Identification of serotypes Pasteurella multocida and Mannheimia haemolytica from cattle and sheep in central Ethiopia

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Abstract

Background: 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 species or serotypes of bacterial agents for optimum control of the disease. This study was conducted with the objectives of identification of bacterial agents causing pneumonic pasteurellosis in cattle and sheep and identify serotypes of P. multocida involved. Methods: Bacteriological and molecular methods were used on 176 pneumonic lungs (93 cattle and 83 sheep) collected from abattoirs and 604 nasal swabs collected from 302 cattle and 302 sheep presented to Asella, Holota and Sheno veterinary clinics. Results: Twenty-five percent, 26.5% and 23.5% of nasal swabs collected from cattle and sheep from Asella, Holota and Sheno, respectively, were positive to one or more species of Pasteurella, Mannheimia and Bibersteinia. Mannheimia haemolytica was the predominant bacteria isolated at all sites. The isolation of these bacterial species was associated with age and pneumonic status of animals and management system. Young animals were more likely to yield positive results than adults (OR = 1.56; 95 % CI: 1.02, 2.38). Similarly, isolation of the three bacterial species was more frequent in animals with signs of pneumonia than those animals without signs of pneumonia (OR = 4.67; 95 % CI: 3.03, 7.19) and from animals under intensive management system than those animals kept under extensive management system (OR = 2.46; 95 % CI: 1.12, 5.39). Out of the total of 176 pneumonic lungs examined isolation was done from 48 (27.27 %) of them. Mannheimia haemolyitica was the predominant species isolated from pneumonic lungs. The identity of P. multocida and M. haemolytica isolated was further confirmed using PCR. Pasteurella multocida serotypes A1 and A3 and M. haemolytica serotype A1 were the predominant serotypes identified. 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. It is also interesting that the serotypes of P. multocida and M. haemolytica identified in the present study are those that are already proven to cause pneumonia in ruminants.

Introduction

Pneumonic pasteurellosis is multi-factorial disease caused by a combination of environmental, husbandry and biotic factors such as viral and bacterial agents [1] although serotypes of *Mannheimia haemolyitica* (*M. haemolyitica*) [2,3] and *Pasteurella multocida* (*P. multocida*) [4] have been considered the leading causes. The disease occurs worldwide but the distribution of the serotypes involved in causing the disease can vary among different areas [5,6]. For instance, 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 [7].

Pneumonic pasteurellosis is implicated as the cause of considerable economic losses in cattle and sheep resulting from mortality and morbidity [8,9]. Losses due to morbidity are more substantial than the less frequently observed mortality in affected animals. In sheep it can occur in both intensive and extensive management systems [7]. However, in cattle it is commonly seen in feed lot animals, accounting for

approximately 30% of mortality worldwide. For instance, in North America pneumonic pasteurellosis is responsible for annual loss of more than one billion USD in beef industry [10].

In Ethiopia pneumonic pasteurellosis has been a topic of frustration for 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 so far. As a result, our knowledge on the composition and distribution of serotypes of bacteria causing pneumonic pasteurellosis is limited. Identification of the serotypes of the bacterial agents is the first step in the understanding of the disease complex. Identification of serotypes of *P. multocida* involved in pneumonic pasteurellosis was not done. The objectives of this study were to identify the serotypes of *P. multocida* and *M. haemolytica* involved in pneumonic pasteurellosis and estimate prevalence of *P. multocida*, *B. trehalosi* and *M. haemolytica* in cattle and sheep in central Ethiopia.

Methods

Field Survey

Study areas

The field study was carried out in Holota, Asella and Sheno, in Oromia Regional State, central Ethiopia (Figure 1). The study areas are typical highlands with elevation ranging from 2000 meters to over 4000 meters above sea level. The mean annual temperature and annual rainfall of the areas, respectively range from 6°C to 25°C and 900 to 1200 mm. The areas are known for their huge livestock population that comprise 436,114 cattle, 455,463 sheep and 49,310 goats [11].

