Vet Res Commun (2013) 37:83–89
DOI 10.1007/s11259-012-9539-5

SHORT COMMUNICATION

Profiling of virulence associated genes of *Pasteurella multocida* isolated from cattle

Subhash Verma · Mandeep Sharma · Shailja Katoch · Lovit Verma · Sandeep Kumar · Vishal Dogra · Rajesh Chahota · Prasenjit Dhar · Geetanjali Singh

Accepted: 13 September 2012 / Published online: 25 September 2012
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Abstract *Pasteurella multocida* is a causative agent of many major diseases of which haemorrhagic septiciemia (HS) in cattle & a buffalo is responsible for significant losses to livestock sector in India and south Asia. The disease outcome is affected by various host- and pathogen-specific determinants. Several bacterial species-specific putative virulence factors including the capsular and virulence associated genes have been proposed to play a key role in this interaction. A total of 23 isolates of *P. multocida* were obtained from 335 cases of various clinically healthy and diseased cattle. These isolates were examined for capsule synthesis genes (*capA, B, D, E* and *F*) and eleven virulence associated genes (*tbpA, pfhA, toxA, hgbB, hgbA, nanH, nanB, sodA, sodC, oma87 and ptfA*) by PCR. A total of 19 *P. multocida* isolates belonging to capsular type B and 4 of capsular type A were isolated. All isolates of capsular type B harboured the virulence associated genes: *tbpA, pfhA, hgbA, sodC* and *nanH*, coding for transferrin binding protein, filamentous hemagglutinin, haemoglobin binding protein, superoxide dismutase and neuraminidases, respectively; while isolates belonging to capsular type A also carried *tbpA, pfhA, hgbA* and *nanH* genes. Only 50 % of capsular type A isolates contained *sodC* gene while 100 % of capsular type B isolates had *sodC* gene. The gene *nanB* and *toxA* were absent in all the 23 isolates. In capsular type A isolates, either *sodA* or *sodC* gene was present & these genes did not occur concurrently. The presence of virulence associated gene *ptfA* revealed a positive association with the disease outcome in cattle and could therefore be an important epidemiological marker gene for characterizing *P. multocida* isolates.

Keywords *Pasteurella multocida* · Isolation & identification · Capsule · Virulence associated genes · PCR

S. Verma (✉) · M. Sharma · S. Katoch · L. Verma · S. Kumar · V. Dogra · R. Chahota · P. Dhar
Department of Veterinary Microbiology,
Dr. G.C. Negi College of Veterinary & Animal Sciences,
CSK Himachal Pradesh Agricultural University,
Palampur, Himachal Pradesh 176062, India
e-mail: sverma8@yahoo.co.uk

G. Singh
Department of Veterinary Biochemistry,
CSK Himachal Pradesh Agricultural University,
Palampur, Himachal Pradesh 176062, India

Introduction

Pasteurella multocida is a pathogenic Gram-negative bacterium that has been classified into three subspecies, five capsular serogroups and 16 serotypes. It is non-motile, coccobacillus, nonsporing and facultative anaerobic microbe. It is a part of normal flora of oral cavity and gastro-intestinal tract of wild and domestic animals (Krieg and Holt 1984). The stress factors like sudden change in the weather, nutrition, overwork, long and stressful journeys etc. lower down the resistance of the animals and the organisms get upper hand resulting into fulminating infection. Once established inside the immunosuppressed animals, the organism may lead to number of primary and secondary infections in a wide range of vertebrate hosts. *P. multocida* is responsible for pneumonia in cattle and sheep (Chanter and Rutter 1989; Frank 1989) and HS in cattle and buffaloes (Carter and De Alwis 1989). The organism is also known to be the causative agent of pasteurellosis in American bison, yak, deer, elephants, camels, horses, elk and other wild animals (De Alwis 1996). It causes fowl cholera in poultry, snuffles in rabbits and atropic rhinitis in swines.

In India, HS has been quantified as the number one bacterial killer disease among cattle and buffaloes (Dutta et al. 1990; Singh et al. 1996).

