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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

ISOLATION AND IDENTIFICATION OF *MANNHEIMIA HAEMOLYTICA*, *BIBERSTEINIA TREHALOSI* AND *PASTEURELLA MULTOCIDA* FROM CATTLE AND SHEEP FROM SELECTED AREAS OF ETHIOPIA

MSc THESIS

BY

ABEBE WIRTU AFATA

JUNE, 2018

BISHOFTU, ETHIOPIA

ISOLATION AND IDENTIFICATION OF *MANNHEIMIA HAEMOLYTICA*, *BIBERSTEINIA TREHALOSI* AND *PASTEURELLA MULTOCIDA* FROM CATTLE AND SHEEP FROM SELECTED AREAS OF ETHIOPIA

A Thesis submitted to the College of Veterinary Medicine, Addis Ababa University in partial fulfillment of the requirements for the Degree of Masters of Veterinary Science in Veterinary Microbiology

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June, 2018

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ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINAEY PUBLIC HEALTH

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: Abebe Wirtu entitled "Isolation and Identification of *Mannheimia haemolytica*, *Bibersteinia trehalosi* and *Pasteurella multocida* from Cattle and Sheep from selected areas of Ethiopia" and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Science in Veterinary Microbiology.

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AUTHOR DECLARATION

This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture. It is my original work and that all sources of material used for this thesis have been duly acknowledged. The thesis will be deposited at the University/College library to be made available to borrowers under rules of the Library.

I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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ACKNOWLEDGMENTS

First of all I would like to thank almighty God who delivered me through ocean of difficulties, always being with me and the most beneficent for giving me strength, patience and health to accomplish this work.

I would like to express my sincere and heartfelt thanks to my wife Misgane Furi and my family as a whole for their endurance and sharing responsibilities, inspiration and patience during my study periods.

Then I would like to thank my major advisor Dr. Gezahegne Mamo for his knowledgeable advice, constructive comments, valuable suggestions and patience throughout the period of this work**.**

Profound thanks are also goes to my co-advisors: Dr.Teshale Sori for devotion of his time to go through this work page by page and making the necessary corrections, suggestions and constructive criticisms and Dr. Bersissa Kumsa for his continuous support and supervisions on the overall activities throughout the period of this work

I highly thank to Dr. Esayas Gelaye (National Veterinary Institute) for his valuable support in the molecular analysis of samples and sequencing activities.

My thanks also extend to members of Microbiology Laboratory staff (CVMA) for all the support and unlimited assistance provided to me.

I thank to Sendafa town animal health department office, staff and animal owners, Abyssinia, ELFORA and Sululta municipal abattoir managements for allowing and rendered help during the study period.

LIST OF ABBREVIATIONS

AA	Addis Ababa
AGID	agar gel immunodiffusion
BWRAD	Bereh woreda rural and agricultural development
BRD	bovine respiratory disease
CSA	Central statistical agency
DNA	Deoxyribo nucleic acid
GTAO	Gonder town agricultural office
LPS	Lipopolysaccharides
MASL	meters above sea level
MM	milimeter
mPCR	Multiplex polymerase chain reaction
NVI	National veterinary institute
OIE	World Organization for Animal Health
OMP	outer membrane protein
PBS	phosphate buffer saline solution
PCR	polymerase chain reaction
PHSSA	*Pasteurella haemolytica* species specific antigens
RAPD	random amplified polymorphic DNA
RBC	red blood cell
Rpt_2	pentanucleotide repeats
rRNA	ribosomal ribonucleic acid
TSB	tryptose soya broth
SDS- PAGE	sodium dodecyl sulfate-polyacrylamide
URT	upper respiratory tract
VP	voges-Proskauer

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ABSTRACT

Pneumonic pasteurellosis is a multifactorial respiratory disease of cattle and sheep caused by combination etiologic agents; hence, reliable information is needed on the inventory of *Pasteurella* species for optimum control of the disease. This study was conducted from November 2017 to May 2018 with objectives of isolation and identification of *Pasteurella* organisms and assessment of their antibiotic sensitivity profiles from cattle and sheep using conventional and molecular methods. A cross-sectional study was carried out on samples collected from abattoirs (Bishoftu and Sululta area) and nasal swabs from cattle and sheep brought to Sendafa veterinary clinic for various reasons. A total of 149 nasal swabs (74 cattle and 75 sheep) were collected and examined using bacteriological methods, of which 33 (22.15%) yielded positive results for *Pasteurella*, *Mannheimia* and *Bibersteinia* species. Out of the 33 isolates 15 (20.27%) were from cattle and 18 (24.00%) were from sheep. The species composition of the isolates showed that 15 (10.07%), 7 (4.7%) and 11 (7.38%) of them were *M. haemolytica*, *B. trehalosi* and *P. multocida,* respectively. *Pasteurella* species were more frequently isolated from pneumonic (36.84%) cattle than non-pneumonic ones (2.78%). Similarly Pasteurella species were more frequently isolated from pneumonic sheep (41.03%) than non-pneumonic counterparts (5.56%). *Mannheimia haemolytica* was isolated from 8.72% of the pneumonic cases, *P. multocida* from 7.38% and *B. trehalosi* from 4.02% of pneumonic cases. From 176 pneumonic lung samples (93 cattle and 83 sheep) collected from abattoirs *Pasteurella* species were isolated from 48 (27.27%) of them comprising 24.73% (23) from cattle and 30.12% (25) from sheep. The isolates comprised *M. haemolytica* (13.07%), *B. trehalosi* (7.39%) and *P. multocida* (6.83%). Molecular characterization also confirmed the identity of *P. multocida* and *M. haemolytica*. In addition, the *P. multocida* isolates were identified to be similar to serovars A1 and A3 found in the Gene bank. The isolates were found to be resistant to the commonly used antibiotics Cloxacillin and Ampicillin (77.78 -100%); whereas they showed sensitivity to Chloramphenicol, Norfloxacillin and Oxytetracycline (71.43 -100%). This study revealed that *M. haemolytica*, *P. multocida* and *B. trehalosi* are commonly circulating in cattle and sheep originated from various parts of the country.

Keywords: *Abattoir, antibiotic susceptibility, Pasteurella species, Cattle, Ethiopia, Isolati on and Identification, PCR*

1. INTRODUCTION

Ethiopia has the largest national livestock populations in Africa. However, the productivity is one of the marginal in the continent due to a number of technical and non-technical factors. Infectious diseases are among the technical factors affecting livestock productivity (Tadele *et al*., 2015). Among the infectious diseases affecting cattle industry, pneumonic pasteurellosis, is one of the common ones.

Pasteurellosis is a respiratory disease that causes pneumonia in cattle and sheep. It is multi-factorial disease caused by a combination of etiologic agents. It is a disease complex that is generally thought to result from invasion of the lung by species of *Pasteurella* and *Mannheimia* when respiratory tract defense mechanisms are broken (Miller *et al.*, 2008). But *Mannheimia haemolytica* is considered a primary bacterial agent associated with the disease (McRae *et al.,* 2016). The bacterial causes of the disease: *M. haemolytica* and *Pasteurella multocida* are commensal organisms of the tonsils and nasopharynx of healthy cattle and sheep; whereas certain factors can trigger the bacteria to move quickly to invade the lungs and cause pneumonic pasteurellosis. Such factors include a variety of stress factors such as transportation, malnutrition, adverse physical, environmental or climatic conditions, previous or co-infection with certain respiratory viruses, mycoplasma or other types of bacteria (Abdelsalam, 2008). It is believed that those 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 bacteria colonizing, escaping clearance in the nasopharynx, and moving to the lungs by gravitational drainage (Zuber, 2009).

Pneumonic pasteurellosis cause death and illness, contributing to losses amounting to millions of dollars through treatment costs, reduced meat yields, and mortalities (Larson, 2005). It is characterized by the presence of acute fibrino-necrotizing pneumonia (Welsh *et al.,* 2004). It is well established that pneumonic pasteurellosis is responsible for huge mortality in feedlot animals accounting for approximately 30% of the total cattle deaths worldwide. The global economic impact of the disease is well recognized and more than US$ one billion are annually lost in beef cattle industry in North America alone (Baundreaux, 2004). The terrible effect of the disease was also evident in sheep farming

and remarkable economic losses were also attributed to massive fatalities in feedlot animals and acute field outbreaks. In addition, substantial amount of money was further lost, almost every year, in improving farm management, animal husbandry and chemotherapeutic and vaccination programs (Radiostitis, 2007).

Distinct serotype associations with specific host species are noted elsewhere in the world and some serotypes have also distinct disease syndromes. The disease is determined by specific serotypes found in cattle and small ruminants. For instance, *M. haemolytica* serotype A1 causes pneumonic pasteurellosis in cattle; *M. haemolytica* serotype A2 causes pneumonic pasteurellosis in sheep and goats and *B. trehalosi* serotypes cause septicaemic pasteurellosis in sheep and goats in abundance (Quinn *et al.,* 2002; Abdulselam, 2008).

In Ethiopia pneumonic pasteurellosis has been a topic of frustration to veterinary practitioners and a topic of liability to ruminant producers. It is a high-priority disease causing significant economic losses through mortality, morbidity, and the high cost of treatment (Marru *et al.,* 2013). There are some studies conducted on pneumonic pasteurellosis in cattle and sheep focusing on the sero-prevalence and bacteriological identification of the agents. The species of bacteria involved has been identified from sheep and cattle in northern, central, eastern and western parts of Ethiopia. Those studies showed that bovine and ovine pneumonic pasteurellosis is a major constraint to sheep and cattle production. So far, the predominant serotypes identified in Ethiopia were *M. haemolytica* biotype A1 in cattle and A2 in sheep (Ayelet *et al.,* 2004; Mekonnen, 2000; Belay, 2007; Deressa *et al*., 2004; Demissie *et al.,* 2010; Abera *et al*., 2014).

Control of pneumonic pasteurellosis is difficult task that requires understanding of its epidemiology, which in turn needs the ability to catalogue the strains of the agents circulating in the country. The previous studies provided valuable information on the occurrence of pneumonic pasteurellosis and its etiology. However, the serotypes identified by previous authors were mostly based on serological evidence. That is, detailed molecular based information on the prevailing serotype (s) is lacking at present. The molecular identification of the serotypes would improve our understanding of the epidemiology of strains involved to the disease as well as provide information on the most appropriate serotypes to be used for successful vaccination against pneumonic pasteurellosis. In countries where, predominant serotypes have been well identified; effective control

measures have been devised. Knowledge of genetic diversity and predominance of the species and serotypes of *Pasteurella* organisms involved in pneumonic pasteurellosisis in Ethiopia is needed for development of effective vaccine in the country. This study was, therefore, aimed at:

- Identification of the species of *Pasteurella* organisms from cattle and sheep slaughtered at abattoirs (Bishoftu ELFORA and Abyssinia export and Sululta municipal abattoirs) using conventional and molecular methods.
- Identification of the species of *Pasteurella* organisms from cattle and sheep brought to Sendafa Veterinary Clinic using conventional and molecular methods
- Determination of antibiotic susceptibility profile of the isolates

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2. LITERATURE REVIEW

2.1. Etiological diversity of pneumonic pasteurellosis

Mannheimia haemolytica, *Bibersteinia trehalosi* and *Pasteurella multocida* were involved mostly as etiological agents of the disease, which are commensally resident in the upper respiratory tract of healthy ruminants (Radiostitis, 2007). These bacteria are gram-negative, facultative anaerobe, non-motile, non-spore forming and small rods or coccobacilli (Hall, 1994). Each species of the bacteria seems to have host preference as depicted in Table 1 but they are often isolated from various host species.

Table 1: Major *Pasteurella* organisms of veterinary importance and their associated diseases (Quinn *et al.,*2002; Abdulselam, 2008).

***Pasturella* species**	**Host**	**disease conditions**
P. multocida Type A	Cattle	Bovine pneumonic pasteurellosis
	Sheep	pneumonia and mastitis
	Pig	pneumonia and atrophic rhinitis
	Poultry	Fowl cholera
P. multocida Type B	Cattle and buffaloes	Hemorrhagic septicemia (Asia)
P. multocida Type D	Pigs	pneumonia and atrophic rhinitis
P. multocida Type E	Cattle and buffalo	Bovine pneumonic Pasteurellosis (Shipping fever complex)
M. haemolytica	Cattle	Shipping fever
	Cattle, sheep & goat	primary and secondary pneumonia
B. trehalosi	Sheep	septicemia/acute systemic disease
	Cattle	Respiratory disease

2.1.1. Pasteurella multocida

Pasteurella multocida, as one of the main etiological agents of pneumonic pasteurellosis, leads to the production of a number of proteins and polysaccharides, which are thought to contribute to its virulence (Chung *et al.*, 1998). The organism spills over into the

nasopharynax and shed in nasal secretions. The species name "*Multocida*" in Latin means killing many which was coined showing mainly massive mortality associated as a result infection by this bacterium. *Pasteurella multocida* is an important animal pathogen of the family Pasteurellaceae that pose serious hazard in livestock industry. Like to other species of the organism *P. multocida* is also a commensal of animal respiratory tract (Ryan and Ray, 2004). The pathogenic role of *P. multocida* was more evident in sheep in which it was responsible for many serious of outbreaks (Quinn *et al.,* 2002). Distinct serotype associations with specific host species are noted and some serotypes have also distinct disease syndrome. Thus, pneumonic pasteurellosos in cattle and sheep caused mainly by *P .multocida* are serotype group A and D, while haemorrhagic septicaemia in cattle and buffalo are caused by *P. multocida* serotypes B: 2 or E: 2 (De Alwis,1999).

Pasteurella multocida is gram-negative, non-motile, coccobacillus of 0.3–1.2 μm in length that does not form spores. It grows on most laboratory media with the exception of bile containing media such as MacConkey agar (Hawari *et al.,* 2008 Kuhnert and Christensen, 2008). It is oxidase and catalase positive and can ferment various carbohydrates. A typical bipolar staining with methylene blue can be seen in smears taken from wounds or tissues rather than from cultures (Hagan *et al.,* 1988). *Pasteurella multocida* has 16 serotypes using lipopolysaccharide antigens as tested by a gel diffusion precipitation test, although it has five sero groups (A, B, D, E, F) using capsular antigens as tested by a passive haemagglutination test (Quinn *et al.,* 1994; Munir *et al.,* 2007).

There are two specific serotypes of *P. multocida*, B: 2 and E: 2 in cattle, which are Asian and African in origin, respectively and predominantly induces hemorrhagic septicemia in cattle and wild ruminants (Waheed *et al.,* 2009). *Pasteurella multocida* strains have also been characterized by outer membrane protein (OMP) typing and 16S rRNA-typing. 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 (Wheeler, 2009). Currently three subspecies of *P. multocida* are recognized Quinn *et al.* (1994) and reported as; *P. multocida subspecies multocida* recovered from domestic animals, *P. multoicda subspecies septica* from (dog, cat and birds) and *P. multocida subspecies gallicida* from birds.