Study population and sample size determination

The study animals were cattle and sheep presented to the respective veterinary clinics of Holota, Asella and Sheno. Both cattle and sheep showing signs of pneumonia and without apparent signs of pneumonia were included in this study. Pneumonic cattle and sheep were those animals with respiratory signs such as coughing, dyspnea, abnormal lung sounds and nasal discharge on clinical examination. 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, M. haemolytica* and *B. trehalosi* between pneumonic and non-pneumonic cattle and sheep. The sample size required to estimate the difference in the proportion of animals shedding these bacteria between pneumonic and non-pneumonic animals was computed as described previously [12]. The expected proportion of non-pneumonic cattle and sheep shedding these bacterial species 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 and 5% significance level was used. Hence, the minimum number of animals

needed was 72 sheep and 72 cattle from each site. As a result, a total 216 cattle and 216 sheep were considered to be sufficient sample size, although 302 cattle and 302 sheep were sampled.

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

Sample collection and bacterial isolation

Nasal swab samples were collected from the nasopharynx of cattle and sheep for isolation of *P. multocida*, *M. haemolytica* and *B. trehalosi* as described previously [13]. The samples were transported to Microbiology Laboratory of the College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu, Ethiopia using ice box [14]. The samples were pre-incubated for 24 hours at 37ºC in tryptone soya broth. Then, a loop full of broth cultures were streaked on blood agar (BM 01448794, HIMEDIA, India) supplemented with 7% sheep blood and incubated at 37ºC for 24 hours [15]. Typical colonies were sub-cultured on MacConkey agar (Oxoid, England) as described by Quinn et al.[15]. Pure cultures of single colony from MacConkey agar were transferred onto nutrient agar (CM0003, Oxoid, England) for conventional biochemical tests [15,16] .

Abattoir survey

The abattoir survey was carried out in Abyssinia and ELFORA export abattoirs, Bishoftu and Sululta Municipal abattoir in Oromia Regional State, central Ethiopia. Apparently healthy cattle and sheep originated from different parts of the country are slaughtered in these abattoirs. 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 edges of pneumonic lesions using sterile forceps and scalpel blade. The specimens were collected in sterile screw caped tubes, labeled individually and stored in ice box and transported to Microbiology Laboratory, College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu for bacteriological investigation. Isolation and identification of species of bacteria was done as described above. A total of 176 pneumonic lung samples (93 cattle and 83 sheep) were collected and analyzed.

Molecular characterization

Extraction of DNA was performed using Qiagen DNeasy (Qiagen, German town, MD, USA) as described by Kumar et al. [17] on pure colonies of *M. haemolytica*, *B. trehalosi* and *P. multocida* grown on nutrient agar (TSA) for 24 to 48 hours in 1mL eppendorf tubes). Serotype A1 specific primers targeting PHSSA gene (Forward: GTTTGTAAGATATCCCATTT and Reverse: CGTTTTCCACTTGCGTGA) and Rpt2 gene (Forward: TTCACATCTTCATCCTC and Reverse: TTTTCATCCTCTTCGTC) of *M. haemolytica* published by Kumar et

al. [17] were used. Multiplex PCR targeting the Rpt2 and PHSSA genes of *M. haemolytica* was employed yielding band sizes of 1022 and 325 base pairs, respectively. The PCR was carried out in a final volume of 25 µL of reaction mixture containing forward and reverse primers (2µL each), DNA template (4µL), IQ super mix (10µL) and RNase free water (3µL). The PCR conditions used include an initial denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 48°C for 1 minute and extension at 72°C for 30 seconds and a final extension cycle at 72°C for 5 minutes. Positive control DNA from *M. haemolytica* and a negative control consisting of reaction mixture except the DNA template were included throughout the analysis.

For confirmation of *P. multocida* standard PCR was carried out using primer sets targeting capsular genes (capA) as described by Townsend et al. [18]. The primers used were: Forward (TGCCAAAATCGCAGTCAG) and Reverse (TTGCCATCATTGTCAGTG) yielding band size of 1044 bp. The PCR reaction was carried out in a final volume of 50 µL containing master mix (Fermentas, Thermo Fisher Scientific, USA), 5 pmol of each primer (Eurofins MWG Operon, Germany), DNA template, and IQ super mix. The reaction conditions comprise an initial denaturation at 95ºC for 5 minutes followed by 35 cycles of denaturation 95ºC for 1 minute, annealing at 55ºC for 1 minute and extension at 72ºC for 30 seconds and a final extension cycle at 72ºC for 7 minutes. A positive control DNA from vaccine strain of *P. multocida* and a negative control consisting of reaction mixture except the DNA template were used.