Pasteurella multocida possesses a number of virulence factors which include polysaccharide capsule, endotoxins or lipopolysaccharide (LPS), outer membrane proteins (OMPs), fimbriae, exotoxins, multocidins or siderophores, extracellular enzymes and plasmids (Harper et al. 2006). On the basis of variation in the cap loci, the pathogen has been classified into capsule type A, B, D, E and F and a relationship exists between the capsular type and disease predilection (Boyce and Adler 2000). Apart from outer membrane proteins and capsular antigens, the virulence associated genes (*tbpA, pfhA, toxA, hgbB, hgbA, nanH, nanB, sodA, sodC, oma87* and ptfA) play important in pathogenesis of *P. multocida* (Ewers et al. 2006). Many such genes of *P. multocida* have been suggested as epidemiological markers and PCR-based methods have been used to ascertain their distribution in strains recovered from wide sources and disease conditions (Lainson et al. 1996; Doughty et al. 2000; Ewers et al. 2006). The present study investigated the distribution of some of these important virulence associated genes in *P. multocida* isolates from dead, diseased or healthy cattle and evaluated the association of these genes in the outcome of disease.

Material and methods

Bacterial isolation and identification

A total of 335 samples were collected from six farms over a period of 1 year. Out of these 335 samples, 50 were collected from 9 animals died from HS suspected disease outbreaks and comprised of whole blood and morbid materials from lung, liver, spleen and heart. Besides these 50 samples, 87 nasal swabs were collected from the live but diseased cattle & 198 nasal swabs were from apparently healthy bovine. Preliminary isolation of the organism was done on 5 % defibrinated sheep blood agar followed by confirmation using traditional bacteriological and biochemical methods as described by Muhairwa et al. 2001 (gram staining, cultural characteristics, oxidase, catalase, methyl red, Voges-Proskauer, sulphide reduction, indole production, motility, triple sugar iron agar, urease production, citrate utilization, nitrate reduction and carbohydrate fermentation reactions). All the biochemical tests were performed in triplicate.

Preparation of chromosomal DNA

Cells from 1.0 ml overnight cultures in Brain Heart Infusion (BHI) broth were harvested by centrifugation for 15 min at

Table 1 Details of primers, PCR conditions and citations used for the detection of virulence associated genes

Primer	Primer sequence (5′–3′)	Gene	PCR conditions (°C/s)			Reference
			Denaturation	Annealing	Elongation	
TbpAF TbpAR	TTGGTTGGAAACGGTAAAGC TAACGTGTACGGAAAAGCCC	*tbpA*	94/30	57/30	72/60	Ewers et al. (2006)
PfhaF PfhaR	TCCATACACTCGGTAATATG TCTAATGTACCAGGCAGATC	*pfhA*	94/30	55/30	72/90	
HgbAF HgbAR	TGGCGGATAGTCATCAAG CCAAAGAACCACTACCCA	*hgbA*	94/30	53/30	72/30	
HgbBF HgbBR	ACCGCGTTGGAATTATGATTG CATTGAGTACGGCTTGACAT	*hgbB*	94/30	54/30	72/45	
ToxAF ToxAR	CTTAGATGAGCGACAAGGTT GGAATGCCACACCTCTATA	*toxA*	94/30	55/30	72/80	
NanHF NanHR	CACTGCCTTATAGCCGTATTCC AGCACTGTTACCCGAACCC	*nanH*	94/30	58/30	72/60	
NanBF NanBR	AGTGTCCGGGAATAGTGGTG CCGTTGTTCACAACGAACC	*nanB*	94/30	58/30	72/60	
SodAF SodAR	TACCAGAATTAGGCTACGC GAAACGGGTTGCTGCCGCT	*sodA*	94/30	55/30	72/40	
SodCF SodCR	AGTTAGTAGCGGGGTTGGCA TGGTGCTGGGTGATCATCATG	*sodC*	94/30	55/30	72/30	Lainson et al. (1996)
Oma87F Oma87R	ATGAAAAAACTTTTAATTGCGAGC TTAGAACGTCCCACCAATGCTG	*oma87*	94/30	55/30	72/150	Ewers et al. (2006)
Fim4F Fim4R	TGTGGAATTCAGCATTTTAGTGTGTC TCATGAATTCTTATGCGCAAAATCCTGCTGG	*ptfA*	94/30	55/30	72/60	Doughty et al. (2000)

4,000 rpm. DNA was isolated by phenol-chloroform-isoamyl alcohol method (Wilson 1987).

