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Molecular Detection and Antibiogram of Pasteurella Multocida and Mannheimia Haemolyticaisolates From Suspected Pneumonic Sheep in Selected Districts of South Wollo, Ethiopia

Mulugeta Legas

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BAHIR DAR UNIVERSITY

COLLEGE OF AGRICUTLURE AND ENVIRONMENTAL SCIENCES

SCHOOL OF ANIMAL SCIENCE AND VETERINARY MEDICINE

DEPARTMENT OF VETERINARY SCIENCE

MSc. IN "MICROBIOLOGY"

MOLECULAR DETECTION AND ANTIBIOGRAM OF *PASTEURELLA MULTOCIDA* AND *MANNHEIMIA HAEMOLYTICA* ISOLATES FROM SUSPECTED PNEUMONIC SHEEP IN SELECTED DISTRICTS OF SOUTH WOLLO, ETHIOPIA

MSc. Thesis

By

Mulugeta Legas

August, 2022

Bahir Dar, Ethiopia

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (MSc.) IN “VETERINARY MICROBIOLOGY”

August, 2022

Bahir Dar, Ethiopia

THESIS APPROVAL SHEET

As member of the Board of Examiners of the Master of Sciences (M.Sc.) thesis open defense examination, we have read and evaluated this thesis prepared by **Mulugeta Legas** entitled **"Molecular detection and antibiogram of *Pasteurella multocida* and *Mannheimia haemolytica* isolates from suspected pneumonic sheep in selected districts of South Wollo, Ethiopia".** We hereby certify that; the thesis is accepted for fulfilling the requirements of the Degree of Master of Sciences (M.Sc.) in "Veterinary Microbiology".

Board of Examiners

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Chair person's name	Signature	Date

DECLARATION

I declare that this thesis entitled **"Molecular detection and antibiogram of *Pasteurella multocida* and *Mannheimia haemolytica* isolates from suspected pneumonic sheep in selected districts of South Wollo, Ethiopia"** submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in **"Veterinary Microbiology"** to the Graduate program of College of Agriculture and Environmental Science, Bahir Dar University by **Mulugeta Legas** (ID. No BDU 120 67 20PR) is an authentic work carried out by him under our supervision. The matter embodied in this project work has not been submitted earlier for award of any degree or diploma to the best of our knowledge and belief.

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DEDICATION

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LIST OFABBREVIATIONS

AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
Bp	Base Pair
CLSI	Clinical and Laboratory Standards Institute
dNTPs	Deoxynucleotide Triphosphate
IHA	Indirect Hemagglutination
LPS	Lipopolysaccharides
µg	Microgram
µl	Microliter
MHA	Muller-Hinton Agar
MIC	Minimal Inhibitory Concentration
mPCR	Multiplex Polymerase Chain Reaction
NVI	National Veterinary Institute
OMP	Outer Membrane Protein
PHSSA	*Pasteurella haemolytica* species specific antigen
TAE	Tris-acetate-EDTA (Ethylenediamine tetraacetic acid)
TSB	Tryptone Soya Broth
TSI	Triple Sugar Iron
UK	United Kingdom
USA	United States of America
UV	Ultraviolet

ABSTRACT

Pneumonic pasteurellosis is the most common economically significance infectious diseases of ruminants and predominantly caused by Mannheimia haemolytica, Bibersteinia trehalosi and Pasteurella multocida. However, M. haemolytica has been recognized as the principal cause of pneumonic pasteurellosis in sheep. Although yearly vaccination is carried out using inactivated P. multocida biotype A, pasteurellosis is still reported. This suggests the need for further research into the species and strains responsible for the disease, which is vital evidence for inclusion and development of a multivalent vaccine. With the aim of molecular detection and antibiogram of P. multocida and M. haemolytica isolates, a cross sectional study was conducted from January 2021 to April 2022 in four selected districts of South Wollo. Based on purposive sampling method, 154 deep nasal swab samples were collected from suspected pneumonic sheep for bacteriological analysis. The result revealed that the overall species recovery rates were 47 (30.52%). Out of 47 isolates, 41 (26.62%) of the isolates were M. haemolytica and 6 (3.90%) were P. multocida. Further molecular analyses of the isolates were conducted using primers targeting PHSSA and Rpt2 genes and revealed, 7/41 (17.07%) isolates of M. haemolytica were positive for PHSSA gene and negative for Rpt2 gene. PCR assay targeting capsular biosynthesis (capA) gene of P. multocida isolates were not detected rather 4/7 (57.14%) of PHSSA gene positive M. haemolytica isolates showed non-specific band size around 650 bp different from expected value. Accordingly, M. haemolytica was primary agent for sheep pneumonia in the study districts. Antibiotic susceptibility test result indicated that M. haemolytica isolates were (100%) susceptible for ampicillin and gentamycin. Hence, they were most effective drugs of choice. However, amoxicillin and erythromycin were (100%) resistant and completely inactive against the isolates.

Keywords: *Antibiogram, Mannheimia haemolytica, Pasteurella multocida, PCR, Sheep*

Chapter 1. INTRODUCTION

1.1. Background and Justification

Ethiopia lies within the tropical latitude of Africa and has an enormous various topography, a wide range of climatic features and a multitude of agro-ecological zone which makes the country suitable for different agricultural production system. This in turn has contributed to the existence of a large diversity of farm animal genetic resource (Marru *et al*., 2013) and the country is home to various indigenous sheep breeds (Yegoraw *et al*., 2017). From the total livestock population of the country, sheep constitute the second major component of livestock (Marru *et al*., 2013).

