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Bacteriological and Molecular Identification of *Mannheimia haemolytica, Pasteurella multocida* and *Bibersteinia trehalosi* from Cattle and Sheep from Selected Areas of Ethiopia

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Abstract

Pneumonic pasteurellosis is a multi-factorial respiratory disease of cattle and sheep caused by combination of etiologic agents; hence, reliable information is needed on the inventory of *Pasteurella* species or serotypes for optimum control of the disease. This study was conducted with the objectives of isolation and identification of *Pasteurella* organisms from cattle and sheep using conventional and molecular methods. Cross-sectional studies were carried out on pneumonic lungs collected from abattoirs and nasal swabs from cattle and sheep brought to Asella, Holota and Sheno veterinary clinics for various reasons. Twenty five percent, 26.5% and 23.5% of nasal swabs collected from Asella, Holota and Sheno, respectively yielded *Pasteurella* species. In all the study sites *Mannheimia haemolytica* was more frequently isolated than *Pasteurella multocida* and *Bibersteinia trehalosi*. The frequency of isolation of *Pasteurella* species is higher in young animals than adults (OR = 1.56; 95 % CI: 1.02, 2.38); in pneumonic animals than those with no signs of pneumonia (OR = 4.67; 95 % CI: 3.03, 7.19) and in animals under intensive management than those managed extensively (OR = 2.46; 95 % CI: 1.12, 5.39). From a total of 176 pneumonic lung samples (93 cattle and 83 sheep) collected and investigated *Pasteurella* species were isolated from 27.27 % (48) of them. *Mannhemia haemolyitica* is the predominant isolate from pneumonic lungs. Molecular characterization also confirmed the identity of *P. multocida* and *M. haemolytica*. In addition, the serovars of *P. multocida* isolates identified were A1 and A3. This study revealed that *M. haemolytica*, *P. multocida* and *B. trehalosi* are commonly circulating in cattle and sheep originated from various parts of the country. Besides, the serotypes of *P. multocida* identified are those known to cause pneumonia in ruminants.

INTRODUCTION

Pneumonic pasteurellosis is frequently the cause of a considerable economic loss in cattle and sheep resulting from mortality and morbidity [1,2]. Although mortality is observed less frequently, losses due to morbidity is more common. In cattle it is commonly seen in feed lot animals, accounting for approximately 30% of mortality worldwide. In North America for instance, it has caused an annual loss of more than USD one billion in beef industry [3]. In sheep it occurs under both intensive and extensive conditions [4]. It is multi-factorial disease caused by interplay of environmental, husbandry and biotic factors such as viral and bacterial agents [5], although serotypes of *Mannhemia haemolyitica* (*M. haemolyitica*) [6,7], and *Pasteurella multocida* (*P. multocida*) [8], have been considered the leading causes. The disease occurs worldwide but the distribution serotypes involved in causing disease can vary [9,10]. In southern Africa *M. haemolytica* serotype 6, 9 and 2 are associated with pneumonic pasteurellosis in sheep and goats whereas serotype 1 is the predominant cause of the disease in cattle [4].

In Ethiopia pneumonic pasteurellosis has been a topic of frustration to veterinary professionals and a topic of liability to ruminant producers. Only limited studies focusing on the isolation of the bacterial agents from pneumonic animals has been carried out. Our knowledge of the composition and distribution of serotypes of bacteria involved in the outbreaks is limited. Effective control of pneumonic pasteurellosis needs the understanding of the epidemiology of the component causes. Identification of the serotypes of the bacterial agents is the first step in the understanding of the disease complex. The objective of this study was to isolate and identify serotypes of *P. multocida* and *M. haemolytica* from cattle and sheep in Ethiopia. First field survey was carried out to isolate and identify serotypes of *P. multocida* and *M. haemolytica* from pneumonic and non-pneumonic cattle and sheep presented to various veterinary

clinics in central Ethiopia. Second, abattoir survey was done for isolation and identification of serotypes of *P. multocida* and *M. haemolytica* from pneumonic lungs of cattle and sheep slaughtered in two export abattoirs and one Municipal abattoir.

