Phenotypic and Molecular Characterization of the Capsular Serotypes of *Pasteurella multocida* Isolates from Pneumonic Cases of Cattle in Ethiopia

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Abstract

Background: *Pasteurella multocida* is a heterogeneous species and opportunistic pathogen associated with pneumonia in cattle. Losses due to pneumonia and associated expenses are estimated to be higher in Ethiopia with limited information about the distribution of capsular serotypes. Hence, this study was designed to determine the phenotypic and capsular serotypes of *P. multocida* from pneumonic cases of cattle.

Methods: A cross sectional study with purposive sampling method was employed in 400 cattle from April 2018 to January 2019. Nasopharyngeal swabs and lung tissue samples were collected from clinically suspected pneumonic cases of calves (n = 170) and adult cattle (n = 230). Samples were analyzed using bacteriological and molecular assay.

Results: Bacteriological analysis revealed isolation of 61 (15.25%) *P. multocida* subspecies *multocida*. Incidence was higher in calves 35 (57.38%) compared to adult cattle 26 (42.62%) at $P < 0.5$. PCR assay targeting *KMT1* gene (~460 bp) confirmed *P. multocida* species. Capsular typing revealed the presence of serogroup A (*hyaD-hyaC*) gene (~1044 bp) and serogroup D (*dcbF*) gene (~657 bp) from 56 (91.80%) and 5 (8.20%) isolates, respectively. Isolation of *P. multocida* serotype A:3 highlights a new evidence in the study areas. Therefore, the current finding suggests further comprehensive studies on microbial diversity of respiratory infection in cattle to design effective control strategy.

Introduction

The genus *Pasteurella* is a member of the *Pasteurellaceae* family, which includes a large and diverse group of gram-negative Gammaproteobacteria. *Pasteurella* species are highly prevalent and cause several economically important endemic and epizootic diseases in a wide range of animals worldwide (Harper *et al.*, 2006; Christensen and Bisgaard, 2010; Wilson and Ho, 2013). Characteristically, the organism is small (0.2 µm up to 2.0 µm), rods/coccobacilli, capsulated, non-spore-forming, non-motile and bipolar in stain. The bacterium is facultative anaerobic, fermentative and grow best with media supplemented in serum or blood (Kehrenberg *et al.*, 2001; Quinn *et al.*, 2002). Among members of this species *Pasteurella multocida* (*P. multocida*) is the most frequently reported heterogeneous species that causes respiratory disease and hemorrhagic septicemia in cattle (Biberstein and Hirsh, 1999).

P. multocida is a commensal and opportunistic pathogen that resides in the upper respiratory tracts of cattle (Dabo *et al.*, 2007; Taylor *et al.*, 2010). It is a common bacterial pathogen implicated with bovine respiratory disease (BRD), or “shipping fever”, non-septicemic pneumonia (Welsh *et al.*, 2004; Confer, 2009). Clinical manifestations range from asymptomatic or mild chronic upper respiratory inflammation to acute pneumonic and/or disseminated disease (Wilson and Ho, 2013). Pathogenesis of this organism emerges in terms of complex interaction with host specific factors such as age, diet, immune status, environment and bacterial virulence factors (Kehrenberg *et al.*, 2001). The major virulence factors of *P. multocida* are capsular protein and lipopolysaccharide (LPS) (Harper *et al.*, 2006). The other factors may include genes encoding structures such as fimbriae and bacterial adhesins or outer membrane proteins (Corney *et al.*, 2007; Hatfaludi *et al.*, 2010).

Strains of *P. multocida* are classified into five capsular serogroups (A, B, D, E, and F) according to Carter (1955) using indirect hemagglutination test and into 16 somatic or LPS serotypes by Heddlestone gel diffusion precipitation assay (Heddlestone *et al.*, 1972). Polymerase chain reaction (PCR) typing has been applied as a rapid and sensitive molecular method for capsular genotyping using primers designed for species and type specific detection that was unique to all *P. multocida* strains (Townsend *et al.*, 1998). Moreover, multiplex PCR assay was employed as an alternative technique for capsular typing with primers designed following identification, sequence determination, and analysis of the capsular biosynthetic loci of each capsular serogroup (Townsend *et al.*, 2001). Serogroups were further classified into eight LPS genotypes (L1 – L8) according to Harper *et al.* (2015).