Several attributes such as adaptability to harsh environmental conditions, less feed and space requirement, high fertility, short reproductive interval, high off-take rate, and low initial investment cost make this segment of livestock a viable option for resource-limited farmers in the country (Ibrahim *et al*., 2022). In spite of large livestock population in Ethiopia, the economic benefits remain marginal due to prevailing of diseases, reproductive inefficiency, management constraints and general lack of veterinary care (Musteria *et al*., 2017). Pneumonic pasteurellosis is among the most common economically significance infectious diseases of ruminants with a wide prevalence all over the world (Bahr *et al*., 2021) .

Pasteurellosis is predominantly caused by three species notably *M. haemolytica*, *B. trehalosi* and *P. multocida* (Legesse *et al*., 2018). However, *M. haemolytica* is considered a primary bacterial agent associated with the disease (McRae *et al*., 2016). These bacterial species are aerobic, fermentative, gram negative rod and cocco-bacilli, catalase and oxidase positive, usually being pleomorphic, grow well at 37°C on 5% sheep blood (Kumar *et al*., 2015).They are commensals in the upper respiratory tract of clinically healthy ruminants; they can gain access to the lungs and are able to induce disease in animals with impaired pulmonary defense mechanisms (Bahr *et al*., 2021).

The name *M. haemolytica* came into being after a number of extensive reclassification designated by Theodore kitt in 1885 and 1932 as *Bacterium bipolare multocidum* and *Pasteurella haemolytica* respectively. Later on *P. haemolytica* was then categorized into two biotypes viz. A and T based on its ability to ferment the sugars arabinose and trehalose

(Legesse *et al*., 2018). In this respect, *P. haemolytica* biotype A was allocated to a new genus and renamed as 'Mannheimia,' the name given by the German scientist Walter Mannheim (Mohamed and Abdelsalam, 2008). Based on the capsular antigen, biotype A houses 12 A serotypes; A1, A2, A5-A9, A12- A14, A16, and A17 (Sahay *et al*., 2020). Whereas, Biotype T (*B. trehalosi)* further subdivided and contains four T serotypes (T3, T4, T10 and T15) (Ferede *et al*., 2013). With respect to surface polysaccharide capsules, *P. multocida* is differentiated into five major serogroups as A, B, D, E, and F (Khalili *et al*., 2016).

M. haemolytica and *P. multocida* that mediate contagion is an important factor in the spread of the disease through the inhalation of infected nasal secretions (Tadesse *et al*., 2017). In addition, *P. multocida* is an important zoonotic agent (Register and Brockmeier, 2019) and humans acquire the infection through animal bites, scratches, licks on skin abrasions, or contact with mucous secretions derived from pets (Wilson and Ho, 2013).

The most effective system for handling *Pasteurella* and *Mannheimia* infections is using antimicrobial agents. However, imprudent antimicrobial usage intensely increases the risk of selecting resistant bacteria (Bahr *et al*., 2021). Therefore, before starting a treatment course, it is necessary to select the most effective antibacterial drugs (Laishevtsev, 2020). Hence, conducting periodically renewed antibiotic susceptibility profile of *P. multocida* and *M. haemolytica* helps to choose appropriate antibiotic against pasteurellosis"(Önat *et al*., 2010; Marru *et al*., 2013). Since pneumonic pasteurellosis is one of the serious problems of small ruminants, effective control and prevention of the disease is mandatory (Legesse *et al*., 2018). However, control of the disease is difficult task that requires integration of various techniques (Marru *et al*., 2013). The most effective preventive method is skillful management practices, and the use of vaccines are of primary importance (Taye *et al*., 2019; Jilo *et al*., 2020). Vaccination is the best alternative practical control strategy to reduce the incidence and burden of the disease and to minimize antimicrobial use (Berhe *et al*., 2017).

1.2. Statement of the Problem

Ethiopia is the home of the largest livestock population in Africa and has an estimated 39.89 million sheep, 50.50 million goats, and 65.35 million cattle. Thus, small ruminants (sheep and goats) are important components of the subsector and have been supporting the national economy of the country by generating hard currency from meat exports (Zewdie *et al*., 2021). Likewise, this sector constitutes a significant portion of livestock production and is a source of cash income, meat, milk and wool for small farm holders (Haile *et al*., 2020). However, the productivity of sheep is unsatisfactory largely due to diseases and poor animal management practices (Legesse *et al*., 2018). Pneumonic pasteurellosis is a high-priority disease causing significant economic losses through mortality, morbidity, and high cost of treatment (Marru *et al*., 2013). Moreover, the geographical distribution of *Pasteurella* is worldwide (Nicholas *et al*., 2008), almost all species of animals including poultry are susceptible for *Pasteurella* infections (Kehrenberg *et al*., 2001) and all age groups can be infected but the most susceptible age group is 6 months up to 2 years (Mukh *et al*., 2011).

