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Isolation and Characterization of *Pasteurella spps* from Pneumonic Cases of Livestock in Three Regional States of Ethiopia: Evidence of Differences between Field and Vaccine Biotypes

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ABSTRACT

Pneumonic pasteurellosis is the leading cause of recurrent morbidity and mortality in ruminant livestock in Ethiopia. Its control is mainly done using an annual vaccination with a monovalent whole broth culture of *Pasteurella (P.) multocida.* However, the multiplicity of the serotypes circulating in the field and the lack of cross-protective immunity hinder the effectiveness of the vaccination program warranting the development of a vaccine with better efficacy. To this effect, the identification, and characterization of the strains from different regions of the country is necessary. In this paper, Pasteurella organisms collected from camels, cattle, goats, and sheep with respiratory signs suggestive of pasteurellosis in the Afar, Tigray, and Benishangul Gumuz regional states were isolated, and characterized. From clinically pneumonic cases, 793 nasal swabs (286 goats, 276 sheep, 168 camels, and 63 cattle) were collected aseptically and cultured on Blood Agar. Bacteria pathogens were identified at the species level by biochemical tests. Culture positivity was 29.3% (243/793). The isolation frequencies of *B. trehalosi*, *M. haemolytica*, and *P.multocida* were 47.7% (116/243), 43.2% (105/243), and 9.1% (22/243), respectively. A higher isolation rate was observed in sheep (37.4%), and the lowest in cattle (6.2%). Mixed infection with *B. trehalosi* and *M. haemolytica* was observed in sheep, goats, and camels. Despite the higher frequencies of isolation of *B. trehalosi* and *M. haemolytica* from all host species, the vaccine currently being used in Ethiopia consists of only *P. multocida* biotypes A for sheep and goats and biotype B for cattle. Moreover, camels are not considered in the pasteurellosis vaccination program in the country. Therefore, the result of the study suggests the need to include *B. trehalosi* and *M. haemolytica* in the vaccine preparation as well as underlines the relevance of considering camels in the vaccination program.

Keywords: Field strains, Vaccine strains, Pneumonic pasteurellosis, Ethiopia.

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1. INTRODUCTION

Respiratory infectious diseases in ruminant livestock are the most significant health constraints halting poor farmers' socio-economics advancement in Sub-Sahara Africa (Tadesse et al., 2017). Pneumonic pasteurellosis is the primary respiratory disease of ruminants, which is responsible for recurrent mortality, morbidity, and substantial economic and production losses (Abdullah et al., 2015; Legesse et al., 2018). The economic impact of pneumonia represents 8% of the total production charges, including medical expenses, poor feed conversion rate, increased production costs, and the declined availability of food for humans (Rico et al., 2017).

In Ethiopia, pasteurellosis is the most over-diagnosed respiratory disease (Ayelet et al., 2004; Tadesse et al., 2017). It is a complex disease that develops when stress factors and concurrent respiratory infections compromise the immune system of the animal. The primary causative agents of pneumonic pasteurellosis include *Mannheimia (M.) haemolytica, Pasteurella (P.) multocida, and Bibersteinia (B.) trehalosi*. Evidence shows that the agents are more frequently isolated from pneumonic animals than animals without pneumonia (Ekong et al., 2014; Afata, 2018), with reported mortality rates significantly higher in lowland agroecologies with minimal infrastructures (Tsegaye et al., 2013). The presence of uncontrolled animal movement and transportation, intensification of animals in quarantines for live animal export, recurrent drought conditions, animal feed shortage, irrational use of drugs, and poor animal health extension systems have been identified as risk factors for the occurrence and severity of the diseases (Tadesse et al., 2017).