MATERIALS AND METHODS

Field Survey

Study areas: The field study was carried out in Holota, Asella and Sheno, central Oromia, Ethiopia (Figure 1).

Holota is located 40 Km west of the capital, Addis Ababa at 9˚30'N and 38˚30'E having an elevation ranging from 2300 - 3800m above sea level. On average the temperature of Holota ranges from 6 to 22ºC. The annual rainfall of the area ranges from 900 - 1100 mm. Holota is known for its potential for livestock production hosting 316,685 cattle, 229,569 sheep and 31,677 goats [11].

Asella is situated 175 Km southeast of Addis Ababa in Arsi zone. Geographically it is situated at 6˚79' to 8˚49'N latitude and 38˚41' to 40˚44'E longitude. The majority of the area has an altitude over 4000m above sea level with annual temperature ranging from 10 to 25ºC. The area is also one of the high rainfall areas receiving rainfall ranging from 901mm to 1200mm per year. It hosts 107,608 cattle, 90,894 sheep and 16,117 goats.

Sheno is found in North Shoa Zone of Oromia Regional State about 74 Km north east of the capital city, Addis Ababa between 9˚20'N latitude and 39˚18'E longitude. On average the altitude of the area is 2918 meters above sea level with annual rainfall of 920 mm. The mean annual minimum and maximum temperatures of the area are 12 to 20ºC, respectively. The area is a host for 11,821 cattle, 135,000, sheep and 1456 goats [11].

Study population and sample size determination

The study animals were cattle and sheep presented to the respective veterinary clinics. Both cattle and sheep having signs of pneumonia and without apparent signs of pneumonia were included in this study. Pneumonic cattle and sheep were those animals with respiratory signs upon clinical examination including coughing, dyspnea, abnormal lung sounds and nasal discharge. All cattle and sheep without apparent respiratory signs listed above were considered non-pneumonic. Purposive sampling was used to compare the proportion of animals shedding *P. multocida* and *M. haemolytica* in pneumonic and non-pneumonic cattle and sheep. The sample size required to detect difference in the proportion of animals shedding *P. multocida* and *M. haemolytica* between pneumonic and non-pneumonic animals was estimated as described by Goddard [12]. Hence, the expected proportion of non-pneumonic cattle and sheep shedding species of *Pasteurella* and *Mannhemia* was assumed to be 50% while for pneumonic ones the proportion was expected to rise to 80%. The power of the study is set at 80% and 95% confidence interval and 5% significance level was used. Hence, the minimum number of animals needed was 36 + 36 = 72 sheep and 72 cattle from each site. That is, 216 cattle and 216 sheep were needed although we included 302 cattle and 302 sheep.

Sample collection and bacterial isolation

Samples were collected from the nasopharynx of cattle and sheep using commercially available deep-guarded culture swabs and processed for isolation of *P. multocida*, *M. haemolytica* and *Bibersteina trehalosi* as described previously [13]. The samples were then transported for bacterial isolation to the Microbiology Laboratory of the College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu, Ethiopia using cold chain system [14]. First the samples were pre-incubated for 24 hrs at 37ºC in tryptone soya broth. Then, a loop full of broth cultures were streaked on blood agar (BM 014 48794, 500G) supplemented with 7% sheep blood and incubated at 37ºC for 24 hrs [15]. In the second place typical colonies were sub-cultured on MacConkey agar (OXOID, 500G) as described by Quinn et al., [16]. Thirdly, pure cultures of single colony from solid media were transferred onto nutrient agar (OXOID CM0003, 500G) for primary and secondary biochemical tests [15,17].