In Ethiopia, the prevalence of previous findings of Marru *et al*. (2013) 31.33%, of which 21.69% *M. haemolytica* and 9.64% of *P. multocida*, Akane *et al*. (2022) *M. haemolytica* (32.62%), and Tewodros and Annania (2016) reported as *M. haemolytica* (25.91%) and *P. multocida* (6.68%). According to those scholars finding, the most cases of ruminant pasteurellosis are caused by *M. haemolytica* of one or more serotypes. However, a monovalent inactivated vaccine containing *P. multocida* biotype A is being produced at the National Veterinary Institute (NVI), Ethiopia for vaccination against ovine pasteurellosis which does not match to the actual causative agents (Berhe *et al*., 2017; Bote *et al*., 2017; Legesse *et al*., 2018). This may suggest the need for the development of a multivalent vaccine using the most prevalent serotypes causing the disease (Ayelet *et al*., 2004). However, there was a gap on the distribution of the prevalent serotypes in the country to be considered for inclusion into multivalent vaccine formation. Therefore, the present study was designed with the following objectives:-

1.3. Objectives of the Study

1.3.1. General objective

- To isolate and molecularly detect *M. haemolytica* and *P. multocida* from suspected pneumonic cases of sheep in selected districts of South Wollo

1.3.2. Specific objectives

- To isolate and identify *Pasteurella* species collected from suspected pneumonic cases of sheep
- Molecular detection of *M. haemolytica* and *P. multocida*
- Determination of antimicrobial susceptibility profiles of *M. haemolytica* and *P. multocida* from suspected pneumonic cases of sheep in the study districts

1.4. Research Questions

- Are there *M. haemolytica* and *P. multocida* in the selected districts of South Wollo?
- Which bacterial species is predominantly circulating in the study districts?
- What are the antimicrobial susceptibility profiles of *M. haemolytica* and *P. multocida* isolates?

Chapter 2. LITERATURE REVIEW

2.1. Ovine Pasteurellosis

Pasteurellosis is a devastating respiratory disease affecting sheep of all ages (Taye *et al*., 2019) and occurs worldwide but with a widespread distribution, occurring in temperate, subtropical and tropical climates where stress is an important trigger mechanism of the disease (Kabeta *et al*., 2015). There are two clinical forms of pasteurellosis: pneumonic and systemic. The pneumonic form is mainly caused by *M. haemolytica* formerly designated as *P. haemolytica*. In sheep in temperate climates, *P. multocida also* rarely causes pneumonic form of infection. *Mannheimia haemolytica* (biotype A) strains are responsible for pneumonic pasteurellosis in sheep of all ages while *P. trehalosi* strain (formerly biotype T) causes a systemic disease (Enzootic septicemia) in 6–10 months old lambs (Aitken, 2007). Although some species cause primary disease, many of the infections are secondary to other infections or result from various environmental and management stress (Tadesse *et al*., 2017).

The causative organisms are commensals of the tonsils and nasopharynx of clinically healthy animals (Jilo *et al*., 2020); whereas stress factors such as adverse environmental or climatic conditions (extremely hot or cold weather with high levels of humidity), dipping, shearing, and poor management practices (overcrowding in a limited space, inadequately ventilation barn, deprivation of feed and water and distant transport or shipping), previous or co-infection can trigger the bacteria to move quickly invade the lungs and cause pasteurellosis in susceptible hosts (Tadesse *et al*., 2017; Taye *et al*., 2019).

In Ethiopia, Pasteurellosis is a common respiratory infection and considerable economic significant causing outbreaks of acute pneumonia in sheep/goats of all ages (Hailu *et al*., 2017). The acute febrile course of pneumonic pasteurellosis is characterized by death of infected animals within few days after the onset of clinical signs if they are not properly diagnosed and treated, while animals that withstand the acute attack can get chronically infected (Bahr *et al*., 2021).

2.2. Taxonomy and Classification

The Mannheimia and Pasteurella are grouped taxonomically in super kingdom (B*acteria*), phylum (*Proteobacteria*), class (*Gammaproteobacteria*), order (*Pasteurellales*), family (*Pasteurellaceae*), and belonging in the genera *Mannheimia* and *Pasteurella* (Christensen *et al*., 2014). The taxon name *M. haemolytica* came into being after a number of extensive reclassification and naming, first as *Bacterium bipolare multocidum* designated by Theodore kitt in 1885 and later in 1932 as *P. haemolytica*. *Pasteurella haemolytica* was then categorized into two biotypes viz. A and T based on its ability to ferment the sugars arabinose and trehalose, respectively (Legesse *et al*., 2018). In this respect, *P. haemolytica* biotype A was allocated to a new genus and renamed as 'Mannheimia' (the name given by the German scientist Walter Mannheim) for his significant contributions in the recent taxonomy of the family Pasteurellaceae (Mohamed and Abdelsalam, 2008); A new genus, Mannheimia, houses the A biovar organisms from the old *'P. haemolytica'* complex with five species: *M. haemolytica, M. glucosida, M. granulomatis, M. ruminalis* and *M. varigena*. On the other hand, *Pasteurella haemolytica* biotype T was first reclassified as *P. trehalosi* (Blackall *et al*., 2007).