2.1.2. Mannheimia haemolytica

Mannheimia haemolytica is an important animal pathogen associated with pneumonic pasteurellosis. *Mannheimia haemolytica* (formerly named *Pasteurella haemolytica*) is commonly isolated from the lungs of cattle with pneumonia (Welsh *et al.,* 2004). Among *Mannheimia haemolytica* serotypes, the A1 is the primary causative agent of bovine pneumonic pasteurellosis, which results in significant economic losses to cattle industries (Whiteley *et al.,* 1992). Serotype A1 alone can cause pneumonia; however pneumonia symptoms are difficult to replicate without adding environmental stress or viral infection (Adlam, 1989).

Mannheimia haemolytica, which is a normal flora of the upper respiratory tract, 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. *Mannheimia haemolytica* is the major cause pasteurellosis among the three species even in Ethiopia as reported by (Abera *et al.,* 2014). However, Marru *et al.* (2013) describes as among the two species *M. haemolytica* is the major causative agent involved in ovine pneumonic pasteurellosis as studied from nasal swab of pneumonic sheep from Haramaya veterinary clinic and counterpart of pneumonic lungs from the slaughtered non-pneumonic sheep.

Based on number of characteristics such as antigenic nature, pathogenicity and Biochemical activity; *Pasteurella haemolytica* can be differentiated in to two biotypes (A and T). Biotype A ferments arabinose, whereas biotype T ferments trehalose. Similarly, Based on genomic characteristics, biological features and analysis of phenotypic content biotype T was reclassified (named) as *P. trehalosi* and biotype A as *M. haemolytica* (Kalian and Fredericksen,1981).These biotypes are 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 passive haemagglution test (Biberstein and Gills, 1999). After years *P. haemolytica* biotype A is allocated to new genus and renamed as *M. haemolytica* while the T serotype as *B. trehalosi*. The T serotypes are T3, T4, T10, T15 and serotype A except the 11A which is considered as *M. glucosida*, all A12 serotypes are (A1, A2, A5-A9, A12-14, A16 and A17) based on capsular antigen typing (Abdelsalam, 2008; Haig, 2011) categorized as *M. haemolytica*.

2.1.3. Bibersteinia trehalosi

Biberstinia trehalosi is complicated by the constantly evolving nomenclature of the organism as it undergoes increasing differentiation from other members of the Pasteurellaceae family (Blackall *et al.,* 2007). The description of the bacteria now known as *B. trehalosi* was first published in 1921 by Jones with an original name of *Bacillus bovisepticus* group I (Jones and Little, 1921). *Biberstinia trehalosi* has been isolated from both healthy and diseased cattle. The organism is carried on nasal, conjunctival and nasopharyngeal mucosa in healthy cattle (Collins, 2011). Conflicting reports exist about the prevalence of *B. trehalosi* in the nasopharynx of healthy cattle. Some reports describe the organism as a ubiquitous commensal organism (Panciera and Confer, 2010). Others report that the organism is not readily isolated from healthy, unstressed cattle (Adlam, 1989). In contrast, Hanthorn *et al.* (2014) reported more supportive of *B. trehalosi*'s role in BRD as secondary and an opportunistic bacterium rather than primary cause of respiratory disease in calves of experimental studies.

2.2. Pneumonic pasteurellosis in cattle and sheep

Bovine pasteurellosis has many synonyms that are descriptive of condition and are relevant to specific circumstances like shipping fever, bovine enzootic pneumonia, transit fever and bovine respiratory disease complex. It is caused by *M. haemolytica* type A1, *B. trehalosi* and *P. multocida* type A. The causative organisms normally reside in upper respiratory tract (De Alwis, 1999). The explosive multiplication of *M. haemolytica* and *B. trehalosi* and also may be *P. multocida* that results from stress leads to two processes. After stress or viral infection, organisms multiplying conditions and their invasion to lungs cause pneumonia. Meanwhile shedding of organisms into environment leads to dissemination of infection in healthy intact animals in their vicinity. The exact mechanisms underlying the rapid proliferation are not completely understood. Good management and avoidance of stress can help to prevent the disease (Radostits *et al.,* 2007).

Pasteurellosis in sheep is probably most economically important disease. The predominant organism that causes disease in sheep in tropical climate is *M. haemolytica*. Biotype A2 causes pneumonia in all age of sheep and septicaemia in young lamb. On the other hand,

Biotype T and *P. multocida* type A have association in both disease syndromes (Abdelsalam, 2008). The causative organism resides in the nasopharnax and tonsils of apparently healthy sheep. Predisposing factors include climatic changes and stressful conditions like transport, dipping and shearing. The prevalence of the disease can be reduced by proper management and by vaccination (De Alwis, 1999).

2.3. Predisposing factors of pneumonic pasteurellosis

2.3.1. Environmental factors

As described in the above *Pasteurella*, *Mannheimia* and *Biberstenia* species are 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 (Hawari, 2008;Al-Ghamdi *et al.,* 2000). 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 (Ahmed *et al.,* 2017; Cyntia, 2011).

Physiological responses to stressors (collectively called stress) include suppression of the immune system; consequently, prolonged stress may increase susceptibility to pathogens and to morbidity and mortality (Bhatti *et al.,* 2012). Environmental stressors most commonly associated with pneumonic pasteurellosis in livestock include heat, cold, wind, chill, humid, crowding, mixing with new animals; poor ventilation barn, handling, transport and deprivation from feed and water are commonly followed by an outbreak of the disease in cattle and sheep. Other predisposing factors, such as lack of sufficient energy or protein, inadequate colostrum consumption, specific vitamins, or certain minerals, also may compromise immunity further (Miller *et al.,* 2008).

2.3.2. Agent factors

Bacterial species incriminated in causing pneumonic pasteurellosis 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 bacteria-host interactions (Hawari *et al.,* 2008). Among the major virulence components of *M. haemolytica* and *P. multocida* are the polysaccharide capsule, OMP, LPS, fimbriae, adhesins, extracellular enzymes and other factors that are still to be investigated and elucidated (Seleim, 2005).

Pneumonic pasteurellosis, or shipping fever, large part 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.,* 1986). Both A1 and A2 possess the ability to colonize the upper respiratory tract of cattle and sheep, they are however often species specific. 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 (Haig, 2011). *Biberstinia trehalosi* mainly causes septicaemia and systemic pasteurellosis in young weaned animals, which is a hyper-acute disease and occurs most commonly in the autumn season, but can also cause pneumonia (Wheeler, 2009).

2.3.3. Host factors

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 (Wheeler, 2009). Bronchopneumonia caused by *P. multocida* or *M. haemolytica* has a cranioventral lung distribution and affects all ages of animals worldwide. It can be particularly devastating in young animals. It is a common cause of morbidity and mortality in young, especially in those that have not received adequate colostrum or in which passive colostral immunity is waning (Cyntia, 2011). Affected animals often die if not treated. The reasons for increased susceptibility to *M. haemolytica* infection in stressed animals are primarily attributed to the breakdown of innate pulmonary immune barriers by stressors (Miller *et al.,* 2008; Martin, 1996).

2.3.4. Concurrent infection

The combined infection with certain respiratory viruses is commonly found to increase the susceptibility of farm animals to secondary bacterial pneumonias (Hodgson et al, 2005). The most important viruses associated with acute respiratory tract infections in farm animals include *Para-influenza virus* type 3, *Bovine herpes virus* type 1 and 5, *Respiratory syncytial virus*, *Ovine herpes virus* type 1 and 2, *Caprine herpes* virus type 1, *Pest des petits ruminants* and *Ovine adenovirus*. Chronic viral respiratory disease caused by *Maedi-visna* and *ovine pulmonary adenocarcinoma*. These virus induced injury to the respiratory epithelium is also believed to enhance bacterial attachment and subsequent colonization of target tissues (Sherril, 2012). Parasitic pneumonia caused lung worms like *Dictyocaulus*, *Filarial*, *Protospronylus rufenscens* and *Mullerius capillaries* (verminous pneumonia), *Mycotic pneumonia* caused by aspergillus species and foreign bodies in the upper respiratory tract, aspiration pneumonia also differential diagnosis (Bell, 2008).

The deleterious effect of certain Mycoplasma species on the respiratory system of ruminant animal has long been recognized. The most important examples of these pathogens include *Mycoplasma mycoides subsp. mycoides, M. mycoides subsp. capri, M. bovis, M. ovipneumoniae* and *M. dispar* (Thirkell *et al.,* 1990). Most of these *Mycoplasma* species are known to contribute to the development of severe pneumonic lesions either alone or in association with pneumonic pasteurellosis of animals (Jones *et al.,* 1997). The synergistic role of some other bacterial organisms in this connection was also evident. Furthermore, a number of other unrelated conditions such as twin pregnancy, selenium deficiency, mycotoxins, and inhalation of foreign material and obstruction of pulmonary airways were also reported to have a predisposing role in the incidence of pneumonic pasteurellosis in susceptible animals (Abdulselam, 2008).

2.4. Method of transmission

Transmission of agents of pneumonic pasteurellosis probably occurs by inhalation of infected droplet, coughed up or exhaled from infected animals which may be clinical case or recovered carriers in which the infection persist in the upper respiratory tract (Kabeta *et al.,* 2015). *Mannheimia haemolytica* is carried in the nasopharynx and tonsils of apparently

healthy animals where interestingly serotyping is most commonly isolated from cattle (Rowe *et al.,* 2001). *Pasteurella multocida* and *Mannheimia haemolytica* are highly susceptible to environmental influences and it is unlikely that mediated contagion is an important factor in the spread of the disease. When conditions are optimal, particularly when cattle are closely confined in inadequately ventilated trains or held for long periods in holding pens and feed lots, the disease may spread very quickly and affect high proportion of the herd within short hours. Animals at pasture are able to move freely and the rate of spread may be slower (Radostitis *et al.,* 2007).

2.5. Distributions and economic significance of pneumonic pasteurellosis

The geographical distribution of *M. haemolytica* is worldwide. However, the microorganis m is reported most frequently in Asia and Africa countries where sheep or goat breeding is widespread. Countries like USA and Canada where cattle breeding for both beef and dairy cattle is also common. In Europe, pasteurellosis or mannheimiosis is also widespread involves many countries where sheep and cattle are present, such as the Netherlands, Germany, Italy and France (Tefera and Smola, 2002; Angen and Bisgard, 2002; Ewers *et al.,* 2004). *Pasteurella multocida* has also a major impact on the livestock industry in countries of Southeast Asia especially in Bangladesh where a severe economic loss has been recorded and is ranked as one of the most important contagious disease of cattle and buffaloes (Ievy *et al.,* 2013). The lower respiratory tract diseases are multifactorial in origin and results in bronchopneumonia causing approximately 75% of the morbidity and over 50% of the mortality in cattle and sheep (Urban-Chmiel and Grooms, 2012) by this *M. haemolytica* and *P. multocida* diseases.

Pneumonic pasteurellosisis an acute infectious disease that causes widespread financial losses because of death, reduced live weight, delayed marketing, treatment costs and unthriftness among survivors (Hawari *et al.,* 2008). As reported by (Griffin, 1997) in USA and Europe, pneumonia caused by *M. haemolytica* is the main cause of financial losses is over US $1 billion in North America in the breeding of calves occur due to death loss, treatment cost and decreased lifetime productivity. In Africa, especially in Ethiopia, bronchopneumonia mainly attributed to *M. haemolytica*, causes both morbidity (18.6%) and mortality (10.6%) in sheep and goats (Sisay and Zerihun, 2003). For instance the

distributions of serotypes of *M. haemolytica* and *B. trehalosi* are summarized in the table 2 below.

Table 2: Serotype distributions of some *M. haemolytica* and *B. trehalosi* worldwide

Prevalence (%)	*M. haemolytica* Serotypes	*B. trehalosi* serotypes	Country Reported	Reference
-	A1*, A2*,A6*, A7*	-	Japan	Imamuraa *et al.*(2014)
25	A1*,A2*,A7*,$A9^{+}$	T4*,$T10^{+}$,$T15^{+}$	New Zealand	Black *et al.* (2011)
12	A1*,A2*,$A6^{+}$,A7*,A 8	T4*	Turkey	Kirkan and Osman (2005)
-	A2*,A7*,$A9^{+}$,A16,	UT	Denmark	Angen *et al.*(2002)
9	A1*,A2*,$A6^{+}$,A7 $A9^{+}$	$T3^{+}$,T4*, UT	UK	Mike (1999)
-	A1*,A2*,A6,$A9^{+}$,A1 1, UT	-	USA	Ghamdi *et al.*(2000)
-	A1*, A2*	$T3^{+}$,T4*	France	Laurence *et al.* (2007)
25.3	A1*,A2*,A5 - 9^{+}	$T10^{+}$, UT	Nigeria	Odugbo *et al.*(2004)

*Most serotypes isolated and distributed among the spesies.

+The Second serotypes in their distribution.

UT= Untypeable

2.6. Status of pneumonic pasteurellosis in Ethiopia

Several studies are conducted in Ethiopia to determine the extent of the problem and the relative distribution of different biotypes and serotypes of *Mannheimia* species. In those studies, there are indications to the prominence of *M. haemolytica* especially serotype A1 and A2 are the most common in the country obtained from nasal and transtracheal swab

with morphology, phenotypical and conventional characterization. However, molecular advances need to know the prevalence and the tangible organisms elaborate bovine and ovine pneumonic pasteurellosis concerning serotype distribution and the etiological diversity of the agent in the country (Kabeta *et al.,* 2015). The studies indicated that pasteurellosis is a major threat in the highlands and also in the lowland hot and humid areas with high death and illness to domestic ruminant production and most serotypes of *M. haemolytica* biotype A are involved in pneumonic pasteurellosis with serotype A1 and A2 in cattle and sheep being the most prevalent respectively (Ayelet *et al*, 2004; Belay, 2007; Mekonnen, 2000; Deressa *et al*. 2004).

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

M. haemolytica	*B. trehalosi*	*P. multocida*	Place	Reference
28.0%	3.2%	2.2%	Bishoftu	Demissie *et al.(*2014)
11.1%	13.4%	1.8%	South Wollo	Belay,(2007)
8.3%	5.6%	25.0%	Debre Zeit	Melese,(2005)
20.0%	-	10.0%	Debre Berhan	Ayelet *et al.(*2004)
19.0%	-	15.0%	Debre Berhan	Deressa *et al.*(2004)
48.0%	7.0%	2.0%	Arsi	Mekonnen,(2000)
46.4%	39.3%	14.3%	Bedele district	Abera *et al.*(2014)
87.4%	-	12.5%	Haramaya district	Marru *et al.(*2013)

From the above summarized table indicated by those scholars, from the 12A serotypes of *M. haemolytica*: A1, A2 and A7 were isolated frequently. A6 and A9 are second in their distribution while A8, A12, A13, A14 in rare case. Serotype A16 and A17 were not reported in the stated study areas. Similarly, *B. trehalosi* serotype T4 is commonly isolated followed by T15, T10 and T3.

