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Paper

Molecular identification and characterization of Pasteurella multocida isolates from pneumonic sheep and goats

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Abstract

Pasteurellosis is an important bacterial disease of small ruminants which is characterized by severe respiratory disease complex causing high morbidity and mortality. The present study was done to know the prevalence of *P. multocida* and serotypes associated with the disease in the southern region of Telangana. The present study observed a prevalence of 15.7% for *P. multocida* by PCR and 16 isolates (8.37%) were recovered in pure cultures. Among the isolates, 56.25% were identified as capsular type A and 43.75% as capsular type B suggesting the involvement of *P. multocida* serotype B in small ruminant respiratory disease. The prevalence of virulence genes were found to be 100% for *ompH, nanB, sodA, oma87, ptfA* and *fur,* 87.5% for *fimA,* 68.75% for *tbpA* and 37.5% for *toxA* indicating the pathogenic potential of the isolates. The high prevalence of virulence associated genes in the isolates indicates the pathogenic potential of the organisms.

Keywords

Pasteurellosis, Pasteurella multocida, Sheep, Goats, India, Virulence genotyping

Introduction

In India, small ruminants are the continuous source of income for rural population and ranks 2nd and 3rd in terms of goat (148.88 million) and sheep (74.26 million) population, respectively. In spite of huge population, frequent occurrence of infectious diseases is a major concern to the sheep and goat farmers. Recently, outbreaks of respiratory infections due to *Pasteurella multocida* have been reported in various states (Kumar et al. 2015, Rawat et al. 2019, Prajapati et al. 2020). *P. multocida* and *M. haemolytica* are found to be the major etiological agents involved in causing respiratory disease complex in small ruminants (Besser et al. 2008, Rawat et al. 2019). These organisms are present as commensals in the upper respiratory tract in cattle, sheep and goat and known to produce the disease in stress conditions or in immune compromised animals. Five capsular types (A, B, D, E and F) and 16 somatic lipopolysaccharide types have been identified among the strains of *P. multocida* (Carter1955). In addition, untypeable strains of *P. multocida* have also been reported (Boyce et al. 2000, Davies et al. 2003, Ewers et al. 2006, Bethe et al. 2009, Einarsdottir et al. 2016). Several workers reported that capsular bacteria are more pathogenic than acapsular strains, indicating that the role of capsule in protecting the bacteria against desiccation, phagocytosis, and bactericidal

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complement action compared to previous work (Boyce et al. 2000, Einarsdottir et al. 2016). Different capsular types of *P. multocida* have been linked to a wide range of diseases in animals and birds. Serogroup A and to a lesser extent, serogroup D, cause fowl cholera in birds (Rimler and Rhodes 1987). Serogroups A and D are associated with pneumonia and atrophic rhinitis in pigs, the latter being associated with toxigenic strains (Chanter and Rutter 1989). Also, serogroup A is associated with pneumonia in cattle (Frank 1989) whereas serogroups B and E are linked to haemorrhagic septicaemia (Carter and De Alwis 1989, Arumugam *et al*, 2011). Capsular types A and D are found to be most common in sheep in certain regions (Zamri-Saad et al. 1996, Prabhakar et al. 2010, Tahamtan et al. 2014).

Pasteurella multocida serotype A is primarily known to cause ovine and caprine pneumonia and predisposing sheep and goats to secondary bacterial, viral, and parasitic diseases. Different serotypes of *P. multocida* are associated with different diseases in animals and birds. Further, multidrug resistant strains of *P. multocida* have been observed. Isolation and identification of the circulating serotypes and virulence genotyping is a prerequisite to develop appropriate treatment and prevention strategies. The linkage between certain pathological conditions or hosts and the capsular serogroup as described above indicates the importance of correct identification of the prevalent capsular serotypes of *P. multocida* to initiate appropriate prevention and control strategies. Although, several studies on *P. multocida* in cattle have been done in India, there is dearth of the available literature on small ruminant pasteurellosis. Moreover, specific serotypes of *P. multocida* are associated with diseases in different host species, cross species infection of serotypes is not uncommon (Prajapati et al. 2020).

The absence of studies on the epidemiology of Pasteurellosis in sheep and goat in the region prompted us to design the present work of molecular identification and characterization of *P. multocida* isolates from pneumonic sheep and goats in the Warangal region of Telangana state to initiate appropriate treatment, prevention and control strategies as the local circulating serotypes are important factors for formulating suitable control strategies.

