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Cloning and Sequence Analysis of Transferrin Binding Protein Gene A of Field and Vaccine Strains of *Pasteurella multocida* B:2

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ABSTRACT

Background: *Pasteurella multocida* is an important bacterial pathogen that causes many major diseases of which haemorrhagic septiciemia (HS) in cattle and buffalo is responsible for catastrophic epizootics in India and South Asia. In India, the disease haemorrhagic septiciemia is considered as the most dreaded bacterial disease. Various host- and pathogen- specific determinants are responsible for disease outcome. Various bacterial virulence genes (*tbpA, pfhA, toxA, hgbB, hgbA, nanH, nanB, sodA, sodC, oma87 and ptfA*) have been proposed to play a key role in this interaction.

Methods: The present study was done to compare the gene and deduced amino acid sequence of transferrin binding protein gene (*tbpA*) gene of field isolates and vaccine strain of *P. multocida* B: 2.

Result: It was observed that *tbpA* gene of field and vaccine strains have similar nucleotide sequence except at positions 574 and 620. The sequence of *tbpA* gene was used for prediction of matured TbpA protein characteristics. The deduced amino acid sequences of 242 amino acids revealed 99% homology with TbpA of *P. multocida* and with a variety of other TonB-dependent receptor proteins, indicating that it belongs to the family of outer membrane receptors. Deduced amino acid sequence was found to be similar in field and vaccine strains except at 207th amino acid. In field isolates Leucine was there while in vaccine strain Phenyl alanine was found. These both amino acids are hydrophobic in nature so no change in physico-chemical property of TbpA is expected. From this study it is concluded that single amino acid difference between field isolate and vaccine strain might not cause change in its binding and physico-chemical property.

Key words: Haemorrhagic septicaemia, Pasteurlla multocida, Transferring binding protein, Vaccine.

INTRODUCTION

Pasteurella multocida is one of the most important bacterial pathogen of cattle and buffaloes which causes fowl cholera in birds, atrophic rhinitis in pigs, haemorrhagic septicaemia (HS) in ungulates, enzootic pneumonia in cattle, sheep and goats, snuffles in rabbits and more rarely wound abscesses and meningitis especially in immunocompromised humans (Bisgaard, 1993., Frederiksen, 1993; Ewers *et al*., 2004., Muenthaisong *et al*., 2020). Among them, In India, the disease HS (caused by serotype B:2 in Asian countries and E:2 in African countries) is considered as the most dreaded bacterial disease and killing approximately 2048 cattle and buffaloes annually (De Alwis, 1992, Singh *et al*., 2014). On the basis of the distribution of disease, India is considered to be endemic for HS (Benkirane and De Alwis, 2002) as outbreaks of HS are reported in bovines mainly during rainy and winter season every year from different states of India (Jindal *et al*., 2002; Shivaprakash *et al.,* 2004; Singh *et al*., 2014).

Iron is an important factor for the growth of bacteria and is made available by iron acquisition proteins including transferrin binding protein, lactoferrin binding protein and haemoglobin binding protein. Bacterial transferrin binding protein (Tbp) is composed of two distinct proteins, TbpA and TbpB (Gray-Owen and Schryvers, 1996; Pogoutse and Morae, 2017). TbpA, an integral outer membrane protein, mediate the transport of iron across the outer membrane efficiently without the involvement of TbpB (Schryvers and Stojiljkovic, 1999; Ogunnariwo and Schryvers, 2001). Expression of transferrin receptors in strains of *P. multocida* has been correlated with HS as *P. multocida* isolated from HS express transferrin receptors whereas the strains of non HS serotypes failed to express this protein (Veken *et.al*., 1996; Ogunnariwo *et al*., 1997; Ognnariwo and Schryvers,

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2001). Iron acquisition related gene *tbpA* has been considered as an important epidemiological marker (Veken *et al*., 1994).

Although HS outbreaks occur commonly in bovines in India (Verma *et al*., 2004; Shome *et al.*, 2019), there is no information about the correlation of tbpA with virulence of *P. multocida.* Therefore, the present study was undertaken for cloning and sequence analysis of *tbpA* gene of *P. multocida* field and vaccine strain.

Highlights

- Nucleotide variation was recorded at two points between field isolates and vaccine strain.
- Amino acid alignment of field isolates and vaccine strain showed variation at single amino acid position

MATERIALS AND METHODS

Characterization of *Pasteurella multocida*

Two field isolates (P1 and P2) and a vaccine strain (VS) of P. *multocida* B: 2 (P52) cryopreserved in Department of Veterinary Microbiology, LUVAS, Hisar were used in present investigation. Both the field isolates and vaccine strain were revived on blood agar plates and characterized by culture characteristics such as morphology, Gram's staining, capsular and bipolar characteristics and biochemical tests. *P. multocida* B: 2 specific PCR (Townsend *et al*., 1998 and Quinn *et al*., 1994). After confirming field isolates and vaccine strain as *P. multocida* B: 2, organisms were incubated in BHI broth for 24 hours at 37°C for extraction of genomic DNA.