Control of pneumonic pasteurellosis through antimicrobial therapy is a difficult task due to drug resistance, and the cost of treatment is beyond the reach of an average farmer (Legesse et al., 2018). According to OIE (2012), vaccination is the recommended control measure against pasteurellosis. In Ethiopia, control of the disease is done through annual vaccination by whole broth culture of *P. multocida* biotype A and B, respectively, for sheep and goats, and cattle (NVI 2020). However, evidence shows frequent outbreaks of the disease in vaccinated sheep and goats in different countries' regional states (Catley et al., 2009). The diversity of serotypes and lack of cross-protective immunity hinder effective vaccine development (Ayalew et al., 2006). In a vaccine trial experiment, against cross-infection with *P. haemolytica* A9 in animals vaccinated with the OMPs of *P. haemolytica A2*, the antibody responses were more specific toward the homologous challenge but generally did not cross-protect against heterologous serotype challenge. On the other hand, the OMPs of *P. haemolytica* A7 was effective in protecting animals against

homologous and heterologous infection by live *P. haemolytica* A2, A7 and A9. Isolation and characterizing the field circulating pathogens is a prerequisite for designing an effective multivalent vaccine. Therefore, this study aimed to isolate and characterize pneumonic pasteurellosis-causing organisms of sheep, goats, cattle and camels collected from selected districts of Afar, Benishangul-Gumuz, and Tigray National Regional States of Ethiopia.

2. MATERIALS AND METHODS

2.1. Description of the Study Areas

This study was conducted in three Ethiopian regional states: Afar, Benishangul Gumuz and Tigray, with different agro-ecological zones (Fig 1), selected based epidemiologic importance of the disease locally and across the international boundaries with Sudan, Eritrea, and Djibouti. Benishangul Gumuz represents a wet ecological zone, whereas Tigray and Afar regions represent arid and semi-arid ecological zones (Gummadi et al., 2018). Afar region is characterized by pastoral (90%) and agropastoral (10%) production systems and is dominated by lowland areas (Simenew et al., 2013). Benishangul Gumuz exhibits an agropastoral production system, whereas a mixed crop-livestock production system dominates the Tigray region. Districts with frequently reported outbreaks within the regions got special attention. Peasant associations within the study district were selected based on convenience and reported disease importance from veterinary field experts.

2.2. Study Design, Sampling Method, and Study Animal Population

This study employed a cross-sectional study design from December 2017 to April 2020. Animals with visible respiratory signs visiting veterinary clinics or water drinking points were considered for sampling irrespective of age, sex, and physiological status (Table 1). None of the animals had a vaccine history for at least nine months before the date of sample collection. The sample size distribution across the regions and animal species was affected based on the dominant animal reared, field level restraining condition, and willingness of owners for the species of animal to be sampled.

The minimal clinical symptoms that define an animal with respiratory syndromes include nasal discharge (serous, purulent, bloody), nasal stuffiness, cough, and fever (Fig 2A). Animals free of these symptoms were considered apparently healthy; hence they were excluded in this study.