Materials and methods

This study was carried out in the Department of Veterinary Microbiology, College of Veterinary Science, Mamnoor, Warangal, P.V. Narsimha Rao Telangana Veterinary University (PVNRTVU), Hyderabad, India, during the period 2020-2021.

Collection and processing of samples

A total of 191 Samples (nasal swabs - 157, lung tissues – 34) were collected from sheep and goat farms in and around Warangal District of Telangana. The nasal swabs were transported in sterile test tubes containing 2 mL of Amies transport medium and placed immediately in an icebox for further analysis (Hawari et al. 2008) and lung tissues were collected in sterile screw capped containers and transported on ice and stored at -20^{o}C until further use. Aseptically, the nasal swabs and lung tissues were directly streaked on Trypticase soya agar (TSA) supplemented with 5% defibrinated sheep blood (Sheep blood agar), Brain Heart Infusion agar (BHI agar) and MacConkey agar (MCA) (HiMedia, Mumbai). The plates were incubated at 37^{o}C for 24 h and examined for the bacterial growth. The colonies showing Gram negative, small, coccobacilli organisms were subjected for various biochemical tests as per the methods described Cruickshank 1975. The isolates were further confirmed by *Pasteurella multocida* polymerase chain reaction method as described by Townsend et al. 2001.

DNA extraction

DNA was extracted from the bacterial cultures by boiling and snap chill method as per the method described (Aror*æt al.* 2006) with slight modifications. The pure genomic DNA was extracted by using HiPurA® Genomic DNA Purification Kit (HiMedia, Mumbai) as per the manufacturer's instructions.

Pasteurella multocida species specific PCR (PM-PCR) and capsular multiplex PCR

The isolates were further confirmed by amplification of *KMT1* gene *P. multocida* using oligonucleotide primers *KMT1T7* (5'-ATCCGCTATTTACCCAGTGG-3') and *KMT1SP6* (5'-GCTGTAAACGAACTCGCCAC-3') as per the method described (Townsend et al. 1998). Multiplex PCR for capsular typing using CAPA, CAPB, CAPD, CAPE and CAPF primers for amplification of *hyaD-hyaC*, *bcbD*, *dcbF*, *ecbJ* and *fcbD* genes respectively, was done as described Townsend et al. 2001. The protocol for PM-PCR and multiplex capsular PCR was as follows, Initial denaturation at 94°C for 5 min, followed by 30 cycles, each cycle consisting of 3 steps- denaturation at 94^{o}C for 30 sec, annealing at 50^{o}C for 30 sec, Extension at 72^{o}C for 60 sec. Final Extension was carried out at 72^{o}C for 10 min. Details of the primers used for capsular PCR were listed in (Table I).

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S.No	Primer	Sequence (5' - 3')	Amplification size
1	CAPA	**F:**TGCCAAAATCGCAGTCAG **R:**TTGCCATCATTGTCAGTG	1044 bp
2	CAPB	**F:**CATTTATCCAAGCTCCACC **R:**GCCCGAGAGTTTCAATCC	760 bp
3	CAPD	**F:**TTACAAAAGAAAGACTAGGAGCCC **R:**CATCTACCCACTCAACCATATCAG	657 bp
4	CAPE	**F:**TCCGCAGAAAATTATTGACTC **R:**GCTTGCTGCTTGATTTTGTC	511 bp
5	CAPF	**F**:AATCGGAGAACGCAGAAATCAG **R:**TTCCGCCGTCAATTACTCTG	851 bp

Table I. Details of the Primers used for Capsular typing of P. multocida.

Virulence genotyping of P. multocida

A total of 9 virulence associated genes (*ompH*, *tbpA*, *toxA*, *fimA*, *nanB*, *sodA*, *oma87*, *ptfA* and *fur*) were targeted using multiplex PCR. First multiplex PCR was carried out for *ompH*, *tbpA* and *toxA* genes, second multiplex PCR for *fimA*, *nanB* and *sodA* genes, duplex PCR for *ptfA* and *oma87* genes while *fur* was amplified in a separate reaction. The primer sequences used for multiplex PCR and protocol for amplification of genes were listed in (Table II and III) respectively.