2.7. Diagnosis

2.7.1. Clinical signs

Pnemonia is an important disease caused by *Pasteurella* and *Mannheimia* species in domestic and agricultural animals. *M. haemolytica* causes two main diseases in sheep and cattle: 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 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, weight loss and have temperatures in the 40.4°C - 42°C range (Radostits *et al*., 2007). 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). Clinical signs alone may not be diagnostic and the diagnostic laboratory is often used to assist the clinician. In addition to the initial examination of live affected animals and obtaining a history, important areas for the veterinarian to gain information includes: assessment of involved organ system by gross and microscopic lesions, identification of the etiologic agents and contributing factors are very important.

2.7.2. Postmortem lesions

There is marked pulmonary consolidation, usually involving at least the antero-ventral part of the lungs. The lung is firm and the cut surface usually reveals an irregular, variegated pattern of red, white and gray tissue due to hemorrhage and necrosis. Occasionally sequestrate of necrotic lung tissue are found. *Pasteurella multicoda* causes fibrino-purulent bronchopneumonia without the multifocal coagulation hemolytic necrosis that characteristics of fibrinous lobar pneumonia associated with *M. haemolytica*. The post mortem findings of lung consolidation and pleurisy are present. The basic post mortem

lesions are acute fibrin hemorrhagic pneumonia with pleurisy adhesion (Schreiber *et al.,* 2000).

The most obvious changes in affected animals are the edema, widely distributed hemorrhages, and general hyperemia. In most cases, there is an edematous swelling of the head, neck, and brisket region. Incision of the swellings reveals a clear or straw-colored serous fluid. The edema is also found in the musculature, and the sub serous petechial hemorrhages, which are found throughout the animal, are particularly characteristic. Blood-tinged fluid is often found in the pericardial sac and in the thoracic and abdominal cavities. Petechial hemorrhages are particularly prominent in the pharyngeal and cervical lymph node (Kopcha, 1997). Histologically the characteristic lung lesion is acute inflammation and emboli in small arterioles and capillaries (Gilmour, 1989). Generally the diagnosis depends on the history of age, recent movement, weaning or housing, isolation and identification of the causative agent was important (Hall, 1994).

2.7.3. Isolation and characterization of the agents

Bacterial culture and species identification: In spite of the molecular advances, morphology and phenotyping are routinely used for primary identification of Pasteurellaceae from clinical pneumonic samples or post mortem lesions (Catry, 2005). All of the *Pasteurella* species can be isolated by culturing appropriate clinical specimens on blood agar. *Pasteurella multocida* will grow at 37^{o}c on blood or chocolate agar. Colonies are smooth, gray, and non-hemolytic after 24 hours of 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. It does not grow on enteric selective media like MacConkey agar.

Mannheimia haemolytica is odorless (indole negative), hemolytic, but grow on MacConkey agar unlike *P. multocida* (Catry, 2005). They are 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_2S gas. They are aerobic or facultative anaerobic with fastidious growth requirements (Quinn *et al.,* 2002). They are oxidase and catalase positives, reduces NO_3 to NO_2, and urease negative. Their 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) (Miflin and Blackall, 2001). 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 (Seleim, 2005).

The conventional method of identification of a suspected isolate as *P. multocida* or *M. haemolytica* involves subjecting the isolate to a range of biochemical tests (Miflin 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. Most of them produce acid from common sugars except lactose (Shawagfeh, 2014). All strains of *M. haemolytica* ferment Mannitol, Gulucose, Maltose, Sorbitol and sucrose without gas production. Indole, Urease, Methyle blue and VP reaction are negative. Catalase and oxidase reaction are positive. Typically they don't to ferment trehalose but ferment L-arabinose (Kaoud *et al.,* 2010). *Biberstenia trehalosi* is gram negative, non-motile rods. All isolates are positive in the nitrate reduction test and negative in Simmons citrate, methyle red and vogas proskauer teat. No isolates produced H2S, urease, gelatinase and indole (Allen *et al.,* 1991).

2.7.4. Serological technique

Serotype differentiation is based on sugar composition of the capsule as well as the composition of LPS component of the cell membrane. 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. 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 Most of these tests are used for capsular typing (OIE, 2008).

Indirect Haemagglutination test (IHA)**:** is a sensitive, reliable and economical serodiagnostic method. It is extensively used for serotyping and detection of antibodies against *Pasteurella, Bibersteinia* and *Mannheimia* species (Biberstien *et al*., 1999). It 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 18-24hours culture of bacteria of known serotypes in tryptose soya broth which is inactivated in a water bath at 60^0C for 1hour. The culture is centrifuged at 2000 rpm for 10 minutes and resuspeneded with equal volume of PBS. Then centrifuged at 5500 (rpm) for 15 minutes. Finally the clear supernatant is collected into sterile test tubes, 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 2000 rpm for 10 minutes, washed twice with phosphate buffer saline (PBS) solution (3 times a total wash). For sensitization of the sheep red blood cells 100µl of packed sheep red blood cell is added to 10ml of capsular extract antigen and then 50µl of glutaraldehyde is added and homogenized with gentle shaking and incubated for 1hour at 37^0c. 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 90µl of PBS solution is added to first row (A) wells and 50µl to the rest wells of 96 well microliter plate. Ten µl of test sera is added to the first row, mix thoroughly and serially diluted by pipetting 50µl up to row H. Fifty micro liters of sensitized RBC is added to each well and incubated for one hour at 37 ^{0}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 (Ayelet *et al*., 2004; Belay, 2007).

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 10µl) is placed on a clean glass surface, and then a small amount of *M. haemolytica* colony from blood agar is picked up on an inoculating needle and mixed with the serum. A strong positive reaction in the form of clumping and clearing occurs as the mixture is stirred with the needle. Negative reactions remain turbid (Hawari *et al.*, 2008).

Agar gel immunodifussion test: 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 (Reggie and Lea, 2007*)*. 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 are 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 serotypes of *M. haemolytica* Al allowed for identification of their major proteins. Therefore this phenomenon has been 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 center well surrounded by six peripheral wells (OIE*,* 2008).

2.7.5. Molecular identification

Conventional polymerase chain reaction (PCR): Molecular methods of bacterial identification have been proved valuable to overcoming some limitations of the conventional biochemical and serological methods and better sensitivity and rapidity (Kumar *et al.,* 2015). Molecular identification is further advance accuracy of characterization in pure and/or mixed cultures, speed of detection, determination of taxonomic position, and indulgent of intra-species genetic relationships. Conventional polymerase chain reaction (PCR) methods of detection of agent in clinical specimen appear increasingly preferred (Catry, 2005). PCR is used to detect or sense a sequence of DNA unique to *Pasteurella* and *Mannheimia* species (Deressa *et al.*, 2004).

Multiplex polymerase chain reaction (mPCR): Assay containing all two or more pairs of primers were specifically amplified serotype specific genetic targets for different *M. haemolytica* target serotypes was indicated by the amplicon sizes in the gel images and testing done for several organism in the same assays. As an extension to the practical use of the PCR, this technique has the potential to produce considerable savings in time and effort with in the laboratory without compromising on the utility of the experiment.

Multiplex PCR has been applied for the detection of PHSSA and Rpt2 genes of *M. haemolytica* using a primer pairs*:* Forward 5'-TTC ACA TCT TCA TCC TC-3', Reverse 5'-TTT TCA TCC TCTT CG TC-3' and Forward 5'-GTT TGT AAG ATA TCC CAT TT-3' Reverse 5'-CGT TTT CCA CTT GCG TGA-3', respectively (Kumar *et al.*, 2015).

Ribotyping: is a molecular technique (molecular typing) for bacterial identification that uses information from rRNA-based phylogenetic analyses (Madigan, 2012). It is rapid and specific method widely used in clinical diagnostics and analysis of microbial communities. All bacteria have ribosomal genes, but the exact sequence is unique to each species, serving as a genetic fingerprint. Therefore sequencing the particular 16S rDNA gene and comparing it to a data base would yield identification of the particular species (Christensen *et al*., 2001). Ribotyping involves the digestion of bacterial genomic DNA with specific restriction enzymes. Each restriction enzyme cuts DNA at a specific nucleotide sequence, resulting in fragments of different lengths. Recently, rRNA gene restriction analysis (ribotyping) of strains of *M.haemolytica* has confirmed the distribution of specific clones (an intraspecific clonal distribution) in bovine or captive bighorn sheep herds (Angen and Bisgard, 2002).

Random amplified polymorphic DNA (RAPD): analysis has been applied for the distinction of strains belonging to the same species. Amplified polymorphic DNA (RAPD) analysis is one of the methods used to characterize and differentiate *Mannheimia* and *Pasteurella* isolates (Chaslus-Dancla *et al*., 1996 and kodjo, 1999). It is a fast, sensitive method for the epidemiological studies and PCR-based method of genetic typing based on genomic polymorphisms (Huber *et al.*, 2002). It involves use of single arbitrary primer in a PCR reaction, resulting in amplification of many discrete DNA. Random Amplified Polymorphic DNA has been used to investigate the genetic differences among *P. multocida* and *M. haemolytica* isolates from cattle and sheep (Ozbey *et al*., 2004).

In veterinary medicine, particularly food animal production, diagnostic testing is used for prevention and control and monitoring as described by (Fulton and Confer, 2012). These include vaccination, antimicrobial treatment for infected animals and implementation of a biosecurity plan. Pneumonic pasteurellosis can be diagnosed by combination of different

techniques. This has been made using conventional methods for centuries. However the conventional methods are time consuming, expensive and non-specific. Nucleic acid-based assays have been established as gold standard for precise molecular identification and phylogenetic relationship within the family as well as on subspecies level. Genotypic characterization has proved beneficial in overcoming limitations of traditional phenotypic procedures, facilitates identification, improves the sensitivity and allows the direct detection of organisms from clinical samples (Hunt *et al.,* 2000). Similarly, there are a lot of open questions concerning the molecular pathogenic mechanisms of the bacterial species. Every diagnostic test has its own merit and demerit as summarized in Table 4.

Table 4: Comparative advantages and disadvantages of diagnostic tests used to diagnosis pneumonic pasteurellosis (Fulton and Confer, 2012)

Test	Application	Advantages	Disadvantages
Serology	Antibody detection	Detect vaccine responses and past infections.	-titers do not necessarily imply disease and are not able to differentiate vaccine induced antibodies from infection acquired antibodies.
Bacteriological techniques	Detect bacteria	-Demonstrate the presence of bacteria in the samples - provide information on the species of bacteria involved -suitable for understanding of the biology of the agents	- Positive culture does not necessarily mean the cause of pneumonia - Sensitivity is not great and may miss true positives due to concurrent infections and antimicrobial therapy. -time consuming and expensive
Insitu hybridization	-detects region of genome of agent in lesions	-identifies infectious agent within the lesion. -provides strong evidence that infectious agent is associated to pneumonia -quick, specific and sensitive	-very expensive -limited to laboratories which have sophisticated facilities -requires high level expertise
Single PCR	-detects genetic material of the agents in sample	-provides specific evidence that infectious agent is associated with pneumonia -fast, specific and sensitive	-cannot differentiate among various serotypes -very expensive
Multiplex PCR	-detects region of several agents' genomes	-with a single test, potential evidence of one or more infectious agent and or serotypes associated with disease can be determined. -fast, sensitive, specific and relatively cheaper.	-primer-dimer formation causes false positives. -performed only in specialized laboratories

2.8. Prevention and control approaches of pneumonic pasteurellosis

Pneumonic pasteurellosis is an acute infectious disease that causes wide financial losses due to death, reduced live weight, delayed marketing, treatment costs and unthriftness 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). On the other hand, Kehrenberg *et al.,* (2001) reported the possibilities to prevent and control infections due to *Pasteurella* and *Mannheimia* species in animals.

Management: The most effective preventive method is good management and avoidance of stress. Because of common occurrence of the disease at the time of shipment from the range to the feedlot, much attention has been given to reduce the incidence of disease at this time. The calves should be transported from the farm of origin directly to the fattening unit. The transport distance should be as short as possible and the animal should be handled in calm and considerate manners at all stages of transport. The calves could weaned and introduced to fattening diet at least two weeks before leaving farm (Frank *et al.,*1986). No single management practice has been effective in controlling this disease complex. Management practices which reduce stress, as well as early diagnosis and antibiotic treatment, are the key approaches of controlling disease within farm, especially during the first two to three weeks after arrival (Filion *et al*., 1984).

Treatment: Antimicrobials are still the tools of choice for prevention and control of infections due to *Pasteurella* and *Mannheimia*. However, indiscreet use of antimicrobials bears a high risk of selecting resistant bacteria, promoting the spread of resistance genes located on plasmids and transposons, and consequently, reducing the efficacy of the antimicrobial agents currently available for the treatment of food animals production (Kehrenberg *et al*., 2001). Early identification of respiratory disease and introduction of effective antibiotic therapy is necessary.

Antibiotic susceptibility tests are important, because resistance to antibiotics is frequent in *P. multocida* and *M. haemolytica*, although it is less common in other pasteurellae (Carter and Wise, 2004). *Pasteurella* are generally susceptible to antibiotics like, penicillin and tetracycline. However, the organism has been found resistant to a variety of antibiotics such as: Ciprofloxacin, Chlortetracycline, Cotrimoxazole, Furazolidone, Lincomycin,

Ampicillin, Augmentin, Kanamycin, Apramycin and Cefatoxime. Penicillin and Tetracycli ne are considered as antibiotics of choice and cephalosporin are acceptable alternative for combating the infection (Munir *et al.,* 2007).

Most cattle are showing some improvement within one to three days of initiating treatment. Antibiotics most commonly used are oxytetracycline at rate 20mg/kg, long acting and 10mg/kg daily for 3 days short acting; Tilmicosin is effective in reducing the population of *M. haemolytica* that colonizing the nasal cavities of calves with respiratory disease (Frank and Duff, 2000). Treatment is frequently unrewarding unless it is begun very early in the disease process because of rapid progression of lung damage and endotoxin release. Parenteral fluids and anti-inflammatory agents are important adjuncts to antibiotic therapy. Although septicemic pasteurellosis has favorable antimicrobial susceptibility, response to therapy is often disappointing. Administering prophylactic antibiotics to at-risk lambs may be beneficial (Merck, 2011).

Vaccination: An alternative non-antibiotic prophylaxis strategy through vaccination is more desirable. This involves the use of specific *Mannheimia* and *Pasteurell*a vaccines. Monovalent vaccine against *M. haemolytica* and *P. multocida* infection has already been in use (Sarah, 2011). Vaccines against pneumonic pasteurellosis involved will help to reduce the severity of the disease, since it is the secondary bacterial phase of the disease that contributes to both its severity and fatality.

