Research in Veterinary Science 98 (2015) 1–6

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Recombinant transferrin binding protein A (rTbpA) fragments of *Pasteurella multocida* serogroup B:2 provide variable protection following homologous challenge in mouse model

Sathish Bhadravati Shivachandra [a,*], Revanaiah Yogisharadhya [b], Abhinendra Kumar [a], Nihar Nalini Mohanty [a], Viswas Konasagara Nagaleekar [c]

[a] Clinical Bacteriology Laboratory, Indian Veterinary Research Institute (IVRI), Mukteswar-263138, Nainital, Uttarakhand, India
[b] National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru-560024, Karnataka, India
[c] Division of Bacteriology and Mycology, Indian Veterinary Research Institute (IVRI), Izatnagar-243122, Bareilly, Uttar Pradesh, India

ARTICLE INFO

Article history:
Received 8 August 2014
Accepted 23 November 2014

Keywords:
Immunogenicity
Pasteurella multocida
Mouse model
Recombinant transferrin binding protein A (rTbpA)
Protective efficacy

ABSTRACT

Transferrin binding protein A (TbpA), an iron acquisition surface protein that also acts as virulence factor, is widely distributed among strains of *Pasteurella multocida*. In the present study, a total of seven clones of TbpA fragments ($_{39}$D to F$_{777}$; $_{39}$D to Q$_{697}$; $_{188}$V to F$_{777}$; $_{188}$V to Q$_{697}$; $_{39}$D to P$_{377}$; $_{188}$V to P$_{377}$ and $_{39}$D to F$_{187}$) belonging to *P. multocida* B:2 were constructed, over-expressed and purified as recombinant fusion proteins from *Escherichia coli* using affinity chromatography. Immunization of mice with rTbpA fragments resulted in a significant ($p < 0.05$) rise in antigen specific serum total IgG and subtypes (IgG1 and IgG2a) tires. All immunized mice challenged with 8 LD$_{50}$ of *P. multocida* B:2 resulted in a variable protective efficacy up to 50%. The study indicated the potential possibilities to incorporate full length TbpA in subunit vaccine formulation composed of synergistic subunit antigens against haemorrhagic septicaemia (HS) in cattle and buffalo.

1. Introduction

Pasteurella multocida, a Gram-negative bacterial pathogen, is widely distributed in almost all parts of world, and is known to be associated with various diseases in domestic as well as wild animal/avian species (Wilkie et al., 2012). Haemorrhagic septicaemia (HS) is one of the major diseases of cattle and buffalo, caused by strains of *P. multocida* especially serogroups B:2 (Asian type) and E:2 (African type) (Shivachandra et al., 2011). Despite the availability of several HS vaccines viz., bacterins with alum precipitated/oil adjuvant, multiple emulsion and modified live vaccines (Verma and Jaiswal, 1998), the incidences of HS outbreaks have not yet been reduced (Ahmad et al., 2014; Biswas et al., 2004; Kumar et al., 2004). Recently, well defined, highly purified recombinant outer membrane proteins (OMPs) of *P. multocida* following target gene sequence analysis (Kumar et al., 2014; Shivachandra et al., 2013, 2014a; Siju et al., 2007), cloning/over-expression and purification in bulk are considered as potential immunogens (Hatfaludi et al., 2010, 2012; Shivachandra et al., 2011, 2012, 2014b; Kumar et al., 2013). One vaccine strategy that has been adopted in several bacteria including the family *Pasteurellaceae*, is to target the surface proteins involved in the acquisition of nutrients from the host as candidate antigens (Loosmore et al., 1996; Potter et al., 1999; Rossi-Campos et al., 1992).

Iron is an essential nutrient required by a majority of microbes in metabolic electron transport chains. In animals, protein carriers like transferrin in blood, lactoferrin in secretory fluids and ferritin within cells make iron non-available for survival of bacteria (Gray-Owen and Schryvers, 1996). However, majority of bacterial species within *Pasteurellaceae* have developed several strategies for obtaining iron from their hosts. Bovine isolates of *P. multocida* are known to encode several high molecular weight OMPs involved in iron acquisition from the host (Hatfaludi et al., 2010; Paustian et al., 2001; Wheeler, 2009). The bacterial transferrin binding protein (Tbp), which mediates iron acquisition from host transferrin, is generally composed of two distinct proteins: TbpA and TbpB (Buchanan et al., 1999). TbpA, an integral TonB-dependent OMP which is significantly larger (~80–100 kDa) than the related siderophore receptor proteins that have been more extensively characterized previously (Buchanan et al., 1999; Ferguson et al., 1998), is proposed to mediate transport of iron across the outer membrane (Schryvers and Stojiljkovic, 1999). Earlier studies have reported the presence of transferrin (Tf) receptors in bovine *P. multocida* strains associated with pneumonia (Ogunnariwo et al., 1997) and HS (Ogunnariwo and Schryvers, 2001; Shivachandra et al., 2005; Veken et al., 1996). In the recent past, a novel Tf receptor (TbpA, a single