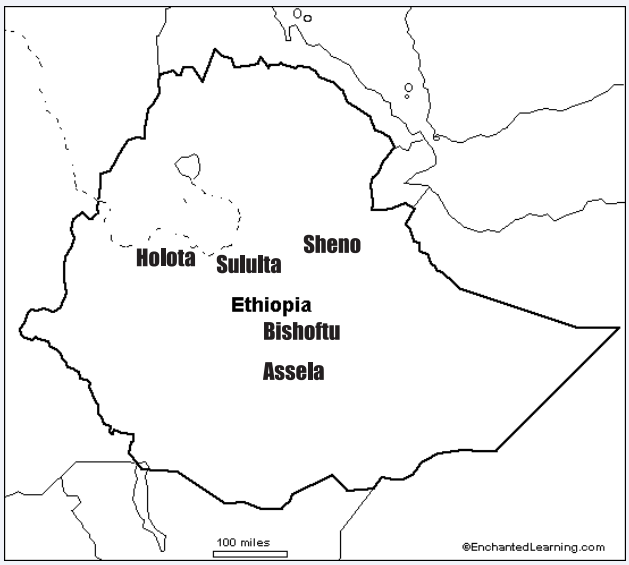

Figure 1 Map of Ethiopia displaying the location of sites for field survey and the abattoirs.

Abattoir survey

The abattoir survey was carried out in Abyssinia and ELFORA export abattoirs located in Bishoftu town, and Sululta Municipal abattoir, Oromia Regional State, Ethiopia. Apparently healthy cattle and sheep are slaughtered and the animals originated from different parts of the country and managed under extensive as well as semi-intensive husbandry system comprising of various age groups were included in this study. Two working days were randomly selected for abattoir visit. Lungs from all cattle and sheep slaughtered on the selected days were thoroughly examined for the presence of pneumonia. On each sampling day at least five lungs with lesions of broncho-pneumonia from cattle as well as sheep were identified. A piece of lung tissue was aseptically taken from the edge of lesions of pneumonic lung using sterile forceps and scalpel blade. The specimen was placed in sterile screw caped tubes, labeled individually and stored in the ice box and transported to the Microbiology Laboratory, College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu for isolation of bacteria. Isolation and identification of species of bacteria was done as described above.

Molecular characterization

DNA extraction was conducted on pure cultures of *M. haemolytica*, *B. trehalosi* and *P. multocida* grown on nutrient agar (TSA) for 24 to 48 using 1mL eppendorf tubes as described by Kumar et al., [18]. The bacterial genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instructions.

Primers targeting PHSSA and Rpt2 genes of *M. haemolytica* published by Kumar et al. [18], were used in this study. Multiplex PCR targeting the Rpt2 and PHSSA genes of *M. haemolytica* was used with the expected band size of 1022 and 325 base pairs. The primers used to target the PHSSA gene were forward- 5'-GTTTGTAAGATATCCCATTT-3' and Reverse 5'-CGTTTTCCACTTGCGTGA-3'; and Forward 5'-TTCACATCTTCATCCTC-3', Reverse 5'-TTTTCATCCTCTTCGTC-3' were used targeting the Rpt2 gene [18]. The PCR was carried out in a final volume of 25 µL of reaction mixture containing ×1 PCR buffer; Primer MH-RPt2 for: 5pM/µL, 2µL (VBC, biotch-7787), Primer Mh-RPt$_2$ REV: 5pM/µL, 2µL; Primer PHSSA For: 5pM/µL, 2µL (VBC, biotch-7887); Primer PHSSA REV: 5pM/µL, 2µL; RNase free water 3µL, IQ Super mix, 10µL (Bio-Rad, USA) and template DNA, 4µL. The PCR conditions used include an initial denaturation at 95ºC for 3 min, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 48ºC for 1 min and extension at 72ºC for 30 seconds and a final extension cycle at 72ºC for 5 min. A positive control of DNA from *M. haemolytica* and a negative control consisting of reaction mixture except the DNA template were included throughout the procedure.

For molecular identification of *P. multocida* conventional PCR was carried out using primer sets targeting capsular genes (capA) as described by Townsend *et al.* (2001). The primers are: Forward 5'-TGCCAAAATCGCAGTCAG-3' and Reverse 5'-TTGCCATCATTGTCAGTG-3' amplifying a segment of about 1044 bp. The PCR reaction mixture (50 µL) containing, master mix (Fermentas, Thermo Fisher Scientific, USA), 5 pmol of each primer (Eurofins MWG Operon, Germany) 6 µL, DNA template, 6 µL and 20 µL IQ super mix were used. The reaction conditions involve an initial denaturation at 95ºC for 5 min, followed by 35 cycles of denaturation 95ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 30 s and a final extension cycle at 72ºC for 7 min. A positive control of DNA from vaccine strain of *P. multocida* and a negative control consisting of reaction mixture except the DNA template were included throughout the procedure.