Humoral immunity plays an important role in protection against the disease. 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). Experimental study indicated that vaccine against *M. haemolytica* A1 provides little or no cross-protection against *M. haemolytica* A2. Vaccines for *M. haemolytica* A2 have been reported to be beneficial in reducing death losses and decreased weight gains from both septicemic and pneumonic forms of pasteurellosis (sherrill, 2015).

Recent studies indicate the use of gamma-irradiated *M. haemolytica* vaccine showed better protective efficacy than the commonly used formalin killed vaccine in laboratory

animals as well as in sheep and hence could be potential alternative method of vaccine production against ovine pasteurellosis (Ahmed, 2015). Exposure to optimum doses of gamma radiation destroys the DNA of the bacterium, making it unable to replicate and establish an infection although some residual metabolic activity may survive. Thus, the irradiated microorganism can still find its natural target in the host (Datta *et al.,* 2006). Gamma irradiated vaccines appear to be more effective than formalin killed vaccines against disease, and has the added advantage of a longer storage life than "live" vaccines (Syaifudin *et al.,* 2011).

In Ethiopia, recent studies indicated that most cases of ruminant pasteurellosis are caused by *M. haemolytica* and a monovalent vaccine (inactivated *P. multocida* biotype B in bovine and inactivated *P multocida* biotype A in ovine) produced at the National Veterinary Institute (NVI) is being used for vaccination against pasteurellosis which cannot match to the actual causative agent. Studies in sheep in central highlands of Ethiopia indicate *M. haemolytica* serotype A2 and A7 were reported to occur at high frequency (Sisay and Zerihun, 2003; Ayelet *et al*., 2004). An experimental study at NVI, Ethiopia showed that vaccination with a formalin killed vaccine prepared from combined A2 and A7 *M. haemolytica* serotypes grown under iron restriction, deliberated relatively good protective efficacy in sheep than either of *M. haemolytica* A7 or A2 monovalent vaccines (Tesfaw *et al*., 2014). However, there is a need of a comprehensive study on the prevalent serotypes in the country to be considered in multivalent vaccine preparation.

3. MATERIALS AND METHODS

3.1. Study Areas

Field Survey

The field study was conducted from November, 2017 to May, 2018 in Sendafa Bake, one of the administrative units of Oromia Regional Government special administration zone surrounding Addis Ababa/Finfinne. It is located in Oromia Regional State, in Northern Shewa; more specifically, in Bereh woreda. The town is situated at a distance of 39Km North east of national capital Addis Ababa. Astronomically, the town is located between 9^0 06'14" to 9^0 10'30" North latitude and 380 57'60" to 390 04'53" East longitudes with an elevation of 2514 meters above sea level (Figure 1). Relatively, it is bordered by Aleltu woreda in the North east, and in the rest direction Berek woreda shares its boundaries. The town has the total area of 880 hectare. Based on the current expansion, the shape of Sendafa Bake town is elongated that extends linearly along the highway that runs from Addis Ababa to Sheno. Concerning weather condition, Sendafa has a moderate temperature; June, July and August are principal rainy season (BWRAD, 2015). Farmers in the vicinity of Sendafa town use a mixed crop and livestock farming system. The livestock production system in the area is both extensive and semi-intensive system. Local sheep, local zebu and cross breeds of cattle are usually reared in the area. The population of cattle, sheep, goat, equine and poultry were about 125,512; 79,026; 3,023; 30,249 and 74,168 respectively (Bereh district Livestock and Fishery Bureau, 2016).

Abattoir survey

The abattoir survey was carried out in Abyssinia and ELFORA export abattoirs located in Bishoftu town, and Sululta municipal abattoir, central part of Oromia Regional State, Ethiopia. The animals originate from various parts of the country as described in Table 5.

Table 5: Description of the areas where animals originate for abattoir

Name of the area	Regional admnistration	Astronomical location	Altitude (m.a.s.l)	Temperature (^{0}C)	Rainfall (mm)	Distance from AA(km)	References
Adamitulu	Oromia	7.58^{0}N &38.43^{0}E	1500 to 2000	12.5 to 27.2	800 to 1100	167	Tibesso *et al.* (2014)
Ambo	Oromia	8^{0}59'N37^{0}51'E	2101	10.76 to 26	1143	112	CSA, (2005)
Birsheleko	Amhara	10^{0}27'to10'42'N & 37^{0}6' to 37^{0}13'E	1400 to 2000	12 to 33.8	400 to 2400	400	Alemayyehu,(2014)
Chancho	Oromia	09^{0}17'84''N 38^{0}75'79''E	2505	10 to 25	8000 to 1500	40	CSA 2015)
Fiche	Oromia	9^{0}48'N38^{0}44'E	2738 to 2782	15 to 19	840 to 1600	147	CSA, (2005)
Gonder	Amhara	12^{0}36'N37^{0}28'E	2220	19.7	1172	734	GTAO, (2016)
Hararghe	Oromia	42^{0}40''&42^{0}22''E 10^{0}25^{0}N	1300 to 2200	8.25 to 23.4	560 to 1260	525	CSA, (2015)
Wolaita	SNNP	6^{0}4'N to 7^{0}1'N 37^{0}4'E to 38^{0}2'E	700 to 2950	11.4 to 34.12	450 to 1446	329	CSA, (2007)
Wollega	Oromia	9^{0}5'N & 36^{0}33'E	2088	10 to 36	800 to 2260	331	CSA, (2007)
Wollo	Amhara	11'8^{0}N 39'38^{0}E	2400 to 2600	7.8 to 21	850 to 1100	263	NMSA, (2005)
Yabello	Oromia	5^{0}23'49N & 39^{0}31'52″E	2000	24 to 29	300 to >700	570	CSA, (2015)

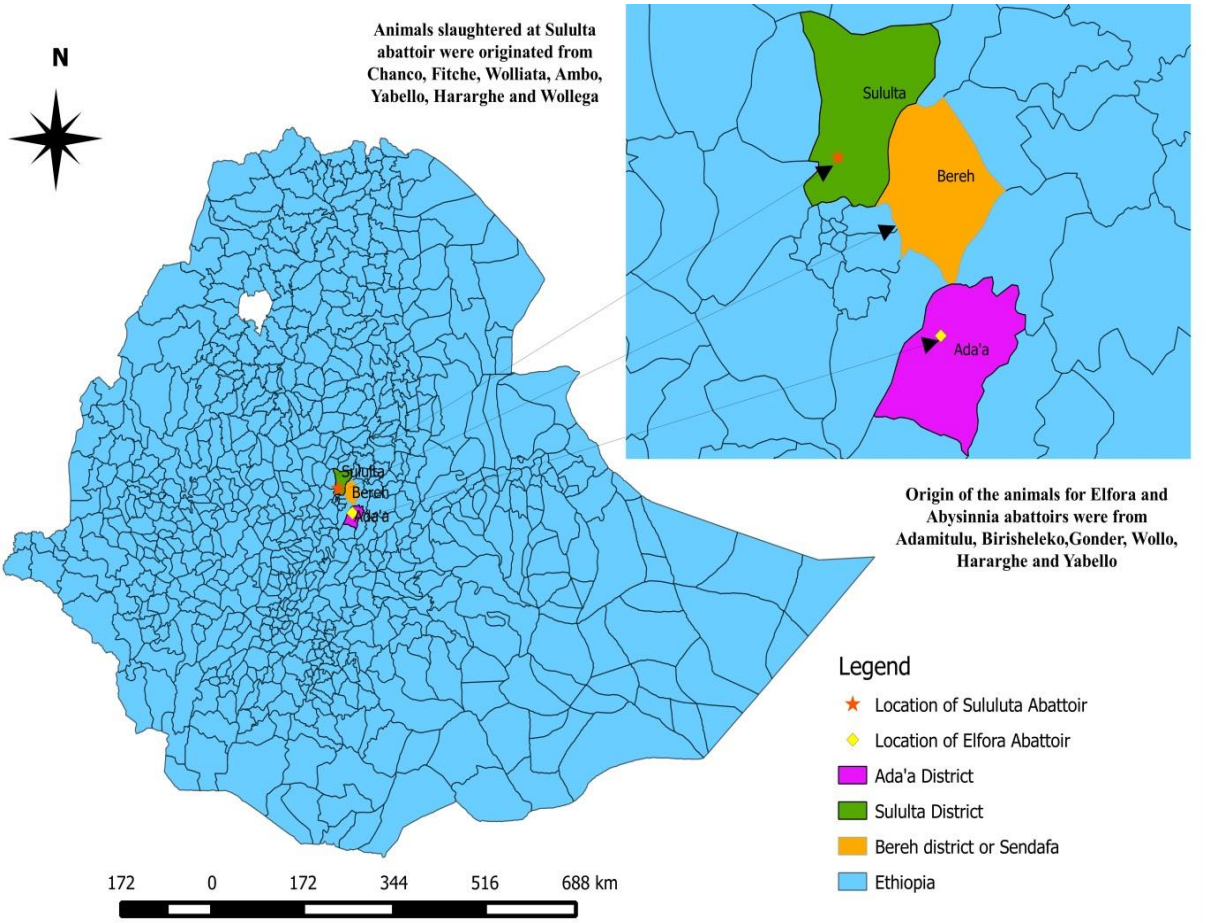

Figure 1: Map of Ethiopia showing the study areas

3.2. Study population

The animals used in this study were comprises apparently health cattle and sheep slaughtered at two export abattoirs located in Bishoftu (Abissynia and Elfora) and one in Sululta municipal abattoir, and animals with clinical manifestations presented to Sendafa veterinary clinic during the study period. The study population constitutes all sheep and cattle kept under different management system (extensive and semi-intensive) belonging vary age group, origin, sex, and health status. Cattle and sheep having respiratory distress presented to the study areas of veterinary clinic found in the study district were included. Cattle and sheep with respiratory distress like irregular breathing pattern, grunting on expiration, coughing, dyspnea, inappetance, lethargy, serous to muco-purulent nasal discharges with fever was suspected as clinically pneumonic (Lopez, 2001). The study population in the abattoirs includes all sheep and cattle slaughtered and after slaughter suspicious pneumonic lungs from slaughter house were recruited. All information on

sampled animal at clinic and abattoir were recorded in a format developed for this purpose. In both cases the animals with pneumonic lung at the abattoir and the mentioned clinical features at veterinary clinic were considered to be study population.

3.3. Study design and sampling

A cross-sectional study using conventional isolation and identification methods was done for both field and abattoir surveys. The sampling method employed was purposive sampling based on the presence of clinical signs of respiratory disease during clinical examination of cattle and sheep brought to veterinary clinics. A nasal swab was collected from pneumonic and non-pneumonic cattle and sheep. Sterile cotton-tipped, 20-25cm long applicator sticks, moistened in sterile tryptose soya broth was directed via the ventral nasal meatus in to nasopharynx, that is, the swabs was carefully inserted into nostril and the mucosa surface; rolled gently; put back to the test tube containing broth; and the tubes was capped. The specimens was labeled individually and kept cooled in the ice box. All collected specimens were transported to Microbiology Laboratory of the college of Veterinary of Medicine and Agriculture, Addis Ababa University for isolation.

For the abattoir survey, two working days were randomly selected for abattoir visit. Lungs from all cattle and sheep slaughtered on the selected days were thoroughly examined for the presence of pneumonia. On each sampling day five lungs with lesion of pneumonia from cattle and five lungs with lesion of pneumonia from sheep were identified. Up on inspection the surface of each suspected lungs with typical lesions of pneumonia were sampled. A piece of lung tissue was aseptically taken from the edge of lesion of pneumonic lung using sterile forceps and scalpel blade. The specimen was placed in to sterile screw caped tubes, labeled individually and kept cooled in the ice box and finally were transported to the Microbiology Laboratory, College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu in ice box for bacteriological procedures.

3.4. Sample size determination

For the field survey sample size was estimated by working on the sampling method, which was applied for both pneumonic and non-pneumonic cases of sheep and cattle. For this purpose the formula given by Goddard, (2003) was used to calculate the sample size and to estimate the difference between proportions. Therefore the expected proportion of cattle and sheep shedding *Pasteurella* and *Mannheimia* species is set at 50% (P_1), while for clinically pneumonic cattle and sheep the expected proportion is estimated at 80% (P_2). The power of the study is set at 80%, 95% confidence interval and significance level of $P<0.05$ was used.

i.e. $$N > \frac{(Z_1 - \alpha/_2 + Z_1 - \beta)^2 [P_1 (1 - P_1) + (P_2 (1 - P_2)]}{(P_1 - P_2)^2}$$

Where $Z_1 - \alpha = 50\%$ significance level

$Z_1 - \beta = 80\%$ power level

P_1 = Expected proportion in healthy sheep and cattle, $P_1=0.5$

P_2 = Expected proportion of diseased sheep and cattle, P2=0.8

$$N_1 > \frac{7.89 \times [0.5 (1 - 0.5) + 0.8 (1 - 0.8)]}{(0.5 - 0.8)^2}$$

$N1 \geq 36$, and $N2 \geq 36$; the total sample required for each species was 36 pneumonic and 36 non-pneumonic animals that is, 72 cattle and 72 sheep, which was 144. For abattoir survey since we used convenience sampling in which all cattle and sheep slaughtered on the selected days were examined and all pneumonic cases were recorded. Those lungs showing fibrinous bronchopneumonia were sampled for isolation and identification of the bacterial agents in question.

3.5. Isolation and identification of bacterial isolates

3.5.1. Bacteriological Methods

Microbiological isolation of *Pasteurella species* and *Mannheimia species* were done as describe by Quinn *et al.* (2002), Hawari *et al*. (2008). For the abattoirs, immediately after arrival, samples were processed near to Bunsen burner to make them ready for inoculation. The lung tissue was transferred into sterile Petridish and the surface of the lung tissue was

seared by hot scalpel blade (incised and minced) with sterile forceps. Then, the specimen was placed in to screw caped test tube containing TSB and pre incubated in the same way as nasal swab. In the laboratory aseptically collected specimens of nasal swab and lung tissue were incubated at 37^0C for 24 hrs. Immediately After 24 hrs pre-enriched incubation of specimens in TSB (Oxoid, Hampshire, England), a loopful of culture was streaked on to blood agar base (BM 014, India) with containing 5% sheep blood and incubated aerobically at 37^0C for up to 24 hours (Annex 1). On culture positive plates, suspected colonies were subjected to gram's staining to study staining reactions and cellular morphology under light microscope at 100x magnifications (Annex 2). Those mixed and gram negative, coccobacilli or short rods bacteria was further sub cultured with due care, on both blood agar containing 5% sheep blood and MacConkey agar (Oxoid, England) for isolation and characterization. The growth of colonies on blood agar and MacConkey agar was characterized using blood agar for the presence of haemolysis, the type of haemolysis, the general appearance of colonies (morphology, color, shape, size and consistency).

