Phenotypic and Molecular Characterization of the Capsular Serotypes of *Pasteurella multocida* Isolated 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 biotypes and capsular serotypes of *P. multocida* from pneumonic cases of cattle.

**Methods:** A cross-sectional study with a 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 the bacteriological and molecular assay.

**Result:** Bacteriological analysis revealed isolation of 61 (15.25%) *P. multocida* subspecies *multocida*. Molecular 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) in 56 (91.80%) and 5 (8.20%) isolates, respectively. Isolation of *P. multocida* A:3 highlights its prevalence in the study areas. Therefore, the current finding suggests further comprehensive studies on *P. multocida* capsular types and microbial diversity of respiratory infection in cattle to design an effective control strategy.

**Key words:** Biovar, Capsular type, Cattle, Ethiopia, *Pasteurella multocida*.

**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 widely prevalent and cause several economically important endemic and epizootic diseases in a wide range of animals worldwide (Harper et al., 2006; Wilson and Ho, 2013). Characteristically, the organism is small (0.2 µm up to 2.0 µm), rods/cocccobacilli, capsulated, non-spore-forming, non-motile and bipolar in a stain. The bacterium is facultatively anaerobic, fermentative and grows best with media supplemented in serum or blood (Quinn et al., 2002). Among members of this species *Pasteurella multocida* (*P. multocida*), 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 (Dabo et al., 2007).

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 Hedleston gel diffusion precipitation assay (Hedleston 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, a multiplex PCR assay was employed as an alternative technique for capsular typing with primers designed following the 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 the respiratory disease of cattle, it continues to result in a great economic impact on 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.
Pasteurella multocida was isolated at species level as one of the major bacterial pathogens from the nasal, trans-tracheal swab and pneumonic lung tissue 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 Pasteurella multocida isolates circulating in the country. Hence, molecular advances are indispensable to understand the capsular Serogroups of Pasteurella multocida representing severe threats to the cattle population in Ethiopia. Therefore, the present study was designed to determine the biotypes and capsular serotypes of Pasteurella 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 agro-ecological zones of highland and lowland areas from 550 to 2492 meters above sea level (Fig 1). Bacteriological and molecular assays were carried out at National Veterinary Institute (NVI), Ethiopia.

Sample size and sample collection

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

Nasopharyngeal swab sample

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

Pneumonic lung sample

An 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. Approximately a 3x3 mm piece of lung tissue was taken aseptically from the edge of the lesion and placed in a sterile universal bottle. All samples were maintained in a cold chain and transported to the Research and Development laboratory of the NVI, Ethiopia.

Isolation and phenotypic characterization

Pasteurella 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 (3260 x g for 3 min at +4°C) and the 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. The 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.

PCR detection of Pasteurella multocida

PCR assay for Pasteurella multocida was carried out using species-specific primers. All amplification and sequencing primers were synthesized by Eurofins Genomics, Austria. Primer for the identification of Pasteurella multocida species was based on a previous report (Townsend et al., 1998; Jonathan and Arora, 2012) and primers were depicted in (Table 1). PCR reaction mix (20 µl) consisted of 2 µl 5 pmol of each primer, 10 µl IQ supermix (Bio-Rad), 3 µl RNase free water and 3 µl DNA template. Amplification was carried out using a thermal cycler (PCRmax™ Alpha Cycler 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 Pasteurella multocida were 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 supermix, 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 the 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 _Pasteurella multocida_ isolates were assigned into subspecies based on sorbitol and dulcitol fermentation. Isolates were further classified into their respective biovars based on carbohydrate/sugar fermentation profiles including glucose, lactose, sorbitol, mannitol, trehalose, maltose, xylose and arabino)se) and ornithine decarboxylase (ODC) (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 were used for analysis and statistical analysis was considered at _P < 0.05_.

### Table 1: Oligonucleotide sequences used in _Pasteurella multocida_ PCR assay.

| Sero-groups | Gene | Primers | Sequence (5' to 3') | Amplicon size (bp) | Reference |
|-------------|------|---------|--------------------|--------------------|-----------|
| All         | KMT1 | KMT1T7- F, KMT1SP6- R | ATCCGCTATTTCACCGATGAG, GCTGTAACGGAAACTGGCCAC | 460 | (Townsend et al., 1998; Jonathan and Arora, 2012) |
| A           | hyaD-hyaC | capA-F, capA-R | TGCCCAACTGAGTGTCAG, TGCCCATGTTGTCAGT | 1044 | (Townsend et al., 2001) |
| B           | bcbD  | capB-F, capB-R | CATTATCCAGTCCAC, GCCCGAGGTTTCAATC | 760 | (Townsend et al., 2001) |
| D           | dcbF  | capD-F, capD-R | TTAAACAGAAGACGAGGCCC, CATCTACCCACTCAACCATACTCAG | 657 | (Townsend et al., 2001) |
| E           | ecbJ  | capE-F, capE-R | TCGCGAGAAAAATTTGACTC, GCTGTCGTTGATTTGT | 511 | (Townsend et al., 2001) |

