

**ADDIS ABABA UNIVERSITY**  
**COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE**

**EPIDEMIOLOGY AND DRUG RESISTANCE OF OVINE PASTEURELLOSIS IN  
SELECTED DISTRICTS OF ARSI ZONE OF OROMIA REGIONAL STATE,  
ETHIOPIA**

**BY**  
**BEHAILU ASSEFA**

**JUNE 2012**  
**DEBREZEIT, ETHIOPIA**

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**A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial  
fulfillment of the requirements for the degree of Master of Science in Tropical Veterinary  
Epidemiology**

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## LIST OF ABBREVIATIONS

<i>hr</i>	<i>Hour</i>
MIC	minimum inhibitory concentration
NCCLS	<i>National Committee for Clinical Laboratory Standards (USA)</i>
ODC	ornithine decarboxylase
P	level of significance
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Spp	Species
TSI	triple sugar iron

## ABSTRACT

This study was conducted from September 2011 to May 2012 in selected districts of Arsi Zone of Oromia Regional State with the objectives of determining the epidemiology of *M.haemolytica*, *P.multocida* and *B.trehalosi* through studying their isolation rates among different contributing host and environmental risk factors. Moreover, the efficacy of Tetracycline, Penicillin, Amoxicillin and Gentamicin against representative isolates was determined. A total of 384 local sheep were examined and 109 isolates were identified. 65 isolates (59.6%) of the total isolates were *M.haemolytica*, 33 isolates (30.3%) *P.multocida* and 11 isolates (10.1%) were *B.trehalosi*. The distribution of general positives among different age groups of sheep were found varying significantly ( $P<0.05$ ) while their distribution across study districts, agroecological zones of study area, sex, health status, treatment condition and stock rates of sheep varies but insignificantly. Antibiotic efficacy results showed that *M. haemolytica* isolates were resistant to Penicillin (33% of the tested isolates) while susceptible to Amoxicillin (all tested isolates) and Gentamicin and Tetracycline (67% of isolates). *P. multocida* isolates were also resistant to Penicillin (33% of the tested isolates) and Tetracycline (67% of the isolates) but susceptible to Amoxicillin (all tested isolates) and Gentamicin (67% of tested isolates). The results of this study indicate that Gentamicin and Amoxicillin are drugs of choice relative to Penicillin and Tetracycline.

Key words: Amoxicillin, Arsi, *B.trehalosi*, Gentamicin, *M.haemolytica*, Penicillin, *P.multocida*, Sheep, Tetracycline

## 1. INTRODUCTION

In sheep respiratory disease is a serious problem that can result in substantial economic losses. One among respiratory diseases of sheep is Pasteurellosis. The term “pasteurellosis” is usually used for disease, often respiratory caused by bacteria in the family *Pasteurellaceae* and genus *Pasteurella* which recently classified in the genera *Pasteurella*, *Mannheimia*, and *Bibersteinia* (Blackall *et al.*, 2007). Bacteria of the family *Pasteurellaceae* are involved in a variety of economically important diseases in food-producing animals. Pasteurellosis is a multifactorial respiratory disorder (Catry, 2005).

*Pasteurella multocida*, *Mannheimia haemolytica*, and *Bibersteinia trehalosi* (all formerly in the genus *Pasteurella*) are the three most commonly isolated bacterial agents from pneumonias that result in high rates of morbidity and mortality in sheep. *Pasteurella multocida* and *Mannheimia haemolytica* are causative agents of several economically significant veterinary diseases. Serious infectious diseases as fowl cholera, bovine hemorrhagic septicemia, and porcine atrophic rhinitis are caused by *P. multocida* whereas *M. haemolytica* is the causative agent of shipping fever or pneumonic pasteurellosis in sheep (Michael, 2008).

*Pasteurella multocida* was first found in 1878 in fowl cholera-infected birds. However, it was not isolated until 1880, by Louis Pasteur - the man whom *Pasteurella* is named in his honor (Katherine, 2008). Now strains of *P. multocida* are grouped serologically into 5 capsular types (A, B, D, E and F) and 16 somatic lipopolysaccharide-types (1–16). *P. multocida* strains have also been characterized by outer membrane protein (OMP)-type and 16S rRNA-type. 16S rRNA-typing revealed that the majority of clinical isolates belong to a single lineage containing seven 16S-types. However, a range of capsular types, OMP-types and host species were represented, indicating significant heterogeneity between closely related strains (Richard, 2009).

*Mannheimia haemolytica* has been the subject of extensive reclassification in the past: first called *Bacterium bipolare multocidum* by Theodore Kitt in 1885, it was renamed *Pasteurella haemolytica* in 1932 and classified into two biotypes (A and T) based on its ability to ferment the

sugars arabinose and trehalose, respectively. These biotypes were further subdivided into 13 A serotypes (A1, A2, A5, A6, A7, A8, A9, A11, A12, A13, A14, A16 and A17) and 4T capsular serotypes (serotypes 3, 4, 10 and 15), based on results from an indirect haemagglutination test (Biberstein and Gills, 2002). After years *Pasteurella haemolytica* biotype A was allocated to a new genus and renamed *Mannheimia haemolytica* while the 4T serotypes named *Bibersteinia trehalosi*. Recently because of serotypes A11 differently classified as *M. glucosida*, *M. haemolytica* is considered having twelve serotypes (A1, A2, A5-A9, A12-14, A16 and A17) based on capsular antigen typing (Sarah, 2011). The genus *Mannheimia* now contains several species including *M. haemolytica*, *M. granulomatis*, *M. glucosida*, *M. ruminalis* and *M. varigena* (Mohamed and Abdelsalam, 2008).

*Pasteurellaceae* are common inhabitants of the tonsils and nasopharynx of a variety of healthy sheep. In sheep, *Pasteurellaceae* are believed to be opportunistic bacteria that colonize the lung after some predisposing insult. The initiating insult can be from respiratory infection by mildly pathogenic agents such as parainfluenza-3 (PI-3) virus, adenoviruses, respiratory syncytial viruses (RSV), *Chlamydia pecorum*, and *Mycoplasma ovipneumoniae*, as well as from mechanical irritants such as dust and lungworms. In most instances, these insults alone do not result in significant epidemics with high morbidity or mortality; however, when these and other stressors are compounded by infection with *Pasteurellaceae*, the result can be increased morbidity and death (Michael, 2008). In general infections result when an animal is compromised by any of a variety of stress factors as inclement weather, shipping(transportation), malnutrition, bacterial invasion of host defense, viral infections, nasopharyngeal colonization and dehydration (Hawari *et al.*, 2008).

The pathogenesis of pneumonic pasteurellosis remains a subject of considerable speculation and controversy due to the complex nature of the disease and the lack of consistency in experimental results (Sarah, 2011). Regardless of the economic importance of this pathogen, relatively little is understood about the precise mechanisms of pathogenesis, specifically how *M. haemolytica* adheres to the respiratory tract epithelium even if previous studies have demonstrated the ability of *M. haemolytica* to adhere to epithelial cells *in vitro* (Kisiela and Czuprynski, 2009).

The application of the advanced diagnostic techniques as electron microscope investigation or DNA analysis of the microorganisms has helped a great deal in the elucidation of the virulence factors of the organism and their role in pathogenesis, which helped in the development of potential candidate vaccines as well as new-targeted generations of antibiotics. Despite the application of advanced investigation and diagnostic techniques on both the organism and the affected animal species, pasteurellosis still continue to contribute to heavy losses in sheep production and remain a hazardous threat to human health worldwide (Ragy, 2005).

So to combat these loses it needs further study on different contributing factors of this disease as it is a multifactorial disease. The different studies conducted in Ethiopia indicated that pasteurellosis is a major threat to sheep production. Some of these studies were those in Amhara Regional State particularly Debre Birhan (Gelagay *et al.*, 2004) and South Wollo (Belay, 2007) and Oromia Regional State particularly selected sites of Arsi Zone (Mekonnen, 2000) as well as those in Debre Birhan, Harshin and Jijiga (Deressa *et al.*). In Arsi, despite annual vaccination against pneumonic pasteurellosis with a monovalent vaccine (inactivated *P. multocida* biotype A), there are high rates of mortality and morbidity following respiratory distress. Pasteurellosis is therefore a high-priority issue at the national level due to the significant economic losses it causes through mortality, morbidity, and the high cost of treatment. The main problem is absence of an extensive study on epidemiology of this disease as well as absence of a cost effective prevention and control methods which suits best for different phenotypes and serotypes of the agent.

This study was aimed:-

- ❖ To determine epidemiology of the common causative agents of ovine pasteurellosis (*M. haemolytica*, *P. multocida* and *B. trehalosi*) in selected four districts of Arsi Zone of Oromia Regional State, Ethiopia through studying isolation rates of the organisms in sheep under different host and environmental risk factors,
- ❖ To evaluate the efficacy of Tetracycline, Penicillin, Ampicillin and Gentamicin against representative isolates obtained from all the four study districts.

## **2. LITERATURE REVIEW**

### **2.1. Microbiology of Pasteurellaceae**

*Pasteurellaceae* is a small, Gram-negative coccobacilli or rods which may show bipolar staining. It may grow on most laboratory media with the exception of bile containing media such as MacConkey agar for *P. multocida* (Hawari *et al.*, 2008). It is non motile and non spore forming, fermentative, with few exceptions; ferment sugars like glucose, sucrose, and maltose and most of them produce acid from common sugar but not H<sub>2</sub>S gas. It is aerobic or facultatively anaerobic with fastidious growth requirements. *Pasteurellaceae* is oxidase and catalase positive, reduces NO<sub>3</sub> to NO<sub>2</sub>, and urease negative. Its growth on artificial media is enhanced by the addition of serum or blood, on which they appear after 24 hours of incubation as round, smooth, greyish colonies of moderate size (1-2 mm in diameter) (Catry, 2005).

### **2.2. Epidemiology**

Pasteurellosis in sheep often is described as a disease complex and generally is thought to result from invasion of the lung by *Pasteurellaceae* following a compromise of the respiratory tract defense mechanisms (Michael, 2008). Both *M. haemolytica* and *P. multocida* are common commensal organisms of the tonsils and nasopharynx of healthy sheep and certain factors can trigger the bacteria to move quickly to invade the lungs or erupt from the tonsils to become systemic and cause various forms of pasteurellosis. The trigger factors causing pneumonic pasteurellosis are stress (e.g. dipping, castration, clipping, docking...etc.), climate (e.g. warm and cold, wet and windy weather) and other infectious diseases. It is believed that these factors seem to alter the upper respiratory tract (URT) epithelium allowing the sequential down-regulation of local pulmonary defense mechanisms such as ciliation and mucous production, resulting in the agent colonizing, escaping clearance in the nasopharynx, and moving to the lungs by gravitational drainage (Zuber, 2009).

Outbreaks of *Mannheimia* are thought to occur when local and systemic defense mechanisms are impaired and virulent strains of the organism undergo massive proliferation prior to invading the nasopharyngeal mucosa or being inhaled in large numbers into the lungs (Kaoud *et al.*, 2010).