The growth cultures of colonies characteristics of round (smooth), greyish color, small to moderate size and mucoid consistency which were either haemolytic or non haemolytic on (blood agar) and on MacConkey agar were characterized. Narrow beta haemolysis on blood agar and grow on MacConkey agar with lactose fermentation was grouped as *M. haemolytica*. While those which were non-haemolytic on blood agar base and didn't grow on MacConkey agar was grouped as *P. multocida*. Pure cultures of single colony type from MacConkey and blood agars were transferred onto nutrient agar (Oxoid, England) for identification of the isolates for a succession of primary biochemical tests (Annex 3): such as catalase, oxidase and urease were performed as described by Quinn *et al.* (2002).

Identification of the bacteria to the species level was proceeded by using the secondary biochemical tests for metabolic end products, and fermentation of sugars; glucose, lactose, maltose, sucrose, arabinose and trehalose following standard procedures Quinn *et al.* (2002), Hawari *et al*. (2008). *M. haemolytica* isolates were selected on the basis of lactose and arabinose fermentation and lack of fermentating of trehalose, whereas *B. trehalosi* isolates utilized trehalose sugars and catalase negative. Other basis of indole production, absence of growth on MacConkey agar and absence of haemolysis on blood agar and characteristics sweetish odour was identified as *P. multocida* isolates (Annex 4).

3.5.2. Molecular Identification

DNA extraction

DNA extraction was conducted at the molecular biology laboratory of the National Veterinary Institute. A few colonies from the phenotypically characterized pure cultures of *M. haemolytica*, *B. trehalosi* and *P. multocida* grown on nutrient agar (TSA) for 24 to 48 were transferred into 1ml of Eppendorf tubes (Kumar *et al.,* 2015). The bacterial genomic DNA was extracted using Qiagen DNeasy Blood and Tissue Kit as per manufacturer's instructions (Qiagen, German town, MD, USA). Briefly, 200µl of bacterial culture suspension was transferred into a labeled 1.5ml micro centrifuge tubes. 20µl proteinase K (Fermentas) and 200µl Buffer AL was added for each tube and mixed by vortexing and incubated at 56ºC for 30 minutes. 200µl 96% ethanol was added per tube and mixed gently by vortexing. The mixture was transferred to a labeled DNeasy mini spin column placed in a 2ml collection tubes and centrifuged for 1 minute at 12000 rpm. The collection tubes were changed by new ones and 500µl Buffer AW1 was added into each spin column and centrifuged for 1 minute at 12000 rpm. The collection tubes was again changed by new tubes and 500µl Buffer AW2 was added each spin column and centrifuged for 3 minutes at 20000 rpm. Finally, the spin columns were transferred into a labeled 1.5ml eppendorf tubes and 40µl Buffer AE was added to each column and the nucleic acids bound to the silica membrane was eluted and the eluted DNA yield was used for PCR amplification.

Multiplex polymerase chain reaction (mPCR)

Primers targeting PHSSA and Rpt2 genes of *M. haemolytica* used in previous studies (Kumar *et al.,* 2015) were used in this study. The primers used both for the PCR and sequencing activities in this study were synthesized by VBC Biotech, Australia and purified by reverse phase high performance liquid chromatography. Multiplex PCR was performed targeting the amplification of Rpt2 and PHSSA genes of *M. haemolytica* with the expected band size of 1022 and 325 base pairs. The pairs of primers used were : forward- 5'- GTT TGT AAG ATA TCC CAT TT- 3', Reverse 5'- CGT TTT CCA CTT GCG TGA-3' and Forward 5'–TTC ACA TCT TCA TCC TC-3', Reverse 5' TTT TCA TCC TCT TCG TC3' for mPCR and sequencing, respectively as described by (Kumar *et al.,* 2015).

The PCR was carried out in a final volume of 25 µl of reaction mixture containing ×1 PCR buffer; Primer MH-RPt2 Fow: 5pM/µl, 2µl (VBC, biotch-7787), Primer Mh-RPt_2 REV: 5pM/µl, 2µl; Primer PHSSA Fow: 5pM/µl, 2µl (VBC, biotch-7887); Primer PHSSA REV: 5pM/µl, 2µl; RNase free water 3µl, IQ Super mix, 10µl (Bio Rad, USA) and template DNA, 4µl. The PCR conditions used to amplify both RPt2 and PHSSA gene fragments included an initial denaturation at 95°C for 3 min, followed by 35 cycles each at 95°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 30 seconds and a final extension cycle at 72°C for 5 min. A negative control consisting of all component of reaction mixture except the DNA template was included in the PCR. Positive control was included in the mPCR using a template of known *M. haemolytica i*solate from NVI collection.

Molecular identification of *Pasteurella multocida*

Pasteurella multocida isolates were tested by conventional PCR for the specific identification of the serotype of *P. multocida*. PCR was carried out using primer sets targeting capsular genes (capA) of *P. multocida* as described by Townsend *et al.* (2001).The primers are: Forward TGCCAAAATCGCAGTCAG and Reverse TTGCCATC ATTGTCAGTG amplifying a segment of about 1044 bp. The PCR reaction mixture (50µl) containing, master mix (Fermentas, Thermo Fisher Scientific, USA), 5pmol of each primer (Eurofins MWG Operon, Germany) 6µl, DNA template, 6µl and 20µl IQ super mix were used.

This reaction mixture was subjected to amplification in a thermal cycler according to standard procedure; an initial denaturation at 95°C for 5 min, followed by 35 cycles each of 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 30 s and a final extension cycle at 72°C for 7 min. A negative control consisting of all component of reaction mixture except the DNA template was included in the PCR. Positive control was included in the conventional PCR from known source, NVI vaccine strain of *P. multocida*. The amplified products were separated by agarose gel electrophoresis that stained with gel red.

Agarose gel electrophoresis of polymerase chain reaction products

Two percent w/v, agarose gel was prepared in 0.5X Tris borate EDTA buffer. 5µl of each PCR product was mixed with 6X loading buffer and loaded into separate well of the pre-prepared gel and 1kb plus DNA molecular marker was also added onto the first and last lane and run at 120 volt for about 60 minutes. The PCR products were analyzed by visualization of desired size of DNA bands in the gel red stained agarose gel under gel documentation system by comparing with molecular marker as described by Sambrook, (2001); Kumar *et al.* (2015).

Purification and sequencing of PCR products

The PCR products yielding positive signals for *P. multocida and M. haemolytica* were purified using the wizard SV Gel and PCR clean-up system kit (Promega, Germany). The concentration of the purified PCR product was quantified using Nanodrop 2000c spectrometer (Thermo scientific, USA). The concentration of each purified product was adjusted and prepared according to the instruction recommended by sequencing company. The purified PCR products were mixed with the sequencing primers and submitted for sequencing to the commercially sequencing LGC Genomics (Berlin, Germany) (Annex 6).

Phylogenetic Analysis

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.17611508 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 737 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

3.6. Invitro antimicrobial sensitivity test

Antibiotic susceptibility test was done by the disk diffusion technique as described by Kirby Bauer. The pure culture colony was made using sterile physiological saline and adjusted to 0.5 McFarland standards then spread to Muller Hinton agar using sterile cotton swab and allowed to stand for 3- 5 minutes to observe any excess moisture from the medium before the antimicrobial disc (OXOID, England) containing single concentration of each antimicrobial agents was then placed onto the inoculated surface using sterile forceps, gently pressed with the point of the forceps to ensure complete contact with the agar surface and left for 30 min for diffusion of the antibiotics in the disc . The plates were inverted upside down and incubated at 37^0C for 18 to 24 hours. The result was evaluated for clear zones produced by antimicrobial inhibition of bacterial growth were measured in mm using measuring caliper and interpreted as susceptible, intermediate and resistant according to Clinical Laboratory Standards Institute, (2015) stated. Each isolates was tested against those antimicrobials detailed in (Annex 5) and selections were based on the availability of the disk.

3.7. Data management and analysis

Data obtained from all investigations were coded and kept in Excel spread sheets. The association among variables collected during this study was computed using various statistical tools in STATA version 13 software. The proportion of species and various serotypes were assessed as factors using descriptive statistics. For all statistical analysis significant level of 0.05 is considered.

3.8. Ethical clearance

Ethical clearance for appropriate sampling of this study was obtained from Addis Ababa University College of veterinary medicine and agriculture minutes of animal research ethics and review committee (Annex 11).

4. RESULTS

4.1. Isolation and Identification

Field Survey

From 149 nasal swab samples (74 cattle and 75 sheep) collected and cultured, *Pasteurella* and *Mannheimia* species were isolated successfully from 22.15% (33) of them. Out of the 33 isolates 20.27% (15) of them were from cattle whereas 24.00% (18) of them were from sheep. The species composition of the isolates showed that 10.07% (15), 4.7% (7) and 7.38% (11) of them were *M. haemolytica*, *B. trehalosi* and *P. multocida,* respectively (Table 6). On the basis of these results it seems that *M. haemolytica* was the dominant bacterial species isolated from cattle and sheep in the study areas.

Out of the 74 nasal swab samples from cattle (38 pneumonic and 36 non-pneumonic) cases *Pasteurella* organisms were isolated from 36.84% (14) and 2.78% (1) of them, respectively (Table 7). The species composition of the bacteria isolated comprises of 8.11 % (6), 5.40% (4) and 6.76 % (5) of *M. haemolytica*, *B. trehalosi* and *P. multocida*, respectively. In the same way, out of 75 nasal swabs from sheep (39 pneumonic and 36 non-pneumonic) cases *Pasteurella* organisms were isolated from 41.03% (16) and 5.56% (2) of them. The species compositions showed that 12.00% (9), 4.00% (3) and 8.00% (6) of them were *M. haemolytica, B. trehalosi* and *P. multocida*, respectively.

Abattoir survey

From 176 pneumonic lung samples (93 cattle and 83 sheep) collected and cultured *Pasteurella* and *Mannheimia* species were isolated from 27.27 % (48) of them. Out of the 48 isolates 24.73% (23) of them were from cattle whereas 30.12% (25) of them were from sheep. The species composition of the isolates showed that 13.07% (23), (7.39% (13) and (6.83% (12) of them were *M. haemolytica*, *B. trehalosi* and *P. multocida,* respectively (Table 8) suggesting predominance of *M. haemolytica* in both cattle and sheep. The highest proportion of *M. haemolytica*, *B. trehalosi* and *P. multocida* came from Birsheleko (75%) followed by Gonder (66.67%), while the lowest was made from samples originated

from Ambo (15%) (Figure 2). Isolation was less frequent in male animals than female counterparts. Animals reared under semi-intensive production system more frequently yielded positive results than those from extensive production system (Table 9).

Table 6: Frequency of isolation of *Pasteurella* species from nasal swabs collected from cattle and sheep

Animal species	No of samples	Species of bacteria isolated			Total	X^2 (P value)
		M. haemolytica	*B. trehalosi*	*P. multocida*		
Cattle	74	6 (8.11%)	4 (5.41%)	5 (6.67%)	15 (20.27%)	0.30 (0.56)
Sheep	75	9 (12%)	3 (4.00%)	6 (8.00)	18 (24.00%)	
Total	149	15 (10.07)	7 (4.7%)	11 (7.38%)	33 (22.15%	

Table 7: Frequency of isolation of *Pasteurella* spp. from pneumonic and non-pneumonic cattle and sheep

Healthy status of the animals	Bacterial species			X^2 (P value)
	M. haemolytica	*B. trealosi*	*P. multocida*	
Pneumonic cattle (n=38)	5 (13.16%)	4 (10.53%)	5 (13.16%)	13.27 (<0.001)
Non-pneumonic cattle (n=36)	1 (2.78%)	0 (0.00%)	0 (0.00%)	
Total (n=74)	6 (8.11%)	4 (5.41%)	5 (6.76%)	
Pneumonic sheep (n=39)	8 (20.51%)	2 (5.13%)	6 (15.38%)	12.91 (<0.001)
Non-pneumonic sheep (n=36)	1 (2.78%)	1 (2.76%)	0 (0.00)	
Total(n=75)	9 (12.00%)	3 (4.00%)	6 (8.00%)	

Table 8: Frequency of isolation of *Pasteurella* spp. from pneumonic lungs collected from abattoir

Animal species	Pneumonic lungs	Species of bacteria isolated			Total isolates	X^2 (P value)
		M.haemolytica	*B.trehalosi*	*P.multocida*		
Cattle	93	11 (11.83%)	7 (7.53%)	5 (5.38%)	23 (24.73%)	
Sheep	83	12 (14.46%)	6 (7.23%)	7 (8.43)	25 (30.12%)	0.64 (0.42)
Total	176	23 (13.07%)	13 (7.39%)	12 (6.83%)	48 (27.27%	

Table 9: Association of culture positive results along with different risk factors

Attribution		No of animals sampled	Percent	X^2 (P Value)
Breed	Cross	115	26.09%	0.13 (0.72)
	Local	210	24.29%	
Sex	Female	102	31.37%	0.30 (0.07)
	Male	223	21.97%	
Age	Adult	230	25.65%	0.22 (0.64)
	Young	95	23.16%	
Prod. system	Extensive	213	23.47%	0.69 (0.41)
	Semi-intensive	112	27.68%	

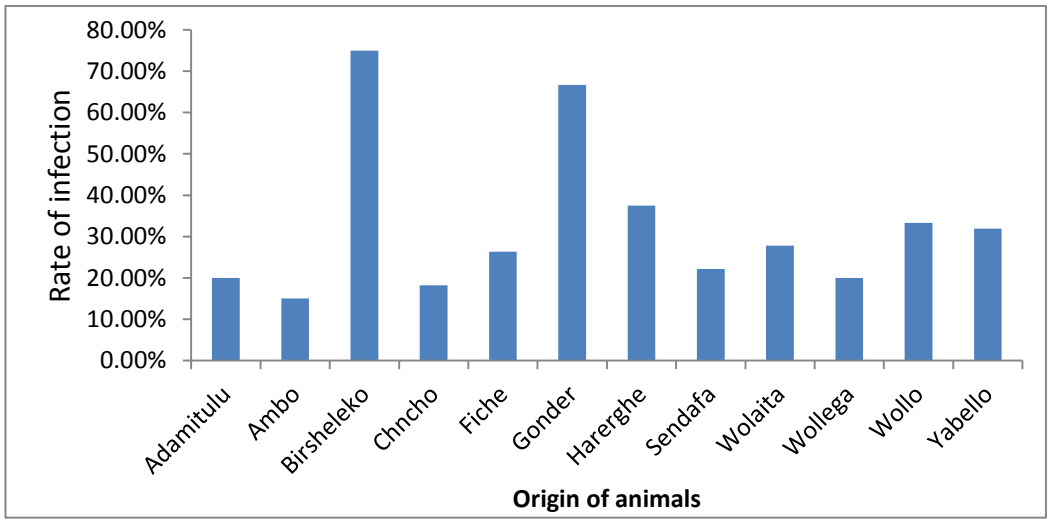

Figure 2: Rate of infections along with animals' origin

4.2. Results of molecular analysis

Twenty two bacterial isolates that were identified as *P. multocida* (7) and *M. haemolytica* (15) by conventional bacteriological methods were selected and tested using PCR based nucleic acid methods. All of the 15 *M. haemolytica* isolates tested were confirmed by the PCR technique (Figure 2). In addition, two *B. trehalosi* isolates were tested alongside the *M. haemolytica* isolates and they gave negative result for the tested bacterial genes.