The PCR products were analyzed by visualization of desired size of DNA bands in the GelRed (Biotium) stained agarose gel as described by Sambrook and Kumar [18].

Sequencing and phylogenetic analysis

The positive PCR products of *P. multocida* and *M. haemolytica* were purified using the Wizard SV Gel and PCR clean-up system kit (Promega, Germany). The concentration of the purified PCR product was measured using NanoDrop 2000c spectrophotometer (Thermo scientific, USA). The purified PCR products were mixed with the sequencing primers and submitted to the commercial sequencing company (LGC Genomics, Berlin, Germany). The current isolates sequences were assembled and edited using Vector NTI Advances™ 10 software (Invitrogen, USA). For comparative multiple sequence analysis the homologous sequences were retrieved from the GenBank and the sequences were aligned using BioEdit version 7.1.3.0 [19]. Multiple sequence alignment was performed using the ClustalW and phylogenetic tree was reconstructed. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion was computed using MEGA version 6 [20].

Data Analysis

The association among variables collected in this study was computed using logistic regression analysis in STATA version 13 software. The frequency of isolation of *Pasteurella* species from cattle and sheep was assessed using descriptive statistics. For all statistical analysis significant level of 0.05 is considered.

RESULTS

Field survey

Out of 200 swab samples collected from Asella 51 (25.50%) were positive for either *Pasteurella* species. Fifty three (26.50%) of the samples tested from Holota were positive for *Pasteurella* species. From samples collected from Sheno 23.53% of them were found positive for one or more of the *Pasteurella* species tested. Overall *Pasteurella* species were isolated from 25.17 % (152/604) of samples collected from cattle and sheep (Table 1). At Asella *M. haemolytica* was isolated from 23.50% of cattle and 37.20% of sheep; *P. multocida* was isolated from equal proportion of cattle and sheep whereas none of the samples gave positive results for *B. trehalosi*. At the remaining sites similar proportion of samples from cattle and sheep yielded *M. haemolytica* and *P. multocida*. In all the study sites *M. haemolytica* was more frequently isolated than *P. multocida* and *B. trehalosi* (Table 2). The frequency of isolation of *Pasteurella* species was higher in young animals than adults (OR = 1.56; 95 % CI: 1.02, 2.38); in pneumonic animals than those with no signs of pneumonia (OR = 4.67; 95 % CI: 3.03, 7.19) and in animals under intensive management

Table 1: Proportion of samples tested positive for one or more Pasteurella species in cattle and sheep from the three study sites.

Site	Asella			Holota			Sheno			Total
	Nº tested	Nº positive	%	Nº tested	Nº positive	%	Nº tested	Nº positive	%	
Species										
Cattle	100	22	22	100	24	24	100	19	19	23.33
Sheep	100	29	29	100	29	29	104	29	27.88	26.97
Age group										
Young	71	21	29.57	101	34	33.66	76	24	31.58	31.85
Adult	112	26	23.21	99	19	19.19	64	14	21.87	21.45
Old	17	4	23.52	0	0	0	64	10	15.63	17.28
Gender										
Female	95	24	25.26	137	38	27.74	101	26	25.74	26.43
Male	105	27	25.71	63	15	23.81	103	22	21.36	23.62
Breed										
Cross	39	12	30.77	28	8	28.57	27	6	22.22	27.66
Local	161	39	24.22	172	45	26.16	177	42	23.73	24.71
Management										
Extensive	133	33	24.81	169	48	27.22	27	6	22.22	25.54
Semi-Intensive1	9	3	15.79	31	7	22.58	177	42	23.73	22.91
Intensive	48	15	31.25	0	0	0		0	0	31.25
Pneumonic										
Yes	109	30	27.52	96	45	46.88	102	41	40.2	37.79
No	91	21	23.08	104	8	7.69	102	7	6.86	12.12
Overall	200	51	25.5	200	53	26.5	204	48	23.53	25.17

Table 2: Proportion of samples yielding positive results for the three Pasteurella species tested.