DNA extraction

The genomic DNA from field isolates and vaccine strain were extracted from overnight incubated BHI broth culture by Gen EluteTM Bacterial Genomic DNA kit (Sigma-Aldrich) as per manufacturer's instructions.

Molecular confirmation of *Pasteurella multocida*

The PCR for *P. multocida* was done to amplify a 620 bp fragment using specific primers (Table 1). Briefly, the PCR master-mix was made for 'n' number of reactions by adding nuclease free water (36.0 µl x n); 10xPCR buffer with 15 mM Mg++ (5.0 µl x n); 10 mM dNTPs mix (1.0 µl x n); 10 µM tbpA-forward primer (1.0 µl x n); 10 µM tbpA-reverse primer (1.0 µl x n); Taq DNA Polymerase (Fermentas), 5 U/µl stock (1.0 µl x n) and template DNA (5.0 µl x n).

The PCR amplification was performed with initial DNA denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min using XP® Gradient Cycler (Bioer Technology, PR China). Finally, the amplification was ended by a final extension at 72°C for 5 min. Furthermore, 5 µl of PCR product was analyzed by electrophoresis in 1% agarose.

PCR amplification and Cloning of tbpA gene (tbpA-PCR)

A 729-bp region of tbpA was amplified by PCR using specific primers (Table 1) and amplification conditions reported previously (Ewers *et al*., 2006). The amplified products of tbpA gene of isolates and vaccine strain were purified using QIAquick gel extraction kit (Qiagen, Germany). The purified products were cloned in pGEM-Teasy vector (Promega, USA). Briefly, the ligation-reaction mixture was made as per manufacturer's instructions and incubated at 25°C for 1 hour and subsequently used for transformation of JM107 cells as described in Sambrook and Russell (2001). Transformed JM107 cells were plated on LB/amp/X-Gal/IPTG agar plates and incubated overnight at 37°C. Transformation was confirmed by *tbpA* specific PCR.

Sequencing and bioinformatics

For sequencing, plasmid DNA was extracted from tbpA / pGEM-Teasy vector transformed JM107 cells by the alkaline lysis method (Sambrook and Russell, 2001). Primers specific to T7 and SP6 promoter regions in pGEM-Teasy vector DNA system were used for sequencing of *tbpA* gene of field isolates and vaccine strain. Optimal alignment of the *tbpA* sequences was done using multiple sequence alignment programs in 'CLC Bio Sequence Viewer version 6.4' software (URL: Http://Www.Clcbio.Com/). The deduced amino acid sequence was aligned in 'CLC Bio Sequence Viewer version 6.4' software (URL: http://www.clcbio.com/). Three-dimensional structural model of the *tbpA* proteins of field isolates and vaccine strain was constructed and transferrin binding sites were located in Phyer2 protein homology/ analogy recognition engine V2.0 software (http://www.sbg.bio.ic.ac.uk/phyre2).

RESULTS AND DISCUSSION

P. multocida organism is a small, non-motile, non-spore forming, Gram-negative coccobacillary rod, facultative anaerobe and belongs to the family *Pasteurellaceae* (Quinn *et al*., 1994). The organism exhibits a characteristic bipolar staining. *P. multocida* causes a wide range of important diseases in domestic animals, being responsible for pneumonia in cattle and sheep (Chanter and Rutter, 1989; Frank, 1989) and HS in cattle and buffalo (Carter and de Alwis, 1989). Five serogroups (A, B, D, E and F), based on capsular antigens, were recognized in *P. multocida* (Harper *et al*., 2006). Isolation of some unusual strains of *P. multocida* with serotypes A:1, A:3, F:3 and F:3,4 from bovines has also been reported in India (Kumar *et al.,* 1996). Significant associations have been observed between HS and *tbpA*, *hgbB* and *pfhA* genes. Similarly, aropic rhinitis was found to be in association with *toxA* gene (Atashpaz *et al*., 2009). Iron is considered to be an important factor for the growth of *P. multocida*. The *tbpA* has been found to be associated with iron acquisition mechanism.

HS can be controlled by vaccination and most countries where the disease is endemic resort to routine prophylactic vaccination (De Alwis, 1999). The case fatality rate (CFR) was comparatively-low in vaccinated animals than unvaccinated animals in buffaloes and cattle, respectively. During 1995-98, a number of outbreaks were also recorded

during winter months in those areas of Haryana where routine vaccination was carried out before start of rainy season (May-June). The cause for occurrence of disease in vaccinated animals might be the low efficacy and under dosing of the available vaccines (Verma *et al*., 2004).

