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Prevalence of virulence associated genes in *Pasteurella multocida* isolates from Tamil Nadu

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ABSTRACT

Pasteurella multocida is an important pathogen responsible for wide range of diseases in different hosts. In this study, 14 *P.multocida* isolates from cattle, goat, rabbit and avian origin were examined for capsular type and 11 virulence associated genes using previously described multiplex PCR. Of the 14 isolates, 11 were capsular type A and 3 were capsular type B. Among the virulence associated genes, *oma87*, *hgbA*, *nanB* and *sodC* were detected in 100% of the isolates and none of the isolates harboured *toxA*. All the isolates from goat origin showed positive (100%) for *phfA* gene. Occurrence of filamentous haemagglutinin encoding *phfA* gene from isolates of goat origin is first reported in this study.

Key words: Goat, *Pasteurella multocida*, *phfA,* Virulence typing

Pasteurella multocida, an opportunistic pathogen, is responsible for fowl cholera in poultry, haemorrhagic septicaemia of buffalo and cattle, pneumonia of lambs and goats, respiratory atrophic rhinitis of swine and purulent rhinitis of rabbit (Gyles *et al*. 2004). *Pasteurella multocida* strains are classified into serogroups (A, B, D, E and F) based on capsule antigens and further classified into 16 serotypes (1–16) based primarily on lipopolysaccharide antigens. There is a clear correlation between the capsular type and disease, also the other factors such as neuraminidases, iron sequestering proteins, fimbriae, bacterial adhesions and outer membrane proteins play key roles in the growth of *P.multocida* within the host (Harper *et al*. 2006). Finding out the virulence factors involved in the mechanism of causing diseases will be helpful for developing effective vaccine strategies against the diseases. In the present study, *P.multocida* isolates collected in Tamil Nadu during 2013–2015 were investigated for their capsular types and presence of eleven virulence associated genes namely *oma87*, *toxA*, *nanB*, *nanH*, *ptfA*, *phfA*, *hgbB*, *exbD-tonB*, *hgbA*, *sodA*, *sodC*.

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MATERIALS AND METHODS

Sample collection and processing: Samples (465) suspected for *Pasteurella multocida* were collected from dead and healthy animals and birds in Tamil Nadu state in the time interval between 2013–2015. Each swab was plated directly on brain heart infusion supplemented with 5% of sheep blood. Biochemical tests were carried out which included IMVIC, sugar fermentation test, catalase and oxidase test for identification.

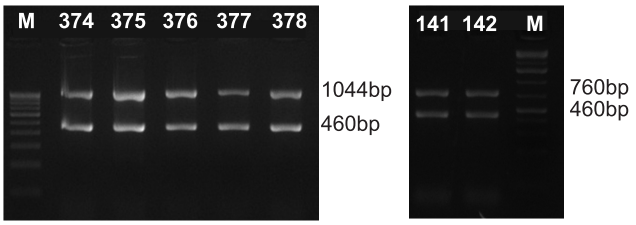
Further DNA was extracted from *P.multocida* isolates inoculated Brain heart infusion broth cultures by high salt method as described previously (Kumar and Ramdass 2001) and stored at –20° C until further use. Previously developed *Pasteurella multocida* specific PCR assay (Townsend *et al*.1998) was used for the further confirmation of the isolates. All the isolates were sent to IVRI, Izatnagar for serotyping.

Multiplex capsular PCR typing: All the *P.multocida* isolates were evaluated for the presence of capsule biosynthesis genes (A, B, D, E and F) using previously described multiplex capsular PCR typing (Townsend *et al*. 2001)

Virulence associated genes multiplex PCR: All the isolates were tested for the presence of 11 virulence associated genes, *oma87*, *toxA*, *nanB*, *nanH*, *ptfA*, *phfA*, *hgbB*, *exbD-tonB*, *hgbA*, *sodA*, *sodC* using previously described multiplex virulence typing PCR (Furian *et al*. 2013). All the amplified products were subjected to electrophoresis in 1.5% agarose gel and stained with Ethidium bomide.