*P. multocida* primarily or secondarily can cause pneumonic pasteurellosis in sheep and worldwide outbreak lead to high mortality and great economic loss. *P. multocida* is responsible for major animal diseases of economic significance in both developed and developing countries. Significantly, development of a carrier state or latent infections plays a critical role in the epidemiology of these diseases. Asymptomatic carriers of *P. multocida* act as a reservoir of infection and transmission occurs by direct contact with infected animals, or dissemination in water supplies (Richard, 2009).

#### 2.2.1. Agent factors contributing to epidemiology of *P. multocida* and *M. haemolytica* and their distribution

*Pasteurella* species are generally extracellular parasites that elicit mainly a humoral immune response. Several virulence factors have been identified both for *P. multocida* and *M. haemolytica* and these virulence factors influence the outcome of bacterial-host interactions (Hawari *et al.*, 2008). Among the major pathogenic components of *M. haemolytica* and *P. multocida* are the polysaccharide capsule, outer membrane proteins (OMP), lipopolysaccharide(LPS), fimbria, adhesins, exotoxines, extracellular enzymes and other factors that still to be investigated and elucidated (Ragy, 2005).

Pneumonic pasteurellosis, or “shipping fever”, is due in large part to *M. haemolytica*. Of the 12A serotypes of *M. haemolytica* described, A1 and A2 are established worldwide, although there have been reports of morbidity and mortality accounted to serotypes A6, A7, A9 and A12 (Quirie *et al.*, 2006). Both A1 and A2 possess the ability to colonize the upper respiratory tract of cattle and sheep, they are however often species specific (Sarah, 2011).

Serotype A1 causes pasteurellosis in cattle and has been the subject of extensive study, while serotype A2 causes disease in sheep and is less-well characterized (Sarah, 2001).

*Bibersteinia trehalosi* mainly causes septicemia and systemic pasteurellosis in young weaned sheep which is a hyper-acute disease and occurs most commonly in the autumn season (Richard, 2009) but can also cause pneumonia.

Table 1: *M. haemolytica* and *B. trehalosi* Serotypes Distribution

Prevalence (%)	<i>M. haemolytica</i> Serotypes	<i>B. trehalosi</i>	Country	Reference
25	A1,A2,A7,A9	T4,10,15	New Zealand	Black <i>et al.</i> (2011)
12	A1,A2,A6,A7,A8,UT	T4	Turkey	Kirkan and Osman (2005)
9	A1,A2,A6,A7,A9,Others,UT	T3,T4,10,15	UK	Mike (1999)
24.5	A1,A2,A5-9,A12-14	T3,4,10,15	Ethiopia, South Wollo	Belay (2007)
13.9	A1,A2,A5-8,A12	T3,4,10,15	Ethiopia, Debre-Zeit (ELFORA)	Mesele (2005)
20	A1,A2,A5-9,A13,A14		Ethiopia, Debre Birhan	Gelagay <i>et al.</i> (2004)
55	A1,A2, A5, A7-9, A11-13	T15	Ethiopia, Arsi Zone	Mekonnen (2000)

From table 1 above it can be seen that of the 12A serotypes of *M. haemolytica* A1, A2 and A7 are found in all countries. A6 and A9 are second in their distribution while A8, A12, A13 and A14 are rare in distribution. But A16 and A17 were not reported in any country. *B. trehalosi* serotype T4 is commonly isolated in different countries followed by T3, T10, and T15.

Table-2 Prevalence of *M. haemolytica* and *B. trehalosi* Serotypes Isolated from Sheep.

Country	<i>M. haemolytica</i> Serotypes												<i>B. trehalosi</i>			
	A1	A2	A5	A6	A7	A8	A9	A11	A12	A13	A14	UT	T3	T4	T10	T15
Turkey <sup>a</sup>	12.5	25	-	21	21	4	-	-	-	-	-	8	-	8	-	-
UK <sup>b</sup>	6	34	-	6	5	-	4	Others (6)				8	3	7	12	9
Ethiopia <sup>c</sup>	18	35	34	27	23	15	9	28	16	6.5	5.5					
Ethiopia <sup>d</sup>	13	36	4	14	16	35	2	29	-	19	-					

Key: <sup>a</sup>Kirkan and Osman, 2005; <sup>b</sup>Mike, 1999; <sup>c</sup>Belay, 2007 and <sup>d</sup>Gelagay *et al.*, 2004

*M. haemolytica* serotypes A2 is the most prevalent serotype in all the studies. Hence, it is the predominant cause of pneumonic pasteurellosis of sheep.

Table 3 below shows that on average pasteurellosis caused by *M. haemolytica* is more prevalent than pasteurellosis caused by *B. trehalosi* and *P. multocida*. It is indicated that ovine pasteurellosis is mostly associated with *M. haemolytica* than *B. trehalosi* and *P. multocida*.

Table-3 Prevalence of *M. haemolytica*, *B. trehalosi* and *P. multocida* in Ethiopia.

Prevalence (%)			Place	Author	Year
<i>M.haemolytica</i>	<i>B.trehalosi</i>	<i>P.multocida</i>			
11.1	13.4	1.8	South Wollo	Belay	2007
8.3	5.6	25	Debre Zeit (ELFORA)	Mesele	2005
20	-	10	Debre Birhan	Gelagay <i>et al.</i>	2004
19	-	15	Debre Birhan, Harshin, and Jijiga	Deressa <i>et al.</i>	2004
48	7	2	Arsi Zone	Mekonnen	2000

#### 2.2.2. Host factors contributing to epidemiology of *P. multocida* and *M.haemolytica*

Disease progression is thought to depend on a complex interaction of host factors including species, age, breed and immune status, and strain-dependent virulence factors of the agent such as production of toxins, adhesins and mechanisms for acquiring nutrients from the host (Richard, 2009). Bronchopneumonia caused by *P. multocida* or *M. haemolytica* has a cranioventral lung distribution and affects sheep of all ages worldwide. It can be particularly devastating in young animals. It is a common cause of morbidity and mortality in lambs, especially in those that have not received adequate colostrum or in which passive colostrumal immunity is waning (Merck, 2011). Affected animals often die if not treated (Michael, 2008).

Table 4: Age as risk factor affecting prevalence rate of Pasteurellosis in Lambs

Age Groups (months)	Prevalence (%)	
	Nasal Swabs	Lung Specimens
1-3	2.1	0
4-8	7.7	10.5
9-12	7.1	13.5

Source: Zuber, (2009)

Even though the study in table 4 covered only lambs up to one year, it shows that as age increases, relative incidence of pasteurellosis also increases. It was zero in lambs of age 1-3 months in lung specimens but lung infection increased with age of the lamb.

Since this data didn't compare lambs with adults on average basis, a clear picture of susceptibility of lambs than adults (Michael, 2008) can't be predicted or may be agent isolation is lower in adults without pneumonia due to development of immunity.

### 2.2.3. Environmental factors contributing to epidemiology of *P. multocida* and *M. haemolytica*

All *Pasteurella* species occur as commensals in the upper respiratory and alimentary tracts of their various hosts. Although varieties of some species cause primary disease, many of the infections are secondary to other infections or result from various environmental stresses. The effects of different environmental stressors are believed to be important components of risk factors for pasteurellosis in many domestic ruminants. Although the effects of stressors are difficult to measure, some indicators including increased body temperature, heart rate, respiratory rate and plasma cortisol have been correlated with disease. The disease appears to occur most often in animals that have undergone recent stress such as transportation, weaning, or commingling with animals from unrelated farms (Merck and Dohme, 2011).

Physiological response to stressors (collectively called "stress") includes suppression of the immune system; consequently, prolonged stress may increase susceptibility to pathogens and to morbidity and mortality. Environmental stressors most commonly associated with pasteurellosis in livestock include heat, cold, wind, chill, crowding, mixing with new animals, poor ventilation, handling, and transport. Other predisposing factors, such as lack of sufficient energy or protein, inadequate colostrum consumption, specific vitamins, or certain minerals, also may compromise immunity further (Michael, 2008).

Most *Pasteurellaceae* of sheep are obligate bacteria that die rapidly in the environment outside a living host. Environmental sources such as water and soil are not thought to be important in maintaining or spreading these bacteria; consequently, transmission is most likely to occur through direct contact among animals. Because many healthy domestic sheep carry strains

associated with disease, transmission of a specific pathogenic *Pasteurellaceae* strain may not be necessary for a disease outbreak to occur. In some instances, however, mixing individuals from different sources and possibly carrying different strains of *Pasteurellaceae* seems to precipitate outbreaks (Michael, 2008).

### **2.3. Diagnostic techniques of ovine Pasteurellosis**

In order to successfully treat pneumonic pasteurellosis, early diagnosis of infected animals is necessary. Diagnosis of pasteurellosis is mainly based on the clinical sign and symptom, laboratory tests and post mortem findings. Confirmatory diagnosis is done by isolation and identification of causative agent (Rex, 2004).

#### **2.3.1. Clinical sign**

Acute and chronic pneumonia produced by *M.haemolytica* and to a lesser extent by *P.multocida* are common disease problems in sheep populations. *M.haemolytica* causes two main diseases in sheep, pneumonic pasteurellosis and systemic pasteurellosis (enzootic septicemia). A wide variety of clinical signs, ranging from sudden death to occasional coughing, may occur in sheep affected with pneumonic pasteurellosis and it is frequently fatal (Graham, 2005). Acute pneumonia which is caused by *M.haemolytica* is a significant cause of mortality. This occurs either as flock outbreaks or as sporadic cases, and a typical pneumonia is a chronic pneumonia of lambs aged from three to twelve months but it is frequently severe where housed lambs are purchased from different sources (Rex, 2004). Affected animal often appear depressed, with a nasal discharge, exhibit inappetance and weight loss, and have temperatures in the 40°C - 42°C (>104°F) range. Most cases occur during the first two weeks after transportation and the course of disease can be rapid with death occurring before the above clinical signs of disease are observed (Rex, 2004).

A figure presented in table 5 bellow shows average measurements (with some variability) of different physiological changes due to pasteurellosis.

Table 5: The Mean Value (M±S.D) of Systemic Reactions in Lambs

Clinical Parameters	Infected Animal
Body temperature, °C	40.2± 0.34
Respiratory rate, breath/min.	48.3± 3.14
Heart rate, beat/ min.	117.53± 6.64

Source: Zuber, (2009)

### 2.3.2. Laboratory diagnosis

A variety of laboratory diagnostic techniques have been developed over the years for pasteurellosis and used routinely in the laboratory. Diagnosis is straightforward if bacteriology is performed well. Detection of agent causing pasteurellosis from clinical specimen by either of culture methods, serological tests, polymerase chain reaction (PCR) and other alternative methods is possible (Rajeev, 2011).