Despite the extensive research conducted over several years on respiratory disease of cattle, it continues to result in great economic impact to the cattle industry (Taylor *et al.*, 2010). Previous studies revealed the extent of respiratory disease problems in the country and losses due to mortality, morbidity and associated expenses are estimated to be higher in Ethiopia. In those studies, *P. multocida* was isolated at species level as one of the major bacterial pathogens from nasal, trans-tracheal swab, and pneumonic lung samples using conventional bacteriological methods (Abera *et al.*, 2014; Musteria *et al.*, 2017; Gebremeskel *et al.*, 2017). However, limited information is available regarding the capsular types and genotypes of *P. multocida* isolates circulating in the country due to lower sensitivity of detection based on phenotypic characterization. Hence, molecular advances are indispensable to understand the

capsular Serogroups of *P. multocida* representing severe threats to the cattle population and to compile adequate epidemiological records. Therefore, the present study was designed to determine the phenotypic and molecular characteristics of *P. multocida* associated with pneumonic cases of cattle in Ethiopia.

Materials And Methods

Study area

The study was conducted in selected areas of Ethiopia. Samples were collected from Asosa (10°04'N, 34°31'E), Bale-Robe (7°7'N, 40°0'E), Bishoftu (8°45'N, 38°59'E), Mekele (13°29'N, 39°28'E), and Yabelo (4°53'N, 38°5'E). The areas are located in different agroecological zones of highland and lowland areas from 550 to 2492 meter above sea level (Fig. 1).

Sample size and sample collection

A cross sectional survey with purposive sampling method was employed to collect samples from reported areas of pneumonic cases in cattle. Nasopharyngeal swabs and pneumonic lung tissue samples were collected from veterinary clinics and abattoir, respectively. A total of 400 samples (n = 170) from calves and (n = 230) from adult cattle were collected during the study period from April 2018 to January 2019.

Nasopharyngeal swab sample

Clinical cases of respiratory infection were inspected, and nasopharyngeal swab samples were collected from clinically sick calves and adult cattle using sterile laryngeal swab. Briefly, swab was directed via the ventral nasal meatus into nasopharynx, rotated vigorously for 30 sec at the contralateral side. The swab was retracted and inserted into sterile screw capped test tube containing transport medium of modified Cary-Blair Medium (Park Scientific, UK).

Pneumonic lung sample

Abattoir survey was carried out on cattle slaughtered and lung with pneumonic cases were inspected for irregularity in shape, cranioventral reddening, marbling, non-friable foci, or fibrinous pleuritis (Caswell *et al.*, 2012). Approximately a 3x3 mm piece of lung tissue was taken aseptically from the edge of the lesion and placed in sterile universal bottle. All samples were maintained in cold chain and transported to the Research and Development laboratory of the National Veterinary Institute (NVI) of Ethiopia.

Isolation and phenotypic characterization

P. multocida strains were isolated employing standard bacteriological assay. Briefly, pneumonic lung tissue samples were minced and suspended in 4 ml sterile physiological saline (pH 7.0 ± 0.2) and centrifuged at 3260 x g for 3 min and supernatant was discarded. The sediment was reconstituted with 100 µl sterile physiological saline. Ten µl of the suspension and nasopharyngeal swabs were streaked comparably onto blood agar base (HiMedia, India) with 5% sheep blood and MacConkey agar (HiMedia, India). Plates were incubated at 37°C for 24 – 48 hrs aerobically. Cultural, morphological, and biochemical assay was conducted to identify *Pasteurella* species according to standard procedure (Quinn *et al.*, 2002).

Molecular Characterization

DNA extraction

Genomic DNA was extracted using DNeasy® Blood and Tissue kit (QIAGEN GmbH, Germany) following the manufacturer's instructions. Briefly, 200 µl sample template was transferred into 1.5 ml microfuge tubes. 20 µl proteinase K and 200 µl buffer AL (lysis buffer) was added, mixed and incubated at 56°C for 10 min. 200 µl 96% ethanol was added, transferred into DNeasy mini spin

column with 2 ml collection tube, and centrifuged at 6,000 x g for 1 min. Then, 500 µl buffer AW1 (washing buffer) was added and centrifuged at 6,000 x g for 1 min. Again 500 µl buffer AW2 (washing buffer) was added and centrifuged at 20,000 x g for 3 min. Finally, samples were transferred into 1.5 ml Eppendorf tubes and 50 µl buffer AE (elution buffer) was added, centrifuged at 6,000 x g for 1 min, and the eluted DNA yield was stored at -20°C until PCR assay.