* Corresponding author. Clinical Bacteriology Laboratory, Indian Veterinary Research Institute (IVRI), Mukteswar-263138, Nainital, Uttarakhand, India. Tel.: +91 05942 286348 ext 4063; Mobile: +91-09756002533; fax: +91 05942 28634.
E-mail address: sbshivachandra@gmail.com (S.B. Shivachandra).

http://dx.doi.org/10.1016/j.rvsc.2014.11.013
0034-5288/

~82 kDa protein) in bovine respiratory strains of *P. multocida* has been characterized and it revealed efficient capability to mediate iron acquisition from bovine transferrins without involvement of a second receptor protein (TbpB) (Ogunnariwo and Schryvers, 2001).

Among several iron-regulated OMPs of *P. multocida* strains, TbpA being a surface exposed antigen stands out as a potential candidate antigen (Shivachandra et al., 2005; Veken et al., 1996) for HS vaccine. However, TbpA does not occur naturally in large quantities. Hence, there is a need for over-expression and high quality purification of rTbpA in bulk to meet the eventual requirements of HS vaccine formulations. In view of these, the study was focused on analyzing the protective immunity elicited by rTbpA fragments in mouse model.

2. Materials and methods

2.1. Bacteria, vector, host cells, primers and mice

P. multocida serogroup B:2 strain P52 (an Indian HS vaccine strain) maintained in the 'Clinical Bacteriology Laboratory', Indian Veterinary Research Institute (IVRI), Mukteswar, Uttarakhand (UK), India, was used. For construction and expression of recombinant proteins, pET32a vector (Novagen, USA), *Escherichia coli* TOP10 and *E. coli* BL21(DE3)pLysS cells were used. All the required primers were synthesized and procured from IDT-DNA, USA. Swiss albino mice (6–8 weeks old) reared in a pathogen-free environment and maintained at the Laboratory Animal Section, IVRI Campus, Mukteswar, India, were used in immunization trials.

2.2. Construction of pTbpA expression clones

A total of seven primer sets targeting either full length mature or partial regions of *tbpA* gene sequence (GenBank Acc # AJ558182) (Shivachandra et al., 2005) were randomly designed. Details of oligonucleotide sequences with their target gene regions are mentioned in Table 1. For construction of clones, purified DNA from *P. multocida* B:2 strain P52 was used as template (50 ng) for amplification of various *tbpA* gene fragments in a PCR mixture comprising 50 pmol of respective primer sets using previously described PCR conditions (Shivachandra et al., 2005). The respective PCR amplified products for each clone was digested with *Bam*HI and *Xho*I; cloned in to pET32a vector and transformed in to *E. coli* TOP10 cells, subsequently in to BL21(DE3)pLysS cells for over-expression in LB medium supplied with antibiotics, ampicillin (50 mg/ml) and chloramphenicol (35 mg/ml).

2.3. Over-expression, purification and Western blot of rTbpA proteins

Each *E. coli* BL21(DE3)pLysS cell harboring respective pTbpA plasmid was grown in 1 liter LB broth with appropriate antibiotics and kept in a shaking incubator at 30/37 °C before induction with 1 mM IPTG. After 3 h post induction, cells were harvested and the pellet was resuspended in a buffer before lysis by sonication (Sonics, USA). Each expressed rTbpA fragment protein was analyzed for its solubility and purified by affinity chromatography using Ni-NTA superflow cartridges (Qiagen, USA) as per the methods described for insoluble (Shivachandra et al., 2012) and soluble fraction (Ahuja et al., 2012). All purified rTbpA proteins were quantified using NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) and stored at –80 °C.

For confirmation, purified rTbpA proteins were transferred onto nitrocellulose membrane using a semidry immunoblot system (Amersham Pharmacia, USA) and detected using anti-*P. multocida* B:2 polyclonal hyperimmune rabbit serum and goat anti-rabbit IgG HRPO conjugate (Sigma, USA) as primary and secondary antibodies, respectively. The blots were incubated in substrate solution (10 mg DAB) for development of color.