The PCR products visualized in 2 % agarose gel stained with GelRed (Biotium) as described by [18,19]. The electrophoresis was allowed to run for 60 minutes at 120 volts.

Sequencing and phylogenetic analysis

PCR products yielding positive results for *P. multocida* and *M. haemolytica* were purified using Wizard SV Gel and PCR clean-up 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 sequences from current isolates 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 [20]. 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 [21].

Data Analysis

The association among variables collected in this study was computed using logistic regression analysis in STATA software version 13. The frequency of isolation of the bacterial species from cattle and sheep

as function of various factors was assessed using odds ratio. For all statistical analysis significant level of 0.05 was considered.

Results

Results of field survey

Out of the 200 swab samples collected from cattle and sheep from Asella 51 (25.50%) were positive for either *Pasteurella* or *Mannheimia*. Fifty-three (26.50%) of the samples collected from cattle and sheep from Holota and 23.53% of the samples collected from Sheno were positive for the three bacterial species. Overall the three bacterial 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 (19.60 %) of cattle and sheep whereas none of the samples gave positive results for *B. trehalosi*. At Holota and Sheno nearly similar proportion of samples from cattle and sheep were positive for *M. haemolytica* and *P. multocida*. *Mannheimia haemolytica* was more frequently isolated than *P. multocida* and *B. trehalosi* (Table 2). The isolation of the three bacterial species was associated with age of animals (more frequent in young animals than adults; OR = 1.56; 95 % CI: 1.02, 2.38); pneumonic status (more frequent in animals with signs of than those animals without signs of pneumonia; OR = 4.67; 95 % CI: 3.03, 7.19) and management system (more prevalent in animals kept under intensive management than those under extensive management system; OR = 2.46; 95 % CI: 1.12, 5.39).

Table 1. Proportion of swab samples yielding positive results for *P. multocida*, *B. trehalosi* and *M. haemolytica* in cattle and sheep from the three study sites

Table 2. Proportion of *P. multocida*, *B. trehalosi* and *M. haemolytica* isolated and identified from cattle and sheep in the study areas

Results of abattoir survey

One or more of the three bacterial species were isolated from 27.27 % (48) of the 176 pneumonic lung samples collected and investigated. 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 the predominance of *M. haemolytica* in both cattle and sheep. The highest proportion of *M. haemolytica*, *B. trehalosi* and *P. multocida* were observed in samples from Birsheleko (75%) followed by Gonder (66.67%), while the lowest was detected in samples originated from Ambo (15%) (Figure 2).

Table 3. Frequency of isolation of *P. multocida, B. trehalosi and M. haemolytica* from pneumonic lungs of cattle and sheep collected from abattoirs

Figure 2. Proportion of lung samples yielding positive result for *P. multocida*, *B. trehalosi* and *M. haemolytica* from various areas of Ethiopia

Results of molecular analysis

Twenty-two bacterial isolates that were identified to be *P. multocida* (7) and *M. haemolytica* (15) by bacteriological methods were selected and tested using PCR. Eleven of the 15 *M. haemolytica* isolates analyzed by the mPCR yielded positive results for *M. haemolytica* serotype A1 (Figure 3 A). four of the isolates did not belong to this serotype. On the other hand, two *B. trehalosi* isolates tested alongside the *M. haemolytica* isolates showed negative results. Similarly, all of the 7 isolate of *P. multocida* tested using PCR targeting the capsular gene of *P. multocida* designated *cap*A produced positive results (Figure 3).

Figure 3. A: Results of Rpt2 and PSSA gene amplification products (approximately 1022 and 325 bp, respectively) of *M. haemolytica*

Nucleotide sequence and phylogenetic analysis

Three isolates that were identified as *P. multocida* by PCR method were sequenced. These isolates were designated as 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 those 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* serotypes A1 and A3 as shown in figure 4.

