

Phenotypic and Molecular Characterization of the Capsular Serotypes of *Pasteurella Multocida* Isolates From Bovine Respiratory Disease Cases in Ethiopia

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Research article

Keywords: Antibigram, Biovar, Capsular type, Cattle, Ethiopia, *Pasteurella multocida*

DOI: <https://doi.org/10.21203/rs.3.rs-61749/v1>

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Abstract

Background: *Pasteurella multocida* is a heterogeneous species and opportunistic pathogen that causes bovine respiratory disease. This disease is one of an economically important disease in Ethiopia. Losses due to mortality and associated expenses are estimated to be higher in the country. Studies revealed that limited information is available regarding the capsular types, genotypes, and antimicrobial sensitivity of *P. multocida* isolates circulating in the country. This suggests, further molecular advances to understand the etiological diversity of the pathogens representing severe threats to the cattle population.

Results: Bacteriological analysis of nasopharyngeal swab and pneumonic lung tissue samples collected from a total of 400 samples revealed isolation of 61 (15.25%) *P. multocida* subspecies *multocida*. 35 (20.59%) were isolated from calves and 26 (11.30%) from adult cattle. Molecular analysis using PCR assay targeting *KMT1* gene (~460 bp) amplification was shown in all presumptive isolates. Capsular typing also confirmed 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. The biovar typing identified 48 (78.69%) isolates of biovar 3. Phylogenetic analysis based on the *hyaD-hyaC* gene nucleotide sequences showed that *P. multocida* serogroup A are genetically closely related to the different *P. multocida* isolates retrieved from the GenBank. Antibiogram of isolates revealed high sensitivity towards chloramphenicol (100%), amoxicillin (95.08%), and amikacin (90.16%). Resistance was observed in cloxacillin (88.52%) and Penicillin-G (77.05%).

Conclusions: In the present study, *P. multocida* capsular type A biovar 3 was predominantly isolated from pneumonic cases in cattle in the study areas. The finding provides useful information for further research and choice of antimicrobials for treatment. Hence, further comprehensive molecular epidemiological investigation is proposed covering wider areas of the country to identify pathogens associated with BRD for an effective control strategy.

Background

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 [1,2,3]. 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 [4,5,6]. 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 [7, 6].

P. multocida is a commensal and opportunistic pathogen that resides in the upper respiratory tracts of cattle [8,9]. It is a common bacterial pathogen implicated with bovine respiratory disease (BRD), or “shipping fever”, non-septicemic pneumonia (10,11). Clinical manifestations range from asymptomatic or mild chronic upper respiratory inflammation to acute pneumonic and/or disseminated disease [3]. 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 [4]. The major virulence factors of *P. multocida* are capsular protein and lipopolysaccharide (LPS) [1]. The other factors may include genes encoding structures such as fimbriae and bacterial adhesins or outer membrane proteins [12,13]. However, the presence of *P. multocida* in the upper respiratory tract of animals is not always associated with disease [14,15].

Strains of *P. multocida* are classified into five capsular serogroups (A, B, D, E, and F) according to Carter [16] using indirect hemagglutination test and into 16 somatic or LPS serotypes by Heddleston gel diffusion precipitation assay [17]. 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 [18]. 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 [19]. Serogroups were further classified into eight LPS genotypes (L1 – L8) according to Harper et al. [20].

Despite the extensive research conducted over several years on BRD, it continues to result in great economic impact to the cattle industry [9]. Previous studies revealed the extent of respiratory disease problems in Ethiopia and losses due to mortality, morbidity and associated expenses are estimated to be higher in the country [21]. 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 [22,23,24,25]. 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 etiological diversity of the pathogens representing severe threats to the cattle population and to compile adequate molecular epidemiology records. Therefore, the present study was designed to determine the phenotypic and genotypic characteristics of *P. multocida* associated with BRD and to evaluate the antimicrobial susceptibility of isolates from Ethiopian cattle.

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 (550 to 2492 meter above sea level) as shown in (Fig. 1).

Sample size and sample collection

A cross sectional survey with purposive sampling method was employed to collect samples. 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 [26]. 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 and phenotypic characterization was carried out on *Pasteurella* presumptive colonies.