Gene	Function	Primer	Primer sequence	Amplicon size	Reference
Protectins *OmpH*	Outermembrane protein H	OmpH	**F:**CGCGTATGAAGGTTTAGGT **R:**TTTAGATTGTGCGTAGTCAAC	438bp	(Van Vliet et al, 1998)
Oma87	Outermembrane protein 87	Oma87	**F**:GGCAGCGAGCAACAGATAACG **R**:TGTTCGTCAAATGTCGGGTGA	838 bp	
Iron acquisition proteins *TbpA*	Transferrin Binding Protein	TbpA	**F:**TGGTTGGAAACGGTAAAGC **R:**TAACGTGTACGGAAAAGCC	728bp	
Fur	Ferric uptake regulation protein	Fur	**F:**GTTTACCGTGTATTAGACCA **R:**CATTACTACATTTGCCATAC	244 bp	
Dermonecrotoxin *ToxA*	Dermonecrotoxin	ToxA	**F:**CTTAGATGAGCGACAAGG **R:**GAATGCCACACCTCTATAG	864bp	
Adhesins *FimA*	Fimbriae	FimA	**F:**CCATCGGATCTAAACGACCTA **R:**AGTATTAGTTCCTGCGGGTG	866bp	
PtfA	Type 4 Fimbriae	PtfA	**F:**TGTGGCATTCAGCATTTTAGTGTGTA **R:**TCATGAAACATTATGCGCAAAATCCTGCTG	488 bp	
Sialidases *NanB*	Outer membrane-associated proteins, an autotranspoter protein	NanB	**F:**CATTGCACCTAACACCTCT **R:**GGACACTGATTGCCCTGAA	555bp	
Superoxide dismutases	Superoxide dismutase	SodA	**F:**TACCAGAATTAGGCTACGC **R:**GAAACGGGTTGCTGCCGCT	362bp	(Farahani M.F et al., 2019)

Table II. Details of the primers used for molecular detection of P. multocida virulence associated genes.

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S.No	Gene	Initial Denaturation (°C/min)	Denaturation (°C/sec)	Annealing (°C/sec)	Extension (°C/sec)	Final Extension (°C/min)
1	*ompH tbpA toxA*	94/5	94/30	47/30	72/30	72/10
2	*fimA nanB sodA*	94/5	94/30	50/30	72/60	72/10
3	*ptfA oma87*	94/5	94/30	55/30	72/45	72/10
4	*fur*	94/5	94/30	45/30	72/45	72/10

Table III. PCR protocols for molecular detection of P. multocida virulence associated genes.

Results

A total of 138 sheep and 53 goat samples were collected. Positive samples for *P. multocida* from sheep and goat were 25 (18.1%) and 5 (9.4%), respectively by PM-PCR showing an expected amplicon of ~460 bp (Figure 1). The overall prevalence was found to be 15.7% (30/191) among the total samples. A total of 16 pure cultures were isolated from 30 PCR positive samples. Among the 16 isolates, 9 were found to be capsular type A (56.25%) and 7 were found to be of capsular type B (43.75%) showing an expected amplicons of ~1044 bp and 760 bp, respectively (Figure 2) by multiplex PCR. None of the isolates were positive for the capsular types D, E and F. The epidemiological importance of nine virulence-associated genes were detected with multiplex-PCRs. Amplification of genes with 438 bp, 728 bp, 864 bp, 866 bp, 555 bp, 362 bp, 838 bp, 488 bp, 244 bp were addressed to the presence of *ompH*, *tbpA*, *toxA*, *fimA*, *nanB*, *sodA*, *oma87*, *ptfA*, and *fur* genes, respectively (Figures 3-6). Further, in the present study *fimA* was amplified at either 866 bp or 788 bp for different isolates (Figure 7). The virulence genes*ompH*, *nanB*, *sodA*, *oma87*, *ptfA* and *fur* were detected in all the 16 isolates (100%) of both capsular types A and B. The amplification of capsular and virulence genes of *P. multocida* KLD4 isolate was shown in (Figure 7). The overall prevalence of other virulence genes *fimA*, *tbpA* and *toxA* were in the order 87.5%, 68.75% and 37.5% (Table IV, Figure 8).