Molecular confirmation of *P. multocida* by PM-PCR

P. multocida were identified by PM-PCR using a pair of *P. multocida* specific primers which amplified the KMT1 gene as described by Townsend et al. (1998a). The PM-PCR was performed using a method detailed by Dutta et al. (2001) with slight modifications in the primer annealing conditions of 54 °C for 45 s.

Maintenance of cultures

The culture were maintained on blood agar slants and stored for a week at 4 °C till further use. These cultures were sub-cultured fortnightly to maintain their viability. For long term storage, *P. multocida* cultures were maintained by

Table 2 Animal number, clinical status, capsule type and presence (+) or absence (−) of virulence associated genes in *P. multocida* isolates

Animal No.	Clinical status	Origin of the strain	Capsule type	Virulence associated genes										
				tbpA	*pfhA*	*hgbA*	*hgbB*	*toxA*	*nanH*	*nanB*	*sodA*	*sodC*	*oma87*	*ptfA*
2205	Apparently healthy	Nasal swab	A	+	+	+	−	−	+	−	−	+	+	+
2215	Apparently healthy	Nasal swab	B	+	+	+	−	−	+	−	−	+	−	−
4351	Haemorrhagic Septicemia	Pooled tissues	B	+	+	+	−	−	+	−	−	+	+	+
4358	Apparently healthy	Nasal swab	B	+	+	+	−	−	+	−	−	+	−	−
4343	Broncho pneumonia	Pooled tissues	B	+	+	+	−	−	+	−	+	+	+	+
201	Haemorrhagic Septicemia	Nasal swab	B	+	+	+	−	−	+	−	−	+	+	+
205	Respiratory symptoms	Nasal swab	B	+	+	+	−	−	+	−	+	+	−	+
206	Broncho pneumonia	Pooled tissues	B	+	+	+	−	−	+	−	+	+	−	+
211	Respiratory symptoms	Nasal swab	B	+	+	+	−	−	+	−	−	+	+	+
224	Apparently healthy	Nasal swab	A	+	+	+	+	−	+	−	+	−	−	+
244	Apparently healthy	Nasal swab	A	+	+	+	+	−	+	−	+	−	+	+
559	Apparently healthy	Nasal swab	B	+	+	+	−	−	+	−	−	+	+	+
590	Broncho pneumonia	Pooled tissues	B	+	+	+	+	−	+	−	+	+	+	+
600	Respiratory symptoms	Nasal swab	B	+	+	+	−	−	+	−	−	+	+	+
609	Apparently healthy	Nasal swab	A	+	+	+	+	−	+	−	−	+	+	−
99	Respiratory symptoms	Nasal swab	B	+	+	+	−	−	+	−	+	+	+	+
P2	Haemorrhagic Septicemia	Pooled tissues	B	+	+	+	−	−	+	−	−	+	+	+
P3	Broncho pneumonia	Nasal swab	B	+	+	+	−	−	+	−	+	+	+	+
P52	Respiratory symptoms	Nasal swab	B	+	+	+	+	−	+	−	+	+	−	+
CP1	Broncho pneumonia	Pooled tissues	B	+	+	+	−	−	+	−	−	+	+	+
CP2	Respiratory symptoms	Nasal swab	B	+	+	+	−	−	+	−	−	+	+	+
BP2	Haemorrhagic Septicemia	Pooled tissues	B	+	+	+	−	−	+	−	−	+	+	+
BP3	Haemorrhagic Septicemia	Pooled tissues	B	+	+	+	+	−	+	−	−	+	+	+

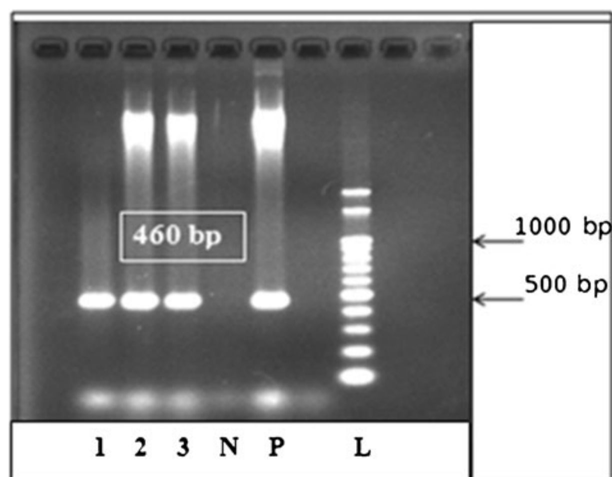

Fig. 1 Confirmation of *P. multocida* using PM-PCR ; Lanes 1–4 460 bp KMT gene amplicons, N- negative control, P-positive control and L- 100 bp Ladder

suspending them in 20 % glycerol stock solution in BHI broth and storing them at −70 °C.