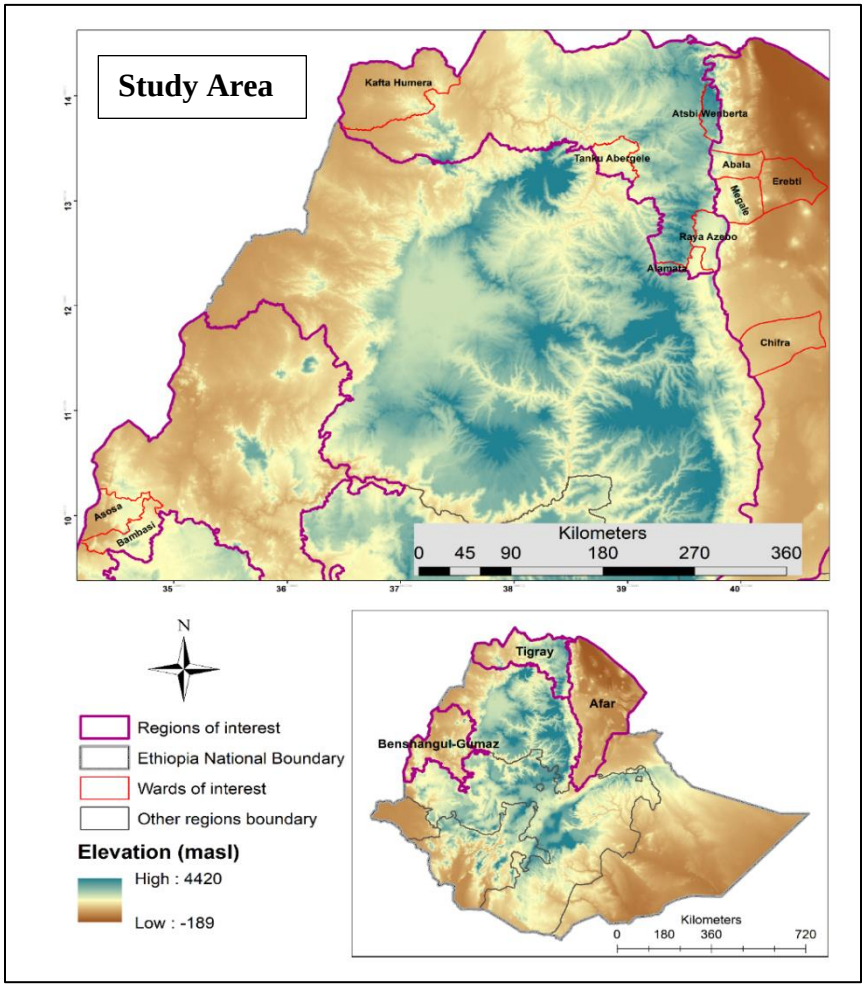

Figure 1. Map of study regions (Afar, Benishangul Gumuz and Tigray) and districts (Source: The map was developed by authors using ArcGIS version 10.3.1).

Table 1. Sample size distribution across districts and animal species

Region	***Study district***	***Sheep***	***Goats***	***Cattle***	***Camels***
Afar	Aballa	21	42	0	60
	Chifra	8	14	0	0
	Erebti	2	23	0	0
	Magaale	0	0	0	54
Benishangul Gumuz	Assosa	26	52	0	0
	Bambassi	44	31	0	0
Tigray	Tanqua Abergelle	36	32	0	0
	Atsbi-womberta	48	10	13	0
	Kafta-Humera	25	42	20	0
	Raya Alamata	19	24	15	0
	Raya Azebo	47	16	15	54
Total		**276**	**286**	**63**	**168**

2.3. Sample Collection and Transportation

Sterile cotton-tipped swabs with applicator sticks were first moistened with nutrient broth (Oxoid, Hampshire, England), transport medium, and were inserted into the nostrils of each camel, cattle, goat, and sheep. Prior to specimen collection, to remove debris and external secretions, the nostril of each animal was thoroughly rubbed with a cotton gauze soaked in 99% savalon. Then, samples were collected by gently rotating the swab 15-20 cm deep in the animals' noses and keeping it in contact with the secretions for up to one minute (Fig 2B) (Marru et al., 2013; Legesse et al., 2018). Nasal swabs were subsequently immediately dipped into the nutrient broth (transport media), labelled, and placed in an icebox. All samples were transported to the laboratory in an icebox and stored at +4°C for not more than 48 hours.