Antimicrobial susceptibility test

All *P. multocida* isolates were tested for antimicrobial susceptibility against twelve types of antibiotics using the disc diffusion method (Quinn *et al.*, 2002). Chloramphenicol (C 30 µg), Amoxicillin (AMC 30 µg), Amikacin (AK 30 µg), Oxytetracycline (OT 30 µg), Streptomycin (S 25 µg), Tetracycline (TE 30 µg), Clindamycin (DA 10 mcg), Polymyxin-B (PB 300 units), Erythromycin (E 15 µg),

Ampicillin (AMP, 10 µg), Penicillin-G (P 10 units), and Cloxacillin (CX 5 µg) disks (HiMedia, India) were evaluated. Clinical and laboratory standard institute breakpoint [27] was used for interpretation of the results.

Molecular Characterization

Bacterial culture preparation

Presumptive isolates of *P. multocida* were streaked on brain heart infusion (BHI) agar (Oxoid, UK) enriched with 10% horse serum. Plates were incubated at 37°C for 24 - 48 hrs aerobically. Loopful colonies of each of the pure isolates were transferred into a sterile 1.5 ml Eppendorf tubes and washed twice in nuclease free water. Cell suspension was centrifuged at 16,100 x g for 3 min and 1 ml of cell suspension was heated at 100°C for 10 min to optimize whole-cell DNA templates for PCR assay.

DNA extraction

Genomic DNA was extracted using DNeasy® Blood and Tissue kit (QIAGEN GmbH, Germany) following the manufacturer's instructions. Briefly, 200µl 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. [18] and Jakeen et al. [28] 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 [19]. 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.

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	[18, 28]
		KMT1SP6- R	GCTGTAAACGAACCTCGCCAC		
A	<i>hyaD-hyaC</i>	capA-F	TGCCAAAATCGCAGTCAG	1044	[19]
		capA-R	TTGCCATCATTGTCAGTG		
B	<i>bcbD</i>	capB-F	CATTTATCCAAGCTCCACC	760	[19]
		capB-R	GCCCGAGAGTTTCAATCC		
D	<i>dcbF</i>	capD-F	TTACAAAAGAAAGACTAGGAGCCC	657	[19]
		capD-R	CATCTACCCACTCAACCATATCAG		
E	<i>ecbJ</i>	capE-F	TCCGCAGAAAATTATTGACTC	511	[19]
		capE-R	GCTTGCTGCTTGATTTTGTC		

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 [29, 30]. 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) [31, 30].

Nucleotide sequencing

PCR amplified *P. multocida* specific DNA band was purified using Wizard® SV Gel and PCR product purification kit (Promega, Germany) following the manufacturer's instructions. Concentration of the purified PCR product was quantified using Nanodrop 2000c spectrometer (Thermo Scientific, USA). The concentration was adjusted according to the instruction recommended by the sequencing company (LGC Genomics, Germany). The resulting sequences were edited, and contig was formed using Vector NTI 11.5 software (Invitrogen). Consensus sequences were generated and aligned using BioEdit 7.1.3.0. Degree of sequence similarity search was conducted using BLAST program (NCBI).

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 and lung samples from abattoir slaughtered animals. Consent was first obtained from the animal owners before sampling.

Data Analysis

Data were analyzed using Graph Pad Prism version 8.4.1 and statistical analysis was considered at $P < 0.05$. Molecular evolutionary genetic analysis (MEGA) version 7 was used for sequence analysis and ClustalW multiple for sequence alignments [32].

Phylogenetic relationships among the current isolates with isolates from other geographical areas were determined by phylogenetic trees construction using the Neighbor-Joining algorithm.

Results

Isolation and phenotypic characterization

Clinically sick cattle exhibited coughing, high fever, depression, anorexia, nasal discharge, lacrimation, breathing difficulty, and inappetence. Nasopharyngeal swab samples revealed 21/125 (16.80%) and 8/75 (10.67%) strains of *P. multocida* from calves and adult cattle, respectively. Pneumonic lung inspection at abattoir showed irregularity of lung shape, cranioventral reddening, marbling, non-friable foci, and fibrinous pleuritis. *P. multocida* incidence from pneumonic lungs was 14/45 (31.11%) from calves and 18/155 (11.61%) from adult cattle (Table 2). Statistical analysis of T-test showed significant association between age and isolation frequency of *P. multocida* ($P < 0.05$).

Table 2. Isolation frequency of *P. multocida*.