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

Discussion

Development and application of optimum strategy for the control of animal diseases starts with understanding of the epidemiology of the diseases in question, which in turn implies being able to catalogue the identity of potential pathogens present within the country. Pneumonic pasteurellosis is one of the diseases that need to be controlled for optimum utilization of ruminant industry in the country. Identification of the serotypes involved and production of vaccines against the serotypes prevalent in the country is important in this regard. Although the prevalence of bacterial species in pneumonic animals has been reported previously [22-28], the focus of those studies was only pneumonic animals. Besides, the previous studies did not identify the serotypes of the bacteria isolated. This is particularly lacking for *P. multocida*. Moreover, the previous authors used only bacteriological methods, which do not discern among strains causing pneumonia and those which do not cause pneumonia. This study used molecular

methods to identify serotypes of *P. multocida* in pneumonic lung samples collected from slaughtered cattle and sheep. Two serotypes of *P. multocida* designated A1 and A3 were identified forthe first time in Ethiopia. Serotypes A1 and A3 of *P. multocida* have been already known to cause pneumonia in ruminants [29,30]in which they produced similar gross and histopathological lesions to pneumonia produced by *M. haemolytica* [31].The results of molecular analysis also revealed the predominance of serotype A1 of *M. haemolytica* in lung tissues and swabs collected from pneumonic cattle and sheep. That is, *P. multocida* serotypes A1 and A3 and serotype A1 of *M. haemolytica* are among important causes of pneumonic pasteurellosis in cattle and sheep. Therefore, the veterinary and livestock authorities should give attention to these serotypes in their disease intervention programs.

Elsewhere in the world studies documented that serotypes of *P. multocida, M. haemolytica* and *B. trehalosi* are common causes morbidity and mortality in cattle and sheep [32]. Field studies conducted so far in Ethiopia also revealed that pneumonia caused by *M. haemolytica* and *B. trehalosi* has been considered to be important constraints to ruminant industry [22,23]incurring considerable economic losses. The previous studies identified serotypes of *M. haemolytica* and *B. trehalosi* from outbreaks of respiratory diseases. Ayelet et al. [24] suggested respiratory problems due to *M. haemolytica* and *B. trehalosi* cause significant mortality and morbidity with consequent huge costs as a result of treatment and control. Although the previous studies did not compare the composition and frequency of isolation between pneumonic and non-pneumonic animals, their findings are in consent to the observation reported in this study. Since ruminant sector remains crucial for poverty alleviation in Ethiopia the findings of this study will be helpful in highland areas where the incidence of pneumonia is relatively higher [22-24]. Thus, we believe that the findings of the current study provide reliable information on the inventory of causative agents of pneumonic pasteurellosis.

Pasteurella multocida, B. trehalosi and *M. haemolytica* were more frequently isolated from pneumonic animals than from non-pneumonic ones. The odd of isolation of these bacterial species from animals with signs of pneumonia is nearly 5 times higher than that from animals without signs of pneumonia. This suggests that although these bacterial species are commensals of the upper respiratory tract, they are more frequently excreted from sick animals. That is, the serotypes that cause pneumonia circulate more frequently among herds or flocks than other serotypes. In consent to our observation Mohamed and Abdelsalam [31] showed that the mean nasal colony count of *P. multocida, B. trehalosi* and *M. haemolytica* was much higher from sick animals than healthy ones. Abera et al. [25] also reported higher frequency of isolation of *P. multocida*, *B. trehalosi* and *M. haemolytica* from pneumonic animals than non-pneumonic ones. Although it is difficult to claim causality as our study was observational and the disease is multi-factorial, the results of this study suggest the potential involvement of *P. multocida*, *B. trehalosi* and *M. haemolytica* in pneumonic pasteurellosis and can potentially hinder the ruminant industry of the country.

The frequency of isolation of *P. multocida*, *B. trehalosi* and *M. haemolytica* was higher in samples collected from animals originating from intensive production system than those from extensive system. This is due to higher density of animals per unit area in intensive production system. This provides

opportunity for frequent contact among animals, which favors spread of the bacterial species from sick animals to healthy ones. Besides, higher density of animals per unit area can cause stress due to overcrowding resulting in increased number and frequency of bacterial shedding. This observation is in agreement with previous reports [30, 31]. However, in contrary to our observation Engdaw and Alemneh [26] reported higher frequency of isolation in animals from extensive production system than those from semi-intensive production system. The later authors studied animals from extensive production systems and semi-intensive system where only few animals are kept per unit area. In addition, the animals they studied from the extensive husbandry system were from marginalized areas where there was feed shortage, which stress animals and confound the effect of management system.