These detection systems provide a wide spectrum of options for rapid diagnosis and for detecting and understanding of latent infections in herd/flock health control programmes. Among these techniques molecular techniques of diagnosis is most important. Molecular technique not only gives diagnosis but it also provides information regarding capsular type of *Pasteurella* (Rajeev, 2011). Phenotyping and serotyping based on variations in fermentation patterns and gene sequencing have been used to distinguish among Pasteurellaceae strains (Michael, 2008). However, PCR methods of detection of agent in clinical specimen appear increasingly preferred (Richard, 2009).

#### Primary identification

In spite of the molecular advances, morphology and phenotyping are routinely used for primary identification of *Pasteurellaceae*. Mostly, the primary identification of *M. haemolytica* is based on the isolation of round greyish colonies of moderate size with a small pronounced surrounding zone of haemolysis after 24h of aerobic incubation on sheep blood agar. *M. haemolytica* does not produce indole and it tolerates bile salts. *P. multocida* typically grows as mucoid confluent colonies, and in routine diagnostic bacteriology it is distinguished from *M. haemolytica* by

production of indole from tryptophan, and by lack of haemolysis. A typical sweetish odour can be present (Catry, 2005).

#### A. Bacteriology (Phenotyping)

##### a. Culture (Colony characteristics)

Although pasteurellosis can be rapidly diagnosed with molecular and serological tests, isolation and accurate species identification are central to epidemiological tracing of outbreak strains. For that matter culturing of the bacteria is important. All of the *Pasteurella* species can be isolated by culturing appropriate clinical specimens on blood agar. *P. multocida* will grow at 37<sup>0</sup>c on blood or chocolate agar. Colonies are smooth, gray, and non-hemolytic after 24hrs incubation. Colonies have a characteristic “chemical” odor (“mousy” odor) on protein containing media (Tryptone broth) due to large amounts of indole produced from the amino acid. *P. multocida* does not grow on enteric selective media. *M. haemolytica* is odorless (indole negative), hemolytic, but grow on MacConkey agar unlike *P. multocida* (Ragy, 2005).

##### b. Stain (Cell morphology)

Up on Gram’s staining they are gram negative, small in size, pleomorphic coccobacilli or short rod in shape and often exhibiting bi-polar staining (Ragy, 2005).

##### c. Biochemical tests

The conventional method for the identification of a suspect isolate as *P. multocida* or *M. haemolytica* involves subjecting the isolate to a range of biochemical tests (Mifflin and Blackall, 2001). Being a facultative anaerobe, they are oxidase and catalase positive except *B. trehalosi* which is catalase negative. They can also ferment large number of carbohydrates in anaerobic conditions (Casolari and Fabio, 2008). Most produce acid from common sugars except lactose.

Both *P.multocida* and *M. haemolytica* are non-motile, urease negative, reduces  $\text{NO}_3$  to  $\text{NO}_2$  but not produce gas ( $\text{H}_2\text{S}$ ) (Mifflin and Blackall, 2001).

All strains of *Mannheimia* ferment mannitol, glucose; maltose, sorbitol and sucrose and they fermented them without gas production. Indole, urease, methyl blue (MB) and Voges-Proskauer (VP) reactions are negative. Catalase and oxidase are positive. Typically they do not ferment trehalose, but ferment L-arabinose (Kaoud *et al.*, 2010).

*B. trehalosi* are Gram-negative, non-motile rods. All isolates are positive in the nitrate reduction test and are negative in Simmons' citrate, methyl red and Voges-Proskauer tests. No isolates produced  $\text{H}_2\text{S}$ , urease or gelatinase. Indole was not produced. The isolates were all negative in the ornithine decarboxylase tests. The isolates showed variable reactions in the catalase and oxidase tests. Variable results were obtained for haemolysis (on bovine blood agar).

#### B. Serology (Serotyping)

Serotypic differentiation is based on sugar composition of the capsule as well as the composition of lipopolysaccharide (LPS). Serotyping of *M.haemolytica* and *B.trehalosi* is based on extractable surface antigens. Serogroups of *P.multocida* is identified based on difference in capsular polysaccharide which is further subdivided in to somatic types based on serological differences of their lipopolysaccharide. Different capsular extraction procedure affected the amount of protein extracted with the capsule. For example, saline extraction revealed fewer protein bands in the sodium dodicyl sulphate polyacrylamide gel electrophoresis analysis (SDS-PAGE) than salicylate extraction which was protein rich extract. Many methods of OMP extractions were reported for use as antigen in serological detection and surveillance of infection as well as in preparation of vaccines (Ragy, 2005).

Conventionally, the identification of the specific serotype is carried out using one or more serological methods. These include rapid slide agglutination, indirect haemagglutination and agar

gel immune diffusion tests (gel-gel precipitation) (OIE, 2008). Most of these tests are used for capsular typing.

1) Rapid slide/plate agglutination test: A single colony is mixed with a drop of saline on a slide, a drop of specific antiserum is added and the slide is warmed gently. A coarse, floccular agglutination appears within 30 seconds. Old cultures may give a fine, granular agglutination that takes longer to appear (OIE, 2008). This is a specific, rapid plate agglutination procedure for serotyping *M.haemolytica*. The procedure did not require special antigen preparation and yielded essentially the same results as the indirect hemagglutination procedure. Simply a drop of antiserum (approximately 10 µl) was placed on a clean glass surface, and then a small amount of *M.haemolytica* colony from blood agar was picked up on an inoculating needle and mixed with the serum. A strong positive reaction in the form of clumping and clearing occurred as the mixture was stirred with the needle. Negative reactions remained turbid (Hawari *et al.*, 2008).

2) Indirect haemagglutination test: Indirect haemagglutination is used for capsular typing using sheep red blood cells coated with bacterial extracts. The test can be carried out in tubes or plates, and is performed in two rows (OIE, 2008). Capsular antigen is extracted from a 24hr culture of bacteria of known serotypes in tryptose soya broth which is inactivated in a water bath at 60<sup>0</sup>c for 30 minutes and centrifuged at 3000rpm for 30 minutes. The clear supernatant is then collected into sterile test tubes to be used as capsular extract antigen. On the other hand fresh sheep blood is collected in Alsever's solution at proportion of 3:5 and the suspension is centrifuged at 2500 revolution per minute (rpm) for 5 minutes, washed twice with phosphate buffer saline (PBS) solution and again centrifuged at 2500 rpm for 5 minutes. For sensitization of the sheep red blood cells 50 µl of packed RBC is added to 5ml of capsular extract antigen and then 50µl of glutaraldehyde is added and homogenized with gentle shaking and incubated for 1hr at 37<sup>0</sup>c. After incubation the suspension is centrifuged and washed twice with PBS solution. Finally the pellet is adjusted with PBS solution to give a 1% suspension of RBC. In v-bottomed micro-plates 50 µl of PBS solution is added to all wells and 50 µl of test sera to the first column and serially diluted by pipetting 50 µl up to column 12. Fifty micro liters of sensitized RBC is added to each well and incubated for one hour at 37<sup>0</sup>c. Result is recorded based on complete or more than 50%

agglutination seen in each well. The titer showing 1/40 dilution and above is taken as positive (Belay, 2007).

3) Agar gel immunodiffusion tests: AGID tests are used for what is described as capsular as well as somatic typing depending on the antigens and antisera used. Somatic typing by agar gel immune diffusion tests uses heat-treated cell extracts. *M.haemolytica* and *B.trehalosi* can be distinguished by their Lipopolysaccharide SDS-PAGE profile.

Examination of OMP preparations by SDS-PAGE showed major differences between strains of *M.haemolytica* isolated from the same or different host species. Yet the individual serotypes were not accurately identifiable by this method due to the great similarity in their protein band resolution. The isolation of outer membranes and inner membranes of *M.haemolytica* A1 allowed for identification of their major proteins. Therefore this phenomenon was used to differentiate between the isolates (Ragy, 2005). The double-diffusion technique is employed. Wells are punched in the solid agar in a circular pattern with one centre well surrounded by six peripheral wells (OIE, 2008).

Secondary identification

Molecular Test

Molecular identification will further improve accurateness of characterization (in pure and/or mixed cultures), speed of detection, determination of taxonomic position, and understanding of intra-species genetic relationships. In an epidemiological context, accurate subtyping is important to recognize particularly virulent strains (in case of outbreaks or monitoring and vaccination programmes) and their source of origin (e.g. cross-transmission). In relation to antimicrobial resistance, molecular typing methods are essential to distinguish between clonal and horizontal spread of resistance genes and to monitor the occurrence and relatedness of resistant strains on an international scale (Catry, 2005).

Techniques which are used for molecular diagnosis are PCR based diagnosis; Restriction endonuclease analysis (REA), Ribotyping, Colony hybridization assay, Random amplified polymorphic DNA (RAPD) analyses and Detection of the agent by Real Time PCR. Polymerase chain reaction (PCR) methods of detection of agent in clinical specimen appear increasingly preferred. PCR is used to detect a sequence of DNA unique to *Pasteurella*. Among these techniques real time PCR is most sensitive and specific (Rajeev *et al.*, 2011). Random amplified polymorphic DNA (RAPD) analyses has been applied for the distinction of strains belonging to the same species. It is a fast, sensitive method for the epidemiological studies and PCR-based method of genetic typing based on genomic polymorphisms. Fourteen RAPD profiles were found in *P. multocida* and 2 profiles were found in *M. haemolytica*. Using more than one RAPD primers could improve the identification of the isolates (Hawari *et al.*, 2008). Potential virulence factors of *M. haemolytica* can be identified, purified and characterized by gene cloning and DNA sequence analysis. These factors include a ruminant-specific leukotoxin, an anti-phagocytic capsule, lipopolysaccharide, iron-regulated outer membrane proteins, lipoproteins, sialoglycoprotease, neuraminidase and potential immunoglobulin proteases (Sarah, 2001).

The identification of *M. haemolytica* and *P. multocida* isolates can be confirmed by polymerase chain reaction (PCR) technique using PMOut primers for *P. multocida* and PHSSA primers for *M. haemolytica* and by random amplified polymorphic DNA (RAPD) using the random primer (OPA-11). PMOut primers used for amplification of genomic DNA from *P. multocida* are (5'-AGG TGA AAG AGG TTA TG-3' and 5'-TAC CTA ACT CAA CCA AC-3') with corresponding anticipated size of 219bp and derived from Omp87 gene while PHSSA primers used for amplification of genomic DNA from *M. haemolytica* are (5'-TTC ACA TCT TCA TCC TC-3' and 5'-TTT TCA TCC TCT TCG TC-3') with corresponding anticipated size of 325 bp and derived from ssa gene (Hawari *et al.*, 2008).