The classification of biotypes A and T of *M. haemolytica* is depending on arabinose and trehalose fermentation respectively (Jilo *et al*., 2020). Based on the capsular antigen biotype A is further subdivided into 12 serotypes; A1, A2, A5-A9, A12- A14, A16, and A17 (Sahay *et al*., 2020) and *M. glucosida* with one serotype, A11 (Berhe *et al*., 2017). Whereas, Biotype T (*B. trehalosi)* contains serotypes of (T3, T4, T10 and T15) which causes systematic pasteurellosis in lambs (Fthenakis, 2007; Ferede *et al*., 2013). *P. multocida* has five serogroups and are designated as (A, B, D, E, and F) using capsular antigens as tested by a passive hemagglutination test and based on somatic (LPS) antigens, 16 serotypes have been identified as tested by a gel diffusion precipitation test (Berhe *et al*., 2017). Currently three subspecies of *P. multocida* are recognized; *Pasteurella multocida* subspecies *multocida*, *Pasteurella multocida* subspecies *septica* and *Pasteurella multocida* subspecies *gallicida* (Jilo *et al*., 2020).

2.3. Etiologies and their Characteristics

Pasteurella multocida, Mannheimia haemolytica and *Bibersteinia trehalosi* belonging to the family Pasteurellaceae of gamma Proteobacteria (Sahay *et al*., 2020). These bacterial species are non-motile, non-sporing, aerobic, fermentative, gram negative rod and cocco-bacilli usually being pleomorphic which measures 0.2µm -2.0µm in length, and grow well at 37°C on 5% sheep blood. They are occasionally characterized by bipolarity that is the staining of only the tips of cells is demonstrable with polychrome stain such as Giemsa stain (Kumar *et al*., 2015; Tadesse *et al*., 2017; Legesse *et al*., 2018). *Pasteurella* and *Mannheimia* species are oxidase-positive, fermentative (Jesse *et al*., 2020), and they are similar with each other but unlike *P. multocida*, *M. haemolytica* does not ferment mannose (Kabeta *et al*., 2015). Most of them are commensals on the upper respiratory mucous membrane and the digestive tract of both domestic and wild animals (Jesse *et al*., 2020). *M. haemolytica, B. trehalosi* and *P. multocida* are etiological agents of pasteurellosis in animals including poultry (Jilo *et al*., 2020). Although pneumonia is multifactorial, it is mainly caused by bacteria such as *M. haemolytica* and *P. multocida*, the main causes of pneumonic pasteurellosis in sheep (Alarawi and Saeed, 2021).

All serotypes of *M. haemolytica* can be involved in pneumonic pasteurellosis in all ages of sheep (Babu *et al*., 2019), but serotype A2 is the most commonly isolated serotype from cases of ovine pneumonic pasteurellosis (Mengstie, 2015). Serotype A2 is most frequently found in both pneumonic sheep and goats (Berhe *et al*., 2017; Babu *et al*., 2019) and A1, A6, A7 (Berhe *et al*., 2017; Taye *et al*., 2019) and A9 being restricted to sheep (Babu *et al*., 2019) and biotype T of (*B. trehalosi*) serotypes cause systemic pasteurellosis in sheep and goats especially in lambs and kids. Serogroups A and D of *P. multocida* have been recognized as incriminated agents of pneumonic pasteurellosis in sheep and goats (Khalili *et al*., 2016). In addition, serogroups A and D causes enzootic pneumonia and shipping fever of cattle and pigs (Berhe *et al*., 2017). *P. multocida* B2 or E2 cause haemorrhagic septicaemia in cattle and buffalo respectively (Jilo *et al*., 2020). Serogroup D causes atrophic rhinitis of pigs and rabbits; and serogroups A and F cause avian cholera of all bird species (Berhe *et al*., 2017).

2.4. Transmission

Pasteurellosis probably acquired through the inhalation of infected nasal secretions, droplets coughed up or exhaled by the infected animal, which may be clinical case or recovered carriers in which the infection persists in the upper respiratory tract (Kabeta *et al*., 2015; Jilo *et al*., 2020). *M. haemolytica* and *P. multocida* that mediate contagion is an important factor in the spread of the disease (Tadesse *et al*., 2017). In addition, *Pasteurella* species are highly susceptible to environmental influences and hence, a close contact is an important factor in the transmission of the disease (Wilkie *et al*., 2012). Particularly, when animals 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 period (Jilo *et al*., 2020).

Interspecies transmission of serotypes among domestic ruminants, as well as between domestic and wild ruminants, has been reported, although some reports suggest that this is a rare epidemiological event (Berhe *et al*., 2017). *P. multocida* infection in humans is commonly causes cellulitis and localized superficial skin abscesses following dog or cat bite, although lick and scratches from these animals have also been described (Chen *et al*., 2002; Maleb *et al*., 2018).

2.5. Clinical Signs

Pneumonic pasteurellosis or shipping fever is the disease that develops within 10-14 days after exposure of stress factors (Jilo *et al*., 2020). An outbreak of per acute pneumonic pasteurellosis usually commences with sudden death in one or two animals, while the next day's occurs the acute form of the disease (Giadinis and Petridou, 2008) in older sheep rather than in lambs with dullness, anorexia, lethargy, fever, dyspnea or hyperpnoea, bilateral serous to mucopurulent nasal and ocular discharge. On auscultation, respiratory sounds are loud and prolonged. In acute cases, death occurs within 2-4 days in untreated animals (Abbott, 2018). A frothy fluid is usually found in the mouth or nostrils in the terminal stages (Aitken, 2007). As an outbreak proceeds and animals that are not sufficiently treated can present the chronic form of the disease that is manifested with weight loss in adults or growth retardation in lambs and kids (Giadinis and Petridou, 2008). In subacute or chronic cases, the clinical signs may be transient and less obvious than in the acute disease (Aitken, 2007). Necropsy findings

is manifested by red and grey consolidation in lungs, catarrhal bronchitis, fibrinous pleurisy, coagulation necrosis of lungs and accumulation of large amounts of effusions and residual lesions of bronchopneumonia in chronic case (Jilo *et al*., 2020).