The higher frequency of isolation of *P. multocida*, *B. trehalosi* and *M. haemolytica* from 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 animals. Weaning is one of the predisposing factors to infection with these bacterial species [31,33], which stresses animals and ultimately compromise their respiratory defense mechanisms. In addition, adult animals could have been infected when they were young and acquired immunity if they survive the infection. Our observation is in agreement with the reports of Alemneh and Tewdros [27].

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. In addition, the serotypes of *P. multocida* and *M. haemolytica* involved in pneumonic pasteurellosis are identified in this study.

Declarations

Ethical approval and consent to participate

This study was submitted to the ethics committee named “Animal Research Ethics Review Committee ‘and approved. Ethical clearance was obtained from the Ethics Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University. The clearance identification number is VM/ERC/24/05/2018. Handling of the study animals throughout the study period was done according to this guideline

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request. The genetic data is available from author number 10 and also deposited in GenBank.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

TST: analyzed the data, wrote up the paper and designed the study

AW: did the bacteriological procedures and involved in collection of samples from abattoirs

EZ and YA: collected samples from abattoirs and assisted laboratory activities

BK, FT and GM: edited the paper and supervised the laboratory work

EG: did the molecular analysis and edited the paper

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Tables

Table 1 could not be included here due to technical issues. It can be found as an image in the Supplemental files.

Table 2. Proportion of *P. multocida, B. trehalosi* and *M. haemolytica* isolated and identified from cattle and sheep in the study areas

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%)

Table 3. Frequency of isolation of *P. multocida, B. trehalosi and M. haemolytica* from pneumonic lungs of cattle and sheep collected from abattoirs

