Isolation and Identification of *Pasteurella* Species from Pneumonic and Apparently Healthy Cattle with Antibiotic Susceptibility Pattern in Dire Dawa Area, Eastern Ethiopia

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Abstract: A cross sectional study was conducted from November 2014 up to April 2015 at Dire Dawa Veterinary Clinic and municipal abattoir, eastern Ethiopia. The aim of the study was to isolate of *Pasteurella* species and assess the associated risk factors from pneumonic and apparently healthy cattle, and antibiotic susceptibility profiles of the isolates. Out of 144 samples (53 nasal swabs from clinic and 91 lung tissues abattoir) examined animals, 48.1% was culture positive. The bacteriological examination revealed 33 (22.9%) overall isolates with 15 (28.3%) were nasal swab positive and 18 (19.8%) from lungs tissues. *M. haemolytica* (19.8%) and *P. multocida* (15.2%) were recovered respectively in which 15 (28.3%) and 18 (19.8%) bacterial isolates were obtained from nasal swabs and pneumonic lungs, respectively. The higher isolation rate of *M. haemolytica* indicated as the major causes in the study area. Among the assessed associated risk factors, age was the potential risk factor in which young animal was highly affected and statistical significant difference was observed (P<0.05). The antibiotic sensitivity tests of the isolates indicated that chloramphenicol and tetracycline were the most effective antibiotics. Thus, an integrated application of overall management and vaccination should be implemented as prevention and control measures.
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Introduction

Bovine pneumonic pasteurellosis is known by a number of synonyms that are descriptive of the condition and are relevant in specific circumstances. The terms shipping fever, transit fever, bovine enzootic pneumonia, and bovine respiratory disease (BRD) complex are all very meaningful terms used to describe the disease. It is caused by *Pasteurella (P). multocida, P. heamolytica, P. canis, P. stomatis* and *P. dagmatis* were occasionally recovered from abscesses and wound infections caused by animal bites or scratches[25, 26]. *P. heamolytica* is the most pathogenic bacterium and more commonly associated with the respiratory disease complex in bovines even if both species are very frequently found to be associated with respiratory diseases besides to variations within different strains regarding their capability to cause diseases in different host animals [1].

The disease is generally fatal and characterized by a serious or severe fibrinous pleuropneumonia which principally affects recently transported less than one year animals. In the BRD complex, more than one species and serotype of *Pasteurellae* are incriminated as playing a role that is secondary to respiratory viruses and 'stress'. This is unlike HS, which is a primary pasteurellosis caused by specific serotypes of the species *P. multocida.* It is associated with BRD are predominantly *P.haemolytica* type A, and *P. multocida* capsular serogroup A [2,3]. The organisms cause pneumonic pasteurellosis are carried in the upper respiratory tract (URT) of calves. In the case of *P. haemolytica* type A1, the 22 bacterium is not easily detected in the URT of healthy calves, but is shed and can be easily isolated in calves that are stressed in some way or affected with another concurrent infection [4].

The disease in cattle is generally recognized as an acute febrile respiratory disease with fulminating fibrinous or fibrino-purulent, bronchopneumonia and fibrinous pleurisy. Observable clinical signs of acute cases usually developed within 10-14 days in adult animals after being exposed to stress but a much earlier onset is more typical. Nevertheless, infected animals in severe cases may die as a result of toxemia even before the development of significant pulmonary lesions. In this case sudden death may be the first sign of acute outbreaks particularly in young calves [5,6].

Pasteurella are the most isolated microorganisms from pneumonic processes in domestic and wild animals; these cause the most significant disease called bovine pneumonic pasteurellosis [7]. Among various Animal diseases, Bovine Pneumonic pasteurellosis is one of the most economically important infectious and the most expensive diseases affecting dairy and beef cattle, and especially in those animals which have been recently included within the herd of animals. Even though the disease is a serious and economically important that raised under poor managed animals, there is well documented information in Ethiopia including Dire Dawa area, eastern Ethiopia [8]. Hence, the objectives of this study were to determine the prevalence of Bovine pasteurellosis, isolate and identify *Pasteurella* species, verify antibiotic susceptibility profiles of the isolates and assess potential associated risk factors from pneumonic and apparently healthy cattle in the study area.