Table 6 - Oligonucleotides used for the amplification of species-specific and virulence associated genes of *Pasteurella* and *Mannheimia* species

Oligonucleotides	Size of product (bp)	Gene	Gene function(Description)	Species
KMT1/SP6T7	460	KMT1-sequence	Capsule serotypes	<i>P. multocida</i>
PomA fwd/rv	921	pomA		<i>M. haemolytica</i>
ToxA fwd/rv	866	tox A	Toxins (Dermonecrotic toxin)	<i>P. multocida</i>
Synth ewd/rv	1264	pmHAS	Hyaluronidase (Hyaluronan synthase)	<i>P. multocida</i>
PMOut/ rv	219	Omp87	Protectins(Outer membrane protein 87)	<i>P. multocida</i>
PHSSA/ rv	325	ssa gene		<i>M. haemolytica</i>

Deressa *et al.*, 2004, Hawari *et al.*, 2008 and Xibiao *et al.*, 2009

## 2.4. Prevention and Control approaches of Ovine Pasteurellosis

Pneumonic pasteurellosis is an acute infectious disease that causes widespread financial losses because of death, reduced live weight, delayed marketing, treatment costs and unthriftiness among survivors (Hawari *et al.*, 2008). These bacteria are part of the normal microbiota in the upper respiratory tract making the disease difficult to prevent (Catry, 2005). However; the following are among alternative prevention and control approaches.

### 2.4.1. Management

No single management practice will be effective in controlling this disease complex. Management practices which reduce stress, as well as early diagnosis and antibiotic treatment, are the key methods of controlling disease within farm, especially during the first two to three weeks after arrival. Animal exhibiting signs of respiratory disease should immediately be separated from other animals. Although antibiotics and vaccines are available for treatment and

prevention of *Pasteurellosis* in sheep they have not been adequately proven to eliminate a potential carrier state in any species (Rex, 2004).

Preconditioning can be important in that it spreads out many of the stressful procedures over a longer time frame and ensures that vaccines can be utilized to generate the greatest level of immunity when animal enter the farm. As part of this procedure, weaning should occur prior to shipping and anticipated poor weather in order to split the stresses associated with separation from the mother and entry into an unfamiliar environment (Rex, 2004). Sound sanitary practices and segregation of affected animals may help limit the spread of the major pasteurelloses.

#### 2.4.2. Antibiotic treatment

To combat pneumonic pasteurellosis, application of antibiotics may be performed for either therapeutic, prophylactic, or metaphylactic purposes. Where curative therapy indicates the treatment of the individual animal in which the diagnosis is made, prophylactic administration is performed in the prevention of shipping fever prior to transport or as element of medicated starter rations after arrival from the native farm. Metaphylaxis is the term used for the treatment of subclinically affected animals in the direct surroundings of diseased animals in order to reduce the morbidity and the mortality of an outbreak and in many cases metaphylactic treatment is administered orally which is liable to result in resistant commensal bacteria in the intestinal tract (Catry, 2005).

The variety of short- and long-acting compounds nowadays available to control pasteurellosis is substantial. Examples are beta-lactam antibiotics such as aminopenicillins (+clavulanic acid) and extended spectrum cephalosporins (cefquinome, ceftiofur), tetracyclines (oxytetracycline, doxycycline), potentiated sulfonamides (trimethoprim + sulfonamides), macrolides (tylosin, tilmicosin, tulathromycin), aminoglycosides (gentamicin), aminocyclitols (spectinomycin), lincosamides (lincomycin), phenicols (florfenicol), and second generation fluoroquinolones (marbofloxacin, danofloxacin, enrofloxacin) (Catry, 2005).

*P. multocida* is somewhat unusual in that it can be effectively treated with beta-lactam antibiotics, despite its Gram-negative structure. It is also often treated with fluoroquinolones or tetracyclines; fluoroquinolones inhibit bacterial DNA synthesis and tetracyclines interfere with protein synthesis by binding to the bacterial 30S ribosomal subunit. This class of antimicrobial agents inhibits bacterial growth by reversely binding to the bacterial ribosome (Andy, 2000).

Early antibiotic therapy is usually effective, and if improvement is not seen within 3 days after administration, another therapeutic agent should be used (Andy, 2000). Mass medication has been demonstrated to be a useful control method, either at farm entry or in the face of a disease outbreak. Antibiotics such as long-acting oxytetracycline (20 mg/kg body weight) or tilmicosin (10 mg/kg), given at farm entry or 72 hours later, have been shown to reduce disease and increase feed conversion and weight gain significantly compared to untreated animals. This method of disease control is one of the few which has been demonstrated to be cost effective and has been reviewed in the scientific literature. Likewise, in the face of an outbreak of respiratory disease, mass medication of coming led animals with long-acting oxytetracycline (20 mg/kg) can reduce disease in a cost effective manner if treatment is initiated early (Sarah, 2011).

However, decreased susceptibility and antimicrobial resistance is frequently reported for many of these agents in *Pasteurella* and *Mannheimia* organisms isolated from pneumonic lungs (Catry, 2005). Tetracycline resistance is among the most frequently reported resistance properties among *Pasteurella* and *Mannheimia* organisms. Ampicillin-resistant bacteria are also now very common and ampicillin-sulbactam has proven effective in the elimination of strains of *M.haemolytica* (Andy, 2000). It is the imprudent use of antibiotics that has driven, and continues to drive, the spread of antibiotic-resistance genes on plasmids and transposon, resulting in the reduced efficacy of these agents (Sarah, 2011).

Table 7: Resistance genes/regions and mechanisms found in *Pasteurella* and *Mannheimia* spp.

Antimicrobial agent	Resistance mechanism	Resistance gene/region	Bacterial source	
			<i>Pasteurella</i>	<i>Mannheimia</i>
Penicillins	$\beta$ -lactamase	<i>blaROB-1</i>	+	+
		<i>blaTEM-1</i>	+	-
		<i>blaPSE-1</i>	+	-
Sulfonamides	dihydropteroate synthase	<i>sul2</i>	+	+
Trimethoprim	dihydrofolate reductase	<i>dfrA20</i>	+	-
Tetracyclines	efflux protein	<i>tet(B)</i>	+	-
		<i>tet(H)</i>	+	+
		<i>tet(G)</i>	+	+
	ribosome protective protein	<i>tet(M)</i>	+	-
Streptomycin	phosphotransferase	<i>strA</i>	+	+
		<i>strB</i>	+	+
Streptomycin/spectinomycin	adenyltransferase	<i>aadA1</i>	+	-
Chloramphenicol	acetyltransferase	<i>catA1</i>	+	-
		<i>catA3</i>	+	+
		<i>catB2</i>	+	-
Chloramphenicol/florfenicol	efflux protein	<i>floR</i>	+	-
Quinolones (naladixic acid)	mutation	<i>gyrA</i>	+	-

Source: Catry (2005)

There are associated issues to be considered at antibiotic treatment that include: treatment protocol for the affected group, duration of treatment, treatment of relapsed cases, role of anti-inflammatory agents and antibiotic resistance (Andy, 2000).

#### Drug resistance epidemiology of the agent

Studies show that there are multiple drug resistance which is frequently encountered in different countries.

Table 8: Drugs resistance epidemiology of *Pasteurella/Mannheimia*

Drug (Antibiotics)	% of resistivity of <i>P. multocida</i> and <i>M. haemolytica</i>			
	Belgium	Netherlands	France	Germany
Ampicillin	0 <sup>a</sup> (0) <sup>b</sup>	9(42)	10(60)	10.4(11.9)
Ceftiofur	0(0)	0(0)	-(-)	0(0)
Oxytetracycline	2.3(0)	45(53)	36(61)	55.7(79.7)
Trimethoprim + sulphonamides	4.6(0)	45(58)	15(32)	8.5(40.7)
Florfenicol	0(-)	0(0)	-(-)	1.9(3.4)
Enrofloxacin	0(0)	5(5)	-(-)	0(0)

Source: Catry, 2005; a- *Pasteurella* and b- *Mannheimia*

Catry's study showed that both *P. multocida* and *M. haemolytica* were susceptible to Ceftiofur (Cephalosporins) in all the countries covered by the study except in France. It indicated Ceftiofur being a first drug of choice. Effective drugs on the second rank include Ampicillin (Beta lactam) and Enrofloxacin (2<sup>nd</sup> generation fluoroquinolones) in Belgium, Florfenicol (Phenicols) in Netherlands and Enrofloxacin in Germany. For the rest drugs even if the degree varies at least some isolates of both *P. multocida* and *M. haemolytica* are found resistant.

Other study conducted by Zuber (2009) in Iraq show that on average *Pasteurella/Mannheimia* isolates in the country were resistant to Carbenicillin (Beta lactam), Cephalexin, Doxycyclin (Tetracyclines) and Nalidixic acid (2<sup>nd</sup> generation fluoroquinolones) at the specified disc concentrations. The case was different in India where isolates found there were resistant to Amoxicillin, Metronidazole, Sulphadiazine and Trimethoprim with percentage of resistivity 100, 67, 100 and 67, respectively (Prabhakar *et al.*, 2010).

Table 9: Drugs resistance pattern of *Pasteurella/Mannheimia*

Drug (Antibiotics)	Disc Conc.	% of resistivity	Country	Author	Year
Amoxicillin	10µg	100	India	Prabhakar <i>et al.</i>	2010
Metronidazole	5µg	67			
Sulphadiazine	300µg	100			
Trimethoprim	5µg	67			
Carbenicillin	10µg	100	Iraq	Zuber	2009
Cephalexin	30µg	80			
Doxycyclin	30µg	80			
Nalidixic acid	30µg	67			

In general one can see that there are multiple drug resistance developed by both *P. multocida* and *M. haemolytica*. From the drug resistance epidemiology one can judge isolates from different areas were not equally susceptible to different antimicrobial agents. For this reason a drug sensitivity test should be conducted before administration of any antibiotics based on commonly isolated strains in an area.

#### Antimicrobial sensitivity testing

The agar disk diffusion method has been used to test common fast-growing bacterial pathogens and is recognized to work well with *P.multocida* and *M.haemolytica*. Reliable results can be obtained with disk diffusion tests that use standardized methodology and zone diameter measurement correlated with minimum inhibitory concentration (MIC) and the behavior of strains among clinically susceptible and resistant categorizations (OIE, 2008).

Table 10: Zone-diameter Interpretive Standards

Antimicrobial Agent	Disc Conc.	Diameter of Inhibition Zone			Results
		Resistant	Intermediate	Susceptible	
Ampicillin	10 µg	<13	14-16	>17	25.5+3.123
gram-negative					
Bacitracin	10 IU	<8	9-12	>13	31.5+3.175
Carbenicillin	10 IU	<19	20-22	>23	0+0
Cephalexin	30 µg	<14	15-17	>18	11.5+4.444
Chloramphenicol	30 µg	<12	13-17	>18	22.4+3.449
Doxycycline	30 µg	<12	13-15	>16	10.7+3.945
Erythromycin	30 µg	<13	14-22	>23	27.7+3.198
Fosfomycin	30 µg	<9	10-12	>13	31.0+3.240
Gentamycin	30 µg	<12	13-14	>15	25.7+1.887
Nalidixic acid	30 µg	<13	14-18	>19	11.7+4.110
Oxytetracycline	30 µg	<14	15-18	>19	19.0+1.225
Penicillin G	10 IU	<14	-	>15	36.2+2.869
Streptomycin(S)	30 µg	<11	12-14	>15	28.5+1.190
Tetracycline	10 µg	<14	15-18	>19	14.7+0.854
Tylosin	30 µg	<16	-	>20	32.0+4.813

Source: Zuber (2009)

Selection of the most appropriate antimicrobial agents to test is a decision best made by each laboratory in accordance with the needs of veterinary practitioners and the drugs available for veterinary use in the country (OIE, 2008).