Similarly all of the 7 isolate of *P. multocida* tested using conventional PCR targeting the capsular gene of *P. multocida* designated *cap*A gave positive results (Figure 3).

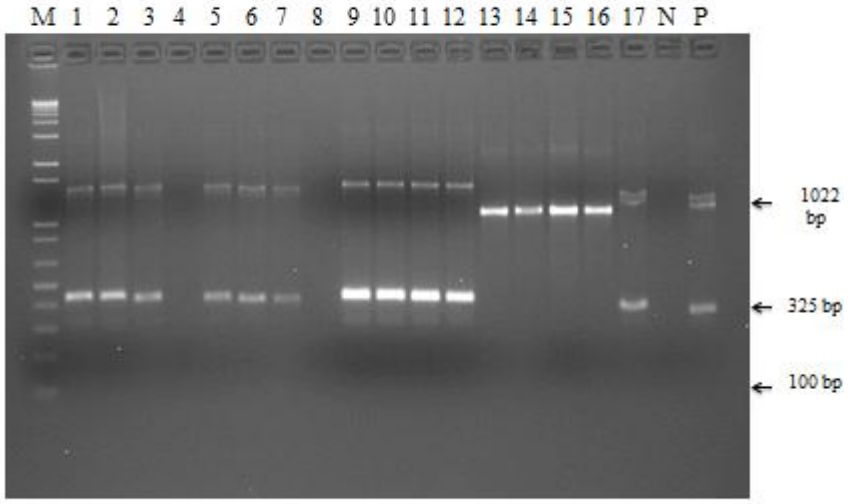

Figure 3: Results of Rpt2 and PSSA genes amplification products (approximately 1022 and 325 bp respectively) of *M. haemolytica*. M = DNA ladder; 1-17: *M. haemolytica* (lane 4 and 8: *B. trehalosi*). N: Negative control; P: positive control.

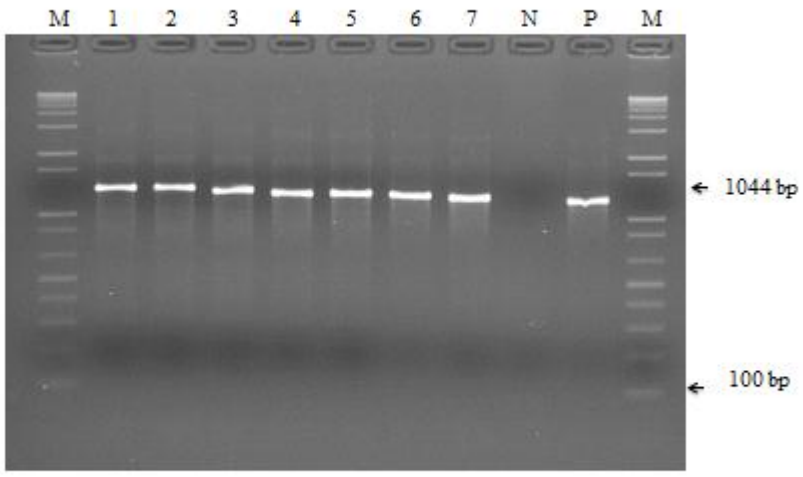

Figure 4: Results of PCR amplification of *P. multocida* product (approximately 1044 bp). Lane M= DNA ladder, Lane 1- 7: *P. multocida*. N: Negative control, P: positive control.

4.3. Nucleotide sequence alignment

Three isolates identified as *P. multocida* were cloned and sequenced. These include isolates designated: Yabello /01/2018, Fiche /01/2018 and Sendafa/01/2018. The results of basic local alignment search tool (BLAST) analysis showed that the P. *multocida* isolates identified in this study were more than 99% similar to strains deposited in gene bank with accession number of: AF036004.2, AF237926.1, AY225345.1, AY225346.1, JF922885.1 and MF417608.1 (Annex 7). The isolates were identical to *P. multocida* serovars A1 and A3 as shown in figure 5.

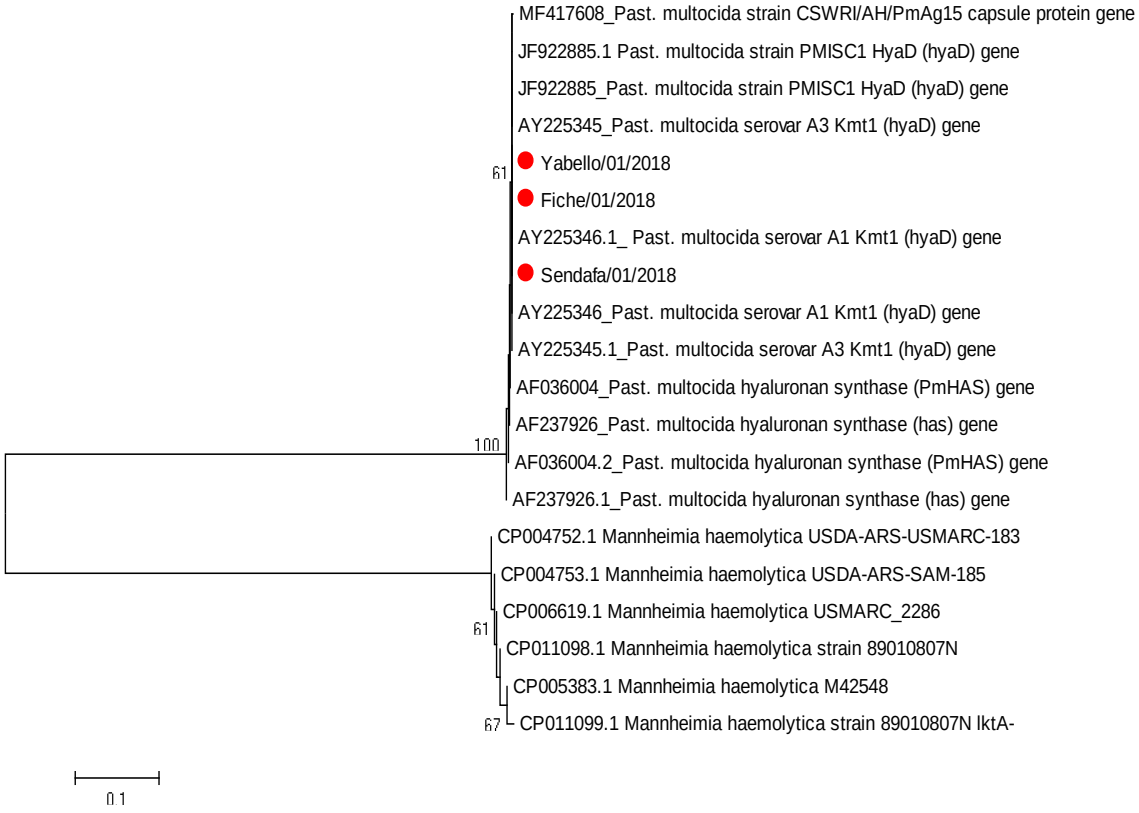

Figure 5: Phylogenetic analysis of 20 *Pasteurella* isolates based on nucleotide sequences of the hyaD gene.

Three current *Pasteurella* isolates and the sequences retrieved from the GenBank were used. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pair wise deletion option was compute using MEGA6. The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the

branches. The *Pasteurella multocida* isolates sequenced in this study are indicated plain red circle

4.4. Results of antibiotic susceptibility test

The results of anti-microbial susceptibility of isolates from pneumonic cases are shown in Table 11. *Mannheimia haemolytica, B. trehalosi* and *P. multocida* isolates were resistant against Cloxacillin (91.67%, 100.00%, and 77.78%) and Ampicillin (83.33%, 85.71% and 100.00%), respectively. On the other hand isolates belonging to the three genera of bacteria were susceptible to Chloramphenicol (100.00%, 85.71% and 88.89%), Norfloxacil lin (100.00%, 100.00% and 88.89%), Oxytetracycline (75.00%, 71.43% and 66.67%) and Tetracycline acts in (75.00%, 71.43% and 66.67%), respectively. Streptomycin was ineffective (50.00%, 57.14% and 55.56%) in *M. haemolytica, B. trehalosi* and *P. multocida*, respectively.

The results of anti-microbial susceptibility of isolates from non-pneumonic cases of cattle and sheep are presented in Table 11. The results showed that all the bacterial isolates tested were resistant to Ampicillin and Cloxacillin whereas nearly 100% of the isolates were susceptible to Chloramphenicol, Norfloxacillin and Streptomycin. Similarly the isolates from the three genera of bacteria appear to be susceptible to Oxytetracycline.

Table 10: Antimicrobial susceptibility pattern of bacteria isolated from nasal swabs and pneumonic lungs in cattle and sheep.

Antimicrobials tested	Performance	Species of bacteria		
		M. haemolytica (n=12)	*B. trehalosi* (n=7)	*P. multocida* (n=9)
Ampicillin	Resistant	10(83.33%)	6(85.71%)	9(100%)
	Intermediate	2(16.67%)	1(14.29%)	0(0.00%)
	Susceptible	0(0.00%)	0(0.00%)	0(0.00%)
Chloramphenicol	Resistant	0(0.00%)	1(14.29%)	1(11.11%)
	Intermediate	0(0.00%)	0(0.00%)	0(0.00%)
	Susceptible	12(100%)	6(85.71%)	8(88.89%)
Cloxacillin	Resistant	11(91.67%)	7(100%)	7(77.78%)
	Intermediate	1(8.33%)	0(0.00%)	2(22.22%)
	Susceptible	0(0.00%)	0(0.00%)	0(0.00%)
Norfloxacin	Resistant	0(0.00%)	0(0.00%)	1(11.11%)
	Intermediate	0(0.00%)	0(0.00%)	0(0.00%)
	Susceptible	12(100%)	7(100%)	(88.89%)
Oxytetracycline	Resistant	2(7.14%)	2(14.29%)	3 (11.11%)
	Intermediate	0(0.00%)	0(14.29%)	0(11.11%)
	Susceptible	10(83.33%)	5(71.43)	6(88.89%)
Streptomycin	Resistant	3(25.00%)	2(28.57%)	2(22.22%)
	Intermediate	3(25.00%)	1(14.29%)	2(22.22%)
	Susceptible	6(50.00%)	4(57.14%)	5(55.56%)
Tetracycline	Resistant	2(16.67%)	2(28.57%)	2(22.22%)
	Intermediate	1(8.33%)	0(0.00%)	1(11.11%)
	Susceptible	8(75.00%)	5(71.43%)	6(66.67%)

Table 11: Antimicrobial susceptibility pattern of bacteria isolated from nasal swabs apparently health in cattle and sheep.

Antimicrobials tested	Performance	Species of bacteria		
		M. haemolytica (*n=2)*	*B. trehalosi* (n=1)	*P. multocida* (n=2)
Ampicillin	Resistant	2(100%)	1(100%)	1(50%)
	Intermediate	0(0%)	0(0%)	1(50%)
	Susceptible	0(0%)	0(0%)	0(0%)
Chloramphenicol	Resistant	0(0%)	0(0%)	0(0%)
	Intermediate	0(0%)	0(0%)	0(0%)
	Susceptible	2(100%)	1(100%)	2(100%)
Cloxacillin	Resistant	2(100%)	1(100%)	2(100%)
	Intermediate	0(0%)	0(0%)	0(0%)
	Susceptible	0(0%)	0(0%)	0(0%)
Norfloxacin	Resistant	0(0%)	0(0%)	0(0%)
	Intermediate	0(0%)	1(100%)	0(0%)
	Susceptible	2(100%)	0(0%)	2(100%)
Oxytetracycline	Resistant	0(0%)	0(0%)	0(0%)
	Intermediate	0(0%)	0(0%)	1(50%)
	Susceptible	1(100%)	1(100%)	1(50%)
Streptomycin	Resistant	0(0%)	0(0%)	1(20%)
	Intermediate	0(0%)	0(0%)	0(0%)
	Susceptible	2(100%)	1(100%)	1(100%)
Tetracycline	Resistant	0(0%)	0(0%)	0(0%)
	Intermediate	1(50%)	0(0%)	2(100%)
	Susceptible	1(50%)	1(100%)	0(0%)

5. DISCUSSION

Ruminant production is one of the important sectors identified by the livestock development master plan of Ethiopian government to achieve food security by the smallholder farmers. Besides, the commercial dairy sector of the country is advised to adopt a low emission dairy production, which entails disease control to reduce what is known as "unproductive emissions". Respiratory disease is one of the diseases that to be controlled in this regard. However, control of diseases including respiratory diseases requires to the ability to make inventory of the potential pathogens present in the country. The present study provided the list of bacterial pathogens commonly incriminated respiratory diseases in cattle and sheep. This study used both conventional and molecular methods to identify *Pasteurella, Mannheimia* and *Bibersteinia* species from samples collected from cattle and sheep. The *P. multocida* strains identified were identical to A1 and A3, which are known to cause pneumonia in cattle Madsen *et al.* (1985); Mukkur, (1979). This preliminary finding is a useful observation to be considered by the veterinary and livestock authorities disease intervention programs.

Respiratory infections of domestic ruminants caused by species of *Pasteurella, Mannheimia* and *Biberstenia* have been documented previously. Particularly infection with *Mannheimia* and *Bibersteinia* is known to cause pneumonia Biberstein, (1999). The prevalence of pneumonia caused by these bacteria has been considered to be important constraints in Ethiopia Tibbo *et al.* (2001); Weldemeskel *et al*. (2002) incurring huge economic loss. Similarly, Ayelet and his colleague (2004) suggested that in Ethiopia, respiratory problems due to *M. haemolytica*, *B. trehalosi* and *P. multocida* cause significant mortality and morbidity and are responsible for huge treatment cost. These previous studies, however, used only conventional methods and collected samples from non-pneumonic animals. Our study was based on both conventional methods of isolation and identification followed by confirmation of the identity of the bacterial species by molecular techniques. Besides, we compared the frequency of isolation of the bacteria from pneumonic and non-pneumonic case and we provided reliable information on the species composition of these respiratory pathogens. The bacteria were more frequently isolated from pneumonic cases than from non-pneumonic cases. Although it is difficult to

claim causality as our study was observational, it is possible to suggest that these bacterial species can potentially hamper ruminant industry.