The systemic form is distinct from the pneumonic pasteurellosis both epidemiologically and pathogenically. The main features of systemic pasteurellosis are sudden death, while recumbence, extremely depressed, dyspnea and frothing at the mouth (Aitken, 2007;Giadinis and Petridou, 2008). So that affected sheep are seldom seen alive. This clinical description is consistent with that of endotoxin shock (Aitken, 2007). Haemorrhagic septicaemia (HS) is an acute, highly fatal septicaemia with high morbidity and mortality, causing major economic losses especially in Asian and African countries. Besides domestic and wild ruminants, domestic pigs and wild boar might also be affected. The disease is an OIE listed systemic pasteurellosis of cattle and buffaloes caused by *P. multocida* (Kutzer *et al*., 2021)and characterized by a sudden onset of fever (41°C-42°C), profuse salivation, sub mucosal petechiation, severe depression, ataxia, localization may occur in subcutaneous tissue, resulting in inflammatory swellings in the submandibular region (throat, dewlap, brisket or perineum), severe dyspnea occurs if respiration is obstructed in ruminants and death in about 24 hours (Jilo *et al*., 2020; Kutzer *et al*., 2021). At necropsy generalized petechial hemorrhages particularly under the serosae, edema of lungs and lymph nodes and subcutaneous infiltration of gelatinous fluid (Jilo *et al*., 2020).

2.6. Epidemiology

Pasteurellosis occurs worldwide (Kabeta *et al*., 2015). However, the microorganism is reported most frequently in Asian and African countries where sheep or goat breeding is widespread. In addition, in USA and Canada cattle breeding are common and pasteurellosis is usual and European countries such as the Netherlands, Germany, Italy and France, where sheep and cattle are present, and the disease has also a major impact (Jilo *et al*., 2020). Out breaks are generally noted at the beginning of the rainy season due to the multiple stressors present during this time and the moist conditions, which prolong the survival time of the organism in the environment (Kabeta *et al*., 2015; Jilo *et al*., 2020). However, the disease can occur throughout the year in the endemic areas (Kabeta *et al*., 2015) and induced by Physical or physiological stressors, including severely bad weather, improper management, co-

mingling, overcrowding, shipping or prior infection with respiratory viruses or other pathogens, may therefore contribute to pneumonic pasteurellosis (Bahr *et al*., 2021).

Almost all species of animals including poultry are susceptible to *Pasteurella* infection. Host range susceptibility order includes buffaloes, cattle, pigs, sheep, goat, birds, rabbits, dogs, cats and humans (Jilo *et al*., 2020). The animal breed, sex, and age, can be effective in the occurrence of pasteurellosis (Karimkhani *et al*., 2011), but the most susceptible age group is 6 months up to 2 years of age (Jilo *et al*., 2020).

2.7. Predisposing Factors

2.7.1. Environmental and management factors

Mannheimia haemolytica and *Pasteurella multocida* 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 and management stressors (Taye *et al*., 2019). The most commonly environmental stressors associated with pasteurellosis in livestock include high temperature and humidity, wind, chill, overcrowding, mixing with new animals, poor ventilation barn, deprivation from feed and water, inadequate colostrum consumption and distant transport may compromise immunity of animals (Miller *et al*., 2008; Taye *et al*., 2019). Weaning is also one of the most stressful procedures in lambs, it is considered as a multifactorial stressor, as it involves nutritional, social, physical and psychological stress (Navarro *et al*., 2019). 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 (Taye *et al*., 2019).

2.7.2. 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 (Taye *et al*., 2019). The main virulence factors of *M.*

haemolytica and *P. multocida* are capsule that inhibits complement mediated destruction of the organisms in serum, fimbriae, that may enhance colonization of upper respiratory tract (URT), outer membrane protein (OMP), adhesins, extracellular enzymes and other factors are still to be investigated. Furthermore, the bacteria possess Lipopolysaccharides (LPS) which is toxic to endothelium and alters leukocyte function and a leukotoxin which is a pore forming cytolysin that affects leukocyte and platelets function. (Tadesse *et al*., 2017; Taye *et al*., 2019).

Pneumonic pasteurellosis has large part of the 12A serotypes of *M. haemolytica* described; A1 and A2 are established worldwide. 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 (Taye *et al*., 2019).