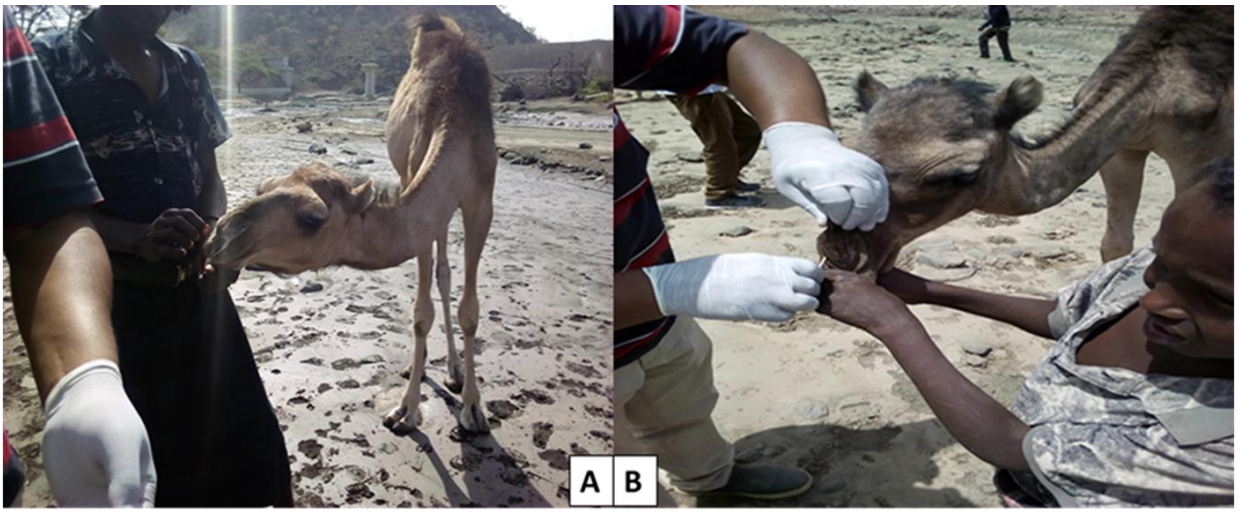

Figure 2. (A) Camel with nasal discharge, (B) Nasal swab sample collection from a Camel.

2.4. Isolation and Identification of Pasteurella Organisms

2.4.1. Culture and Isolation

Swabs containing nutrient broths were incubated at 37°C for 24 hours and used for bacterial isolation. Loop-fulls of each gently vortexed broth culture were streaked on blood agar (Titan Biotech Limited, Bhiwadi) supplemented with 7% sheep blood and incubated aerobically at 37°C for 24 hours. Based on Gram's staining reaction and potassium hydroxide (KOH) string test, gram-negative bacteria were selected. From culture-positive plates, typical colonies were sub-cultured into blood agar to get pure colonies. Further analysis was made based on the general appearance of colonies (morphology, colour, shape, size, and consistency) and lactose fermentation on

MacConkey agar (Titan Biotech Limited, Bhiwadi). *P. multocida* doesn't grow on MacConkey agar, and *M. haemolytica* ferment lactose, whereas *B. trehalosi* doesn't ferment lactose. Primary and secondary biochemical tests were performed on pure colonies sub-cultured on nutrient agar (Quinn et al., 2002; PHE, 2015) (Table 2).

Table 2. Primary and secondary biochemical test characteristics of isolated bacteria.

Biochemical test types	***M. haemolytica***	***B. trehalosi***	***P. multocida***
OxidaseOxidas test	+	+	+
Catalase	+	+	+
Indole	-	-	+
Urease	-	-	-
Motility SIM	-	-	-
O-F test	-	-	-
L-Arabinose	+	-	-
D-Trehalose	-	+	-
Maltose	+	+	-
Sucrose	+	+	+

+: test positive; -: test negative

2.4.2. Biochemical Tests

Primary biochemical tests including catalase, oxidase, Oxidation Fermentation (O-F), indole, and motility tests were performed. The secondary biochemical tests were conducted by urease and sugar fermentation tests (sucrose, maltose, D+-trehalose, and L+ arabinose). All the sugar fermentation tests were prepared in O-F basal medium at 1% concentration and evaluated for the production of acids as described by Villard et al. (2008) and PHE (2015). Further interpretation of both primary and secondary biochemical tests was made according to Quinn et al. (2002).

2.4.3. Preservation of Isolates

Pure isolates were inoculated into 2 ml Brain Heart Infusion (BHI) broth enriched with **1**0% glycerol. Cultures were allowed to grow overnight at 37°C and cryopreserved at -20°C till future use (Brockbank et al., 2001).

2.5. Data Management and Analysis

Data related to animal species, study regions, and bacterial isolates were collected regularly and managed using a Microsoft Excel version 2013 spreadsheet. Statistical analysis was made using STATA version 15 statistical software. Descriptive statistics were used to determine the frequency

of isolates across regions and animal species. Chi-square (χ^2) was used to assess the significance of statistical associations between predictor variables (animal host species and regional states) and the outcome variables (*Pasteurellaceae* isolates). Multivariable logistic regression analysis was performed to determine the strength of the association between predictor and outcome variables. At all levels, $p<0.05$ was considered statistically significant. The Hosmer-Lemeshow test was used to evaluate the fitness of data in logistic regression analysis. In this test, we assessed how well the logistic regression model fits the observed data by comparing the expected and observed frequencies in different groups.