**RESULTS AND DISCUSSION**

_Pasteurella_ species are highly prevalent among animal populations and economically important pathogen that causes diseases in a wide range of hosts. _P. multocida_ is opportunist, associated with acute and chronic infections that can lead to morbidity and mortality any time when lower airway defense mechanisms are compromised (Harper et al., 2006; Peek et al., 2018). The present study was conducted to identify and characterize the most prevalent capsular serotypes of _P. multocida_ from pneumatic cases of cattle in Ethiopia using standard bacteriological and molecular methods. Clinically sick cattle exhibited coughing, high fever (> 39.5-0°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_ associated pneumatic samples were confirmed with the bacteriological assay.

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 pneumatic lungs of calves showed 14 (31.11%) and 18 (11.61%) from adult cattle (Table 2). _P. multocida_ incidence was higher in calves compared to adult cattle (_P < 0.05_). 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%), and Asosa (8.75%). While individual cases revealed an incidence rate as high as 44.4% from calves’ lung in Bishoftu.

Despite 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 pneumatic cases in cattle. Presumptive _P. multocida_ isolates were confirmed by PCR assay targeting _KMT1_ gene fragment of species-specific detection. Thus, the finding revealed ~460
Phenotypic and Molecular Characterization of the Capsular Serotypes of *Pasteurella multocida* Isolated from Pneumonic...

bp size product in all *P. multocida* isolates (Fig 2). 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 3). 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 the 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 48 (78.69%) *P. multocida* biovar type 3 as a predominant isolate and comprised 43 (89.58%) capsular type A isolates and 5 (10.42%) capsular type D. Moreover, *P. multocida* A:1, A:2 and A:12 were isolated at a rate of 7 (11.47%), 4 (6.56%) and 2 (3.28%), respectively (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 (Dabo *et al.*, 2007). Hence, the findings of *P. multocida* capsular type A:3 as the most prevalent pathogen from identified cases calls for further microbiological investigation and assessment of the economic impact of this pathogen at the national level.

The present study revealed remarkable evidence in the distribution of *P. multocida* capsular types 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 an 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 a wider area of the country, based on the outbreak report, should be carried out to assess the serotypes and genotypes of *P. multocida* isolates circulating in the country.

Table 2: Frequency of isolation of *P. multocida* from study areas.

| Study area     | Calves (n = 170) | Adult cattle (n = 230) | Total (n = 400) | X²-square (P-value) |
|---------------|------------------|------------------------|----------------|---------------------|
|               | Swab (n = 125)   | Lung (n = 45)          | Swab (n = 75)  | Lung (n = 155)      |
| Asosa         | 2 (8.0%)         | 2 (22.2%)              | -              | 3 (9.6%)            | 7 (8.75%) |
| Bale-Robe     | 4 (16.0%)        | 3 (33.3%)              | 3 (20.0%)      | 4 (12.9%)           | 14 (17.5%) |
| Bishoftu      | 7 (28.0%)        | 4 (44.4%)              | 4 (26.67%)     | 5 (16.13%)          | 20 (25.0%) 0.02 |
| Mekele        | 3 (12.0%)        | 2 (22.2%)              | -              | 2 (6.45%)           | 7 (8.75%) |
| Yabelo        | 5 (20.0%)        | 3 (33.3%)              | 1 (6.67%)      | 4 (12.9%)           | 13 (16.25%) |
|               | 21 (16.8%)       | 14 (31.1%)             | 8 (10.67%)     | 18 (11.61%)         | 61 (15.25%) |

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

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

Fig 2: 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: test samples; lane E: extraction control; lane N: negative control.
Moreover, the microbial diversity of pathogens associated with respiratory infection of cattle need to be compiled to make a 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. Therefore, the current finding suggests further comprehensive studies on *P. multocida* capsular types covering wider area of the country to understand its impact and assessment of microbial diversity of respiratory infection in cattle to design an effective control strategy.

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