Materials and Methods

Study area

A cross sectional study was conducted from November 2014 up to April 2015 at Dire Dawa Veterinary Clinic and Municipal abattoir. It is located in the eastern part of the country specifically lying between 900 27' and 900 49'N latitudes and between 4100 38' and 4200 19'E longitudes and the town is 515 Km from east of Addis Ababa the capital city of Ethiopia and 333 Km from the international port of Djibouti and it is enclosed by the state of Somalia and the state of Oromia on estimated area of 128,802 hectares. It is found on an altitude of 950 meters above sea level. The mean annual rainfall and the mean day-night air temperature ranges are respectively 550-850 mm and 14-30°C [9]. Based on the 2007 census conducted by the central statistical agency of Ethiopia, the town has a total population of 341,834 of whom 171,461 are men and 170,461 women; 233, 24 or 68.23% of the population are urban inhabitants those are relatively sparsely populated lowland exhibiting agro-pastoral system and with mixed farming ranges on about 10,370 hectares. The area is characterized by an arid and semi-arid climate with low and erratic rainfall which is a bi-modal type of rainfall with April as a peak for the small rains and July for the big rains i.e. small rains in spring, big rains in summer with June as a dry month, the rainy season is from February to May and from July to September and the dry season is from October to January. Out of the rural population about 4% are pure pastoralists and they are engaged in livestock production. The total livestock population in the area is 219,323. The survey results of the population and Housing Census (2007/08) of the study area, showed that goats comprise the heights proportion, 54.2% of the total livestock, followed by sheep 21.1%, cattle 18.4%, asses 4%, and camel 2.3% some poultry and honeybees are also kept.

Study animals

The study population was indigenous zebu cattle that vary in sex, age, and body condition score which kept under extensive husbandry system with communal grazing and watering points. All cattle with respiratory signs, pneumonic (53 animals) presented to the Dire Dawa Veterinary Clinic were included. For comparative study, apparently health cattle (91) from slaughter house were also included in the study. Totally 144 animals were sampled.

Sampling methods

Purposive sample technique was used to sample clinically pneumonic animals and lung tissues from slaughtered animals at the Dire Dawa Municipal Abattoir. Each study animal was individually identified and restrained by an assistant and kept fixed before the sampling procedures. A total of 53 nasal swabs were collected aseptically from clinically pneumonic cattle at the clinic showing respiratory signs such as an irregular breathing pattern and grunting on expiration, coughing, serous nasal discharge. Similarly, a total of 91 lung tissue samples from slaughtered animals were collected immediately after slaughter. All samples were processed bacteriologically.

The methods followed were based on the procedures of Quinn *et al*.[7]. Sterile cotton-tipped, 20-25 cm long nasal swabs, moistened in sterile tryptose soya broth, were directed via the ventral nasal meatus in to nasopharynx i.e. the swabs were carefully inserted in to nostril; rolled gently; put back to the test tube containing broth; and the tubes were capped. The swabs were then kept in an ice box (4°C**)**. The specimens collected were transported to Dire Dawa Veterinary Regional Laboratory for bacteriological analysis. At a time of sample collection aseptic procedures were implemented to avoid picking contamination from the external nares; thus, the external nares were decontaminated with cotton soaked with 70% ethanol. The collected tissue samples were placed in separate sterile universal bottles, labeled and kept cooled in the ice box and transported to the laboratory.

Sample processing and culture

Nasal swab samples streaked on sheep blood agar plates and incubated at 37°C for 24-72 hrs. The same

samples were streaked on MacConkey agar for primary differentiation of the pathogen following standard procedures. Colonies were characterized and those giving gram-negative coccobacilli or short rods with or without bipolar staining on smears were sub-cultured for identification. The pure *Pasteurella* suspected culture (isolate) was subjected to biochemical tests using standard procedures.