However, the prevalence of 3 virulence genes varied among the capsular types A and B. The *fimA* gene was found to be present in all the 9 (100%) isolates of capsular type A but was detected in only 5 (71.4%) capB isolates. The *tbpA* gene was observed to be present in 4 (44.4 %) isolates of capsular type A but was found in all the 7 (100%) capB isolates. The *toxA* gene was found to be detected in 6 (66.6%) capA isolates while none of the capB isolates possessed *toxA* gene. Details of the association of capsular types to virulence genes were shown in (Table V) and described in (Figure 9).

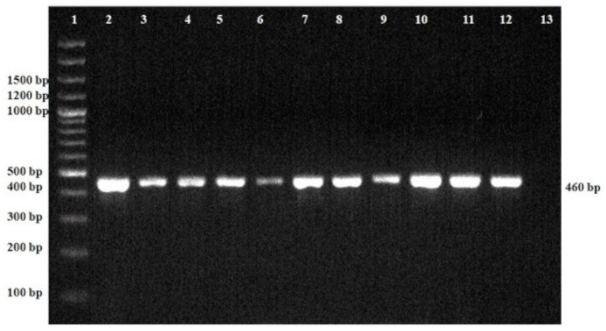

Figura 1. PM-PCR for detection of KMT gene of P. multocida. Lane 1: 100 bp DNA ladder, Lane 2: Positive control (P. multocida ATCC 12945), Lane 13: No template control, Lanes 3-12: P. multocida isolates showing 460 bp amplicon.

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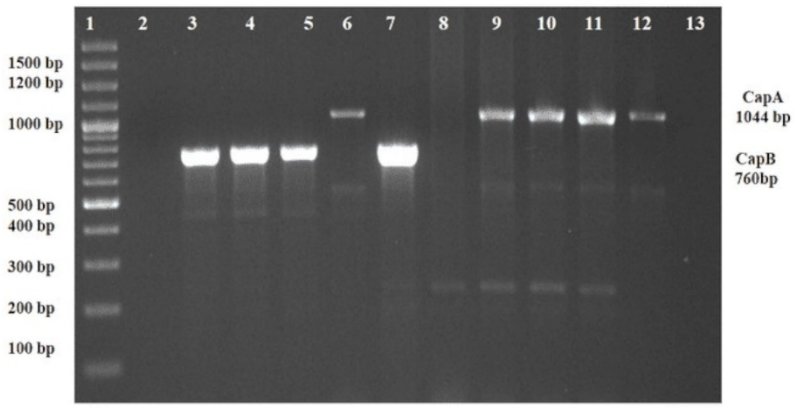

Figure 2. Multiplex PCR for detection of capsule biosynthesis genes of P. multocida. Lane 1: 100 bp DNA ladder, Lanes 2, 8 and 13: No template controls, Lanes 3, 4, 5 and 7: CapB isolates showing an amplicon of 760 bp, Lanes 6, 9-12: CapA isolates showing an amplicon of 1044 bp.

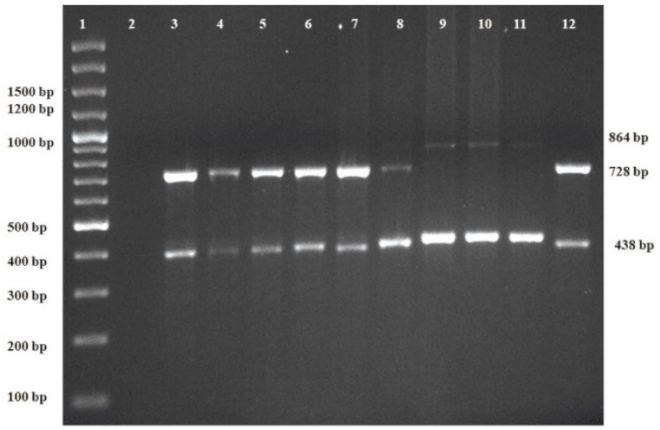

Figure 3. Multiplex PCR for detection of ompH, tbpA and toxA genes of P. multocida. Lane 1: 100 bp DNA ladder, Lane 2: No template control, Lanes 3-8 and 12: Isolates positive for ompH (438 bp) and tbpA (728 bp), Lanes 9-11: Isolates positive for ompH (438 bp) and toxA (864 bp).