Capsule typing

The capsular types of the isolates were determined by multiplex capsule-PCR with the capsule- specific primer pairs specific for *capA, capB, capD, capE* and *capF* gene as described by Townsend et al. (2001).

Detection of virulence associated genes by PCR

The DNA of *P. multocida* isolated from dead, diseased or apparently healthy bovine was used as a template to amplify virulence associated genes for which PCR-based protocols called virulence genotyping as described by Lainson et al. (1996) for *sodC*, Doughty et al. (2000) for *ptfA* and Ewers et al. (2006) for *tbpA, pfhA, toxA, hgbB, hgbA, nanH, nanB, sodA and oma87* virulence associated genes were used. The sequences of oligonucleotide primers, amplification conditions and references are listed in Table 1 that has been adapted from Ewers et al. 2006. In brief, for all PCR reactions, a total of 100 ng DNA template was added to the reaction mixture (25 µl) containing 2.5 µl 10XPCR buffer, 2.5 µl of 25 mM magnesium chloride, 0.4 µl of 25 mM dNTPs, 0.5 µl of each primer pair in a 10 pmol concentration and 1unit of *Taq*-polymerase (Promega corporation, Madison, USA). The samples were then subjected to 25 cycles of amplification in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, U.S.A). Amplification products were resolved by gel electrophoresis on 1.2 % agarose gel, stained with ethidium bromide (1 µg/ml) and visualized on UV transilluminator (Alpha Innotech, USA).

Statistical analyses

A statistical analysis was performed using GraphPad QuickCales software to establish association between virulence associated genes, *P. multocida* isolates and origin of samples i.e. diseased or apparently healthy animals.

Results

Bacterial isolation and identification

A total of 23 (6.87 %) *P. multocida* isolates were recovered from 335 samples collected from dead, diseased or apparently healthy bovine. The origin, source and capsule type of *P. multocida* isolates have been summarized in Table 2. All the isolates of *P. multocida* showed positive reactions for biochemical tests: catalase, oxidase and nitrate reduction; while negative reaction for urease production, sulphur reduction, motility, MR and VP test. These isolates yielded variable patterns for indole production and citrate utilization. In triple sugar iron agar slants, acid slant and acid butt was produced by all the isolates. Besides this, all were positive for glucose fermentation, however variable results were obtained for other sugars viz. sucrose, lactose, maltose, mannitol, galactose, dulcitol, sorbitol, salicin, arabinose and trehalose.

Molecular confirmation by PM-PCR

All 23 isolates were found positive for PM-PCR. The primer pair KMT1SP6 and KMT1T7 amplified KMT1 gene fragment from *P. multocida* that was electrophoresed to approximately 460 bp (Fig. 1).

Fig. 2 Distribution of virulence associated genes in *P. multocida* based on the health status of animals

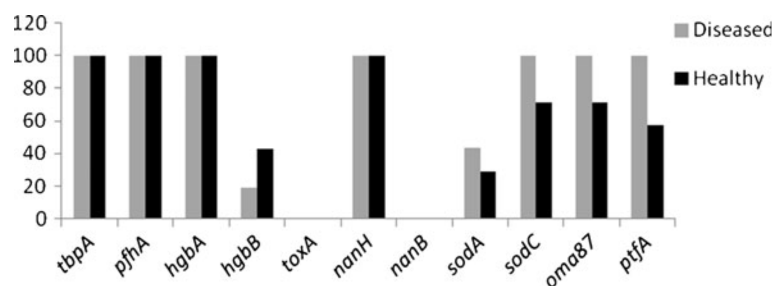

Table 3 Percent distribution of virulence associated genes in *P. multocida* isolates