Study animal	Sample		<i>P. multocida</i> isolates					Total	T- test (P-value)
	Type	Size	Asosa	Bale-Robe	Bishoftu	Mekele	Yabello		
Calves	Swab	25/125	8.0%	16.0%	28.0%	12.0%	20.0%	16.8%	0.0378
	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%	

Cultural and biochemical characteristics

P. multocida presumptive colonies were identified as small, white-creamy, mucoid, and smooth. Colonies were showed non-hemolytic growth on blood agar (Fig. 2B) and failed to grow on MacConkey agar. Isolates revealed Gram negative and small rod/coccobacilli by Gram's staining (Fig. 2A). Biochemical profile of isolates showed positive reaction for catalase, oxidase, indole production, and nitrate reduction but found negative for urease. Isolates produce acid from sorbitol and glucose but not from dulcitol, lactose, and maltose. *P. multocida* subspecies *multocida* were identified and showed higher incidence in Bishoftu (25.0%) followed by Bale-Robe (17.50%), Yabelo (16.25%), Mekele (8.75%), and Asosa (8.75%).

Antimicrobial susceptibility

P. multocida isolates showed high susceptibility to chloramphenicol (100%), amoxicillin (95.08%), amikacin (90.16%), oxytetracycline (88.52%), streptomycin (83.61%), tetracycline (78.69%), clindamycin (77.05%) and polymyxin-B (67.21%). Intermediate resistance was recorded to erythromycin (62.30%) and ampicillin (55.74%). Isolates were resistance to Penicillin-G (77.05%) and cloxacillin (88.52%) showed in Figure 3 ($P < 0.0001$).

PCR assay

All presumptive *P. multocida* isolates were confirmed by PCR assay and revealed amplification product of approximately 460 bp band size for species specific detection (Fig. 4). *P. multocida* capsular typing confirmed the presence of *hyaD-hyaC* gene of serogroup A specific and amplified product showed approximate band size around 1044 bp in 56 (91.80%) isolates (Fig. 5).

Subspecies and biovar identification

The identification of biovars of the capsular types among the studied *P. multocida* isolates described in Table 3. All 61 isolates were identified as *P. multocida* subspecies *multocida*, which produce acid from sorbitol and glucose but not from dulcitol, lactose and maltose. Ornithin decarboxylase (ODC) producing isolates belonged to biovar 3 (78.69%), followed by biovars 1 (11.47%), biovars 2 (6.56%), and biovars 12 (3.28%). Biovar 3 isolates comprised capsular type A (70.49%) and D (8.20%), whereas Biovars 1, 2, and 12 comprised capsular type A (Table 3).

Table 3. Biovar characteristics of the current *P. multocida* isolates

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

Sequence analysis

The *hyaD-hyaC* gene nucleotide sequences of three Ethiopian outbreak isolates (Assosa, Bale Robe and Bishoftu) and 26 sequences retrieved from the GenBank were used. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option was computed using MEGA version 7 software. The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches (Fig. 6).

Discussion

Bovine respiratory disease (BRD) is an infectious respiratory disease of cattle with multifactorial causes that encompasses multiple viral and bacterial pathogens, a multitude of environmental and management factors and their interactions are responsible for the outbreak of disease [33, 34]. The bacterial pathogens associated with BRD include *P. multocida*, *Mannheimia haemolytica*, *Mycoplasma* species, *Histophilus somni*, and *Trueperella Pyogens* [35, 36]. *Bibersteinia trehalosi* is also considered as an emerging pathogen to cause BRD but not a primary pathogen to cause BRD [36]. Though, bacterial pathogenic diversity has yet to be further investigated; the current study emphasized on isolation and molecular characterization of *P. multocida* from BRD cases to show the prevalence and its contribution among all other pathogens in the study areas.

The present study revealed remarkable evidence in the distribution and occurrence of *P. multocida* infection in different agroecological regions of Ethiopia. Clinical findings and lung lesion are consistent with clinical features associated with BRD as reported in previous studies [8, 36]. In the current study, *P. multocida* was recovered from 61 (15.25%) of BRD cases. Isolation based on age showed 20.59% and 11.30% was identified from calves and adult cattle. This finding is higher than previous report of 3.34% [22] and lower than Musteria *et al.*, [24] reported 39.2%. Similar finding 13.29% was reported by Gebremeskel *et al.* [25]. As is evident from the study, clinically sick calves showed (16.80%, 31.11%) and adult cattle (10.67%, 11.61%) *P. multocida* strains from nasopharyngeal and pneumonic lungs, respectively. Calves are more prone to *P. multocida* infection than adult cattle ($P < 0.05$) and this may be due to prior exposure of adult cattle to the pathogen [8, 9].