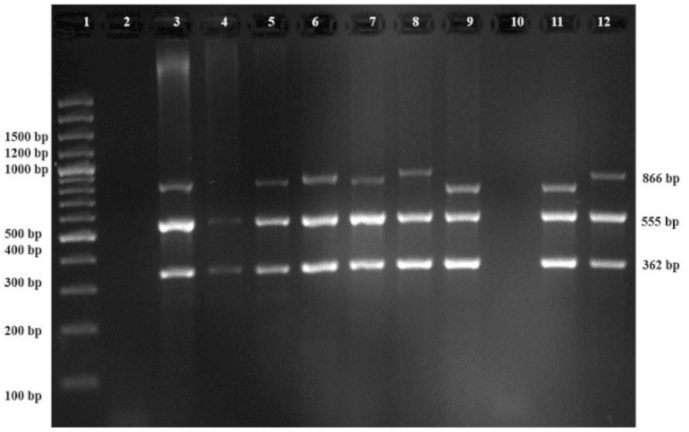

Figure 4. Multiplex PCR for detection of fimA, nanB and sodA genes of P. multocida. Lane 1: 100 bp DNA ladder, Lane 2 and 10: No template controls, Lanes 3-8 and 12: Isolates positive for sodA (362 bp), nanB (555 bp) and fimA (866 bp), Lanes 9 and 11: Isolates positive for sodA (362 bp), nanB (555 bp) and fimA (788 bp).

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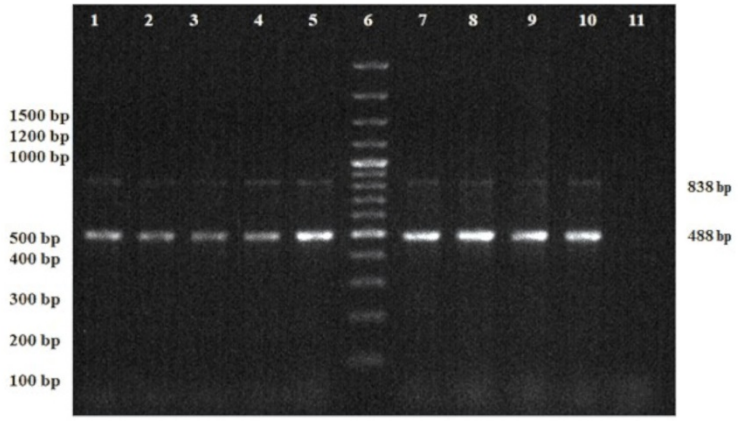

Figure 5. Duplex PCR for detection of ptfA and oma87 genes of P. multocida. Lane 6: 100 bp DNA ladder, Lane 11: No template control, Lanes 1-5 and 7-10: Isolates positive for ptfA (488 bp) and oma87 (838 bp).

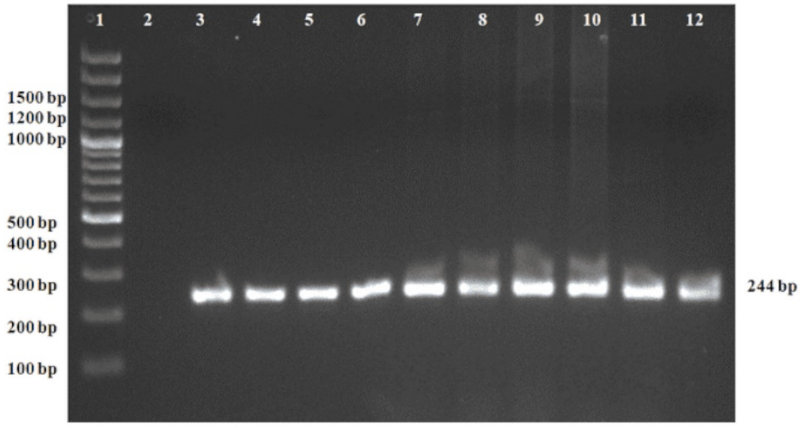

Figure 6. PCR for detection of fur gene of P. multocida. Lane 1: 100 bp DNA ladder, Lane 2: No template control, Lanes 3-12: Positive isolates showing an amplicon of 244 bp.