2.7.3. Host factors

Disease progression is thought to depend on a complex interaction of host factors including species, age group, breed variation, sex differences and immune status. Male sheep seem to have higher risk of mortality associated with respiratory disease than females (Navarro *et al*., 2019). Moreover, strain-dependent virulence factors of the agent such as production of toxins, adhesins and mechanisms for acquiring nutrients from the host. Bronchopneumonia caused by *P. multocida* or *M. haemolytica* has a cranioventral lung distribution and affects all ages of animals worldwide. 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 (Taye *et al*., 2019). The disease occurs most commonly in young growing animals from six months to two years of age but all age groups are susceptible. Calves, lambs and kids those are non-immune to *M. haemolytica* are considered to be more susceptible to the disease than calves, lamps and kids that have serum neutralizing antibodies to the organism and its cytotoxin. Animals that are recovered from the experimental disease are resistant to naturally occurring disease (Tadesse *et al*., 2017).

2.7.4. Concurrent infections

Pneumonic pasteurellosis can occur either alone or in conjunction with other organisms (Taye *et al*., 2019). It is generally accepted that every disease has an impact on the immune system; however, there are some processes which specifically cause immunosuppression as part of their pathogenic mechanisms. These diseases may play a special role as predisposing factors for other diseases (Navarro *et al*., 2019). Primary infections with respiratory pathogens such as parainfluenza type 3, adenovirus, respiratory syncytial virus, or in particular *Mycoplasma ovipneumoniae* (Taye *et al*., 2019), Orf, coccidiosis, lungworms and diptera as *Oestrus ovis* (Navarro *et al*., 2019) appear to predispose to secondary infection with *Pasteurella* and *Mannheimia.* The combined infection with certain respiratory viruses is commonly found to increase the susceptibility of sheep to secondary bacterial pneumonia's (Taye *et al*., 2019).

Respiratory viral infections affect mucociliary clearance mechanisms in lungs for removing the pathogens that reach the lower respiratory tract and thus increase the susceptibility of sheep to secondary bacterial infections. The respiratory viral agents create a favorable environment in the lungs supporting the bacterial growth by interfering with the mucociliary clearance mechanism of the respiratory tract and by down regulating the phagocytosis by the pulmonary macrophages (Rawat *et al*., 2019).

2.8. Economic Significance

Pneumonic pasteurellosis is an acute infectious disease that causes widespread financial losses because of death, reduced live weight, delayed marketing, treatment costs and unthriftiness among survivors (Taye *et al*., 2019). In Ethiopia it has been a topic of frustration to veterinary practitioners and a topic of liability to ruminant producers and outbreaks of the disease are often associated with wet humid weather during the rainy season and other stress factors (Jilo *et al*., 2020). It is well established that pneumonic pasteurellosis is responsible for the largest cause of mortality in feedlot animals in which the disease accounts for appropriately 30% of the total cattle death worldwide (Kabeta *et al*., 2015). 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). Morbidity depends on the

immune status of the herd, either acquired naturally or induced by vaccination. (Jilo *et al*., 2020).

Pasteurella multocida is an important zoonotic agent (Register and Brockmeier, 2019); Humans acquire the infection primarily through contact with animals, most usually through animal bites, scratches, licks on skin abrasions, or contact with mucous secretions derived from pets (Wilson and Ho, 2013). Skin and soft tissues are the most common sites for Pasteurella infections and, less commonly, the respiratory tract (Maleb *et al*., 2018). *P. multocida* is not a usual constituent of the human upper respiratory tract, but strains genetically identical to those found in swine are frequently isolated from pig farmers and from inhabitants of regions with intensive pig breeding. Water from scalding tanks is also a potential source of exposure for abattoir workers and it has been proposed that the infection be considered an occupational disease. Most human carriers remain healthy, but *P. multocida* may also be associated with acute or chronic respiratory disease. Human pasteurellosis most often presents as skin or soft tissue infection, typically with rapid onset, characterized by inflammation, swelling, and purulent exudate. More serious manifestations such as septicemia, osteomyelitis, endocarditis, pneumonia, meningitis, and peritonitis are limited to immunocompromised patients (Register and Brockmeier, 2019).

2.9. Diagnosis

The diagnosis of pasteurellosis can be performed with wide variety of clinical signs, and necropsy findings with the history of stress factors (Jilo *et al*., 2020). The isolation of bacteria from heavily contaminated specimens of blood, swabs and tissues of infected animals is the gold standard technique for diagnosis of pasteurellosis (Sebbar *et al*., 2018; Alarawi and Saeed, 2021). The bacteriological differential diagnosis is based on Gram-staining, growth on blood agar and growth characteristics of pink to red colonies on MacConkey agar (Sebbar *et al*., 2018), positive oxidase test, may reveal bipolar-staining organisms on Giemsa or Leishman methods,; and the identification criteria is based on biochemical profile of *P. multocida* and *M. haemolytica* isolates (Quinn *et al*., 2002a). Moreover, Indirect Hemagglutination test (IHA), Rapid slide/plate agglutination test, agar gel immunodiffusion test and counter immune-electrophoresis are common serological tests applied in diagnosis (Sebbar *et al*., 2018; Jilo *et al*., 2020).

More recently, molecular techniques, including pulsed field gel electrophoresis, southern blots and polymerase chain reaction (PCR)-based assays are used (Sebbar *et al*., 2018). 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 (Singh *et al*., 2018; Taye *et al*., 2019). This method is further advance accuracy of characterization in pure and/or mixed cultures, speed of detection, determination of taxonomic position and indulgent of intraspecies genetic relationships. PCR is also used to detect or sense a sequence of DNA unique to *Pasteurella* and *Mannheimia* species (Taye *et al*., 2019).