Isolation and identification of *P. multocida* based on the cultural and biochemical characters revealed the preponderance of *P. multocida* subspecies *multocida* from pneumonic cases of cattle. Higher incidence was identified in Bishoftu (25.0%) followed by Bale-Robe (17.50%), Yabelo (16.25%), Mekele (8.75%), and Asosa (8.75%). Though, the overall incidence of *P. multocida* in the current study was 15.25%, individual cases revealed an incidence rate as high as 44.4% from calves' pneumonic lung tissue in Bishoftu. Analysis of *P. multocida* infection in recent years indicated that the frequency of this pathogen ranges from 3.4% to 39.2% [22, 24, 25] in different study areas of Ethiopia. *P. multocida* could be found in mixed infections of the lung along with *M. haemolytica*, *H. somni*,

T. pyogenes, *B. trehalosi*, *Mycoplasma* species, or various viruses of cattle [36]. Thus, the current finding suggests further investigation to isolate and identify other pathogens associated with bovine pneumonic cases.

In previous studies, in spite of the distribution of *P. multocida* species [22, 24, 25] it is not much known about the capsular types and serotypes of *P. multocida* strains associated with BRD in Ethiopia. Capsular types and serotypes of *P. multocida* differ based on the type of disease and host species affected. In this study, Capsular typing revealed that *P. multocida* serogroup A (91.80%) is the predominant strain followed by serogroup D (8.20%). This finding is consistent with Kong et al. [37] who identified serogroup A as a predominant isolate from cattle. In similar study, Katsuda et al. [38] detected capsular type A (93.7%), D (6.3%), and Ewers et al., [39] identified capsular type A (93.2%) and D (3.3%) from cattle. Higher incidence of capsular type A (99.3%) has also been reported by Davies et al. [40] and lower prevalence of serogroup A (68.19%) and D (22.72%) has been identified from cattle and buffalo [41].

The current finding showed that *P. multocida* capsular type A biovar 3 was identified as the predominant isolate in the study areas, which account for 78.69% followed by biovar A:1, A:2, and A:12 at rate of 11.47%, 6.56%, and 3.28%, respectively. Phylogenetic analysis based on the *hyaD-hyaC* gene nucleotide sequences showed that the three *P. multocida* serogroup A current isolates from Ethiopia (Assosa, Bale Robe and Bishoftu) are genetically closely related to the different *P. multocida* isolates retrieved from the GenBank. The nucleotide sequences of the current isolates submitted to the GenBank to get gene accession number.

In this study, *P. multocida* isolates were found to be highly susceptible to chloramphenicol (100%), amoxicillin (95.08%), and amikacin (90.14%). Susceptibility was also observed in oxytetracycline (88.52%), streptomycin (83.61%), tetracycline (78.69%), and clindamycin (77.05%). Isolates revealed an intermediate resistance to erythromycin (61.30%) and ampicillin (55.74%). Isolates were resistance to Penicillin-G (77.05%) and cloxacillin (88.52%). Similar results were reported by Musteria et al. (2017), where susceptibility of chloramphenicol (89.4%) and tetracycline (80.9%) were recorded. Ampicillin (53.2%) was found as an intermediate drug. Penicillin-G (10.6%) and streptomycin (14.9%) were inefficient, while vancomycin was inactive against isolates. Likewise, study by Abera *et al.* [22] showed isolates of *P. multocida* were susceptible to amoxicillin, chloramphenicol, cephalexin, polymyxin-B, kanamycin and florfenicol; however, moderate resistance was observed to tetracycline, erythromycin and Penicillin-G.