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%)**

Figures

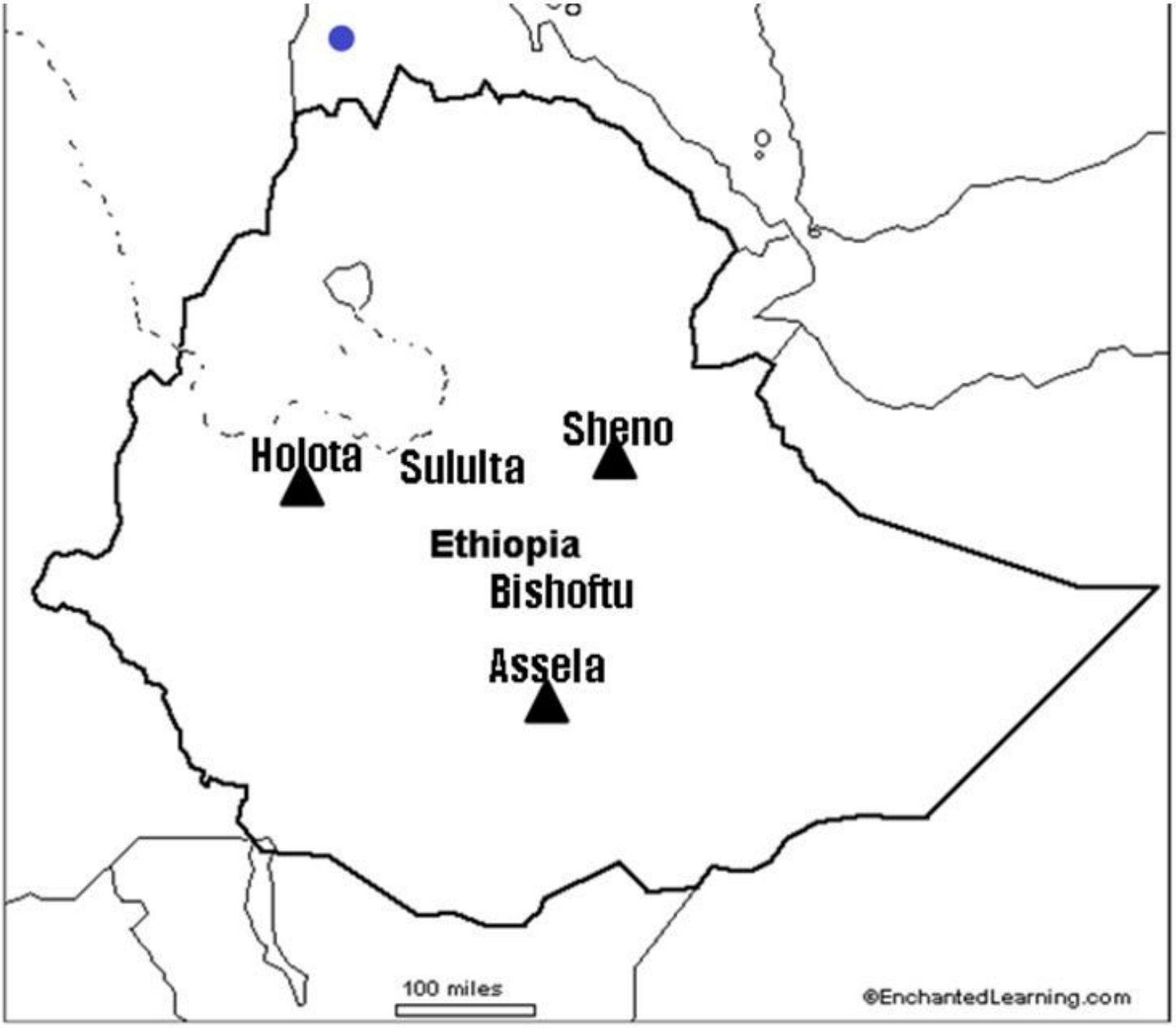

Figure 1

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

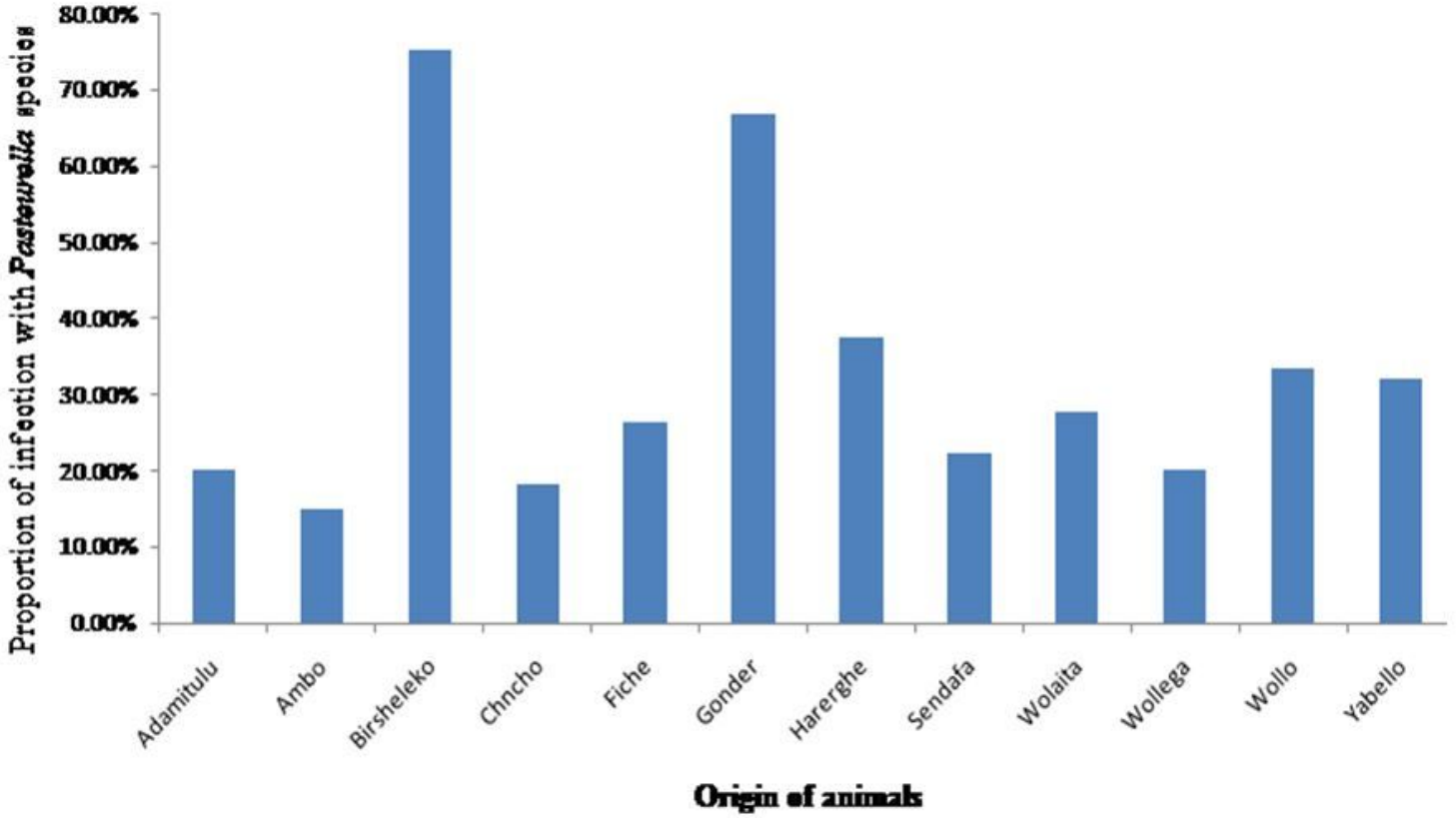

Figure 2

Proportion of lung samples yielding positive result for P. multocida, B. trehalosi