3. RESULTS

The three species of the target bacteria were isolated from the four animal host species within this study. The overall isolation rate of at least one of the Pasteurellacae organisms from the sampled animals was 29.3% (232/793). The isolation rate was 47.6% in camel, 33.0% in sheep, 23.8% in cattle, and 19.9% in goats. Out of the 243 isolates, *B. trehalosi* was the dominant (47.7%, 116/243), followed by *M. haemolytica* (43.2%, 105/243). Eleven animals (seven sheep, two goats, and two camels) had a mixed infection of *B. trehalosi* and *M. haemolytica*. *B. trehalosi* was the dominant isolate of goats, sheep, and camels, whereas *M. haemolytica* was the dominant cattle isolate (Table 3).

Table 3**.** Proportion of pasteurellacae organisms isolated from pneumonic cases of animals.

Host animal species	***Number of animals sampled***	***Animals positive for at least one of the species of bacteria***	***Species of bacterial isolates***				***Number of animals with mixed infection of M. haemolytica and B. trehalosi***
			B. trehalosi	***M. haemolytica***	***P. multocida***	***Total isolates per animal host species***	
Goats	286	55 (19.2%)	29 (25%)	23 (21.9%)	5 (22.7%)	57 (23.5%)	2
Sheep	276	84 (30.4%)	48(41.4%)	40 (38.1%)	3 (13.6%)	91 (37.4%)	7
Camels	168	78 (46.4%)	34(29.3%)	33 (31.4%)	13(59.1%)	80 (32.9%)	2
Cattle	63	15 (23.8%)	5 (4.3%)	9 (8.6%)	1 (4.5%)	15 (6.2%)	0
Total	**793**	**232 (29.3%)**	**116 (47.7%)**	**105 (43.2%)**	**22 (9.1%)**	**243 (100%)**	11

All the Pasteurella isolates were fermentative, positive for oxidase, catalase, and sucrose, and negative for urease and motility tests. *M. haemolytica* isolates were L-arabinose fermentative, whereas *B. trehalosi* isolates were D-Trehalos fermentative.

A statistically significant difference in isolation rates of the three bacteria species among regions and animal species was observed. The isolation rate of *B. trehalosi* showed a statistically significant association among regions ($\chi^2 = 48.71$, $p < 0.001$) and host species ($\chi^2 = 12.79$, $p < 0.005$). Similarly, the isolation rate of *M. haemolytica* had a statistically significant variation among regions ($\chi^2 = 12.42$, $p = 0.002$) and host species ($\chi^2 = 13.16$, $p = 0.004$). Unlike the other two bacterial species, the isolation rate of *P. multocida* showed statistically significant variation only with host species ($\chi^2 = 19.70$, $p < 0.001$) (Table 4). However, in a multivariable logistic regression analysis with an adjusted odds ratio (AOR), the predictor variable sampled region showed a statistically significant association with the isolation rate of the organisms. The odds of *B. trehalosi* isolation in Afar and Benishangul-Gumuz regional states were 5.03 and 3.66 times higher than Tigray region.

Table 4. Chi-square test result of *Pasteurella species* against study regions and animal species.

Variable	***Category***	***B. trehalosi***		***M. haemolytica***		***P. multocida***	
		Yes (%)	***χ^2 (p-value)***	***Yes (%)***	***χ^2 (p-value)***	***Yes (%)***	***χ^2 (p-value)***
Region	Tigray (n=416)	27 (6.5)	48.71 (<0.001)	63 (15.1)	12.42 (0.002)	7 (1.7)	5.64 (0.060)
	Afar (n=224)	58 (25.9)		35 (15.6)		11 (4.9)	
	Benishangul (n=153)	31 (20.3)		7 (4.6)		4 (2.6)	
Animal Species	Cattle (n=63)	5 (7.9)	12.79 (0.005)	9 (14.3)	13.16 (0.004)	1 (1.6)	19.70 (<0.001)
	Goat (n=286)	29 (10.1)		23 (8.0)		5 (1.8)	
	Sheep (n=276)	48 (17.4)		40 (14.5)		3 (1.1)	
	Camel (n=168)	34 (20.2)		33 (19.6)		13 (7.7)	

Note: Yes means, the bacteria was isolated.

Similarly, the odds of isolation of this species from camel were 2.94 times higher than its isolation rate from cattle. Unlike *B. trehalosi,* the odds of the isolation rate of *M. haemolytica* in the Benishangul Gumuz region was 0.27 times lower than in the Tigray region. The odds of the isolation rate of *P. multocida* in the Afar region was 3.02 times higher compared in the Tigray region (Table 5).