Isolation and distribution of *P. multocida* serotypes vary in different areas over time. Therefore, subsequent monitoring on emerging serotypes of *P. multocida* is essential for the development of effective control strategy. In Ethiopia, a monovalent vaccine prepared from inactivated *P. multocida* capsular type B at the National Veterinary Institute (NVI) has been used for long period of time. Despite the use of this vaccine, there is high rate of morbidity and mortality following respiratory infection in different parts of the country [21]. This may be due to limited protection of the vaccine for heterologous serotypes of *P. multocida* strains [42, 43]. Although the findings in the current study which depicts the frequent isolation of *P. multocida* serogroup A is by no means conclusive, it highlights useful information vis-à-vis the use of monovalent vaccine comprising *P. multocida* serogroup B for the control of pneumonic pasteurellosis in Ethiopia. Hence, serotyping and genotyping of isolates and genetic relatedness is required to compile information on molecular epidemiology of *P. multocida* and other pathogens causing BRD in the country for the improvement of the existing prevention and control strategy.

Conclusion

Although many pathogens are responsible for majority of the cases which are yet to be determined, the current study revealed that *P. multocida* capsular type A biovar 3 is the most common serogroup associated with bovine respiratory cases in the study areas. The current finding suggests further comprehensive study on the possible pathogens involved in precipitating BRD at national level. The current finding provides useful information for further research, improvement of currently used vaccine, and choice of antimicrobials for treatment.

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.

Funding

Laboratory analysis of this research was supported financially by National Veterinary Institute (NVI) of Ethiopia.

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.

Acknowledgments

The authors highly acknowledge the animal owners and abattoir staff for allowing samples collection. Great appreciation forwarded to the Research and Development laboratory of the National Veterinary Institute of Ethiopia for providing the required facilities and support to conduct this research. Appreciation goes to the Joint FAO/IAEA Division of the Animal Production and Health Section, Austria for covering the sequencing cost. The authors also thank the Department of Biotechnology, University of Koneru Lakshmaiah Education Foundation (KLEF) for supporting the study.

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Figures

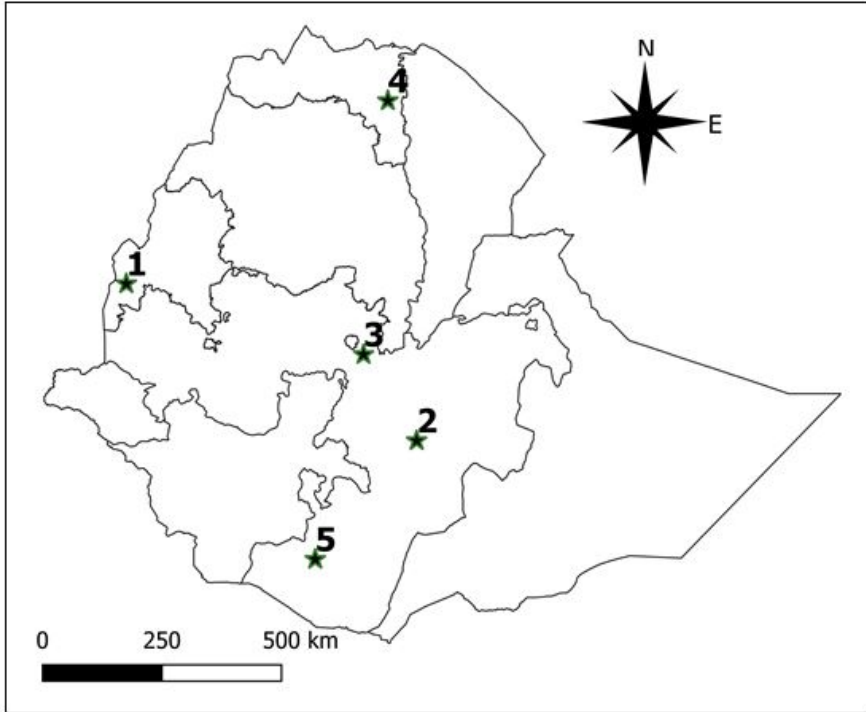


Figure 1

Map of Ethiopia indicating 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)