and M. haemolytica from various areas of Ethiopia

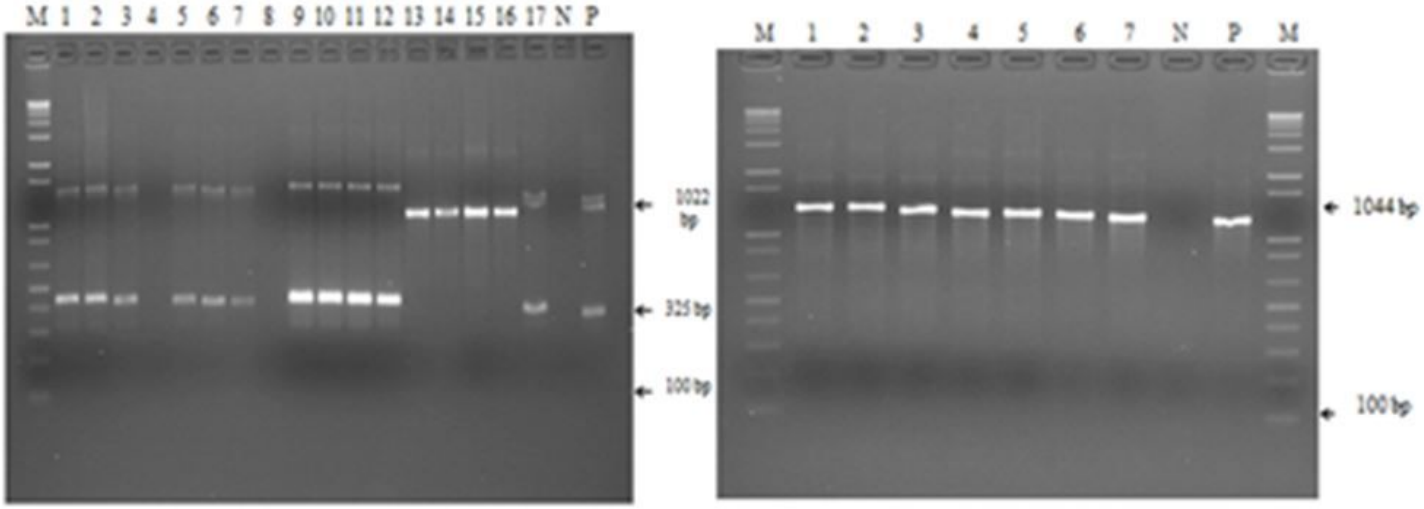

Figure 3

Results of Rpt2 and PSSA gene amplification products (approximately 1022 and 325 bp, respectively) of M. haemolytica (M = DNA ladder; 1,2,3,5,6,7,9-12 and 17: M. haemolytica serotype A1; lanes 4 and 8= B.