PCR detection of *P. multocida*

PCR assay for *P. multocida* was carried out using species-specific primers. All amplification and sequencing primers were synthesized by Eurofins Genomics, Austria. Primer for the identification of *P. multocida* species was based on previous report by Townsend et al. (1998) and EL-Jakee et al. (2016) as described in (Table 1). PCR reaction mix (20 µl) consisted of 2 µl 5 pmol of each primer, 10 µl IQ super mix (Bio-Rad), 3 µl RNase free water and 3 µl DNA template. Amplification was carried out using thermal cycler (PCRmax™ Alpha Cyclor 2, AC296, UK). Briefly, initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1:30 min, and final extension at 72°C for 7 min.

PCR for capsular typing

The capsular antigens of *P. multocida* was assayed in multiplex PCR using serogroup specific primers targeting serogroups A, B, D, and E as described previously (Townsend *et al.*, 2001). The oligonucleotides used were described in (Table 1). PCR reaction mix was prepared in 40 µl final volume of 6 µl 5 pmol of each primer, 20 µl of IQ super mix, 2 µl RNase free water, and 6 µl template DNA. Amplification was carried out at initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 30 sec, and final extension at 72°C for 7 min.

Agarose gel electrophoresis of PCR products

Amplification of PCR product was carried out in 2% (w/v) agarose gels prepared in 1x Tris borate EDTA (TBE) electrophoresis buffer. Ten µl of each PCR product was mixed with 6x gel loading dye and loaded into separate wells of gel. Ten µl of DNA ladder (100 bp or 1 kb plus, Fermentas) was added into the last lane. Gel electrophoresis was conducted at 120V for 60 min and PCR products were visualized under gel documentation system (UVI TEC, UK) stained with GelRed (Biotium, Inc).

Subspecies and biovar identification

Confirmed *P. multocida* isolates were assigned into subspecies based on sorbitol and dulcitol fermentation (Mutters *et al.*, 1985; kim *et al.*, 2019). Isolates were further classified into their respective biovars based on carbohydrate/sugar fermentation profiles including glucose, lactose, sorbitol, mannitol, trehalose, maltose, xylose, and arabinose) and ornithin decarboxylase (ODC) (Blackall *et al.*, 1997; kim *et al.*, 2019).

Ethical statement

Requirement compliance for animal ethics approval from University and Institution was not required for this study. Due to this reason, samples were collected from animals without experimental intervention. Consent was first obtained from the animal owners before sampling.

Data analysis

Data collected during the study period were analyzed using STATA software version 11. Descriptive statistics was used for analysis and statistical analysis was considered at $P < .05$.

Results And Discussion

Pasteurella species are highly prevalent among animal populations and economically important pathogen that cause wide range of diseases in livestock. *P. multocida* is a normal inhabitant of the upper airway of cattle and calves. It is likely opportunist, associated with acute and chronic infections that can lead to morbidity and mortality any time when lower airway defense mechanisms are compromised (Peek *et al.*, 2018; harper *et al.*, 2006; Davies *et al.*, 2004). The present study was conducted to identify and characterize the most prevalent serotypes of *P. multocida* from pneumonic cases of cattle in Ethiopia using phenotypic and molecular methods. Clinically sick cattle exhibited coughing, high fever ($> 39.5.0^{\circ}\text{C}$), depression, anorexia, nasal discharge, lacrimation, breathing difficulty, and inappetence. Postmortem findings showed irregularity of lung shape, cranioventral reddening, marbling, non-friable foci, and fibrinous pleuritis. However, suspected cases of *P. multocida* pneumonia were confirmed with bacteriological assay.

Cultural and biochemical assay of *P. multocida* was conducted according to standard procedures (Quinn *et al.*, 2002). Presumptive isolates were identified as small, white-creamy, mucoid, smooth, non-hemolytic growth on blood agar, and failed to grow on MacConkey agar after 24 hrs incubation at 37°C (Fig. 2B). Isolates appeared as Gram-negative and small rod/coccobacilli by Gram's staining (Fig. 2A). Biochemical assay of isolates showed positive reaction for catalase, oxidase, indole production, and nitrate reduction but found negative for urease, citrate, methyl red (MR), and Voges-Proskauer (VP). Carbohydrate fermentation assay revealed identification *P. multocida* subspecies *multocida* (Table 2).