Therefore the present study was done to compare the gene and deduced amino acid sequences of *tbpA* gene of field isolates and vaccine strain of *P. multocida* B:2.

Characterization of both *P. multocida* B:2 isolate and vaccine strain

Both field isolates and vaccine strain formed smooth, greyish glistening translucent dew drop like non-haemolytic colonies on blood agar after 24 hours incubation at 37°C (Fig 1A). Field isolates and vaccine strain of *P. multocida* were Gram-negative coccobacilli (Fig 1B) when examined by Gram's staining. The organisms showed a thick capsule on capsule staining and bipolarity on methylene blue staining (Fig 1C). Organisms were found indole, oxidase, nitrate and glucose and sucrose fermentation test positive.

Both field isolate and vaccine strain were also confirmed by *P. multocida* B: 2 specific PCR yielded amplified product size of 620 bp (Fig 1D).

Cloning, sequencing and bioinformatics of *tbpA*

Transformed clones were screened by their colour for vector insert and white colonies (Fig 2) were picked up and tested by tbpA-PCR (Fig 3). Clones having vector insert were

Table 1: Detail of primers used in this study.

Target organism	Gene	Primer sequence	Annealing temperature	Amplimer size (bp)	Reference
P. multocida serotype B	Not known	ATCCGCTAACACACTCTC AGGCTCGTTTGGATTATGAAG	50	620	Townsend *et al*., 1998
P. multocida	tbpA	TTGGTTGGAAACGGTAAAGC AACGTGTACGGAAAAGCCC	55	729	Ewers *et al*., 2006

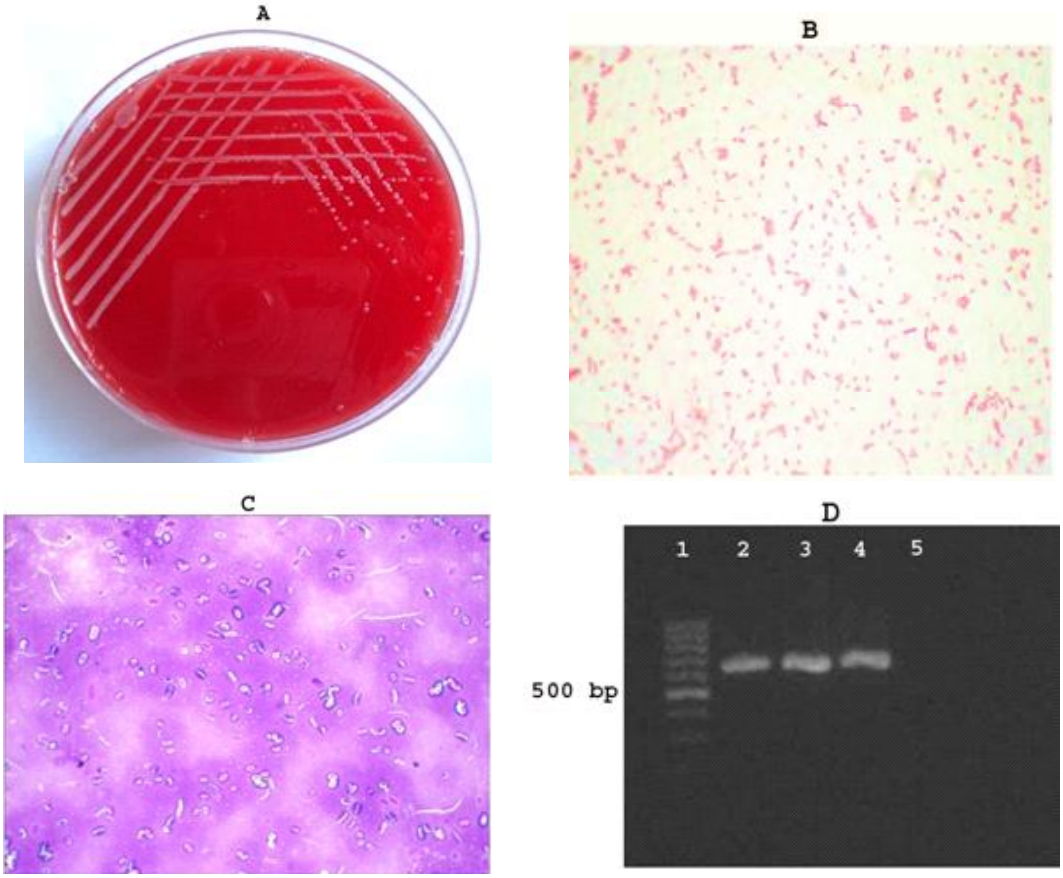

Fig 1: confirmation of *P. multocida* by (A) Blood agar plate showing dew drop like colonies; (B) Gram's staining showing gram negative bacilli; (C) methylene blue staining showing thick capsule; (D) Agarose gel electrophoresis of *P. multocida* serotype B PCR products. Lane 1: 100 bp DNA ladder; Lane 2: field isolate 1 (F1), Lane 3: field isolate 2 (F2), lane 4: vaccine strain (VS), lane 5: non-template control