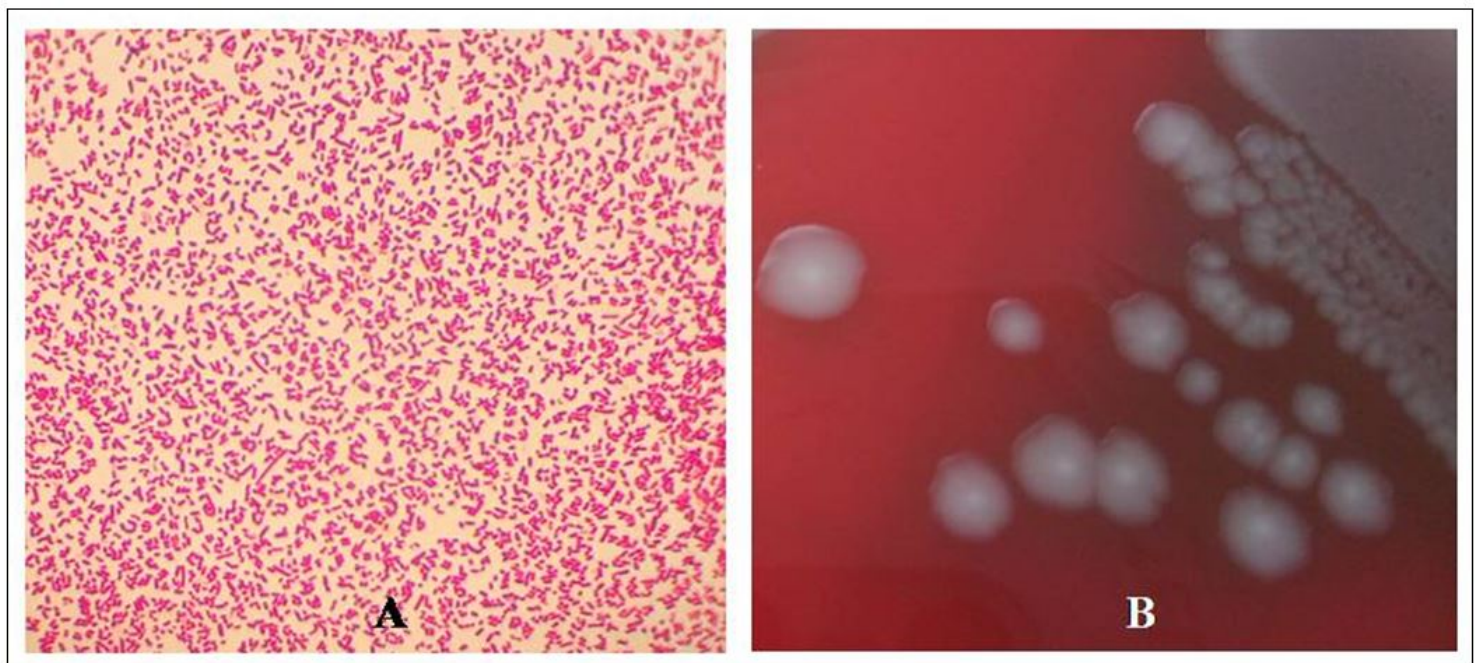


Figure 2

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