A section of lung samples were aseptically taken from the edge of the lesion in case of pneumonic cases and the samples were processed before inoculation in to appropriate media as follows:- the surface of the lung tissue passed through the Bunsen burner several times to avoid surface contamination and transferred to petridish. Then the surface was heated with spatula and incised and minced with sterile forceps, scalpel blade. Then the tissue specimens were placed in to screw capped test tubes containing to TSB was incubated for 2hrs at 37^{o}C and then streaked on to both blood agar plate (BAP, 5% blood in blood agar base) and MacConkey Agar plates. The plates were incubated at 37^{o}C for 24 hrs. The grown colonies were characterized and sub-cultured on nutrient agar plate to get pure culture for further biochemical tests following standard protocol [10].

Isolation and identification of Pasteurella species

To obtain, pure cultures of a single bacterial colonies type from blood agar and MacConkey agar. Bacterial colonies characteristics of rod, coccobacilli, rough or smooth, gram negative, with or with no growth on MacConkey, presence or absence of heamolysis on blood agar were subjected to sub culturing. Then, each bacterial colony was characterized and further bacterial identification was made using series of primary and secondary biochemical tests following standard procedures [11].

Bacterial cultures were firstly characterized based on primary identification characteristics such as the cellular morphology of the bacteria; Gram staining, oxidase, catalase and oxidation fermentation tests to determine the genus of the bacterial isolates. After, pure colonies were obtained, transferred to nutrient agar media. Furthermore, species of the bacterial isolates were determined using of secondary biochemical tests. Tryptophan Broth was used for Indole production conducted according to the procedure described by Quninn *et al*. [7]. All activities from streaking primary media to secondary biochemical tests conducted for final identification of bacterial isolates to the species level. *P. multocida* on blood agar the coloniesnon-heamolytic round, smooth or mucoid, all the isolates failed to grow on MacConkey on the basis of Gram staining the isolates were found to be grow Gram negative, Coccobacillary rods with or without bipolar staining on primary identification [8,12].

In Vitro Antimicrobial Sensitivity Test

Antimicrobial susceptibility profiles of the isolates were determined using standard disc diffusion methods as described by Kirby -Bauer on Mueller-Hinton agar supplemented with defibrinated 5% sheep blood. The antibiotic susceptibility tests of *Pasteurella species* from pure culture was carried out by standard disk diffusion methods and interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines [13]. The following common antimicrobials applied in the country were assayed: Chloramphenicol (CAF 30Ng), Penicillin- G (10 IU), Tetracycline (30 Ng), Ampicillin (10Ng), Sulfamethoxazole (25Ng), Vancomycin (10Ng), Streptomycin (10Ng) and Gentamycin (30Ng). About 4-5 similar colonies from the blood agar were suspended in a propagating medium of nutrient broth and incubated for 2-8 hour duration of incubation then the bacterium culture was taken and re suspended into saline water then it is adjusted until similar to 0.5 McFarland standards according to the guide line [14]. Using sterile cotton swab with applicator stick the suspended bacteria from the broth was uniformly distributed on the surface of Muller Hinton agar plate, containing 5 % of sheep blood, at three different planes.

The plates were dried for some minutes by putting upside down. Antibiotic discs were applied on the plate using sterile forceps ensuring complete contact with the agar surface and 4-5 discs regularly placed in one plate 3 cm apart and 1.5cm from the edge antibiotic impregnated paper discs and the plates were incubated at 37 ^{0}C for 8-12 hours. Finally, the diameters of the zone of inhibition was measured using a transparent ruler to the nearest mm. Based on the zone of inhibition the isolates were categorized descriptively as resistant, intermediate, and susceptible.

Data analysis and interpretation

The collected data were entered and analyzed by using SPSS version 20. Descriptive statistics called person Chi-Square test was used to determine the statistical significance for categorical data analyzed statistically,

confidence level was held at 95% and *P<0.05* was set for significance.

Results

Bacterial isolation

In this study, out of 144 examined cattle (53 nasal swabs from pneumonic and 91 apparently healthy), *Pasteurella* was isolated in 33 (22.9%) in which 15 (28.3%) were nasal swab positive and 18(19.8%) from lungs tissues (Table 1).