Asella	*M. haemolytica*	*P. multocida*	*B. trehalosi*
Cattle	12 (23.50%)	10 (19.60%)	0 (0.00%)
Sheep	19 (37.20%)	10 (19.60%)	0 (0.00%)
Holota			
Cattle	13 (24.53%)	5 (9.43%)	6 (11.32%))
Sheep	14 (26.42%)	6 (11.32%)	9 (16.98%)
Sheno			
Sheep	14 (6.86%)	6 (2.94%)	10 (4.90%)
Cattle	11 (5.39%)	6 (2.94%)	2 (0.98%)

than those managed extensively (OR = 2.46; 95 % CI: 1.12, 5.39).

Abattoir survey

From a total of 176 pneumonic lung samples (93 cattle and 83 sheep) collected and investigated *Pasteurella* species were isolated from 27.27 % (48) of them. Out of the 48 isolates 24.73% (23) of them were from cattle whereas 30.12% (25) of them were from sheep. The species composition of the isolates showed that 13.07% (23), 7.39% (13) and 6.83% (12) of them were *M. haemolytica*, *B. trehalosi* and *P. multocida,* respectively (Table 3), suggesting predominance of *M. haemolytica* in both cattle and sheep. The highest proportion of *M. haemolytica*, *B. trehalosi* and *P. multocida* came from Birsheleko (75%) followed by Gonder (66.67%), while the lowest was detected from samples originated from Ambo (15%) (Figure 2).

Molecular analysis

Twenty-two bacterial isolates that were identified as *P. multocida* (7) and *M. haemolytica* (15) by conventional bacteriological methods were selected and tested using PCR based nucleic acid methods. All of the 15 *M. haemolytica* isolates tested were confirmed by the PCR technique (Figure 3A). In addition, two *B. trehalosi* isolates were tested alongside the *M. haemolytica* isolates and they gave negative results. Similarly all of the 7 isolate of *P. multocida* tested using PCR targeting the capsular gene of *P. multocida* designated *cap*A gave positive results (Figure 3B).

Nucleotide sequence and phylogenetic analysis

Three isolates identified as *P. multocida* were sequenced. These include isolates designated: Yabello/01/2018, Fiche/01/2018 and Sendafa/01/2018. The results of basic local alignment search tool (BLAST) analysis showed that the *P. multocida* isolates identified in this study were more than 99% similar to strains deposited in GenBank with accession number AF036004.2, AF237926.1, AY225345.1, AY225346.1, JF922885.1 and MF417608.1. The isolates were identical to *P. multocida* serovars A1 and A3 as shown in Figure 4.

DISCUSSION

The Government of Ethiopia has made the development of ruminant sector, particularly the smallholder operations, a prior-

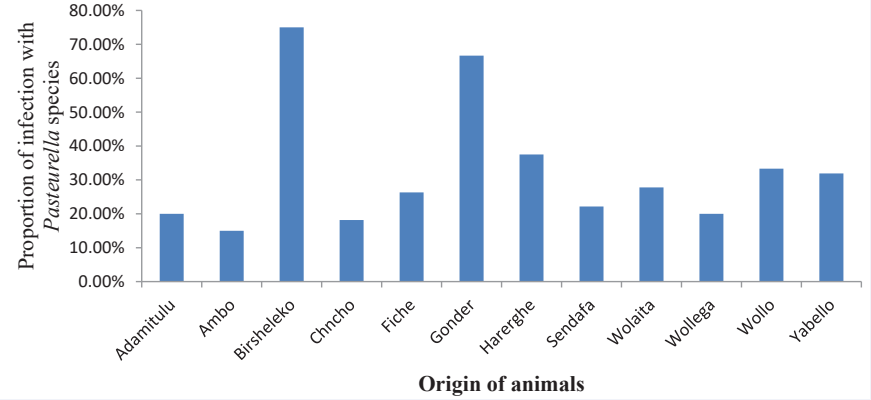

Figure 2 Proportion of samples yielding positive results for one or more of Pasteurella species from various areas of Ethiopia.