2.10. Antimicrobial Susceptibility Profiles of *M. haemolytica* and *P. multocida*

Bacteria as a group or species are not necessarily uniformly susceptible or resistant to any particular antimicrobial agent. Levels of resistance may vary greatly within related bacterial groups (Martinez, 2014). *In vitro* antimicrobial susceptibility testing (AST) is performed to predict how a bacterium may respond to an antimicrobial agent *in vivo* (clinical response) or to monitor changes in susceptibility in relation to time and geographic location. In both instances, results may be reported qualitatively, as susceptible, intermediate, or resistant (S-I-R), or quantitatively, as the minimal inhibitory concentration (MIC) (Michael *et al*., 2018).

Mannheimia haemolytica showed 100% resistance to gentamycin and vancomycin while they were 100% sensitive to chloramphenicol followed by 89.3% and 83.9% to sulfamethoxazole and tetracycline consequently. Similarly *P. multocida* showed 100% sensitivity to chloramphenicol followed by sulfamethoxazole and tetracycline. However, *P. multocida* is more susceptible to sulfametrioxazole and tetracycline (Marru *et al*., 2013). Chloramphenicol (89.4%) and tetracycline (80.9%) were the most effective drugs; whereas ampicillin (53.2%) was the intermediate drug while penicillin-G (10.6%) and streptomycin (14.9%) were inefficient drugs and vancomycin was totally inactive against both isolates (Musteria *et al*., 2017). According to Bahr *et al*. (2021), 100% of *M. haemolytica* isolates were resistant to ampicillin, amoxicillin, and penicillin-G. Resistance to tetracycline (83.3%) and streptomycin (75%), followed by cefotaxime and chloramphenicol (58.3%) while they were 100% sensitive to gentamycin followed by 83.3%, trimethoprim-sulfamethoxazole. Similarly, *P. multocida* isolates showed 100% resistance to ampicillin, amoxicillin, penicillin-G, and tetracycline, followed by streptomycin (91.6%), cefotaxime (87.5%).

2.11. Prevention and Control

Since pneumonic pasteurellosis is one of the serious problems of small ruminants, effective control and prevention of the disease is mandatory (Legesse *et al*., 2018). The most effective preventive method is skillful management practices, hence avoidance of environmental and management stressors and the use of vaccines is of primary importance (Taye *et al*., 2019; Jilo *et al*., 2020). Vaccination is the best alternative practical control strategy to reduce the incidence and burden of the disease and to minimize antimicrobial use (Berhe *et al*., 2017). Recent studies indicated that most cases of ruminant pasteurellosis are caused by *M. haemolytica* and vaccine produced by NVI, Ethiopia against the disease is from *P. multocida serotype A* and *B* which does not correspond to the real causative agent (Tadesse *et al*., 2017). Problems with vaccination arises where there is multiple serotypes of *M. haemolytica* as well as *B. trehalosi* without cross protection becomes a challenge for the development of vaccine that is effective worldwide (Berhe *et al*., 2017; Tadesse *et al*., 2017).

The use of antibiotics for the treatment of the disease could be effective (Scott, 2011). Before starting a treatment course, it is necessary to select the most effective antibacterial drugs (Laishevtsev, 2020). Oxytetracycline is the antibiotic of choice for pasteurellosis, as there are few antibiotic resistant strains in sheep, unlike in cattle (Scott, 2011). One treatment with long-acting oxytetracycline intramuscularly at 20 mg/kg is effective in controlling the development of pneumonic pasteurellosis for four days in animals with early clinical signs or in animals which are post-exposure but preclinical. Retreatment after three to four days is advisable because relapses occur. Feeding of broad-spectrum antibiotics, especially tetracycline, to feedlot lambs is often done for recently weaned lambs, in an effort to reduce the incidence of acute pasteurellosis (Abbott, 2018). Also other antibiotics as penicillin-streptomycin, tylmaicosin and florfenicol can be used (Giadinis and Petridou, 2008).

Chapter 3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was conducted between January 2021 to April 2022 in four selected sheep rearing South Wollo Zone districts namely Dessie Zuria, Kuta Ber, Haik and Kombolcha (Fig.3.1). South Wollo is located in the south east corridor of the Amhara region in northern Ethiopia and it lies within 10°12′N, and 11°40′N latitude and 38°30′E and 40°05′E longitude (Cafer and Rikoon, 2017; Mohammed *et al*., 2018). Its zonal capital, Dessie, is found 401 km Northeast of Addis Ababa (Yimer and Asseged, 2007). South Wollo zone experiences three distinct seasons, namely winter, summer, and spring. Winter is a dry season from October to January. Spring is the short rainy season that occurs between mid-February and mid-May locally called 'belg', while summer is the main rainy season that extends from mid-June to mid-September and locally called 'kiremt'(Assefa *et al*., 2008; Agidew and Singh, 2018). The altitude of the area ranges from 1500 to 3500 meters above sea level (Rosell and Holmer, 2007; Rosell and Holmer, 2015). The mean annual temperature and mean annual rainfall of the area ranges from 14 to 20 °C and from 680 to 1200 mm, respectively. The main farming system is mixed farming and sheep are the predominant animal species kept in the area (Agidew and Singh, 2018).