Table 1: The overall prevalence of *Pasteurella* in study area

No of examined Animal	**+Ve Result**	**Prevalence (%)**
Pneumonic	15	28.3%
Apparently healthy	18	19.8%
Total (144)	**33**	**48.1%**

Of the 33 isolates, 28 (84.8%) and 5 (15.2%) were found to be positive for *P. heamolytica* and *P.multocida*, respectively. From, 53 nasal swab samples examined from respiratory tracts of cattle, 5 (15.2%) and 18 (19.8%) of *P. multocida, M. haemolytica* were isolated respectively. While 91 lung tissues samples were collected from Dire Dawa Municipal Abattoir, 18 (100%) *M. haemolytica* were isolated. The total culture results of isolates from both the nasal swabs and lung tissues were 22.9 % that comprises of two different bacterial species **(**Table 2).

Table 2: Distribution of *Pasteurellacae species* isolated from nasal swabs and lung tissues of cattle in Dire Dawa area

Types of Sample	***Pasteurella* species isolated**		**Total**
	P.multocida	***M.heamolytica***	
Nasal Swab (Clinic)	5 (15.2%)	10(30.3%)	15 (45.5)
Lung tissues (Abattoir)	0 (0%)	18 (54.5%)	18 (54.5%)
Total	**5 (15.2%)**	**28 (84.8%)**	**33 (100)**

However, the result of present study revealed that, higher bacteriological confirmed isolates were found in clinical cases than that of, in apparently healthy animal, at abattoir. Concerning with, the agreement between the host factors of study animals and, entire bacteriological confirmed cases; body condition has significance association statistically (P=0.02); in similar fashion, *P. hemolytica* was isolated in higher abundance frequency in moderate body condition at abattoir next to age old animals. Age was also significantly associated with *P. hemolytica* infection at (P=0.01). However, sex has no any relation for both *Pasteurella* isolates at clinic and abattoir which was contributed in equal magnitude in both male and female (P>0.05) (Table 3).

Table 3. Demographic association of culture positive result of examined animals samples from clinic and abattoir with their risk factors in study area

Variables		**Nasal +Ve**	**Lung +Ve**	**Total**	**P-Value (X^2)**
Sex	Female	8	9	17(51.51%)	0.09 (4.527)
	Male	7	9	16(48.48%)	
Age	Young	9	6	15(45.45%)	0.01 (2.092)
	Adult	3	9	12(36.36%)	
	Old	3	3	6 (18.18%)	
Body Conditions	Good	3	5	14(42.42%)	0.02 (7.705)
	Moderate	4	10	11(33.33%)	
	Poor	8	3	8 (24.24%)	

Bacterial Isolates

The antibiotic susceptibility tests of bacterial species isolated during the study period from nasal swabs and lung tissues as carried out by disc diffusion method showed that almost *P. multocida* were found to be sensitive to most of the drugs tested. Bacterial isolates were more susceptible to five antibiotic discs used even if the degrees of susceptibility vary. Gentamycin, Streptomycin and Vancomycin were 100% failed under resistance to *P.multocida* whereas Chloramphenicol,Tetracycline, Sulfamethoxazole and penicillin-G were scored 100%, 80%, 60%, and 60% sensitivity patterns respectively to be sensitive for *P.multocida* isolates, which indicated that chloramphenicol and Tetracycline were the most effective antibiotics. But Sulfamethoxazole and penicillin G accepted as moderately effective drugs (Table 4).