RESULTS AND DISCUSSION

Molecular typing of *P.multocida* using REP-PCR, ERIC-PCR, MLST analysis confirmed the diversity of *P. multocida* circulating in India and also the possibility of transboundary spread of strains across evolutionary time (Sarangi *et al*. 2014). Virulence gene occurrence in *P.multocida* has a strong positive association with the outcome of infection with the organism in cattle (Katsuda *et al*. 2013). Therefore virulence gene profiling from different hosts and different regions will contribute in finding out the pathogenesis of *P.multocida.* In this study, 14 *P.multocida* isolates were recovered from various hosts during 2013–2015 and further confirmed by *P. multocida* species specific PCR (PM–PCR) assay. Out of the 14 isolates, 11 isolates showed positive for Capsular A serotype and 3 isolates were positive for capsular B serotype in the laboratory (Table 1 and Fig 1a and b). These results also coincided with the serotyping results obtained from Indian Veterinary Research Institute (IVRI), Izatnagar. Further. all the *P.multocida* isolates were screened for the presence of



Figs 1a-1b. **1a.** Agarose gel electrophoresis showing amplified product of capsular A gene (1000 bp) and Kmt1 gene (465 bp). Lane 1: Molecular weight marker 100 bp ladder, Lane 2–6: 374, 375,376,377,378 isolates from cattle origin belongs to capsular A type. **1b.** Agarose gel electrophoresis showing amplified product of capsular B gene (760 bp) and Kmt1 gene (465 bp). Lane 1–2: 141,142 isolates from cattle origin belongs to capsular B type, Lane 3: Molecular weight marker 100 bp ladder.

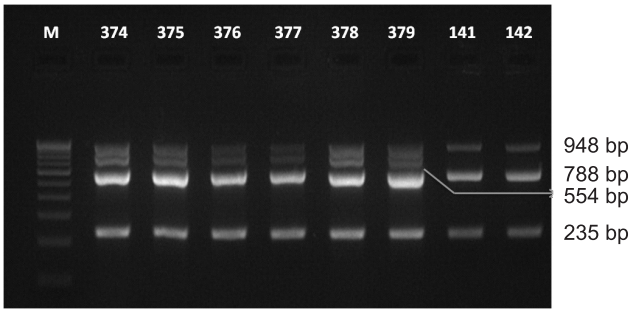


Fig. 2. Agarose gel electrophoresis showing amplified product of *oma87* (948 bp), *sodC* (235 bp), *hgbB* (788 bp), *nanB* (554 bp). Lane 1: Molecular weight marker 100 bp ladder, Lane 2–9: 374, 375, 376, 377, 378, 379, 141,142 isolates from cattle origin.

11 virulence associated genes of *P.multocida* (Table 2 and Fig 2). Among the 14 *P.multocida* isolates, the percentage of prevalence of eleven virulence genes ranged from 0% (*toxA*) to 100% (*oma87*, *hgbA*, *nanB* and *SodC*).

Distribution of virulence genes according to capsular types shows 100 % prevalence of *oma87*, *sodC*, *hgbA*, *exbD-tonB*, *nanB*, *nanH*, *ptfA*, *phfA*, and other three genes *sodA*, *toxA*, *hgbB* were absent in capsular B type isolates whereas in capsular A type isolates, all the virulence genes except *toxA* was regularly distributed (Table 2). The frequency related to the cattle and goat origin was also recorded (Table 3).