Phenotypic and molecular characterizations of *P. multocida* revealed an overall incidence of 61 (15.25%) *P. multocida* subspecies *multocida*. 21 (16.80%) and 8 (10.67%) isolates were recovered from calves and adult cattle nasopharyngeal swab samples, respectively. *P. multocida* incidence from pneumonic lungs of calves showed 14 (31.11%) and 18 (11.61%) from adult cattle (Table 3). *P. multocida* incidence was higher in calves compared to adult cattle ($P < 0.05$) Hence *P. multocida* infection was significantly associated with age of cattle. Previous reports of *P. multocida* incidence ranges from 3.4% to 39.2% in Ethiopia (Abera *et al.*, 2014; Musteria *et al.*, 2017; Gebremeskel *et al.*, 2017). In the present study higher incidence was identified from Bishoftu (25.0%) followed by Bale-Robe (17.50%), Yabelo (16.25%), Mekele (8.75%), and Asosa (8.75%). While individual cases revealed an incidence rate as high as 44.4% from calves' lung in Bishoftu.

In spite of the distribution of *P. multocida* species in Ethiopia, it is not much known about the capsular types and serotypes of *P. multocida* strains associated with pneumonic cases. Presumptive *P. multocida* isolates were confirmed by PCR assay targeting *KMT1* gene fragment of species specific detection. Thus, finding revealed ~460 bp size product in all *P. multocida* isolates (Fig. 3). Although capsular typing confirmed the presence of *hyaD-hyaC* gene (~1044 bp) of serogroup-A in 56 (91.80%) isolates and *dcbF* gene (~657 bp) of serogroup-D in 5 (8.20%) isolates (Fig. 4). The current finding is in accordance with Kong *et al.* (2019) who identified serogroup A as a predominant isolate from cattle. Similarly, Katsuda *et al.* (2013) reported isolation of capsular type A (93.7%), D (6.3%), and Ewers *et al.*, (2006) identified capsular type A (93.2%) and D (3.3%) from cattle.

P. multocida biovar assay revealed *P. multocida* A:3 as a predominant isolate from 48 (78.69%) cases in the study areas. *P. multocida* A:1, A:2, and A:12 were also isolated at rate of 7 (11.47%), 4 (6.56%), and 2 (3.28%), respectively. *P. multocida* A:3 isolates comprised capsular type A 43 (89.58%) and 5 (10.42%) capsular type D. All the rest isolates (A:1, A:2, and A:12) were belonged to capsular type A (Table 3). *P. multocida* capsular type A is a principal bacterial respiratory pathogen in cattle, causing morbidity and mortality with consequent social and economic cost (Hotchkiss *et al.*, 2010; Dabo *et al.*, 2007). Hence, the findings of *P. multocida* capsular type A as the most prevalent pathogen from identified cases calls for further microbiological investigation and assessment of the economic impact of this pathogen at national level.

The present study revealed remarkable evidence in the distribution of *P. multocida* infection in Ethiopia. However, *P. multocida* is not the only pathogen associated with pneumonic cases of cattle and other respiratory disease bacteria, Mycoplasma species, and viruses might involve. Hence, subsequent monitoring on emerging pathogens and serotypes of *P. multocida* is essential for the development of effective control strategy in the country. The current study depicts isolation of *P. multocida* A:3 strain. Thus, the finding highlights the molecular epidemiology of isolates in the study areas. Therefore, microbiological investigation covering wider area of the country, based on outbreak report, should be carried out to assess the serotypes and genotypes of *P. multocida* isolates circulating in the country. Moreover, microbial diversity of pathogens associated with respiratory infection of cattle need to be compiled to make definite conclusion in the improvement of the existing prevention and control strategy.

Conclusion

Although many pathogens are responsible to cause pneumonia in cattle are yet to be determined in Ethiopia. The present study revealed that *P. multocida* A:3 strain is the most common serotype isolated from pneumonic samples of cattle in the study areas. The current finding suggests further comprehensive studies on microbial diversity of respiratory infection in cattle to design effective control strategy.

Abbreviations

BHI: Brain heart infusion; DNA: Deoxyribose nucleic acid; NVI: National Veterinary Institute; PCR: Polymerase chain reaction

Declarations

Ethics approval and consent to participate

This study has been approved by the doctoral committee of Koneru Lakshmaiah Education Foundation, department of biotechnology for requirement compliance of ethical standards in handling and specimen collection from animals. Consent was first obtained from the animal owners before sampling.

Consent for Publication

Not applicable

Availability of data and materials

All data supporting the findings of this study can be obtained from the corresponding author upon formal request. Nucleotide sequence data generated in this study is submitted to GenBank Database to get accession numbers.

Competing Interest

Authors declare that they have no competing interest.

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Authors' contributions

All authors participated in the conception and design of the study; MA conducted all laboratory works and drafted the manuscript. BV, TA, and EG analyzed the data and rigorously revised the manuscript. AB, GD, and TD participated in laboratory work. MY coordinated and revised the paper. All authors read and approved the final manuscript.