Table 4. Summary of antimicrobial sensitivity test result descriptively

Antimicrobials Used	***P.multocida* isolates**		
	Sensitive	**Resistant**	**Intermediates**
Ampicillin (10Ng)	2(40%)	2(40%)	1(20%)
Gentamycin (30Ng)	-	5(100%)	-
Tetracycline (30Ng)	4(80%)	-	1(20%)
Penicillin- G(10Ng)	2(40%)	3(60%)	-
Streptomycin(S)(10Ng)	-	5(100%)	-
Sulfamethoxazole (25Ng)	3(60%)	1(20%)	1(20%)
Vancomycin (30Ng)	-	5(100%)	-
Chloramphenicol (30Ng)	5(100%)	-	-

Discussion

In this study, the overall prevalence of bovine pneumonic pasteurellosis was 48.1% in study area. This finding was slightly lower than [1,8,15] who reported 50.2%, 63.8% and 67.6%, respectively. However, the result was higher than that of Tilaye [16] who reported 28.4%. This might be due to the difference management system, laboratory facilities and predisposing factors.

The prevalence of *M. haemolytica*, 18 (54.5%) was only isolated from at abattoir whereas *P. multocida* was isolated from nasal swabs and lung tissues. This result was lower than the findings of [17] who reported as 40.8% and 56%, respectively in pneumonic lung tissues. This difference might be due to the type of sample taken from purely pneumonic lung tissues and improved health facilities in the current study area.

About 33 (22.9%) overall isolates: *M. haemolytica*, 28 (84.8%) and *P. multocida*, 5 (15.2%) were recovered on bacteriological analysis respectively. Comparing the two Pasteurella species, *M. haemolytica* (84.8%) was the major causative agent involved in bovine pneumonic pasteurellosis. This was consistent with previous reports of [16,18]. It is a normal flora of the URT may play a secondary role after the primary initiating agent suppressed the host defense mechanism, and favors the multiplication of Pasteurella species leading to bronchopneumonia in purely pneumonic animal [19]. It constituted higher percentage of the total isolates this implying that it was the major causative agent involved in bovine pasteurellosis. This finding was consistent with other previous reports [20,21] that indicated *Pasteurella* species are the causative agent of pneumonic pasteurellosis in most animal species of all climatic zones in which ruminant's usually asymptomatic carriers. In the present study, the results revealed *M. haemolytica* was isolated both from pneumonic and apparently healthy animals whereas *P.multocida* was only isolated from pneumoniconce [8, 22].

In this study, higher rate of infection was associated with young age groups as compared to adults ($P<0.05$). This might be due to the immune status of the animal being able to predispose to the bacterial infection and other predisposing etiological agents [21].

According to antibiotic sensitivity test finding report before by Esra *et al*.[23] who reported as *P.multocida* were susceptible to Chloramphenicol and Streptomycin (90%), and 80% to Tetracycline and Gentamycin, 75% to Sulfamethoxazole from bacterial examination in nasal cavity of apparently healthy and unhealthy Holstein cattle, in Afyon Kokatepe University, Turkey; Kamran who reported that, sensitivity of *P.multocida* isolates to Gentamycin and Chloramphenicol 72.73% and to Ampicillin 45% [24]. Antibiotic sensitivity patterns of the isolates were determined in this study. The antimicrobial susceptibility tests indicated that chloramphenicol and tetracycline were the most effective antibiotics. The

finding was in agreement with the result of Abera *et al.* [8] which was conducted at Illubabor Zone, in Bedelle District of Oromia regional state, western Ethiopia. One of the interesting finding of this study was demonstration of *P. multocida* isolates to be highly resistance for Gentamycin (100%), Vancomycin (100%), and streptomycin (100%), that strength the finding of [21] so this finding is forwarded streptomycin as the drug of choice for gram positive bacteria rather than for gram negative bacteria. Additionally, this study indicates that, *P.multocida* is susceptible to Chloramphenicol, penicillin G, Tetracycline and Sulfamethoxazole.

Conclusion

In this study, bovine pneumonic pasteurellosis was the major disease of cattle in the study area and *M. haemolytica* is the most common cause. Being young animal was a risk factor for the disease. Furthermore, the antibiotic sensitivity tests of the isolates indicated that chloramphenicol and tetracycline were the most effective antibiotics. Thus, Avoiding of the predisposing factors and use of broad spectrum antimicrobials as a prophylactic and treating sick animals should be suggested.

Competing Interests

The authors declare that there is no competing interest.

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