All the *P.multocida* isolates showed positive for the *oma87* gene, an 87-KDa outer membrane protein which is previously identified as one of the possible adhesin protein in *P.multocida*. All the previous studies with the *P.multocida* isolates from cattle, avian and other different host origin showed 100 % frequencies which is similar to the present study (Furian *et al*. 2013; Khamesipour *et al*. 2014, Ewers *et al*. 2006). In almost all the previous studies, the frequency of filamentous haemagglutinin encoding *phfA* gene was reported to be at low prevalence with varying frequencies in between 46–52%, 45–60%, and 15–40.5% among the *P.multocida* isolates from cattle, poultry and pig origin

Table 1. Prevalence of virulence associated genes among *P. multocida* isolates recovered from different host origin

Sample name/ host	Serotype	Place	Year	*sodA*	*hgbA*	*ptfA*	*phfA*	*exbD-tonB*	*nanH*	*toxA*	*oma87*	*sodC*	*hgbB*	*nanB*
374/Cattle	A	Chennai	2013	-	+	-	-	-	+	-	+	+	+	+
375/Cattle	A	Chennai	2013	-	+	+	-	+	+	-	+	+	+	+
376/Cattle	A	Chennai	2013	-	+	-	-	+	+	-	+	+	+	+
377/Cattle	A	Chennai	2013	-	+	-	+	+	+	-	+	+	+	+
378/Cattle	A	Chennai	2013	-	+	-	+	-	-	-	+	+	+	+
379/Cattle	A	Chennai	2013	+	+	+	+	+	+	-	+	+	+	+
141/Cattle	B	Vellore	2014	-	+	+	+	+	+	-	+	+	-	+
142/Cattle	B	Vellore	2014	-	+	+	+	+	+	-	+	+	-	+
G61/Goat	A	Chennai	2014	+	+	+	+	+	+	-	+	+	+	+
G23/Goat	B	Chennai	2014	-	+	+	+	+	+	-	+	+	-	+
G58/Goat	A	Chennai	2014	-	+	+	+	+	+	-	+	+	-	+
R1/Rabbit	A	Chennai	2015	-	+	+	+	+	+	-	+	+	-	+
Poultry1/15/ Chicken	A	Chennai	2015	-	+	+	+	+	+	-	+	+	-	+
D1/Duck	A	Chennai	2015	-	+	+	+	+	-	-	+	+	-	+

-, absence of virulence gene; +, presence of virulence gene.

respectively whereas recent study from India with *P.multocida* isolates from different host origin reported 85 % (pig) to 100% (avian) and they suggested that *phfA* gene might be providing survival advantage to the bacterium in the host and the occurrence of horizontal gene transfer has led to such high prevalence among Indian strains (Sarangi *et al*. 2014). In the present study, 79 % of isolates showed positive for this gene. Interestingly, all the isolates from goat showed positive for this gene which is in contrast to the previous study which reported the complete absence of this gene in the *P.multocida* isolates from goat origin (Shayegh *et al*. 2014). The present study was the first to report the presence of *PhfA* gene in the isolates from goat origin. Only 62.5 % isolates from cattle showed positive for this gene which fade away the fact that the *phfA* gene as an epidemiological marker in bovine diseases. Also in the previous studies with 46 *P.multocida* isolates from rabbit, it was reported that none of the isolates harboured *phfA*, while this gene was reported in all the Indian isolates from rabbit origin (Ferreira *et al*. 2012, Sarangi *et al*. 2014). In the present study also, one isolate from rabbit origin showed positive for this gene.

The bacterial iron acquisition haemoglobin binding proteins A and B, play an important role in increased proliferation of the bacterium by binding hemin as iron source (Ewers *et al*. 2006). Among the hemoglobin binding proteins, *hgbA* was present in 100 % of the isolates which was similar to the previous report that the gene was regularly distributed among *P.multocida* isolates (Khamesipour *et al.* 2014). Whereas the presence of *hgbB* was 50% overall and it varies among different hosts which correlates with the previous report (Furian *et al*. 2013). All the six capsular A type isolates of cattle showed the presence of *hgbB* gene, which is in agreement with previous studies in which higher frequency of *hgbB* was reported from diseased cattle than healthy cattle and association of *hgbB* gene in bovine diseases was also reported (Katsuda *et al*. 2013; Khamesipour *et al*. 2014).