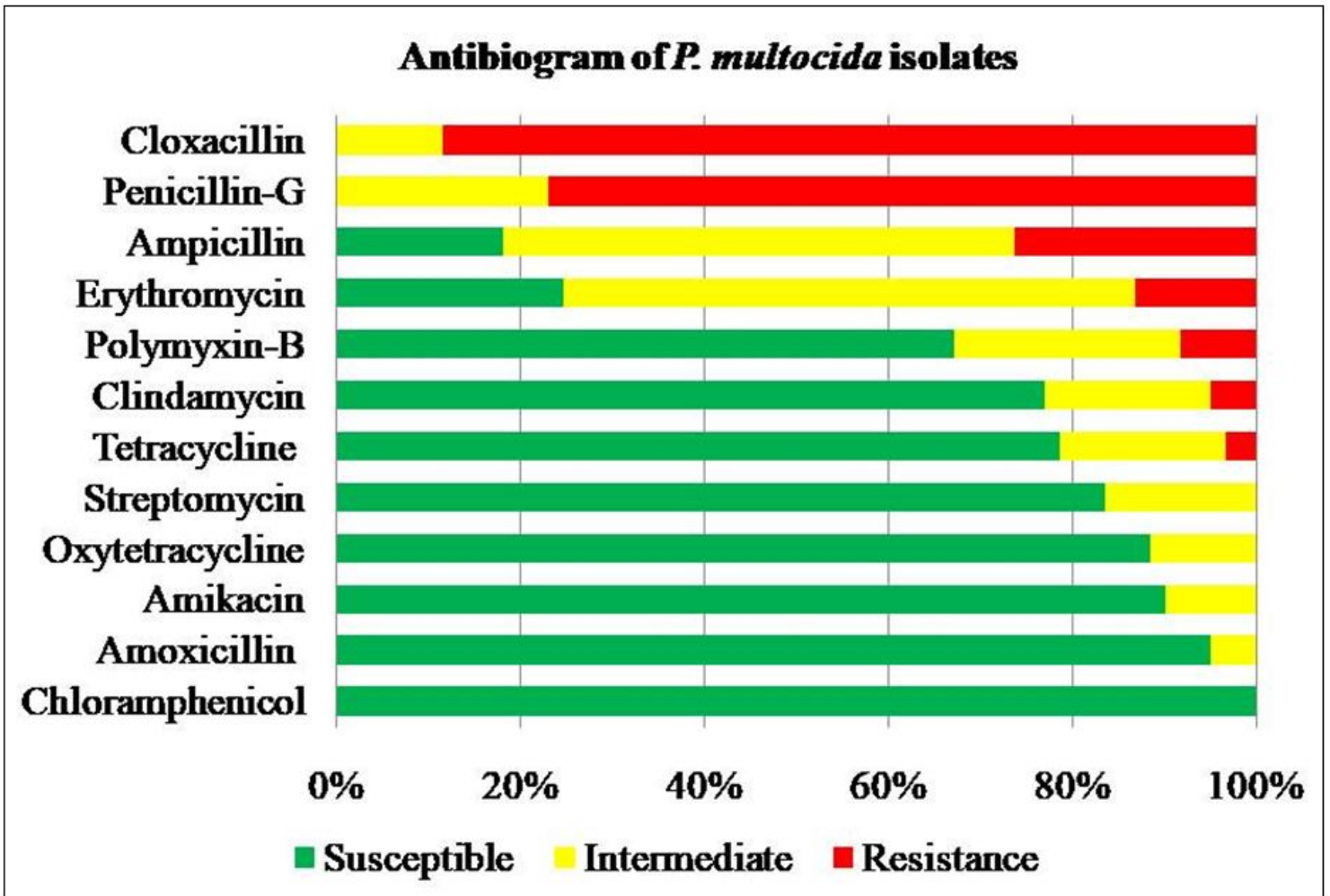


Figure 3

Antimicrobial susceptibility pattern of *P. multocida* isolates.