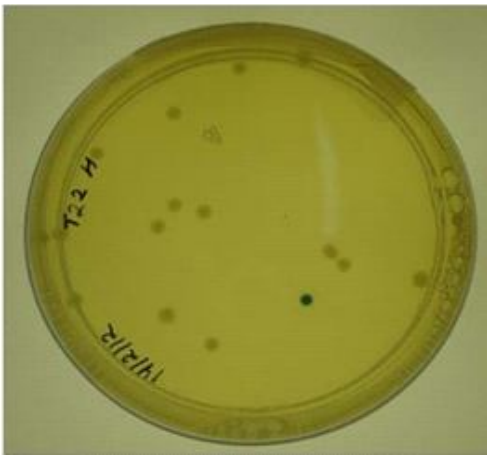

Fig 2: Luria-Bertani Agar plate showing blue and white colonies of JM107 transformants. White colonies have *PM-tbpA*/pGEM-Teasy vector inserts while white colonies lack this vector.

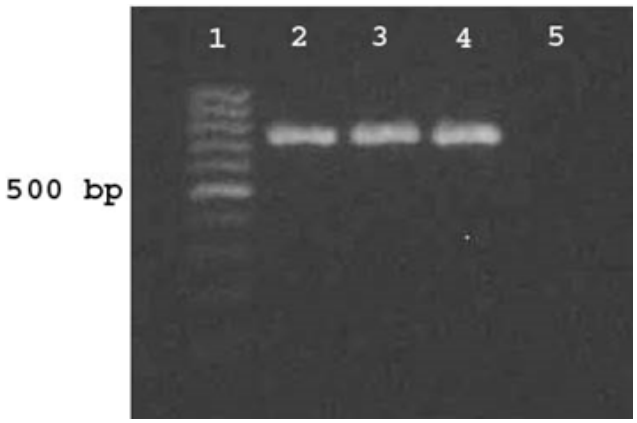

Fig 3: Agarose gel electrophoresis of *PM-tbpA* PCR products amplified from JM107 transformants. Lane 1: 100 bp DNA ladder (Fermentas); Lane 2-4: PCR product of clone #1-3, lane 5: non-template control

sequenced. Nucleotide sequences were found to have 729 nucleotides. All the three sequences were blasted at NCBI and showed 99% homology with *tbpA* of *P. multocida* P52, *tbpA* of *P. multocida* D: 1 and complete genome of *P. multocida* 36950.

The sequences were aligned using CLC Bio Sequence Viewer version 6.4' software programme. Alignment showed differences in nucleotides at positions 574 and 620. At 574 position nucleotide, F2 and VS have thymine while F1 has cytosine and at 620 position nucleotide, F1 and F2 have thymine while VS has cytosine. Similar to this, in a study, conducted by Shivachandra *et al*. (2005), comparison of *tbpA* of *P. multocida* B:2 (P52) was made with *P. multocida* A:1 and 98.4% homology and 1.5% divergence was reported.

The sequence of *tbpA* gene from F1, F2 and VS was used for prediction of matured TbpA protein characteristics using the 'CLC Bio Sequence Viewer version 6.4' software. Deduced amino acid sequence of 242 amino acids revealed 99% homology with TbpA of *P. multocida* and with a variety of other TonB-dependent receptor proteins, indicating that it belongs to the family of outer membrane receptors. This finding coincides with that of Ogunnariwo and Schryvers (2001). Deduced amino acid sequence alignment of showed a single amino acid variation at 207th position. In both field isolates Leucine was there while in vaccine strain Phenyl alanine was found. These both amino acids are hydrophobic in nature so no change in physico-chemical property of TbpA is expected. The finding of Shivachandra *et al*., (2005) also indicated that TbpA has a very high antigenic index, hydrophilicity and surface probability.

The amino acid variation was not found at the binding site residues (D 72, L 141, L 142), predicted by Phyer2 protein homology/analogy recognition engine V2.0 software. 3-D protein model constructed from Phyre2 software that used PDB: c2iahA as template showing 99.6% homology with TbpA protein.

Further three binding sites at amino acid residue 72 (ASP), 141 (LEU) and 142 (LEU) of TbpA protein, obtained by using Phyer^{2} protein homology/analogy recognition engine V2.0 software, were predicted. This indicates that the amino acid change is not found at these binding sites.

From this study it is concluded that single amino acid difference between field isolate and vaccine strain might not cause change in binding and physico-chemical property of TbpA.

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