Virulence associated genes	Total no. of isolates (n=23)	Capsular type A (n=4)	Capsular type B (n=19)	Isolates recovered from diseased animals (n=16)	Isolates recovered from apparently healthy (n=7)
tbpA	100	100	100	100	100
pfhA	100	100	100	100	100
hgbA	100	100	100	100	100
hgbB	26.09	75	15.79	18.75	42.86
toxA	0	0	0	0	0
nanH	100	100	100	100	100
nanB	0	0	0	0	0
sodA	39.13	50	39.84	43.75	28.57
sodC	91.30	50	100	100	71.42
oma87	91.30	75	94.74	100	71.42
ptfA	86.95	75	89.47	100	57.14

Distribution of capsular types

The isolates of *P. multocida* belonging to capsular type A were obtained from 4 (17.4 %) animals and those of capsular type B were obtained from 19 (82.6 %) animals. Capsular type D, E and F were not detected in the population sampled.

Prevalence of virulence associated genes in *P. multocida* isolates

The distribution of virulence associated genes in relation to the origin of isolates (healthy *vis-a vis* diseases) is shown in Fig. 2.

Their distribution in *P. multocida* isolates ranged from 26.09 % (*hgbB*) to 100 % (*tbpA, pfhA, hgbA* & *nanH*). All the isolates possessed *tbpA, pfhA, hgbA and nanH* gene. The gene *hgbB, sodA, sodC, oma87* and *ptfA* were present in 26.09 %, 39.13 %, 91.30 %, 91.30 % and 86.95 % isolates, respectively. The *nanB* and *toxA* were absent in all 23 isolates. *P. multocida* isolates belonging to capsular type B carried virulence genes *tbpA, pfhA, hgbA, sodC* and *nanH* whereas those belonging to capsular type A were harbouring *tbpA, pfhA, hgbA* and *nanH* genes as detailed in Table 3. Only 50 % of capsular type A isolates had *sodC* gene while 100 % of capsular type B isolates contained *sodC* gene. In capsular type A isolates, either *sodA* or *sodC* gene was present and these genes did not occur concurrently.

Discussion and conclusion

The present study was carried out to isolate *P. multocida* from samples collected from apparently healthy, diseased and dead cattle from suspected HS disease outbreaks and to study the prevalence of virulence associated genes among these isolates. Traditional methods depends on using biochemical characterization as one of the means to confirm *P. multocida* identity but variable reactions shown by some of the strains with some of these biochemical tests create difficulties in arriving at conclusive decisions. This means additional assays have to be carried out to ensure fool-proof identity. Many such variations have been described by Fegan et al. 1995; Blackall et al. 1997; Townsend et al. 1998b and Ekundayo et al. 2008. In the present study, the results of catalase, oxidase and sugar-fermentation were very similar to *Flavobacterium spp*. and *Neisseria spp.* whose members are oxidase and catalase positive and were fermentative (Kim et al. 2006; Parija 2009). Molecular confirmation by PCR therefore, proved to be quick, specific and sensitive assay for the confirmation of *P. multocida*. Higher percentages of isolates belonging to capsule-type B as compared to capsule-type A were recovered from bovines.

Although the molecular basis of the pathogenicity and host specificity of *P. multocida* is not well understood, the organism is known to possess a number of virulence factors which have integrated role in pathogenesis (Hunt et al. 2000a & Harper et al. 2006). The present study was thus carried out to study the prevalence of virulence associated genes in the bovine isolates of *P. multocida.* The prevalence of 11 virulence associated genes which included genes coding for iron acquisition factors (*hgbA, hgbB & tbpA*), adhesion related genes (*ptfA, nanB, nanH and pfhA*), outer membrane and porin proteins (*oma87*), superoxide dismutases (*sodA* & *sodC*) and dermonecrotoxin (*toxA*) were studied.