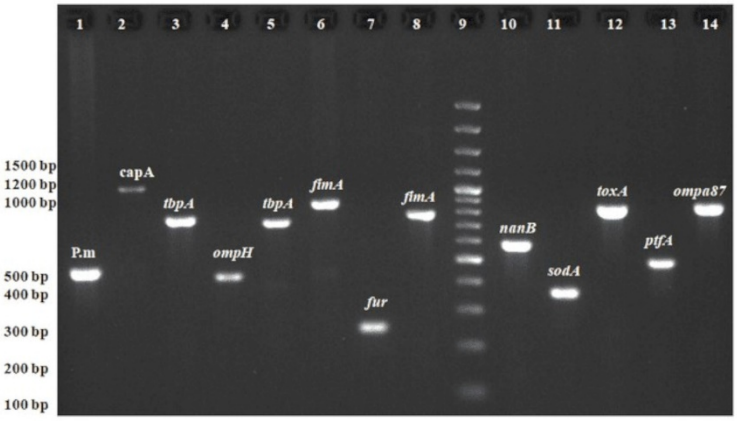

Figure 7. Capsular typing and virulence gene profiling of P. multocida KLD4 isolate. Lane 1: Positive for Kmt1 gene (460 bp), Lane 2: Positive for CapA (1044 bp), Lanes 3 and 5: Positive for tbpA gene (728 bp), Lane 4: Positive for ompH gene (438 bp), Lane 6: Positive for fimA (866 bp) (KLD4 isolate), Lane 7: Positive for fur gene (244 bp), Lane 8: Positive for fimA gene (788 bp) (MNR5 isolate), Lane 9: 100 bp DNA marker, Lane 10: Positive for nanB gene (555 bp), Lane 11: Positive for sodA gene (362 bp), Lane 12: Positive for toxA gene (864 bp), Lane 13: Positive for ptfA gene (488 bp), Lane 14: Positive for oma87 gene (838 bp).

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Isolate id	Capsular type	Virulence genotyping								
		ompH	*tbpA*	*toxA*	*fimA*	*nanB*	*sodA*	*oma87*	*ptfA*	*fur*
MNR 5	B	+	+	-	+	+	+	+	+	+
MNR 13	B	+	+	-	+	+	+	+	+	+
MNR 22	B	+	+	-	-	+	+	+	+	+
MNR 30	B	+	+	-	-	+	+	+	+	+
ADDL 1	B	+	+	-	+	+	+	+	+	+
ADDL 16	B	+	+	-	+	+	+	+	+	+
MDK 3	A	+	+	-	+	+	+	+	+	+
MDK 16	B	+	+	-	+	+	+	+	+	+
MDK 22	A	+	-	+	+	+	+	+	+	+
VCC 19	A	+	-	+	+	+	+	+	+	+
SMD 14	A	+	-	+	+	+	+	+	+	+
KLD 4	A	+	+	+	+	+	+	+	+	+
PNT 11	A	+	-	+	+	+	+	+	+	+
PNT 21	A	+	-	+	+	+	+	+	+	+
KZP 8	A	+	+	-	+	+	+	+	+	+
KZP 12	A	+	+	-	+	+	+	+	+	+
Percentage (%)		100	68.75	37.5	87.5	100	100	100	100	100

Table IV. Prevalence of virulence genes among different capsular types of P. multocida isolates.

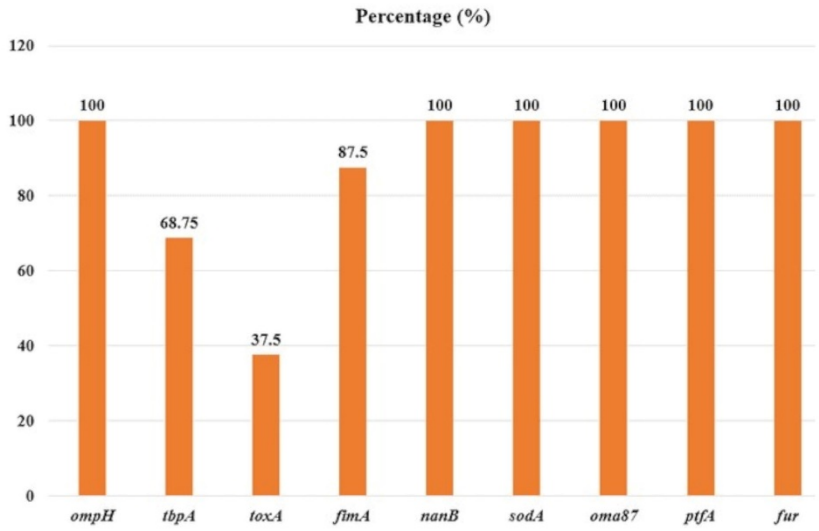

Figure 8. Prevalence of virulence genes among all the P. multocida isolates in the study.