#### 2.4.3. Vaccination

Due to the pathogenic nature and distribution of these organisms control of pasteurellosis is particularly difficult and it is generally accepted that vaccination is the desired approach to prevention (Willie, 2003). The most effective control measure for any infectious disease is the development of immunity, and prior exposure to *M. haemolytica* usually provides protection against subsequent infection.

There is no doubt that vaccination against pasteurellosis offers a safe, reliable and practical means of control (Stephen, 2001). Therefore, vaccination should be effective but some of the older vaccines did not perform well. A new generation of vaccines based on sound scientific principles and knowledge of the pathogenesis of *M. haemolytica* infection are now available and some of these have been shown to be effective under field conditions, following either one or two

administrations (Sarah, 2011). However, in order to realize the full potential of these products and ensure that optimal protection is induced, two immunizations are prudent for high risk groups. Ideally the first vaccination should occur on the ranch and the second either when the animal is shipped or at the farm, according to the manufacturer's instructions (Bowland and Shewen, 2000).

The key to control is an integrated approach beginning on the ranch with vaccination and exposure to farm conditions followed by subsequent revaccination after entry into the farm. One study has suggested that vaccination at branding and time of shipping provides optimal immune responses to the particular vaccine. In the past, this approach has not shown a significant beneficial effect, but newer vaccines are now available which should prove to be more effective. However, their cost-effectiveness has yet to be determined.

Numerous attempts have been made to develop efficacious vaccines against *M. haemolytica* all of which have been partially successful. Immunological cross-reactivity was demonstrated between the leukotoxins and the hemolysins. Moreover, the toxin and its gene have been sequenced and recombinant derivative of the toxin have been assessed for the efficacy as much potent vaccine production. A pure DNA sequence encoding *M. haemolytica* leukotoxin has now been patented and *E. coli* transformed by a plasmid vector containing the leukotoxin gene sequence and used for production of recombinant leukotoxin crude leukotoxin which is administered through gut to induce a pulmonary immune response in sheep (Ragy, 2005).

Further, injectable, edible and mucosal vaccines against pneumonic pasteurellosis are currently commercially available. Live vaccines and bacterins (killed bacteria) are used for the prevention of some strains of the disease. Commercial vaccines that include formaldehyde-treated whole cells or formaldehyde-detoxified crude bacterial extracts of toxigenic organisms were available (Ragy, 2005). However, it should be stressed that vaccine development is problematic and there is no single vaccine that provides protection from all the various strains (Andy, 2000).

### 3. MATERIALS AND METHODS

#### 3.1. Description of study Area

The study was conducted in Arsi zone, Oromia Regional State. Asella town is the administrative centre of the zone, and located 175 km southeast of Addis Ababa. Arsi zone is one of the 18 administrative zones of Oromia regional state. It is found in the central part of the region. It is located at 6°79' and 8°49' N and 38°41' and 40°44' E. It has an area of 2,118,675 hectares, of which 39.7% is highland, 29.1% is lowland and 27.5% is mid-altitude. The altitude of the area is ranging between 500 (Awash and Wabe valley) and 4245 (Mount Kaka) meters above sea level. The annual temperature varies between 10°C and 25°C. The average annual rainfall ranges between 901mm and 1200mm, with some spatial and temporal variability in quantities and distribution. Its pattern is of a bimodal type with 60% occurring in the long rainy season extending from June to September and the short rainy season from December to February. The other two seasons are the cool dry season extending from October to November and the major dry season from March to May. Arsi Zone has 24 Districts. The sheep population for each District as of the 2011/2012 census by Arsi Zone Animal Development, Protection and Marketing Agency is as follows:

Table-11 Sheep Population of the 24 Districts of Arsi Zone

District	Sheep Population	District	Sheep Population
Amigna	63,332	Limu and Bilbilo	297,792
Aseko	8,497	Lode Hetosa	97,624
Bele	45,556	Merti	25,696
Chole	41,067	Munesa	163,400
Dodota	21,859	Robe	61,535
Digelu and Tijo	103,952	Seru	4,759
Diksis	99,874	Shirka	72,950
Gololcha	14,347	Sire	63,848
Guna	63,772	Sude	85,801
Hetosa	53,000	Tena	55,000
H/Wabe	37,389	Tiyo	55,237
Jeju	107,683	Ziway Dugda	28,798

Source: Arsi Zone Animal Development, Protection and Marketing Agency (2012)

To represent the study area four districts namely Digelu and Tijo, Hetosa, Munesa, and Tiyo were selected. These districts were selected on the basis of past studies conducted and history for presence of ovine pasteurellosis by the Zonal Animal Development, Protection and Marketing Agency. To represent the agro-ecological zones of the study area; highland, mid-altitude and lowland agro climatic zones, were chosen.

**Digelu and Tijo:** It is found in the southeastern part of Arsi zone. Digelu and Tijo district is mainly characterized by highlands and plateaus. It has highland (90%) and mid-altitude (10%) agro climatic zones.

**Hetosa:** The district is found in the northeastern part of the zone. Hetosa district has highland (32%), mid-altitude (48%) and lowland (20%) agro climatic zones.

**Munesa:** This district is found in the southern part of Arsi Zone. It is characterized mainly by highlands (54%) and mid-altitude (44%). Lowland is only 2%. Forest, shrub and bush, and manmade forests are available in the district.

**Tiyo:** It is found in the Central part of Arsi Zone. The administrative seat of the district is Asella town which is also the administrative centre of the zone. Mount Chilalo is the major feature in the district. The district is classified into highland (38%), mid-altitude (45%) and lowland (17%) agro climatic zones. High forest, woodland, shrub and bush and man-made forests are available in Tiyo (Arsi Zone Animal Development, Protection and Marketing Agency, 2012) .

### 3.2. Study animals

For the determination of contributing factors to epidemiology of ovine Pasteurellosis, indigenous sheep breeds belonging to different localities, age, sex, health status, treatment condition and herd size were sampled. For the efficacy evaluation of the commonly used antibiotics in the area, representative *Mannheimia/Pasteurella* organisms which were collected and phenotyped were used.

### **3.3. Study design**

The study design was a cross-sectional study and it was designed to determine prevalence of *Pasteurella/Mannheimia* using isolation rates on culture and biochemical tests and to identify their distribution among different age groups, sex, district and ecological zones, health status and herd size as well as to compare the case between vaccinated and none vaccinated and antibiotic treated and none treated groups of sheep. The other part of the study design was a controlled experimental study, which is to evaluate the efficacy of Tetracycline, Penicillin, Ampicillin and Gentamicin against representative *Mannheimia haemolytica* and *Pasteurella multocida* isolates obtained from different districts and agroecology.

### **3.4. Sampling methods and sample size**

This study was conducted to determine prevalence of *Mannheimia/Pasteurella* phenotypes and their distribution dynamics within and among different agro-ecology in the four study districts and relate their distribution to different host and environmental risk factors and also to determine a drug resistant isolate if any. The districts were selected by purposive sampling technique where each district has three agro-climatic zones except one which does not have low land agro-climatic zone. Sheep were sampled from each study district proportional to their sheep population which employed a weighted average sampling technique. Then, the number of sheep to be sampled from each study district was divided equally for the agro ecological zones of the districts and individual samples were selected by convenient sampling technique.

Since the prevalence of ovine pasteurellosis in the selected districts were not known, the sample size were determined using the formula given by Thrusfield (1995) based on maximum expected prevalence of 50%. For this study 95% level of significance was considered. Hence,

$$N = 1.96^2 \times P_{\text{exp}} (1 - P_{\text{exp}}) / d^2, \text{ Where } N = \text{required sample size}$$

$P_{\text{exp}}$  = expected prevalence

$d$  = desired absolute precision, 5%

$$\Rightarrow N = 384$$

Therefore, during the study period a total of 384 sheep were sampled as follows: The sheep population of D/Tijo, Hetosa, Munesa and Tiyo were 103,952, 53,000, 163,400 and 55,237 respectively. The sampling fraction which is equal to sample size (384) divided by total population of the four districts (375,589) is 384/375589. Hence proportional to the total number of sheep population they possess, the number of sheep sampled from each study district was 106 sheep, 54 sheep, 168 sheep and 56 sheep respectively. Again the number of sheep to be sampled from each agro-climatic zone of the districts was determined simply by equally dividing the share of the districts for the number of agro ecological zones of the district.

Finally individual sheep sample was selected purposely from each agro ecological zones of study districts and then bacterial samples were collected from each sample individual at time of visit.

### 3.5. Study methodology

Sample for phenotyping and determination of prevalence of the disease in sheep was collected by nasal swab sampling method. For the evaluation of efficacy of the commonly used antibiotics, disc diffusion test was conducted on selected isolates. Information on ecology and other environmental factors of the study area, host factors (age, sex, health status,), sheep production system, management (medication/vaccination, stocking density, transportation, housing, feeding, mixing and replacement) as well as others which would be used in the analysis and interpretation were recorded for each sheep at sampling.

### 3.5.1. Sample Collection and Culture

Sample collection procedure involves taking nasal swab using sterile cotton-tipped swabs after disinfecting the nasal area by 70% ethyl alcohol. Sample collection was made at different times throughout the study period. Samples were collected from sheep sampled from the three agro-ecological zones of each study districts and the collected samples were placed in a separate labeled (labeled with code) test tubes containing a transport media, nutrient broth. The collected samples were transported packed in ice to Asella Regional Veterinary Laboratory for culturing and then identification and phenotyping. In the laboratory nasal swabs were incubated immediately at 37 °C for 24 hours. After 24 hours incubation of nasal swabs in nutrient broth the swabs were streaked onto sheep blood agar plates and again incubated at 37°C for 24 hrs.

Presumptive *Pasteurella* colonies which were identified on the basis of colony morphology (color, shape, size) and presence and absence of haemolysis were Gram stained and sub-cultured to Sheep blood and MacConkey agar for subsequent genus and species verification. Samples which were difficult for identification based on colony morphology, haemolysis and gram stain were further identified using other major discriminatory tests.

### 3.5.2. Biochemical tests

For further identification of the cultured and Gram stained bacteria which were suggestive of *pasteurella/Mannheimia/Bibersteinia*, different biochemical tests were conducted by selecting those which aid in differentiation among *Pasteurella/Mannheimia/Bibersteinia* species. These include indole and catalase tests, fermentation of lactose, glucose, sucrose, trehalose and arabinose, oxidase, hydrogen sulfide and motility tests. The data obtained were recorded and compared for confirmation of the isolates to which species they belong.