Another iron acquisition *exbD-tonB* protein encoding gene was present in 86% of the isolates which is below the frequency (100%) normally expected for the genes encoding the proteins of this complex (Bethe *et al*. 2009). All the three isolates from goat showed presence of this gene. The regular distribution of *nanB* and *nanH* genes among the *P. multocida* strains allows one strain to express two different sialidases characterized by a high pH tolerance enabling bacterial activity in different host cell compartments. This could confer the bacterium not only growth benefits but also the property to adhere to and invade host cells by unmasking host receptors (Mizan *et al*. 2000). Out of the two sialidases encoding genes, 86% of isolates were positive for *nanH* which correlates with the previous finding that the prevalence of *nanH* varied according to host origin and geographical location (Tang *et al*. 2009). In the case of *nanB*, 100 % of the isolates were positive and consistent with the previous reports (Sarangi *et al*. 2014, Furian *et al*. 2013).

Detection of two superoxidase dismutase genes *sodA* and *sodC* among *P.multocida* isolates from India was first reported in this study. *sodC* shows 100 % prevalence in all the isolates. One of the previous report with *P.multocida* strains isolated from cattle with pneumonic lung showed 100 % occurrence of *sodC*. In an another report with *P.multocida* isolates from rabbit with respiratory disease also similar frequency was observed and it was lesser in carrier animals (Ferreira *et al*. 2012, Khamesipour *et al*. 2014, Furian *et al*. 2013).This reveals that *sodC* gene may play a role in pathogenicity, further studies in finding out the role of *sodC* in pathogenicity of *P.multocida* isolates are required.

Another superoxidase dismutase gene, *sodA* was present in lesser frequency only. The absence of *toxA* gene in all the isolates tested proves the previous fact that the gene encoding dermonecrotic toxin was more frequent in the cases of atrophic rhinitis of swine (Harper *et al*. 2006).

Though in the present study only limited numbers of isolates were tested, it proved the presence of *phfA* gene in *P.multocida* strain isolated from goat and also provided the evidence for *sodC* and its role in *P. multocida* pathogenesis.

Table 2. Frequency of virulence-associated genes in 14 isolates of *Pasteurella multocida* from different hosts

Virulence gene	Frequency (n=14)	Cap A (n= 11)	Cap B(n=3)
Superoxide dismutase			
SodA	14 %(2)	18 % (2)	0% (0)
sodC	100% (14)	100 % (11)	100 % (3)
Iron acquisition			
hgbA	100% (14)	100 % (11)	100 % (3)
hgbB	50% (7)	64 % (7)	0% (0)
exbD-tonB	86% (12)	82 % (9)	100 % (3)
Adhesins			
ptfA	71% (10)	64 % (7)	100 % (3)
phfA	79% (11)	73 % (8)	100 % (3)
Sialidases			
nanH	86% (12)	82 % (9)	100 % (3)
nanB	100% (14)	100% (11)	100 % (3)
Toxins			
toxA	0% (0)	0% (0)	0% (0)
Protectins			
oma87	100% (14)	100 % (11)	100 % (3)

Table 3. Frequency of virulence-associated genes in *Pasteurella multocida* isolates related to the cattle and goat origin

Host	*SodA*	*hgbA*	*ptfA*	*phfA*	*exbD-tonB*	*nanH*	*toxA*	*oma87*	*sodC*	*hgbB*	*nanB*
Cattle(n=8)	12.5 %	100 %	50 %	62.5 %	75 %	87.5 %	0 %	100 %	100 %	75 %	100 %
Goat(n=3)	33.3 %	100 %	100 %	100 %	100 %	100 %	0 %	100 %	100 %	33.3 %	33.33 %

Further studies on larger sample from different hosts and different capsular types would contribute more for understanding the pathogenesis of *P.multocida* in the host.

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