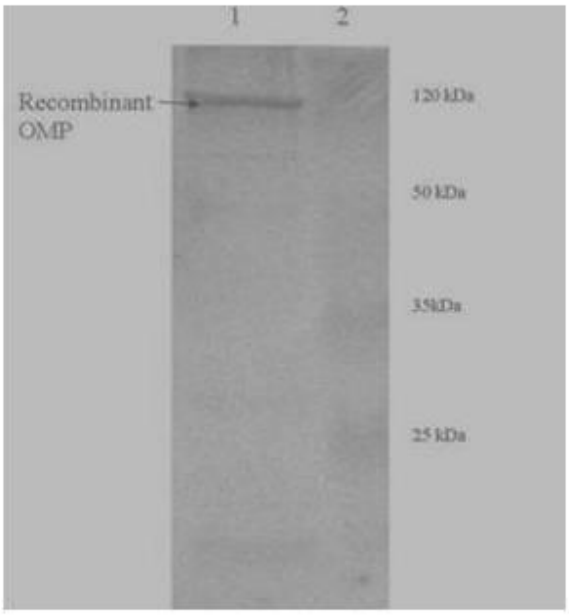

Fig 4 II: Western Blot of Recombinant Omp
Lane 1: Recombinant Omp
Lane 2: Marker

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