Virulence genes	CapA (n=9)	CapB (n=7)
ompH	100%	100%
tbpA	44.4%	100%
toxA	66.6%	0%
fimA	100%	71.4%
nanB	100%	100%
sodA	100%	100%
oma87	100%	100%
ptfA	100%	100%
fur	100%	100%

Table V. Prevalence of virulence genes among different capsular types of P. multocida isolates.

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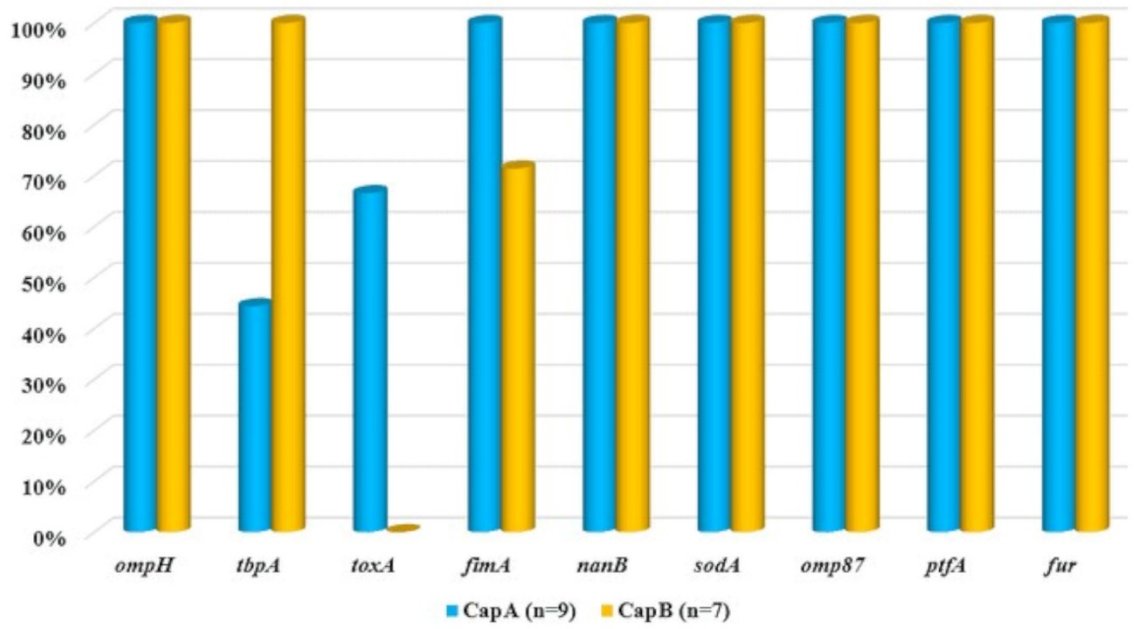

Figure 9. Prevalence of virulence genes among different capsular types of P. multocida isolates in the study.

Discussion

The present study was conducted to investigate the prevalence of *P. multocida* infections and to identify the circulating capsular types in Telangana, India. All the 30 PCR positive samples produced an expected amplicon of ~460 bp similar to the amplicon of standard reference culture of *P. multocida* subsp. *P. multocida* 12945 strain. In agreement with other studies that reported the amplification of ~460 bp fragment specific for *P. multocida* (Dutta et al. 2004, Kumar et al. 2004, Ewers et al. 2006, Rajkhowa et al. 2012, Tahamtan et al. 2014, Al-Maary et al. 2017). Further, they opined that PM-PCR assay can provide rapid, sensitive, and specific identification of *P. multocida* isolates with relatively easier methodology. Also, in the present study the isolation percentage was found to be 8.37%. These results indicate the diagnostic sensitivity of PCR in comparison to isolation and identification of the organisms which is even time consuming. This was in accordance with several other workers (Rajkhowa et al. 2012, Al-Maary et al. 2017, Hussain et al. 2017, Sujatha et al. 2018). Multiplex capsular PCR was found to be a simple, sensitive, rapid, and extremely reliable technique (Al-Maary et al. 2017) The present study revealed the presence of capsular types A (56.25%) and B (43.75%) among the isolates from sheep and goat in the study region. None of the isolates harboured capD, capE or capF. Capsular type A was found to be the predominant type than the capsular type B The high prevalence of capsular types A and D were documented (Ewers et al. 2006, Shayegh et al. 2008, Shayegh et al. 2009, Prabhakar et al. 2010, Tahamtan et al. 2014, Fernandez et al. 2018, Mombeni et al. 2021). Similar to our findings, capB isolates were recovered from pneumonic sheep and goat (Prabhakar et al. 2012, Aski and Tabatabaei 2016). Whereas the presence of capE isolates in sheep were documented (Al-Maary et al. 2017). Although *P. multocida* type A is commonly associated with pneumonic pasteurellosis in small ruminants, the identification of capB isolates from the sheep and goat in the study region warrants the need fora detailed investigation by studying a greater number of samples for developing suitable control strategies. The high prevalence of type B isolates in the sheep and goats in the region warrants the incorporation of both type A and B strains in the vaccine development.