### 3.5.3. Antibiotic efficacy evaluation

For this study, bacterial cultures that were already identified by their species were used to check species which are resistant or susceptible to commonly used antibiotics in the area and to identify

a drug which is efficacious. In the laboratory, isolated colonies were suspended in nutrient broth (approximately 0.5 McFarland turbidity) and were swabbed onto a media (Mueller-Hinton agar) which was dispensed on a petridish. Then antimicrobial discs were fixed individually at different sites on the surface of inoculated agar plate evenly. Finally it was allowed to grow at 37 °c overnight and its zone of complete inhibition was measured to nearest millimeter using a ruler by holding on the back of inverted petridish. The result was compared to the standards (Zone Diameter Interpretive Standards and equivalent Minimum Inhibitory Concentration Break Points of the NCCLS Performance Standards for Antimicrobial Susceptibility Testing) and the species of the isolate was related with a drug effective against them or with a drug to which they are resistant.

### **3.6. Data analysis**

For interpretation of the results, after entry of the collected data into the Microsoft Excel sheet, it was summarized by descriptive statistics and then displayed by tables and graphs to illustrate the relationships between the dependent variables (each *Pasteurella* species and their total) and independent variables (district, agro ecology, age, sex, health status, medication and number of sheep owned). Chi-square ( $X^2$ ) tests for repeated measures were used to test relationship between dependent variable (*Pasteurella* species distribution) and different independent host and environmental factors. For these analyses SPSS statistics 20 and Epi Info were used. For the antimicrobial efficacy evaluation test, the dependent variables were the diameter of zone of inhibition. Antibiotic efficacy was determined by comparing zone of inhibition of each drug with the standard. The 95% confidence intervals was the threshold for the determination of significances.

## **4. RESULTS**

In the present study characterization of the organism implicated for ovine pasteurellosis was conducted based on colony morphology, gram staining and biochemical tests to subsequently classify the agents and to relate isolation of the agents with contributing factors for epidemiology of the agents.

### **4.1. Culture Characteristics**

After 24 hours incubation of a nasal swab sample on blood agar a mixed bacterial colony was observed in almost all of the samples. Upon sub-culturing of colonies suggestive of *pasteurella* and *Mannheimia/Bibersteinia* on to blood agar and MacConkey agar plates simultaneously, cultures with colony characteristics of round (smooth) edge, greyish colour, small to moderate size and mucoid consistency which were either hemolytic or non haemolytic, and not grow on MacConkey agar or grow on MacConkey agar were observed. Further sub-culturing and Gram staining of the isolates yielded Gram negative cocci and coccobacilli. Based on the above results the isolates which were hemolytic on blood agar and grow on Mac Conkey agar with lactose fermentation were grouped as *M.haemolytica* and those which were non lactose fermenter on Mac Conkey agar keeping the other results the same as that of *M.haemolytica* were grouped as *B.trehalosi* while those which were non hemolytic on blood agar and didn't grow on Mac Conkey agar were grouped as *P.multocida* (Table 12).

### **4.2. Biochemical activities of the isolates**

Further identification and discrimination tests conducted with a series of confirmatory biochemical tests such as oxidase, indole test, fermentation and gas production tests on TSI agar as well as fermentation of glucose, sucrose, and arabinose and trehalose sugars together with catalase, motility and H<sub>2</sub>S production tests yielded different results. Those which were indole and

catalase positive, yellow butt indicating fermentation of glucose and sucrose but red slant (non lactose fermentation) in TSI agar, negative to fermentation of arabinose and trehalose sugars, motility and hydrogen sulfide tests were identified as *P.multocida*. Those biochemical tests which yielded catalase positive, indole negative and fermentative with gas production forming a crack, positive to glucose and sucrose fermentation but negative to H<sub>2</sub>S production on TSI media and negative to motility tests on SIM media were classified as *M.haemolytica* but those which were catalase and indole negative, yellow butt and red slant indicating non lactose fermentation on TSI media, arabinose –ve but trehalose +ve while the rest of the test results similar to that of *M.haemolytica* were identified as *B.trehalosi* (table 12).

Table 12: Phenotypic culture and biochemical test characteristics

Phenotypic characteristics	<i>M. haemolytica</i>	<i>B. trehalosi</i>	<i>P. multocida</i>	Total isolates
Blood agar (hemolysis)	+	+	-	
MacConkey (lactose fermenter)	+	-	No growth	
Gram stain	-	-	-	
Colony colour	gray	gray	gray	
Colony size	Small to medium	Small to medium	Small to medium	
Oxidase	+	+	+	
TSI (lactose fermenter)	+	-	-	
Catalase	+	-	+	
Indole test (SIM)	-	-	+	
H <sub>2</sub> S Production	-	-	-	
Motility	-	-	-	
Arabinose	+	-	-	
Trehalose	-	+	-	
Number of isolates identified of the total sample (N = 384)	65	11	33	109
Relative prevalence of the isolates (No. each spp of 109 isolates)	65/109 (59.6%)	11/109 (10.1%)	33/109 (30.3%)	100%
Absolute prevalence of the isolates	65/384 (16.9%)	11/384 (2.9%)	33/384 (8.6%)	109/384 (28.4%)

Based on these of the 384 nasal swab samples collected only 109 isolates were identified, which gives an isolation rate of 28.4%. This nasal carrier rate is statistically lower than the hypothesized 50% prevalence in the population of sheep of the four selected districts. Here it was found that 65 isolates (16.9%) were *M. haemolytica*, 11 isolates (2.9%) were *B. trehalosi* and 33 isolates (8.6%) were *P. multocida* regardless of the districts, agroecology, age, sex and other attributes.

But when the case was seen for each of the above attributes the situation was looked like the one seen in table below.

Table 13: Distribution of total positivity among different epidemiological risk factors

Risk factor	Level	No examined	No positive	Prevalence (CI)	X <sup>2</sup> , P-value
District	Digelu-Tijo	106	30	28.3(19.7, 36.9)	7.25, 0.0643
	Hetosa	54	19	35.2(22.5, 47.9)	
	Munesa	168	38	22.6(16.3, 28.9)	
	Tiyo	56	22	39.3(26.5, 52.1)	
Agro-ecology	Highland	146	43	29.5(22.1, 36.9)	0.3247, 0.8502
	Mid-altitude	146	39	26.7(19.5, 33.9)	
	Lowland	92	27	29.3(20.0, 38.6)	
Age	< 3mth	57	9	15.8(6.3, 25.3)	27.65, 0.0001
	4-6mth	45	15	33.3(19.5, 47.1)	
	7-9mth	64	19	29.7(18.5, 40.9)	
	10-12mth	36	17	47.2(30.9, 63.5)	
	1-3year	83	20	24.1(14.9, 33.3)	
	4-6year	66	11	16.7(7.7, 25.7)	
	7-9year	33	18	54.5(37.5, 71.5)	
Sex	Female	319	93	29.2(24.2, 34.2)	0.547, 0.460
	Male	65	16	24.6(14.1, 35.1)	
Health status	Coughing	34	11	32.4(16.7, 48.1)	2.319, 0.509
	Dead	5	1	20.0(-15.1, 55.1)	
	Healthy	317	86	27.1(22.2, 32.0)	
	Weak	28	11	39.3(21.2, 57.4)	
Medication	No	300	94	31.3(26.1, 36.5)	5.909, 0.052
	OxyTTC	13	2	15.4(-4.2, 35.0)	
	Vaccine	71	13	18.3(9.3, 27.3)	
No owned	< 5	83	24	28.9(19.1, 38.7)	1.046, 0.790
	6-[10]	209	62	29.7(23.5, 35.9)	
	10-[15]	55	15	27.3(15.5, 39.1)	
	38	37	8	21.6(8.3, 34.9)	

Regardless of species of the isolates and agroecology the isolation rate of the agent in Tiyo was 39.3%, Hetosa 35.2%, Digelu-Tijo 28.3% and Munesa 22.6%. These show there were variations in distribution of the agent among the four study districts but the variation is not statistically significant. By the same way the agroecological distribution of the agent shows that it was 29.5% in high land, 29.3% in lowland and 26.7% in mid-altitude indicating there were no statistically significant variations among agroecological zones.

Age distribution of the total positivity shows that the isolation rate of the agent varies significantly among different age groups ( $P < 0.05$ ). The isolation rate of the agent increases from age categories 1-3mth (3) to 4-6mth (6), from 7-9mth (9) to 10-12mth (12) and from 4-6year (72) to 7-9years (109) (Fig 1).

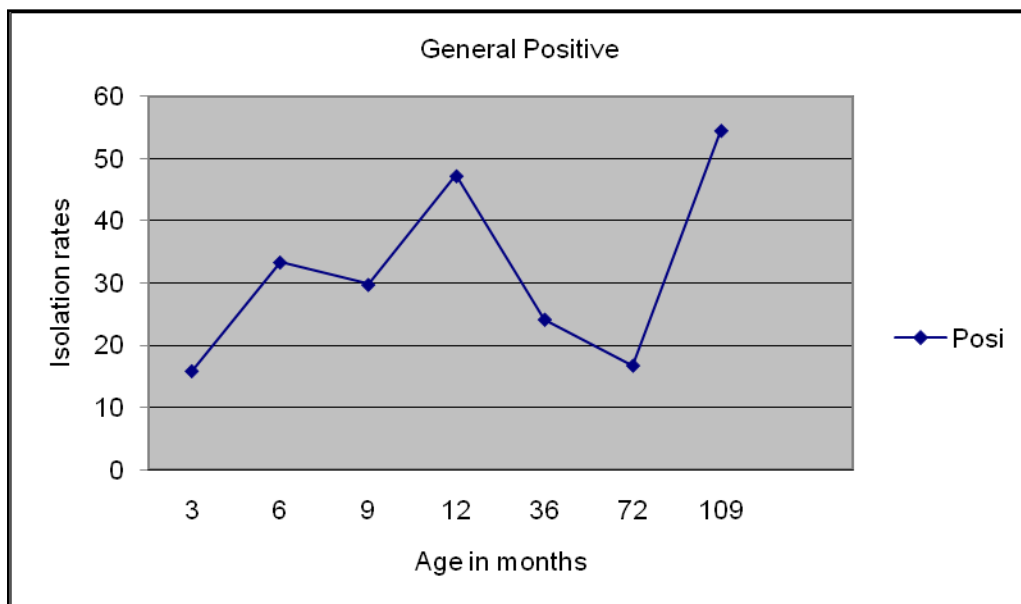


Fig 1: Age distribution of total positivity

Sex, Health status, Medication and No of sheep owned per house hold distributions of the agent all show no statistical significant variation even if there were variations observed.