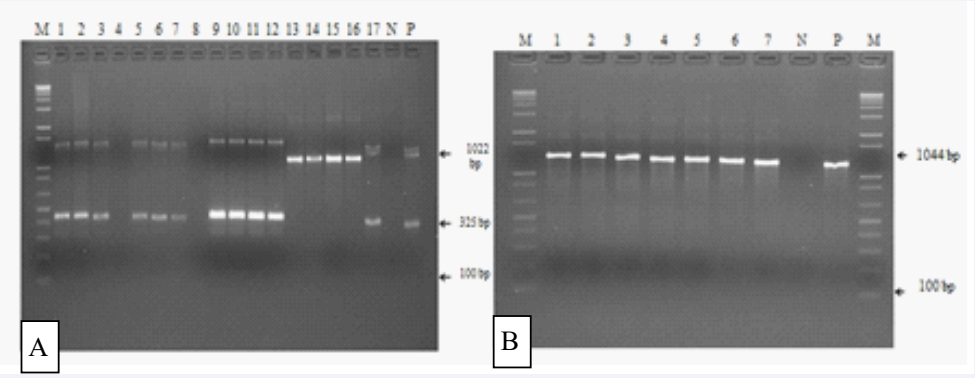

Figure 3 Results of molecular analysis of *M. haemolytica* (A) and *P. multocida* (B) isolates. .

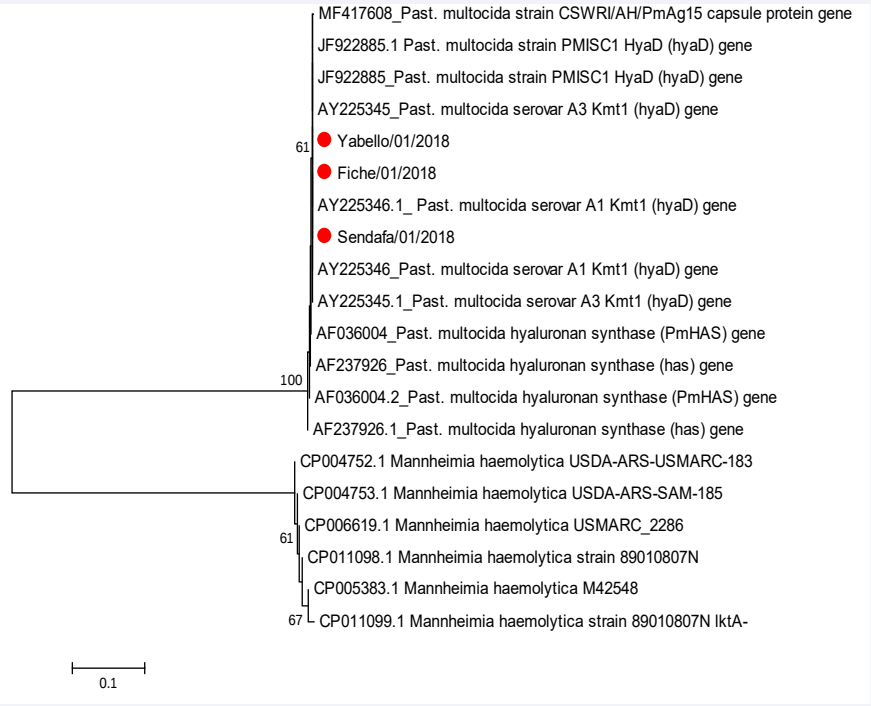

Figure 4 Phylogenetic analysis of 20 *P. multocida* isolates based on nucleotide sequences of the hyaD gene. The *P. multocida* isolates sequenced in this study are indicated plain red circle.

Table 3: Frequency of isolation of *Pasteurella* species from pneumonic lungs of cattle and sheep collected from abattoir.