2.4. Mice immunization and challenge studies

Thirty six healthy Swiss albino mice were divided into six groups (I–VI) with each having 6 mice. A total of five proteins (rTbpA Frg #1, 4, 5, 6 and 7) were selected for immunization of each group of mice. Each mouse in a respective group was inoculated with an antigen amount of 30 μg/100 μl/mouse with Freund's complete adjuvant (FCA) by subcutaneous route. After 21 days, a booster dose with rTbpA proteins (30 μg/100 μl/mouse) was given to all the immunized mice using Freund's incomplete adjuvant (FIA). Control group mice received PBS only. All the animals were provided with feed and water *ad libitum* and monitored regularly.

For challenge studies, *P. multocida* B:2 strain P52 passaged once in mice with LD_{50} calculated as per the standard procedure described previously (Kumar et al., 2013; Shivachandra et al., 2014b) was used. On 56th day post-immunization, each immunized mice as well as control group mice were challenged with 0.2 ml of culture containing 8 LD_{50} (120 CFU) by subcutaneous route and observed

Table 1
List of primers used to construct various fragments of transferrin binding protein (TbpA) of *P. multocida* serogroup B:2 strain P52 based on *tbpA* gene sequence (AJ558182) (Shivachandra et al., 2005).

SL #	Construct (TbpA regions)	Primer sequences[a] (5′————3′)	*tbpA* target regions	Amplicon size (bp)	Expressed protein size[b]
1	rTbpA-Frg #1 (minus signal sequence)	TNF: 5′-cgcGGATCCGATTCGGATTCAACAAGAACAACGCCA-3′ TR4: 5′-gtgCTCGAGTTAAAAACGAATAACCACCGAGGCA-3′	115–2332 nt ($_{39}$D to F_{777})	~2244 bp	~103 kDa (904 aa)
2	rTbpA-Frg #2 (minus β20–β22)	TNF: 5′-cgcGGATCCGATTCGGATTCAACAAGAACAACGCCA-3′ TR3: 5′-gtgCTCGAGTTATTGTTTGACAACCTTTTTATC-3′	115–2088 nt ($_{39}$D to Q_{697})	~1980 bp	~93 kDa (823 aa)
3	rTbpA-Frg #3 (β1–β22)	TBF: 5′-cgcGGATCCGTCGAACCTGGTCGCCATTTGGGCT-3′ TR4: 5′-gtgCTCGAGTTAAAAACGAATAACCACCGAGGCA-3′	562–2332 nt ($_{188}$V to F_{777})	~1785 bp	~86 kDa (754 aa)
4	rTbpA-Frg# 4 (β1–β19)	TBF: 5′-cgcGGATCCGTCGAACCTGGTCGCCATTTGGGCT-3′ TR3: 5′-gtgCTCGAGTTATTGTTTGACAACCTTTTTATC-3′	562–2088 nt ($_{188}$V to Q_{697})	~1530 bp	~77 kDa (674 aa)
5	rTbpA-Frg #5 (N-plug + β1–β8)	TNF: 5′-cgcGGATCCGATTCGGATTCAACAAGAACAACGCCA-3′ TR2: 5′-gtgCTCGAGTTAAGGACTCGTTGAAAATTCTC-3′	115–1131 nt ($_{39}$D to P_{377})	~1035 bp	~56 kDa (504 aa)
6	rTbpA-Frg #6 (β1–β8)	TBF: 5′-cgcGGATCCGTCGAACCTGGTCGCCATTTGGGCT-3′ TR2: 5′-gtgCTCGAGTTAAGGACTCGTTGAAAATTCTC-3′	562–1131 nt ($_{188}$V to P_{377})	~585 bp	~40 kDa (354 aa)
7	rTbpA-Frg #7 (N-plug region)	TNF: 5′-cgcGGATCCGATTCGGATTCAACAAGAACAACGCCA-3′ TR1: 5′-gtgCTCGAGTTAAAAGTCCTCAATATCTTTTGT-3′	115–561 nt ($_{39}$D to F_{187})	~465 bp	~34 kDa (314 aa)

[a] At 5′ of each sequence: small letters denote primer tags; underlined region denote restriction sites for *Bam*HI and *Xho*I enzymes; forward primers (TNF, TBF) and reverse primers (TR1, TR2, TR3, TR4).
[b] All constructs had N-terminal 6× histidine tag, which included additional 165 aa accounting for 17.7 kDa to TbpA target regions.