Iron acquisition and uptake are essential for bacterial survival and as a result pathogenic bacteria have developed different strategies for their uptake. *P. multocida* produces both iron chelating siderophores and outer membrane receptors such as transferring binding protein and haemoglobin binding protein for the iron binding host molecules, transferrin and haemoglobin (Choi-Kim et al. 1991; Ogunnariwo et al. 1991; Ogunnariwo and Schryvers 2001; Cox et al. 2003; Bosch et al. 2004). *P. multocida* grown under iron depleted media or in vivo expressed three iron regulated OMPs with molecular masses of 76, 84 and 94 kDa, respectively with all three having affinity for siderophore binding (Choi-Kim et al. 1991). Haemoglobin binding proteins A and B help bacteria by using haemin as iron source. Although the bacterium does not produce a classical haemolysin, it carries esterase gene causing a haemolytic phenotype as seen in *E. coli* under anaerobic conditions (Cox et al. 2000; Hunt et al. 2000b). By inducing lysis of erythrocytes, haemoglobin is released and is thought to be bound by *P. multocida* haemoglobin binding proteins. The high prevalence of iron acquisition genes in the *P. multocida* as well as their significant role in pathogenesis suggests that their presence in *P. multocida* provide the bacterium an

added advantage for enhanced pathogenicity (Venken et al. 1994; Ogunnariwo and Schryvers 2001). Veken et al. (1994) reported the presence of *tbpA* in bovine isolates of *P. multocida* associated with pneumonia and haemorrhagic septicaemia. In another study, Ogunariwo and Schryvers found that *tbpA* (−) strains are commensal or at least cause other diseases. Many previous studies have shown that iron acquisition related gene *tbpA* is an epidemiological marker (Cox et al. 2003; Bosch et al. 2004; Ewers et al. 2006) in addition to an important virulence factor in *P. multocida* isolates of cattle. Current study also found high occurrence of *pfhA* and *tbpA* among *P. multocida* isolates from diseased as well as healthy cattle. These findings are although at variance with results obtained in previous studies advocating *tbpA* and *pfhA* as virulence factors (Ewers et al. 2006) but are in agreement with the results of Shayegh et al. 2010.

The adhesion related genes *nan*H was found to be regularly distributed in *P. multocida* irrespective of the capsular type A or B or the health status of cattle. In this study, the presence of *ptfA* and an association between its distribution and bovine disease ($P<0.05$) was observed. High prevalence of the *ptfA* gene (type 4 fimbriae) in isolates from diseased bovine (100 %;16/16) as compared to isolates from healthy bovine (57.14 %; 4/7) was expected given the fact that this gene is supposed to be a key element in fixing bacteria on the surface of the epithelial cells (Ewers et al. 2006).

Components of bacterial outer membrane such as transmembrane proteins and lipoproteins play key role in the interaction of pathogen with host environment and in the host immune response to infection. OMPs of gram negative bacteria have a role in disease processes as they act at an interface between the host and pathogen (Lin et al. 2002). High prevalence of the gene coding for *oma87* in the isolates points towards their important role in host-pathogen interaction. Since many genes were also harboured by isolates originated from healthy animals; how and under what circumstances the gene or its pathways and products contribute to pathogenesis is a matter of investigation.

Superoxide dismutases (SODs) are virtually ubiquitous in bacteria, catalysing the conversion of O_2^- generated by macrophages & neutrophils into hydrogen peroxide and oxygen (McCord and Fridovich 1969). In the present study, the virulence associated genes *sodA & sodC* were found in a higher percentage among isolates from diseased animals as compared to isolates recovered from apparently healthy animals. However, the association of these genes with disease in bovine was not statistically significant.

All isolates of *P. multocida* from dead, diseased or apparently healthy bovine were carrying the virulence associated genes *tbpA, pfhA, hgbA, sodC* and *nanH.* Among the five virulence associated genes; *tbpA* and *pfhA* have been considered as epidemiological markers in the past studies and supposed to have strong positive association to the outcome of disease in cattle. However, their presence in isolates arising from healthy bovines warrants detailed investigation about their role in disease outcome. The virulence associated genes *sodA, sodC, oma87* and *pfhA* were found in a higher percentage in isolates from diseased animals as compared to isolates from apparently healthy animals; however strong association between genes and the isolates recovered from diseased animals was confirmed only for *ptf*A. Future studies should focus on the role of these genes in health & disease and how their expression is influenced and regulated under immunosuppression.

Acknowledgements Authors wish to thank Indian Council of Agricultural Research for providing grant to carry out this work; H.P. Animal Husbandry Department & HPKV, Palampur for logistic support and Dr. Varun Sankhyan for his help with statistical analysis.

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