Animal species	Pneumonic lungs	Species of bacteria isolated			Total
		M. haemolytica	*B. trehalosi*	*P. multocida*	
Cattle	93	11 (11.83%)	7 (7.53%)	5 (5.38%)	23 (24.73%)
Sheep	83	12 (14.46%)	6 (7.23%)	7 (8.43)	25 (30.12%)
Total	176	23 (13.07%)	13 (7.39%)	12 (6.83%)	48 (27.27%)

ity to achieve food security [21]. Simultaneously the Government has been advised to adopt a "low emission development" for its commercial dairy industry, which entails genetic improvement and disease control to reduce what is known as "unproductive emissions" [22]. Pneumonic pasteurellosis is one of the diseases that have to be controlled. However, control of diseases including respiratory diseases requires to the ability to make inventory of the potential pathogens present in the country. The present study provided the preliminary list of bacterial pathogens commonly incriminated in pneumonic pasteurellosis in cattle and sheep. In this study we used molecular methods to identify serotypes of *P. multocida* in addition to the conventional bacteriological techniques from samples collected from cattle and sheep. The strains of *P. multocida* identified in this study were confirmed to be A1 and A3. This is the first report of identification of these strains in Ethiopia. Strains A1 and A3 of *P. multocida* have been known to cause pneumonia in ruminants [23,24]. Both *M. haemolytica* and *P. multocida* have been known to cause pneumonia with similar gross and histopathological lesions [25]. Therefore, the veterinary and livestock authorities should take this into consideration in their disease intervention programs.

Pasteurellosis of cattle and sheep caused by serotypes of *P. multocida, M. haemolytica* and *B. trehalosi* have been documented, the latter two being the dominant cause of pneumonia Biberstein [26]. Field studies have revealed that pneumonia caused by *M. haemolytica* and *B. trehalosi* has been considered to be important constraints in Ethiopia [20,27], incurring considerable economic loss. Ayelet and his colleague [28], also suggested respiratory problems due to *M. haemolytica*, *B. trehalosi* and *P. multocida* cause significant mortality and morbidity with consequent huge treatment cost. These previous studies, however, used only conventional methods on samples from non-pneumonic animals. Our study was based on both conventional methods of isolation and identification followed by confirmation of the identity of the bacterial species by molecular techniques. Besides, we compared the frequency of isolation of the bacteria from pneumonic and non-pneumonic cases. Hence, we suggest that this study provided reliable information on the inventory of *Pasteurella* species causing respiratory problems.

The three bacteria were more frequently isolated from pneumonic animals than from non-pneumonic ones. The odd of isolation from pneumonic animals is nearly 5 times higher than that of non-pneumonic animals. In consent to our observation Mohamed and Abdelsalam [25], reviewed that *Pasteurella* organisms were frequently isolated from the nasopharynx and trachea of sick animals though the mean nasal colony count was much higher in sick animals than in healthy ones. Similarly Abera *et al*. [29], reported higher frequency of isolation of *Pasteurella* species in pneumonic animals than non-pneumonic ones. Although it is difficult to claim causality as our study was observational and the disease is multi-factorial, it suggests the possible involvement of these bacteria in the genesis of pneumonia and can potentially hinder ruminant industry.

The frequency of isolation of *Pasteurella* species was higher in samples collected from animals originating from intensive production system than from those from extensive system. This is due to higher density of animals per space in intensive production system. This provided opportunity for frequent contact among animals, which favors spread of *Pasteurella* species among animals. Besides, higher density of animals per unit area can cause stress due to overcrowding resulting in increased number of and frequency of bacterial shedding. This observation is in agreement with the reports of Chowdhury [30] Radostitis [31]. However, in contrary to our observation Engdaw and Alemneh [32], reported higher frequency of isolation in animals from extensive production system than those from semi-intensive production system.

The higher frequency of isolation of *Pasteurella* species in young animals than adults could be due to difference in the immune status of the animals. The young cattle and sheep used in this study were recently weaned groups. Weaning is one of the predisposing factors to infection with *Pasteurella* species [31,33], which stresses animals and ultimately compromise their respiratory immunity. Our observation is in agreement with the reports of Alemneh and Tewodros [34], and that of Hailu et al. [19].

CONCLUSION

In conclusion this study revealed that *M. haemolytica*, *P. multocida* and *B. trehalosi* are commonly circulating in cattle and sheep originated from various parts of the country. Besides, the serotypes of *P. multocida* identified are those known to cause pneumonia in ruminants. The *Pasteurella* species were more frequently isolated from pneumonic animals than from animals without pneumonia [35].

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