Our findings showed that similar proportion of cattle and sheep were carrying *M. hemolytica, B. trehalosi* and *P. multocida*. This is mainly due to co-herding of cattle and sheep that allowed circulation of bacteria among these animals. Sometimes calves and sheep are housed together at night. Our observation is agreement with previous reports made from different parts of Ethiopia such as that of Demissie *et al.* (2010) in Bishoftu, Marru *et al*. (2013) in Haramaya, Ayelet *et al. (*2004) in Debre Birhan, and Belay (2007) in South Wollo. However, our result is the proportion of cattle and sheep found positive for these bacteria in our study is lower than that of Abera *et al.* (2014) in Bedele district. This might be due to difference in ecological conditions where animals are raised and the occurrence of concurrent infections. The later author conducted the study in Bedele, where the incidence of trypanosomoisis and tick-borne diseases is high. The later diseases are known to cause stress to infected animals and compromise their immunity. These diseases are known to suppress the immunity of animals Asmaa *et al.* (2014). The effect of ecological factors on the occurrence of various species of *Pasteurella* has been shown previously Gilmour, (1989). Our findings are higher than that of Nurhusien (2005) in Ethiopia who had reported 8.7% prevalence of the bacterial species considered. Our observation is also in agreement with reports made elsewhere in Africa such Egypt Kaoud *et al*. (2010) but differs from that of Khalili *et al*. (2016) in Iran. This difference might be due to variations in the husbandry practices, season of the year and herd size. Diseases caused by *M. haemolytica, B. trehalosi* and *P. multocida* are associated with stress and other predisposing factors. The occurrence and magnitude of these predisposing factors vary from place to place including husbandry practices. The role of such factors on the incidence of disease caused by these bacteria has already been described Quinn *et al.* (1999).

In this study significant variation was observed in prevalence of *Pasteurella* spp. between pneumonic and non-pneumonic animals. This suggests the possible involvement of these bacteria in the genesis of pneumonia. In consent to our observation Abera *et al*. (2014) reported higher prevalence of *Pasteurella* spp. in pneumonic animals than non-pneumonic counterparts.

The highest prevalence was observed in Birsheleko (75%) followed by Gonder (66.67%) whereas the lowest observed in Ambo (15%). This difference might be due to distance from the abattoir and shipment conditions. Birsheleko and Gonder are away from Bishoftu, where the abattoir is located. That is animals are transported long range and in truck is overloaded there will be stress and close contact between animals, which facilitates transmission and spread of the agents among animals. The effects mixing of cattle and sheep from different sources at auction of markets increases stress and shipping long distance by train has been demonstrated elsewhere Ribble, (1995); Powers *et al.*(2014).

Our observation revealed that prevalence of *Pasteurella* species is higher in female animals than males even though the difference was not statistically significant. This is in consent with the findings of Marru *et al*. (2013) in Haramaya district who has also observed no association between *Pasteurella* infection and gender. In the present study, the association of animals in semi-intensive and extensive production system was compared. The bacterial species were more frequently isolated from animals originating from semi-intensive production system than from those from extensive system. The possible reason for this could be due to crowding and frequent contact among animals in semi-intensive husbandry systems than animals, which roam freely in the range. This situation is likely to favor spread of *Pasteurella* species among animals. Similar observation was reported previously by Radostitis *et al.*(2007); Chowdhury *et al*.(2002). However in contrary to our observation Engdaw and Alemneh, (2015) reported high prevalence in animals from extensive production system than those from semi intensive production system.

The results of this study demonstrated that the *Pasteurella* species isolated were resistant to commonly used antibiotics. This has significant implications. At present antibiotic therapy is the affordable and feasible option for control of pasterurellosis. The multiplicity of the species and serotypes, having no cross-protection among each other, preclude the use of vaccines to control respiratory disease caused by these. The veterinary and livestock authorities should take this into account when attempting to control diseases caused by *Pasteurella* species. Regular monitoring and rational use of antibiotics is needed to avoid the emerging antibiotic resistance. According to the present research result, Chloramphenicol, Oxytetracycline and Tetracyclines are effective drugs against *Pasteurella* species. Aminoglycosides (Streptomycin) and Beta-lactams (Ampicillin and

Cloxacillin), which are known to be most effective antibiotics in veterinary field Muktar *et al.* (2017) and which are commonly used under Ethiopian settings are not much effective. Despite diverse in the site of origins, the isolates seems to exhibited uniformity in sensitivity to a majority of the antibacterial agents. This is due to the fact that only few antibiotics are used widely in all parts of Ethiopia and possibly in the globe as many bacteria have developed resistance to many of the antibiotics. Developments of resistance to antibiotics by all *M. haemolytica*, *B. trehalosi* and *P. multocida* isolates have been reported by different scholars Catry *et al*. (2006); Welsh, (2004). The result of current study was coincide with the literature which stated as Chloramphenicol is highly effective and well tolerated broad spectrum antibiotic to many genera of gram negative and gram positive bacteria Sisay, (1997). Moreover, the present result was consistent with the previous report Kaminski *et al.* (2016) that Tetracycline exhibited moderate activities against the tested isolates.

In consent with the present results, Guler *et al.* (2013) reported the occurrence of resistance against commonly used antimicrobial agents for treatment of respiratory diseases of livestock such as Norfloxacin and Oxytetracycline although they showed that the resistance to these antibiotics by *P. multocida* isolates was generally low. However, antibiotic susceptibility studies should be renewed periodically. In addition pre-existing resistances in which the cellular mechanisms required for antimicrobial susceptibility are absent from the chromosomal mutation and accusation of transferable genetic material. Treatment with a specific antimicrobial agent selects those micro-organisms that have pre-existing or acquired resistance Quinn *et al.* (1994).

6. CONCLUSIONS AND RECOMMENDATIONS

This study revealed that *M. haemolytica, P. multocida* and *B. trehalosi* are commonly circulating in cattle and sheep originated from various parts of the country. Besides, the serotypes of *P. multocida* identified are those known to cause pneumonia in cattle. The three species of bacteria were more frequently isolated from pneumonic animals than from animals without pneumonia. All the three species of bacteria isolated were resistant to some of the antibiotics and susceptible to few of them. Therefore, the following recommendations are suggested:

- Further serotyping of the isolate from wide range of agro-ecology is needed
- The exact role of the bacterial species isolated in the genesis of pneumonia should further studied
- Animal transportation should be considered while transporting animals to abattoirs so as to reduce stress
- There should be regulation and guidance in rational use antibiotics at national level.

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8. ANNEXES

Annex 1: Media used and preparations for bacteriological examination

A. Tryptose Soya broth (Oxoid, Hampshire,England)

Composition (g/l): Pancreatic digest of casein 17.0; pancreatic digest of soya bean meal 2.0; sodium chloride 5.0; Di-basic potassium phosphate 2.5 and Glucose 2.5.

Preparation: Dissolved 30.0gm in one liter of distilled water and distributed in to final containers, sterilize by autoclaving at 121 ^{0}C for 15 minutes and dispensed in 5 ml amount in sterile 10ml screw capped tubes.

The tubes were stored at 4^{0}c until used.

B. Blood agar plate

- 37 gms of blood agar base was suspended in 1 liter of distilled water and heat boiling until completely dissolved.
- The dissolved medium was autoclaved for 15 minute at 121^{0}c, cooled at 45-50 ^{0}c followed by aseptically mixed with 5 % sheep blood and dispensed in 20 ml amount in sterile 90 mm petridishes.
- The plates were stored at 4^{0}c until used.

C. MacConkey agar

- 51.53 gram Mac Conkey agar was suspended in 1 liter of distilled water and boiled it until completely dissolved.
- The dissolved medium was autoclaved for 15 minute at 121^{0}c and dispensed in 20 ml amount in sterile 90 mm petridishes.
- The plates were stored at 4^{0}c until used.

D. Nutrient Agar

- 41gm nutrient agar was suspended in 1 liter of distilled water and heat boiling until completely dissolved.
- The dissolved medium was autoclaved for 15 minute at 121^{0}c, cooled at 45-50 ^{0}C and dispensed in 20 ml amount in sterile 90 mm petridishes.
- The plates were stored at 40c until used.

E. Triple Sugar Iron agar

- 65 gm Triple sugar Iron agar was suspended in 1liter distilled water and dissolved by heating.

- 6ml of the aliquot was distributed into test tubes and autoclaved for 15 minutes at 121^0c.
- The tubes were solidified in slanting position so that butt was formed.

Annex 2: Grams staining procedure

The procedures followed were as described by Quinn *et al.* (2002).

- Clean, cooled glass slides were used to make thin smearing from suspected colony of the specimens
- Sterile distilled water was used to emulsify a colony or portion of colony on the previously flamed side of the slide.
- Air dried and heat fixed the slide by passing it through a flame two or three times.
- The slides were Flooded with crystal violet and allowed them to remained for 1minutes. Rinsed the slide gently with cold tap water.
- Gram's iodine solution was applied and allowed it to remain for 1 minute.
- While holding the slide at a slight angle, allowed drops of alcohol to run over the smear (15-seconds). Quickly rinsed well with water.
- Safranin was applied and allowed it to counter stain for 60 seconds. Rinsed with water until all free stain is removed. Blotted (did not wipe) the slide were dried with bibulous paper.
- Smears were examined under oil immersion

Annex 3: Procedures for biochemical tests used for Identification of Mannheimia and Pasteurella species.

A. Catalase test

Principle: the breakdown of hydrogen peroxide in to oxygen and water is mediated by the enzyme catalase.

Procedure: A loop full of the bacterial growth was taken from the colonies of nutrient agar medium. Bacterial cells were placed on a clean microscopic slide and a drop of 3% H_2O_2 was added effervescence of oxygen gas, with a few seconds, indicates appositive reaction.

B. Oxidase test

- Principle: The cytochrome oxidase enzyme is able to oxidize the substrate tetramethyl –p-phenylenediamine dihydrochloride, forming a colored end product, endophenol.
- Procedure: prepare a solution 1% of tetramethyl-p-phenylenediamine hydrochloride, then apiece of filter paper is moistened in a petri dish with fresh reagent and the test bacterium is streaked firmly across the filter paper with a glass rod. A dark purple colour along the streak line with in 10 seconds indicates appositive reaction.

C. Indole test

- Principle: Indole positive bacteria possess an enzyme tryptophanase which converts tryptophan to indole.
- Procedure: stab inoculate SIM medium with test bacterium and incubated at 37^0Cfor 18 to 24 hours. Then Kovac's reagent (0.2 ml) was added to tube and stand for 10 minutes.
- Interpretation: the formation of dark red ring indicates positive reaction while in negative reaction a yellow ring is formed.

Annex 4: Summary of Culture characteristic and Biochemical tests used as reference for isolation and identification of *Pasteurellacae* organisms (Quinn *et al.,* 2002; Ragy, 2005)

Features	M. haemolytica	B. trehalosi	P. multocida
Haemolysis	+	+	-
MacConkey	+	+	No growth
TSI	+	+	+
Catalase	+	-	+
Indole (SIM)	-	-	+
Odor	-	-	+
Arabinose	+	-	-
Trehalose	-	+	-
Maltose	V	V	+
Oxidase	+	+	+
Glucose	+	+	+
Sucrose	+	+	+
Lactose	+	-	V
H_2S Production	-	-	-
Motility (SIM)	-	-	-
Urease	-	-	-

+ = indicates positive reaction

- = indicates negative reaction

V= variable

Annex 5: Zone interpretive chart for antimicrobials (inhibition zone diameter in mm

Antimicrobial agent	Disk Potency (mm)	Resistance (≤)	Intermediate	Susceptible (≥)
Ampicillin	AMP-25	13	14-16	17
Chloramphenicol	C-30	12	13-17	18
Cloxacilin	COX-10	10	11-12	13
Norphloxacine	NOR-10	12	13-16	17
Oxytetracycline	OT-30	14	15-18	19
Streptomycine	S-25	11	12-14	15
Tetracycline	TE-10	14	15-18	19

Annex 6: Polymerase chain reaction

a. Master mix preparation for *M.haemolytica* (Multiplex PCR)

Type of Reagent	For one reaction	Total 17 reactions
RNase free water	8 µl	136µl
Primer MH-RPt2 FOR, 5pm/µl	4 µl	68µl
Primer MH-RPT2 REV, 5pm/ µl	4 µl	68µl
Primer PSSA FOW, 5pm/ µl	4µl	68µl
Primer PHSSA REV, 5pm/ µl	4µl	68µl
IQ Super mix	20µl	340µl
Template (DNA)	6µl	
Total Volume	50µl	

b. Run Multiplex PCR Reaction

Steps	Temperature	Time	Cycle
Initial Denaturation	95^0C	3 min	1 Cycle
Denaturation	95^0C	1min	35 Cylce
Annealing	48^0C	1min	
Elongation	72^0C	1min	
Final Elongation	72^0C	5 min	1 Cycle
Hold at	4^0C	Until machine off	

C. Master Mix for capsular type of *P.multocida*

Type of Reagent	For one reaction	Total 7 reactions
RNase free water	3 µl	21µl
Primer capA FOW, 5pm/µl	2µl	14 µl
Primer capA REV, 5pm/ µl	2 µl	14µl
IQ Super mix	10 µl	70 µl
Template (DNA)	3 µl	
Total Volume	20µl	

d. Amplification cycle of conventional PCR reaction

Steps	Temperature	Time	cycle
Initial Denaturation	95^0C	5 min	1 Cycle
Denaturation	95^0C	1min	35 Cycles
Annealing	55^0C	1min	
Elongation	72^0C	1min	
Final Elongation	72^0C	7 min	1 cycle
Hold at	4^0C	Until machine off	

e. Agarose gel of PCR product for multiplex

- Prepared 100ml of 2% agarose in 0.5X TBE buffer.
- The mixture was boiled until completely dissolved
- Allowed it to cool to about 45^0C
- The gel was poured and inserts well former (comb). Allowed to set on a flat surface for about 15 min
- The tank was filled with 1X TBE buffer and removed comb from gel
- 4 µl of loading buffer containing gel red was added in to 20 µl of PCR product
- Samples (10 µl of PCR product) and 10 µl of 100 bp plus molecular markers were loaded in to appropriate gel wells.
- Run electrophoresis at 120 volts for 60 minutes.
- Viewed and photographed the gel on UV- trans illuminator. Use UV-safety spectacles.

f. Agarose gel of PCR product for conventional

- Processing of PCR amplification
- Equal volume membrane binding solution was added in to PCR amplification.
- Binding of DNA
- Insert minspan columns in to collection tubes
- Prepared PCR product mixture was transferred to minicolumn assembly. Incubated it at room temperature for 1 minute.
- Centrifuges at 16000 X g for 1 minute. Discarded flow through and minicolumn was inserted in to collection tubes.