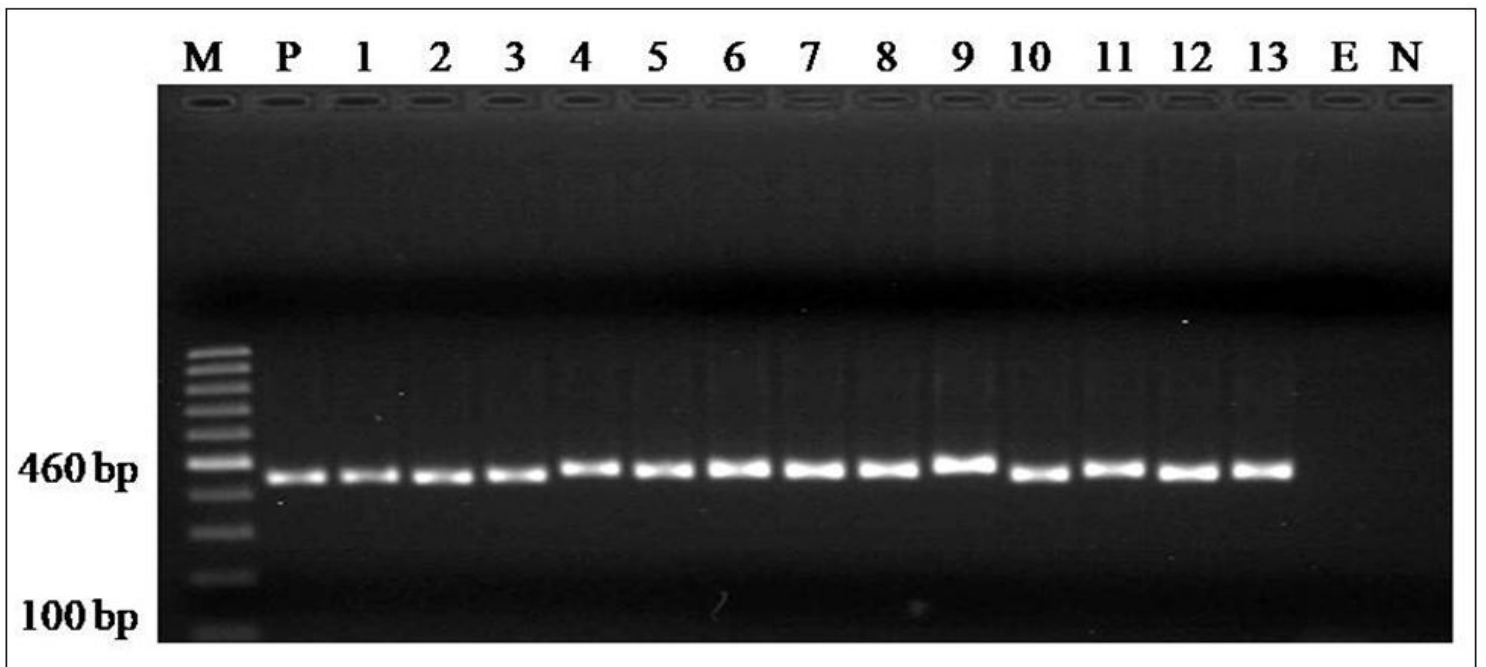


Figure 4

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.

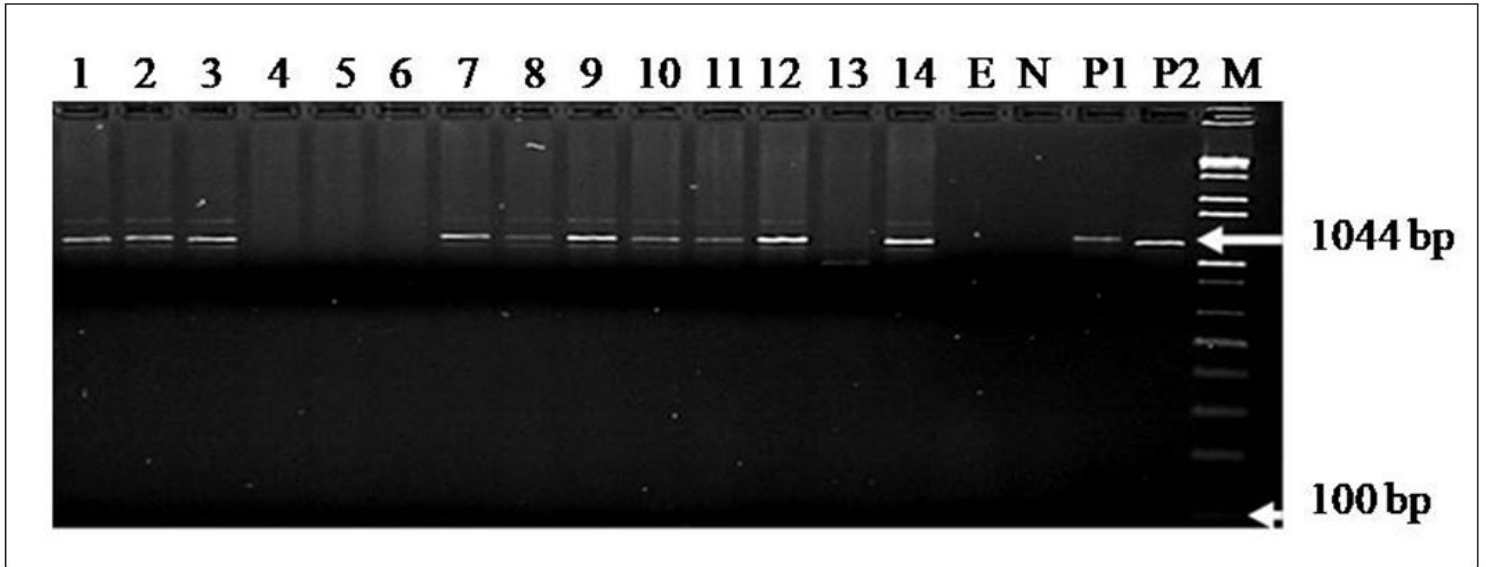


Figure 5

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



Figure 6

Phylogenetic analysis of the 29 *P. multocida* isolates based on the nucleotide sequences of the *hyaD-hyaC* gene (approximately 1044 nt). The isolates sequenced in this study are indicated with colored rectangle.