Table 5. Multivariable logistic regression analysis of *Pasteurella species* against study regions and animal species.

Variable	***Category***	***B. trehalosi***		***M. haemolytica***		***P. multocida***	
		Yes (%)	***AOR (95%CI)***	***Yes (%)***	***AOR (95%CI)***	***Yes (%)***	***AOR (95%CI)***
Region	Tigray (n=416)	27 (6.5)	Ref	63(15.1)	Ref	7(1.7)	Ref
	Afar (n=224)	58 (25.9)	5.03 (3.08-8.22)	35 (15.6)	1.04 (0.66-1.62)	11 (4.9)	3.02 (1.15-7.90)
	Benishangul (n=153)	31 (20.3)	3.66 (2.10-6.37)	7 (4.6)	0.27 (0.12-0.60)	4 (2.6)	1.57 (0.45-5.43)
Animal Species	Cattle (n=63)	5 (7.9)	Ref	9 (14.3)	Ref	1 (1.6)	Ref
	Goat (n=286)	29 (10.1)	1.31 (0.49-3.52)	23 (8.0)	0.52 (0.23-1.20)	5 (1.8)	1.10 (0.12-9.61)
	Sheep (n=276)	48 (17.4)	2.44 (0.93-6.41)	40 (14.5)	1.02 (0.47-2.22)	3 (1.1)	0.68 (0.07-6.66)
	Camel (n=168)	34 (20.2)	2.94 (1.10-7.91)	33 (19.6)	1.47 (0.65-3.26)	13 (7.7)	5.20 (0.67-40.60)

Note: Yes means, the bacteria was isolated.

4. DISCUSSION

Pathogen isolation and characterization is the central point of marker selection for diagnosis and vaccine. In this study, *Pasteurella species* were isolated and characterized from cattle, sheep and goats, and camels in Tigray, Afar and Benshangul Gumuz. Once the dominant biotypes are fully known, the intension was to cryopreserve isolates for future use in the development and evaluation of local biotyoes for vaccine and diagnosis.

The percentage of isolation for at least one major bacterial agent of pasteurellosis was 29.3%. This finding was lower compared to previous studies conducted by Marru et al. (2013) in Haramaya district, Eastern Hararghe, Ethiopia, and Alemneh and Tewodros (2016) in Fogera district, Northwest Ethiopia, which reported rates of 40.6% and 55.9%, respectively. Conversely, higher isolation rates were reported by Legesse et al. (2018) in central Ethiopia and Haftu et al. (2014) from pneumonic lungs of small ruminants slaughtered in Gondar, with an isolation rate of 36.84% and 48.28%, respectively. However, the isolation rate in this study was higher than the findings reported by Sadia et al. (2016) at 21.1%, from nasal swabs of small ruminants in the east Shewa zone of Oromia region and Aditi et al. (2014) at 8.51%, in Bedelle district of western Ethiopia. This variation could be ascribed to the fact that we isolated the Pasteurella species only from animals exhibiting visible clinical respiratory symptoms, whereas they included apparently health animals in their studies. Moreover, the influence of environmental factors, host

characteristics, and climatic differences should not be overlooked as potential factors contributing to these differences.

The higher isolation rate of *B. trehalosi* and *M. haemolytica* in the present study agreed with previous reports (Marru et al., 2013; Afata, 2018). Similarly, the higher isolation rate of *B. trehalosi* compared to *M. haemolytica* was consistent with Sisay and Zerihun's (2003) finding in South Wollo, North East Ethiopia, and Assefa and Kelkay (2018) from Kola Tembien and Tanqua Abergelle districts of Tigray regional state. The relatively higher isolation rate of *B. trehalosi* in this study may be due to its ability to grow faster and shorter doubling time (10 min versus 27 min) than *M. haemolytica*, enabling this organism to achieve a higher final cell density. In line with this, it has been reported that the logarithmic Colony Forming Unit per ml (CFU/ml) count for *B. trehalosi* at 24 hours was three times more elevated than that of *M. haemolytica* (Bavananthasivam et al., 2012). Growth inhibition of *M. haemolytica* by several bacterial species is also likely to contribute to the infrequent detection of this bacterium compared to *B. trehalosi* (Kugadas et al., 2014).