Washing

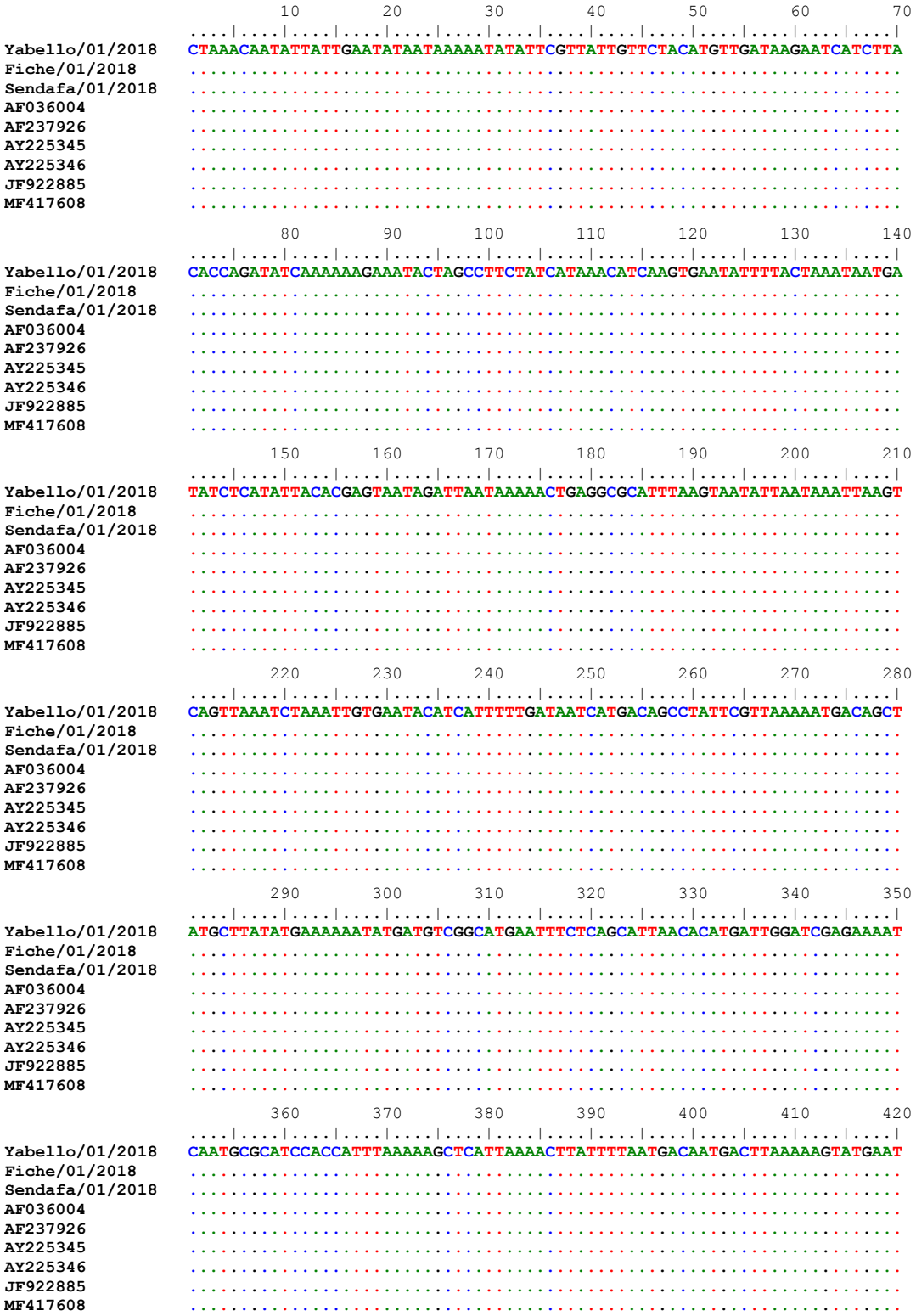
- 700µl membrane wash solution containing ethanol was added and centrifuged at 1600X g for 1 minute. Reinserted minicolumn in to collection tubes.
- Steps 2 repeated with 500µl membrane wash solution and centrifuged at 1600 X g for 5 minutes.
- Made empty the collection tubes and recentrifuged the column assembly for 1 minute with opened micro centrifuge for allowing evaporating residual ethanol.

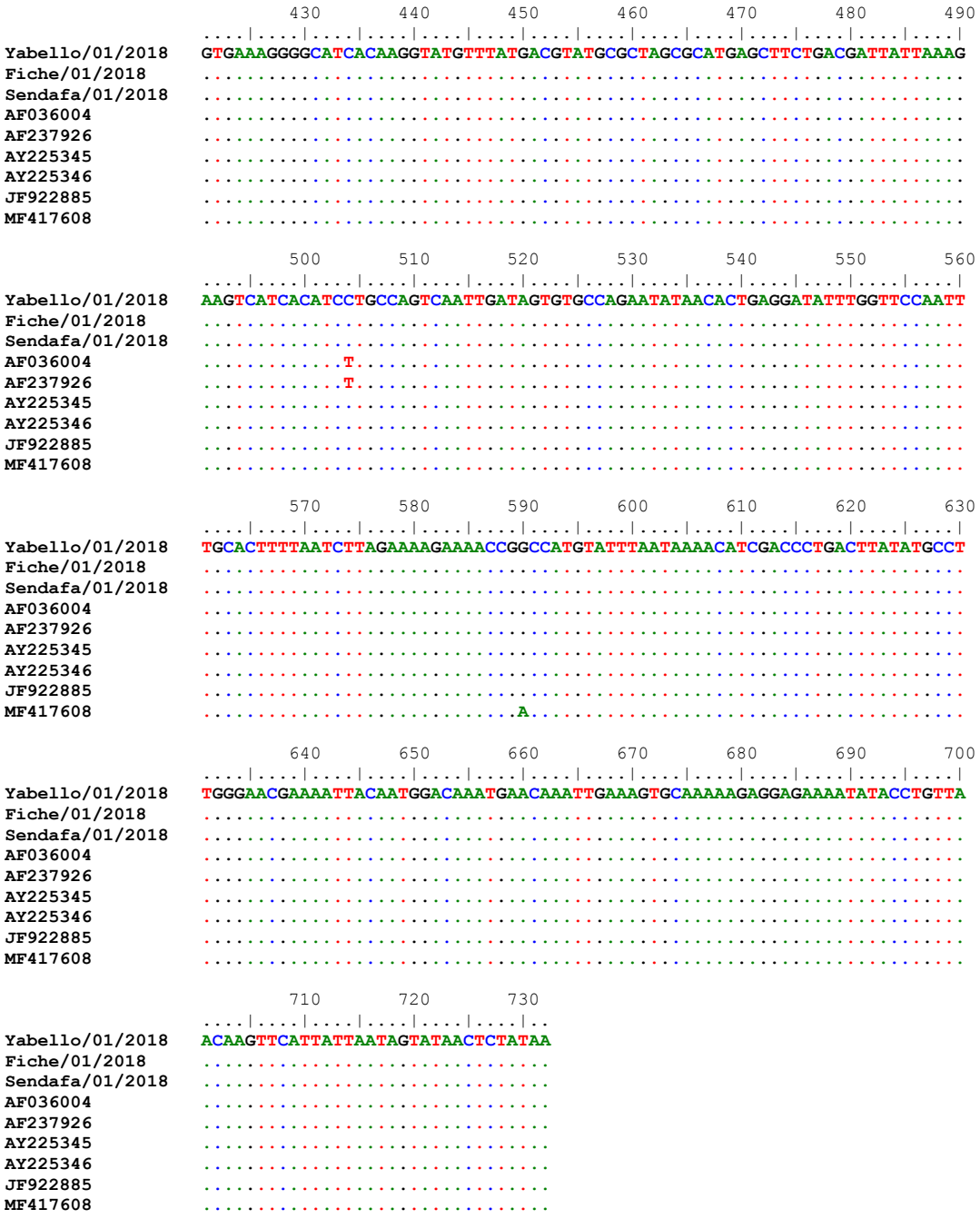
Elution

- Carefully the minicolumn was transferred to a 1.5ml clean microcentrifuge tubes
- 50µl nuclease free water was added to minicolumn, incubated at room temperature for 1 minute and centrifuged at 1600X g for 1 minute.

  Discarded minicolumn and stored the DNA at -20 ^{0}C until submitted for sequencing.

Annex 7: Nucleotide Sequence alignment result of *cap*A gene, *P. multocida* (n=3)

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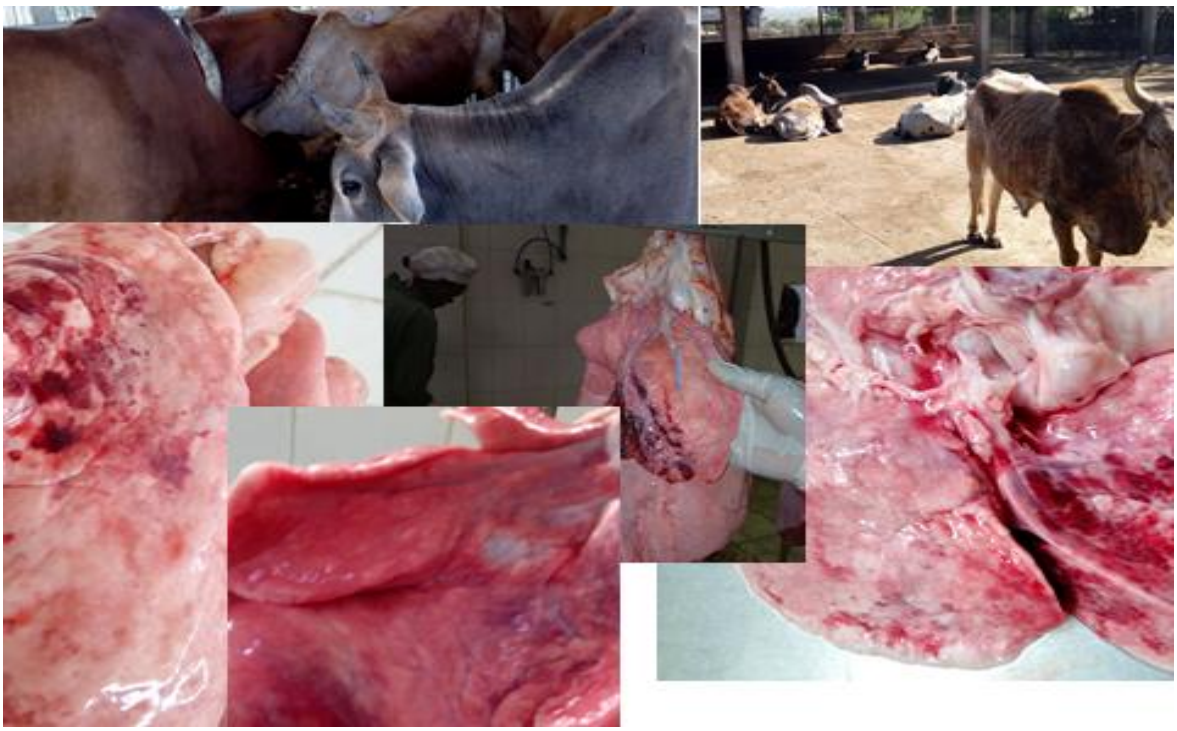
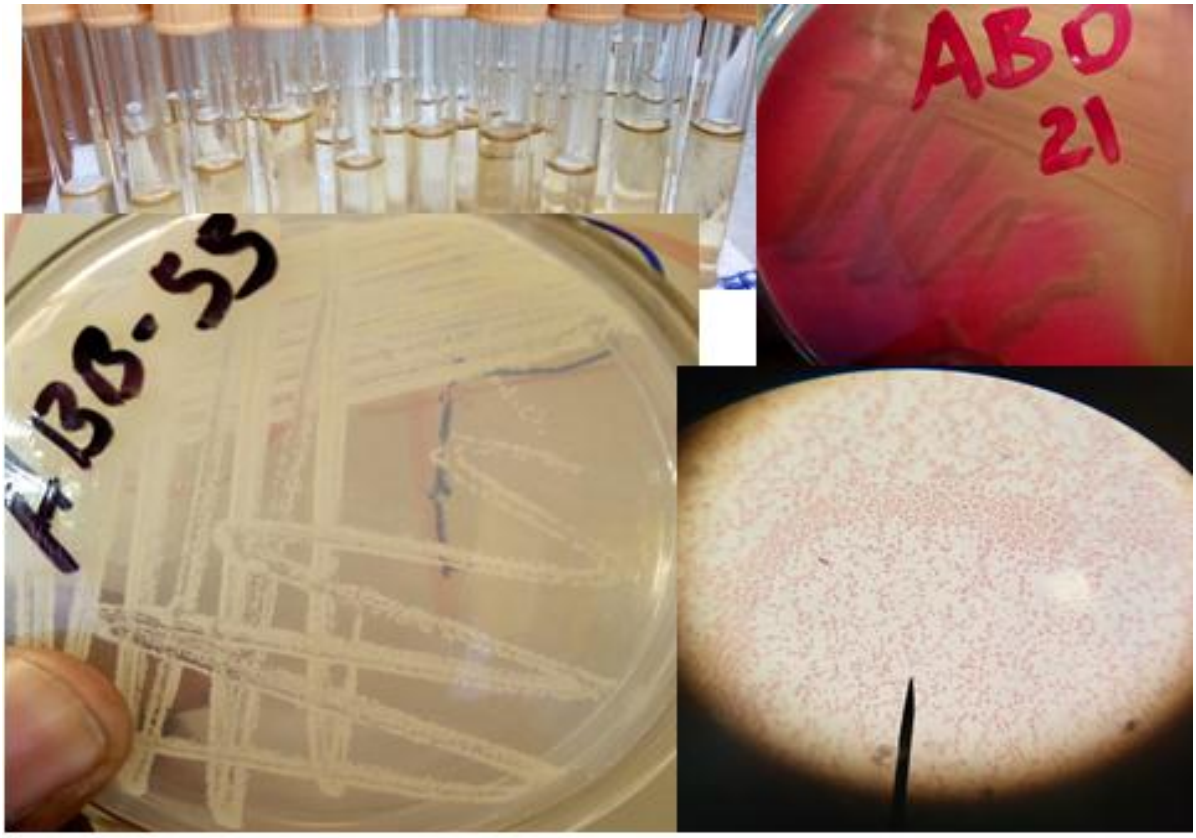
Annex 8: Different pictures of clinical signs, cultural and Biochemical characteristics

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OXIDASE

Annex 9: Field data collection Format

Date	Sample code	Species	Oigin	Age	Sex	Breed	Main clinical signs observed	Origin	Health sstatus	Management	Type of Samples	Transport media used

Annex 10: Laboratory test results recording format

Code	Growth on	Heamolysis on	Odour	Gram stain	catalase	oxidase	motility	Genus	Indole	Lactose	Maltose	Sucrose	Glucose	Arabinose	Trehalose	Species

Annex 11: Ethical clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/24/05/10/2018

Name of Applicant: Abebe Wirtu (BSc in VLT, MSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: Isolation and identification of *Mannheimia haemolytica, Biberstinia trehalosi and Pasterulla multocida* from cattle and sheep from selected areas of Ethiopia

Date of application: 02/11/2017

Nature of the project: non-invasive
Target animal species: Cattle and Sheep
Number of animals involved: 149
Study area: Ethiopia

Minutes No. and date of review: VM/ERC/05/10/018, 03/01/2018

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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Please quote Our Ref. No. When replying

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