trehalosi; N= Negative control; P= positive control; lanes). B: Results of PCR amplification of P. multocida (M= DNA ladder; Lanes 1-7= P. multocida; N= Negative control; P= positive control)

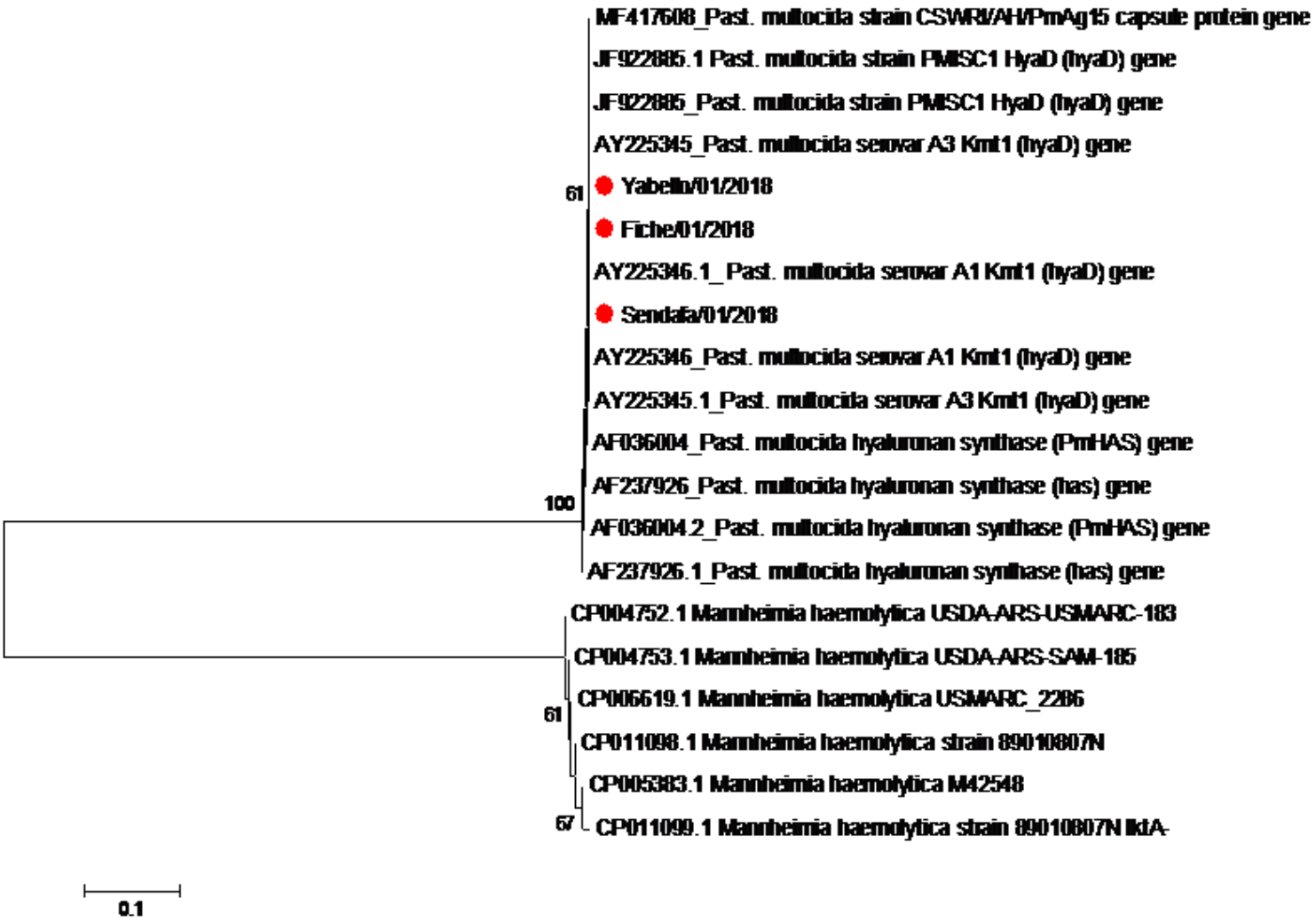

Figure 4

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.jpg