The relative lesser isolation of *P. multocida* in this study could be explained by the fact that *M. haemlytica* and *B. trehalosi* dominate in the upper respiratory tract (nasal swab) while *P. mulotocida* dominates nasopharynx to bronchi of the respiratory system (Ackermann and Brogden, 2000; Mohamed and Abdelsalam 2008). Previous studies reported that *P. multocida* occasionally causes pneumonic pasteurellosis (Assefa and Kelkay, 2018). On the other hand, a higher rate of isolation of *P. multocida* from goats, sheep, and cattle was reported in various areas of Ethiopia (Assefa and Kelkay, 2018). Isolation of *P. multocida* from the lung is higher than nasal swabs (Mohamed and Abdelsalam, 2008). Moreover, the difference in transport media used (Tefera and Smola, 2002; Sisay and Zerihun, 2003), and area-specific predominance of isolates could be additional factors that contributed to this variation. The relatively lower isolation rate of *P. multocida* should not be underestimated due to its pathogenic potential and its possible role in the aetiology and pathogenesis of pneumonia (Berhe et al., 2017; Hailu et al., 2017).

Mixed isolates of *B. trehalosi* and *M. haemolytica* were obtained from sheep, goats, and camels. Still, there was none for mixed isolates of *B. trehalosi* and *P. multocida* or *M. haemolytica* and *P. multocida*. In a culture media, a proximity-dependent inhibition of the growth of *M. haemolytica* by *B. trehalosi* (Dassanayake et al., 2010) and *P. multocida* (Bavananthasivam et al.,

2012) was reported. Repeated outbreaks due to mixed infection by *M. haemolytica, B. trehalosi,* and *P. multocida* have been reported (Assefa and Kelkay, 2018).

This study indicated the importance of camels in the epidemiology of the disease. The isolation rate of pasteurellosis agents in camels (46.4%) was higher than in other host species. With their browsing behaviour, goats consume relatively uncontaminated matter, so less exposure to infection may have a lower prevalence (Marru et al., 2013; Daphal et al., 2018). The alveolar surface-to-metabolic weight ratio in sheep is lower than in other species (Omer et al., 2012) and may explain the higher respiratory disease occurrence. In line with this study, *B. trehalosi, P. multocida,* and *M. haemolytica* were isolated from lung samples of camels in Egypt (Abo-Elnaga and Osman, 2012) and Nigeria (Abubakar et al., 2010).

5. CONCLUSION

In this study, *M. heamoltyica, B. trehalosi,* and *P. multocida* were isolated and preserved for future use. Though a higher isolation rate of all Pasteurella species was recorded in camels than other host species, unfortunately, these animals are not usually included in the pasteurellosis vaccination strategy of the country. Hence, it is of paramount importance to have them in the vaccine biotypes. In addition, considering the higher isolation rate of the organism from camels, including camels in the regular pasteurellosis vaccination program is warranted. Despite the higher isolation rate of *M. hemolytica* and *B. trehalosi,* Ethiopia's existing vaccine against pasteurellosis contains only formalin-killed whole-cell bacterin of *P. multocida*. Hence, designing a new country-level or area-specific vaccine against pasteurellosis should encompass predominant isolates circulating in the country. Limitation of this study was that isolates were not further characterized through serotype-specific markers for serology, molecular, or Biolog analysis.

6. ACKNOWLEDGEMENTS

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Ethiopia, the former Ministry of Science and Technology (MoST), now the Ministry of Innovation and Technology (MiNT).

7. CONFLICT OF INTEREST

No conflict of interests.

Availability of data and material

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable

Authors contributions

BH, HTM, BA, AD, MM, MY, and AB collected samples and processed them in the laboratory. BH and HTM analysed data and wrote the first draft manuscript. BH, HTM, BDF, AB, GG, AG, and SK have conceived the study and contributed to the write-up of the manuscript. All have read and approved the final manuscript.

Ethics approval and consent to participate

Full ethical approval for sample collection and preservation was obtained from the College of Veterinary Sciences of Mekelle University and the Animal Research Ethical Review Committee of Addis Ababa University. Experienced and qualified professionals collected samples. Animals were handled with kindness and proper care by minimising discomfort, distress, or pain.

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