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Tables

Table 1. Oligonucleotide sequences used in *P. multocida* PCR assay.

Sero-groups	Gene	Primers	Sequence (5' to 3')	Amplicon size (bp)	Reference
All	KMT1	KMT1T7-F	ATCCGCTATTTACCCAGTGG	460	(Townsend <i>et al.</i> , 1998; EL-Jakeen <i>et al.</i> 2016)
		KMT1SP6-R	GCTGTAAACGAACTCGCCAC		
A	<i>hyaD-hyaC</i>	capA-F	TGCCAAAATCGCAGTCAG	1044	(Townsend <i>et al.</i> , 2001)
		capA-R	TTGCCATCATTGTCACTG		
B	<i>bcbD</i>	capB-F	CATTTATCCAAGCTCCACC	760	(Townsend <i>et al.</i> , 2001)
		capB-R	GCCCGAGAGTTTCAATCC		
D	<i>dcbF</i>	capD-F	TTACAAAAGAAAGACTAGGAGCCC	657	(Townsend <i>et al.</i> , 2001)
		capD-R	CATCTACCCACTCAACCATATCAG		
E	<i>ecbJ</i>	capE-F	TCCGCAGAAAATTATTGACTC	511	(Townsend <i>et al.</i> , 2001)
		capE-R	GCTTGCTGCTTGATTTTGTG		

Table 2. Cultural and biochemical profile of *P. multocida* subspecies *multocida* isolates

Characteristics	<i>P. multocida</i>
Growth on blood agar	Non- haemolytic growth
Growth on MacConkey	No growth
Oxidase	Positive
Catalase	Positive
Ornithin decarboxylase (ODC)	Positive
Indole production	Positive
Nitrate reduction	Positive
Urease	Negative
Methyl Red (MR)	Negative
Citrate	Negative
Voges-prokauer (VP)	Negative
Glucose	Positive
Sucrose	Positive
Dulcitol	Negative
Mannitol	Positive
Maltose	Negative
Sorbitol	Positive
D-xylose	Positive

Table 3. Isolation frequency of *P. multocida* from study areas

Study animal	Sample		<i>P. multocida</i> isolates and study area					Total	x ² square (P-value)
	Type	Size	Asosa	Bale-Robe	Bishoftu	Mekele	Yabelo		
Calves	Swab	25/125	8.0%	16.0%	28.0%	12.0%	20.0%	16.8%	0.02
	Lung	9/45	22.2%	33.3%	44.4%	22.2%	33.3%	31.11%	
Total		34/170	11.76%	20.59%	32.35%	14.7%	23.53%	20.59%	
Adult cattle	Swab	15/75	-	20.0%	26.67%	-	6.67%	10.67%	
	Lung	31/155	9.68%	12.9%	16.13%	6.45%	12.9%	11.61%	
Total		46/230	6.52%	15.22%	19.56%	4.35%	10.87%	11.30%	
Total		80/400	8.75%	17.5%	25.0%	8.75%	16.25%	15.25%	

Table 4. Biovar characteristics of the current *P. multocida* isolates.

Sugar Fermentation									Biovar type	Isolation percentage
Trehalose	Galactose	D-Xylose	Arabinose	Mannitol	Lactose	Sorbitol	Maltose	Glucose		
+	+	+	-	+	+	+	-	+	1	7 (11.47%)
+	+	+	-	+	-	+	-	+	2	4 (6.56%)
-	+	+	-	+	-	+	-	+	3	48 (78.69%)
-	+	+	-	+	+	+	-	+	12	2 (3.28%)

Figures

Figure 1

Map of Ethiopia indicating study areas where samples were collected; 1- Asosa; 2- Bale-Robe; 3- Bishoftu; 4- Mekele; and 5- Yabelo. The map was sketched using ArcGIS 9 software (ArcMap™ version 9.3, California, USA)

Figure 2

P. multocida isolate; (A) Gram staining smear and (B) non-hemolytic growth on blood agar.

Figure 3

Agarose gel electrophoresis of PCR amplified product of KMT1 gene (~460 bp) specific for *P. multocida*. Lane M: 100 bp DNA ladder; lane P: Positive control; lanes 1-13: tested samples; lane E: extraction control; lane N: negative control.

Figure 4

Agarose gel-electrophoresis of PCR amplified of serogroup A *hyaD-hyaC* gene (~1044 bp). Lanes 1 to 3; lanes 7 to 14 are sample positive; lanes 4 to 6 and 13 are sample negative; lane E: extraction control; lane N: negative control; lane P1 and P2: Positive control; lane M: 1 kb plus DNA ladder.