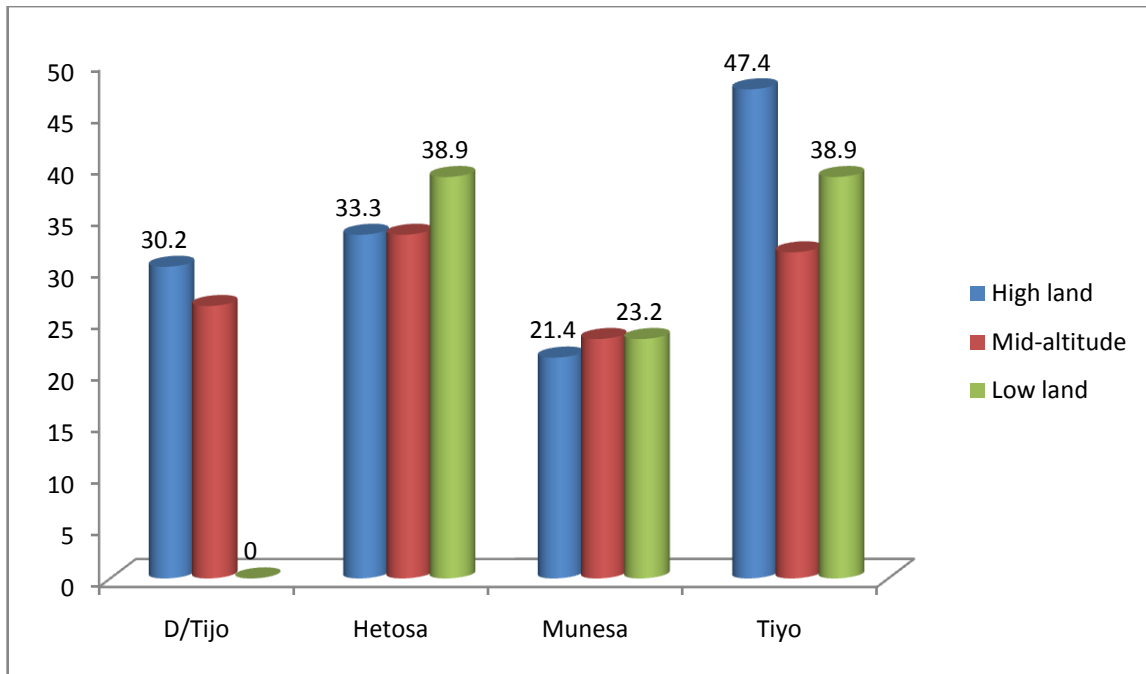


Figure 2: Distribution of isolates among agroecologies within districts.

When the distribution of the agent by their species was seen among districts and agroecology there were variations observed in distribution of a single species of the isolate among districts and there were also variations in distribution of isolates in a district. Agroecological distribution of species of the agent also varies. That is species of the agent was not equally distributed among high land, low land and mid-altitude agroecologies. At the same time distribution of different species of the isolates within an agroecology of the study area varies.

A statistical test was conducted to see if there were any dependencies of distribution of isolates on districts as well as on agroecological zones and to see if the variations observed among and within districts and agroecologies were statistically significant. A Pearson chi-square test was conducted by cross tabulating isolate verses district and isolate verses agroecology. Based on these there were no dependencies observed between isolates and both district and agroecology. Pearson chi-square shows p value = 0.064 (2-sided) for association between isolates and district and p value = 0.850(2-sided) for isolates verses agroecology. This implies that there was no association between Isolates and Districts and Agroecology.

That is distribution of isolates was independent of district and agroecology which means they were independent of each other.

Likewise age and sex distribution of isolates were examined for similarity in distribution of isolates among different age groups of sheep and between female and male sheep. A Pearson chi-square test computed for existence of dependency between isolates and age of sheep shows that there was dependency between age of sheep and distribution of the disease agents which implies that at 95% confidence level distribution of isolates is dependent on age of sheep (P-value<0.05). That is statistically there is significant association between distribution of isolates and age of sheep. Sex isolate cross tabulation shows that there was no significant variation in distribution of isolates both within female and male sheep and also between female and male sheep. That is distribution of isolates was independent of sex of sheep; sex of sheep does not limit distribution of isolates. This implies that sex (host factor) is not a determinant factor of the disease pasteurellosis in sheep.

Isolates versus health status of sheep, isolates versus medication and isolates versus stocking density (number of sheep owned by a single owner) shows that there were no dependencies between isolates and each of health status, medication and number of sheep owned indicating that distribution of isolates were independent of each of the three factors listed above.

Table 14: Distribution of phenotypes of the agent among different epidemiological risk factors

Risk factor	Level	No examined	<i>M.haemolytica</i> n(%)	<i>P.multocida</i> n (%)	<i>B.trehalosi</i> n (%)	X <sup>2</sup> , P-value
District	Digelu-Tijo	106	19(17.9)	9(8.5)	2(1.9)	10.419,0.318
	Hetosa	54	10(18.5)	6(11.1)	3(5.6)	
	Munesa	168	21(12.5)	12(7.1)	5(3)	
	Tiyo	56	15(26.8)	6(10.7)	1(1.8)	
Agro-ecology	Highland	146	24(16.4)	15(10.3)	4(2.7)	4.243,0.644
	Mid-altitude	146	26(17.8)	11(7.5)	2(1.4)	
	Lowland	92	15(16.3)	7(7.6)	5(5.4)	
Age	< 3mth	57	5(8.8)	4(7)	0 (0)	35.609,0.008
	4-6mth	45	10(22.2)	5(11.1)	0(0)	
	7-9mth	64	10(15.6)	5(7.8)	4(6.3)	
	10-12mth	36	9(25)	5(13.9)	3(8.3)	
	1-3year	83	13(15.7)	6(7.2)	1(1.2)	
	4-6year	66	7(10.6)	3(4.5)	1(1.5)	
	7-9year	33	11(33.3)	5(15.2)	2(6.1)	
Sex	Female	319	57(17.9)	29(9.1)	7(2.2)	4.639,0.200
	Male	65	8(12.3)	4(6.2)	4(6.2)	
Health status	Coughing	34	8(23.5)	3(8.8)	0(0)	12.972,0.164
	Dead	5	0(0)	1(20)	0(0)	
	Healthy	317	47(14.8)	28(8.8)	11(3.5)	
	Weak	28	10(35.7)	1(3.6)	0(0)	
Medication	No	300	56(18.7)	28(9.3)	10(3.3)	6.267,0.394
	OxyTTC	13	1(7.7)	1(7.7)	0(0)	
	Vaccine	71	8(11.3)	4(5.6)	1(1.4)	
No owned	< 5	83	14(16.9)	7(8.4)	3(3.6)	2.454,0.982
	6-[10]	209	39(18.7)	17(8.1)	6(2.9)	
	10-[15]	55	8(14.5)	6(10.9)	1(1.8)	
	38	37	4(10.8)	3(8.1)	1(2.7)	

### 4.3. Antimicrobial susceptibility testing

After overnight incubation of representative isolates on Mueller Hinton agar plates with attached antibiotic impregnated discs (Tetracycline, Penicillin, Amoxicillin and Gentamycin) fixed at different sites, a zone of bacterial growth inhibition was measured to the nearest millimeter and the result was as presented in the table below.

Table 15: Drug sensitivity tests (Inhibition zones in mm)

District	Agroecology	Species	Amoxicillin 10µg =MIC (< 13 =R)	Gentamicin 10 µg=MIC (< 12 =R)	Penicillin 10Iu=MIC (< 14 =R)	Tetracycline 30 µg=MIC (< 14 =R)
Tiyo	Highland	<i>M.haemolytica</i>	22	30	22	13
Tiyo	Lowland	<i>M.haemolytica</i>	20	14	0	20
Hetosa	Mid-altitude	<i>M.haemolytica</i>	30	19	20	30
		<i>Average</i>	24	21	14	21
D/Tijo	Highland	<i>P.multocida</i>	25	35	19	23
Munesa	Highland	<i>P.multocida</i>	30	20	20	5
Munesa	Mid-altitude	<i>P.multocida</i>	20	10	0	5
		<i>Average</i>	25	22	13	11

The drug sensitivity test conducted disclosed that all isolates (100%) were susceptible to Amoxicillin, 66.7% susceptible to Gentamicin, 66.7% susceptible to Penicillin and 50% susceptible to Tetracycline. On average both *M.haemolytica* and *P.multocida* are susceptible to Amoxicillin and Gentamicin but resistant to Penicillin. *M.haemolytica* is susceptible to Tetracycline while *P.multocida* is resistant. From this result it can be said that Amoxicillin is a drug of choice while Tetracycline and Penicillin are drugs to which some isolates developed resistance relative to other drugs.

## 5. DISCUSSION

The present study was conducted in four districts of Arsi zone each comprised of highland, mid-altitude and lowland agroecologies except one district, D/Tijo which does not have lowland agroecology. Among agroecologies high land agroecology covers larger proportion (53%) which is known by having high sheep population followed by mid-altitude (37%) and lowland (10%). In this study it was found that total nasal isolation rate of agents implicated for ovine pasteurellosis was 28.4%. This is lower than the expected 50% prevalence hypothesized at the beginning of this study. However, the result of present study is in agreement with that of Deressa *et al.* (2004) in Debre Birhan, Harshin and Jijiga which were 34%, Gelagay *et al.* (2004) in Debre Birhan of 30%, Belay (2007) in South Wollo of 26.3% and Black (2011) in New Zealand of 25%. But it is lower than that of Mekonnen (2000) in Arsi zone which was 57% and that of Mesele (2005) in Debre Zeit (ELFORA) which was 38.9% while the result of this study is higher than that of Mike (1999) in UK which was 9% and that of Kirkan and Osman (2005) in Turkey which was 12%.

On bacteriological examination a total of 109 isolates was found comprised of *M. haemolytica* (16.9%), *P. multocida* (8.6%) and *B. trehalosi* (2.9%) with significant difference among rates of isolation of the three species. These rates for *M. haemolytica* are in agreement with that of Gelagay *et al.* (2004) which were 20%, Deressa *et al.* (2004) which was 19% and Belay (2007) which was 11.1%. But it is higher than the result of the study conducted by Mesele (2005) which was 8.3% and lower than the one identified by Mekonnen (2000) which was 48%. For *P. multocida* the present rate is in agreement with that of Gelagay *et al.* (2004) which was 10% while it is higher than that of Mekonnen (2000) which was 2% and Belay (2007) which was 1.8% but lower than that of Mesele (2005) which was 25% and Deressa *et al.* (2004) which was 15%. The case of *B. trehalosi* was identified only by some studies made so far. Anyway the present *B. trehalosi* isolation rate is lower than those rates identified by Belay (2007) which was 13.4%, Mekonnen (2000) which was 7% and Mesele (2005) which was 5.6%. Accordingly there was a significant difference existing among isolation rates at different time in different areas even if there is no significant difference in isolation rates among the three species in all of the studies at different time and areas. This implies that there must be a continuous survey to be held in

different areas to know a recent rate for each of the species implicated for ovine pasteurellosis and to design a cost effective and efficient prevention and control strategies suited for each area.

An agroecological distribution of the isolated agents revealed that there was variation in distribution of the species from agroecology to agroecology with highland distribution rate of 29.5%, lowland 29.3% and mid-altitude 26.7%. That is the distribution rate was higher in highland than lowland followed by mid-altitude. However, there was no dependency of distribution of the species on agroecological zones of the study area.