In epidemiological studies now-a-days, virulence associated genes are being extensively targeted for bacterial pathogen detection and differentiation (Prajapati et al. 2020). The overall percentage of virulence genes among the isolates were found to be 100% for *ompH*, *nanB*, *sodA*, *oma87*, *ptfA* and *fur*, 87.5% for *fimA*, 68.75% for *tbpA* and 37.50% for *toxA* among all the isolates. The association of virulence genes to capsular types A revealed 44.44%, 66.66% for *tbpA* and *toxA*, respectively whereas capB isolates revealed 100% for *tbpA*, 71.40% for *fimA* and none of the capB isolates harboured *toxA* gene. In our study, all the isolates (100%) harboured *ptfA* and *nanB* gene irrespective of capsular types indicating the pathogenic potential of the isolates. Similar findings (100%) have also been reported by previous studies on sheep and bovine isolates of *P. multocida* in India (Hatfaludi et al. 2010, Prabhakar et al. 2012, Verma et al. 2013, Sarangi et al. 2015).

The present study observed 100% prevalence of *ompH* and *oma87* among all the capsular types. Similar findings were also reported (Ewers et al. 2006, Tang et al. 2009, Ghanizadeh et al. 2015, Aski et al. 2016, Mombeni et al.

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2021). In contrast to other genes, the prevalence of *tbpA* was found to be 68.75% which was in agreement with the studies (Shayegh et al. 2009). Sarangi et al. (2014) reported prevalence of 80% suggesting significant association of the gene with small ruminant's *P. multocida* isolates. The prevalence of *toxA* gene varied among the capA (37.15%) strains while none of the capB isolates possessed this gene. Similar prevalence rate was documented by (Harper et al. 2006, Ferreira et al. 2012, Ghanizadeh et al., 2015). Whereas (Shayegh et al. 2008, Sarangi et al. 2015, Aski and Tabatabaei et al. 2016, Prajapati et al. 2020) recorded high prevalence (90% -100%) of *toxA* genes among the capA strains and capD strains (Ewers *et al.* 2006). *sodA* gene product is known to destroy radicals which are normally produced within the cells and toxic to biological systems. In the current study, all the capsular types possessed *sodA* gene indicating 100% prevalence of *sodA* and its pathogenic nature. The results of the present findings are in line with those of (Ewers et al. 2006, Aski and Tabatabaei et al. 2016, Mombeni et al. 2021). The prevalence of *fimA* gene which encodes for fimbrillin (*fimA*), a subunit protein of fimbriae and *fur* gene associated with iron uptake in Gram negative bacteria (Van Vliet et al. 1998) was found to be 100% among the isolates which is in accordance with the report of other workers (Tang et al. 2009, Farahani et al. 2019, Prajapati et al. 2020 indicating the pathogenic potential of the isolates.

Conclusion

Pasteurella multocida is implicated with respiratory infections of sheep and goats in the study region. PM-PCR was found to be sensitive in the diagnosis of pasteurellosis than isolation and identification of the organism. Present study recorded high prevalence of *P. multocida* infections among sheep and goats, this warrants immediate emphasis to develop suitable vaccine against pasteurellosis in small ruminants. *P. multocida* capsular types A and B were identified, and type A was found to be predominant than type B capsular types. However, the identification of capsular type B strains in the region warrants the incorporation of both types A and B strains in the vaccine for effective prevention and control of small ruminant pasteurellosis.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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