Age distribution of the three species associated with ovine pneumonic pasteurellosis disclose that there was significant variation in distribution of the species among different age groups of sheep and at the same time distribution of the species was dependent on age groups of sheep. According to the present study different age group distribution of the isolates looked like as follows: within age group 1-3mth it was 15.8%, 4-6mth (33.3%), 7-9mth (29.7%), 10-12mth (47.2%), 1-3year (24.1%), 4-6year (16.7%) and 7-9year it was (54.5%). This was not in agreement with that of the result obtained by Zuber (2009) in Iraq with slight fluctuation in age grouping which was in 1-3mth age category it was 2.1%, 4-8mth (7.7%) and 9-12mth (7.1%).

Sex wise distribution also shows that there was no significant variation in distribution of the species between female and male sex and that the distribution was independent of sex of sheep. In the present study isolation rate of the agent in female sheep was 29.2% while its rate in male was 24.6%. Similar studies shows that there was no significant sex variation ( $P>0.05$ ) in goats where the average pulmonary consolidation score in female was 8.7% while that of male was 10.8% (Emikpe and Akpavie, 2010).

Other direction was also searched if there was association between distribution of isolates and health status of sheep and different prevention and treatment interventions and again to see if there was any variation in distribution whether that variation was significant or not. For that matter an independent association was observed between distribution of the isolates and both that of health status of sheep and different interventions made either to prevent or to control the

disease caused by those agents. There were no significant variations in distribution of the isolates encountered among sheep with different health status. Dependency of distribution of the isolates on health status of sheep was evaluated by grouping them as those which were coughing, dead, apparently healthy and weak with poor body condition. Accordingly 32.4% isolation rate was observed in those sheep with coughing, 20.0% in dead, 27.1% in healthy and 39.3% in those sheep which were weak. Results showed no difference in distribution of the isolates among sheep with different health status but this may not mean there is no biological difference among sheep with different health status. There were similar studies conducted in different time at different areas among which results of Belay (2007) are not in agreement with those of the present study in that in apparently healthy sheep he found 13.8% prevalence rate which is lower than the present result but in agreement on results for sick ones which was 32.7%

Regarding prevention and treatment interventions so far applied in the area, they didn't bring any difference in reducing the bacterial burden when sheep vaccinated against pasteurellosis and those treated with Oxytetracycline, a commonly used antibiotic in the area, were compared with those which were not given any vaccine and not been treated with any antibiotic against pasteurellosis. In those which were given no treatment the isolation rate was 31.3%, in those treated with OxyTTC 15.4% and in those vaccinated with *Pasteurella multocida* A vaccine it was 18.3% showing no significant variation. In a similar study conducted by Black *et al.*, 2011 it was found that there was no evidence that vaccination reduced total lamb mortality when comparison of total lamb mortality was made between vaccinated and control groups. This is why different researchers who made study in this area frequently recommend a recheck on effectiveness of the available vaccine and antibiotics against ovine pasteurellosis and recommend production of efficient polyvalent vaccine which can act against different strains of the agent and recommend development of best drugs to which different strains are susceptible.

Based on number of sheep owned per house hold there was no regular pattern in distribution of the rate of isolation across different stocking rates. Any way there was no statistical association between stocking densities and distribution rate of isolates and there was no significant variation of the distribution of the isolates among different stocking rates of sheep. The rates range from

28.9% for stocking rates < 5, 29.7% for 6-[10], 27.3% for 10-[15] and 21.6% for herd size of 38. This may not be the case in real case on ground and this may be because there were confounding variables like size of house and other management situations which mask a significant variation among different herd sizes.

Drug sensitivity evaluation for commonly used antibiotics shows that all tested isolates (100%) were susceptible to Amoxicillin, 66.7% susceptible to Gentamicin, 66.7% susceptible to Penicillin and 50% susceptible to Tetracycline. This is in agreement with the results of Catry (2005) for Amoxicillin and Tetracycline with some exceptions but not in agreement with that of Prabhakar *et al.* (2010) for Amoxicillin where there were Amoxicillin resistance detected. In this study it was found that Amoxicillin is a drug of choice while Tetracycline is a drug to which some isolates developed resistance relative to other drugs. Other studies also showed that Tetracycline resistance is among commonly reported drug resistance (Andy, 2000). But in the study area Tetracycline was a commonly used drug without considering its efficacy and this might be one reason for the continuing threat of the disease in the area.

## 6. CONCLUSION AND RECOMMENDATIONS

*M.haemolytica* was detected as a common isolate in the area followed by *P.multocida* and *B.trehalosi*. There was variation in isolation rates of the agents within and among different host and environmental risk factors even if some of those variations were statistically insignificant. Regarding drug susceptibility it was found that Amoxicillin was a drug of choice while Penicillin and Tetracycline were drugs to which resistance were developed by many tested isolates relative to other drugs tested. But in the study area Tetracycline was a commonly used drug without considering its efficacy. This may be due to misuses of the drugs which lead to development of resistance.

Knowing epidemiology of the disease and contributing factors to the disease has a paramount importance in designing a cost effective prevention and control method. For that matter knowing prevalence of the disease to strain level helps one to design a best intervention method using vaccine or antibiotics. Management practices directed at reducing stress are important in preventing *Pasteurella/Mannheimia* associated disease in sheep. For a veterinary practitioner, knowledge of the pathogen involved and its antimicrobial resistance profile is essential information for an adequate treatment.

Based on results of this study the following points are recommended

- ❖ It should be known to the public that pasteurellosis is a disease which can be prevented by implementing a management strategy which can avoid stress.
- ❖ Further investigation and confirmation of strains involved which help to apply a cost effective and efficient prevention and control options is mandatory.
- ❖ A continuous monitoring and evaluation of drug usage and drug resistance as well as vaccination programs is needed to be implemented.
- ❖ It is better to develop a polyvalent vaccine from locally isolated common strains which will suit best in the prevention and control of this disease.

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## 8. ANNEXES

### Annex 1: Field Data Collection Format

Date \_\_\_\_\_

District \_\_\_\_\_ Agroecology \_\_\_\_\_

Breed of sheep \_\_\_\_\_ Season \_\_\_\_\_

Code	Owner	Breed of sheep	Age	Sex	Health status	Rx/Vaccine/Other	Herd size	Type of barn	Feeding system

### Annex 2: Laboratory Test Results Recording Format

Code	Blood agar	Mac Conkey	Gram stain	Oxidase	Tentative Dx	SIM media	TSI media	Catalase test	Sugar fermentation	Dx

Annex 3: Summary of Culture characteristic and Biochemical tests used as reference at identification

Features	<i>P. multocida</i>	<i>M. haemolytica</i>	<i>B. trehalosi</i>
Haemolysis	-	+	+
Mac Conkey	-	+	+
Lactose (TSI)	-	+	-
Catalase	+	+	-
Indole (SIM)	+	-	-
Odor	+	-	-
Arabinose	V	+	-
Trehalose	V	-	+
Xylose	-	+	-
Oxidase	+	+	+
Glucose	+	+	+
Sucrose	+	+	+
H <sub>2</sub> S Production	-	-	-
Motility	-	-	-

Source: Ragy, (2005); Casolari and Fabio, (2008); Mifflin, (2001); Belay, (2007)

Annex 4 Gram Staining Procedure

A clinical sample is placed on a slide and rinsed with:

Crystal Violet

Iodine Solution (the Mordant)

Decolorizer (ethanol)

Safranin (the Counter stain)

Water

## Annex 5 Antibiotic Susceptibility Testing Procedures/Techniques

### Techniques of Antibiotic Sensitivity Test

Bacteria cultured on a solid medium and then appropriate suspension prepared from few grown colonies.

Suspension with bacteria swabbed over agar.

Discs impregnated with various antibiotics are placed onto agar and incubated for 24 hours at 37 °C.

Zones of inhibition of bacterial growth, measured around discs, should be compared with those in the National Committee for Clinical Laboratory Standards (NCCLS) Document M100 (M2).

Results obtained may then be reported as resistant, intermediate or susceptible.

Annex 6 Odds of Pasteurellaceae positivity as compared between risk factors following exclusion (removal) of confounder effect

<b>Term</b>	<b>Odds Ratio</b>	<b>95%</b>	<b>C.I.</b>	<b>Coefficient</b>	<b>S. E.</b>	<b>Z-Statistic</b>	<b>P-Value</b>
<b>Age (2/1)</b>	2.5897	0.9881	6.7869	0.9515	0.4916	1.9357	0.0529
<b>Age (3/1)</b>	1.9661	0.7861	4.9176	0.6760	0.4678	1.4453	0.1484
<b>Age (4/1)</b>	4.4402	1.6504	11.9459	1.4907	0.5050	2.9521	0.0032
<b>Age (5/1)</b>	1.5129	0.6208	3.6870	0.4140	0.4545	0.9110	0.3623
<b>Age (6/1)</b>	0.9602	0.3597	2.5632	-0.0406	0.5010	-0.0811	0.9354
<b>Age (7/1)</b>	5.7676	2.0738	16.0412	1.7523	0.5219	3.3575	0.0008
<b>Agro-ecology (2/1)</b>	0.8682	0.4789	1.5739	-0.1414	0.3035	-0.4657	0.6414
<b>Agro-ecology (3/1)</b>	1.1206	0.5436	2.3100	0.1139	0.3691	0.3085	0.7577
<b>District (2/1)</b>	1.1553	0.5275	2.5302	0.1443	0.4000	0.3608	0.7182
<b>District (3/1)</b>	0.6588	0.3275	1.3253	-0.4173	0.3566	-1.1702	0.2419
<b>District (4/1)</b>	1.3841	0.6443	2.9733	0.3251	0.3901	0.8332	0.4047
<b>Health status (2/1)</b>	0.6166	0.0546	6.9680	-0.4835	1.2372	-0.3908	0.6959
<b>Health status (3/1)</b>	0.7130	0.3159	1.6091	-0.3383	0.4153	-0.8146	0.4153
<b>Health status (4/1)</b>	1.0105	0.3268	3.1249	0.0105	0.5760	0.0182	0.9855
<b>No. owned1 (1/0)</b>	1.1286	0.6140	2.0747	0.1210	0.3106	0.3896	0.6968
<b>No. owned1 (2/0)</b>	1.0778	0.4725	2.4586	0.0749	0.4208	0.1781	0.8586
<b>No. owned1 (3/0)</b>	1.3469	0.4246	4.2733	0.2978	0.5891	0.5056	0.6131
<b>Sex</b>	0.8080	0.4183	1.5608	-0.2132	0.3359	-0.6346	0.5257
<b>CONSTANT</b>	*	*	*	-1.0406	0.7397	-1.4068	0.1595

## **9. CURRICULUM VITAE**

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## 10. SIGNED DECLARATION SHEET

I under sign, declare that the thesis is my original work and has not been presented for a Degree in any University.

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Signature \_\_\_\_\_

Date of Submission \_\_\_\_\_

This thesis has been submitted for examination with my approval as University advisor

Dr. Reta Duguma \_\_\_\_\_