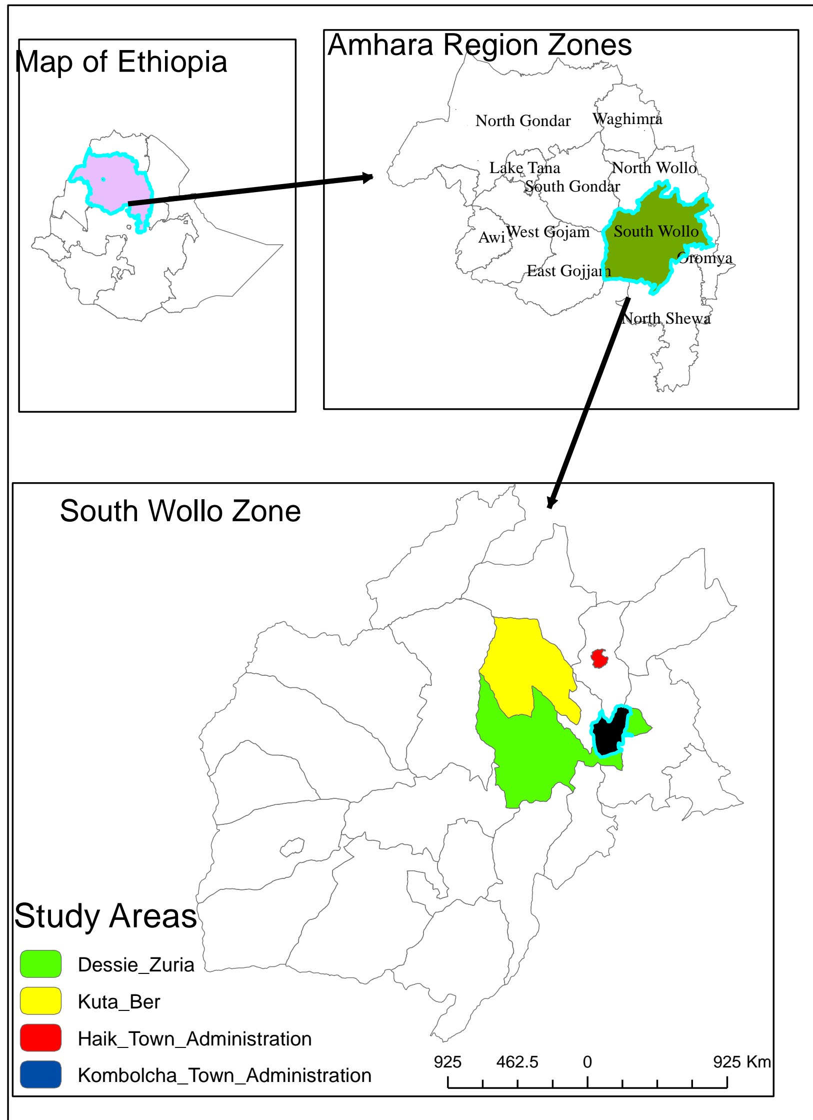


Figure 3. 1. Map depicting the different study districts of South Wollo (EthioGIS, 2007)

3.2. Study Animals

The study animals were sheep suspected from pneumonic pasteurellosis and presented to the selected districts of veterinary health posts/veterinary clinics during the study period. All sheep were examined at each veterinary clinic for the evidence of pneumonic pasteurellosis. Up on clinical examination, all sheep manifesting; anorexia, coughing, dyspnea, lethargy, serous to muco-purulent ocular and nasal discharge, and fever were considered. Accordingly, pneumonic individual cases presented to veterinary clinics of the study sites /districts were considered for sampling.

3.3. Study Design and Sampling Technique

A cross-sectional study was conducted on ovine pneumonic pasteurellosis from January 2021 to April 2022 in four selected districts of South Wollo Zone. Specimens from suspected pneumonic cases were subjected to bacteriological and molecular analysis to identify *Pasteurella* and *Mannheimia* species. In the current study, a total of 154 samples were collected for bacteriological analysis based on the principle of purposive/judgmental sampling technique. At each stage of sampling, access of suspected cases of sheep with pneumonic pasteurellosis, transport access to study sites and resource considerations in respective to study sites were considered. Since all cases were sampled from different farmers, it is assumed that each case originated from a different flock or herds.

3.4. Sampling Procedures

Each study animal was individually identified and restrained by an assistant and kept fixed before the sampling procedures. After were well disinfecting the nostrils of animals with cotton soaked in 70 % ethyl alcohol, sterile cotton-tipped, 20-25cm long applicator sticks moistened with tryptose soya broth (Himedia, India) were directed via the nostrils of each pneumonic pasteurellosis suspected sheep. That is, the swabs were carefully inserted into nostril and the mucosa surface; rolled gently to take specimens as described by Carter (1990). The swabs were then placed back into the test tube containing 3 ml of sterile tryptose soya broth (Himedia, India) and clearly labeled, and then the tubes were capped. Finally, all collected specimens were packed in ice box and transported to Kombolcha Veterinary Laboratory for bacteriological analysis.

3.5. Bacterial Isolation and Identification

Pre-enriched in tryptose Soya broth (Himedia, India) nasal swab specimens were incubated for 24 hrs at 37°C. Then, the isolation and identification of *P. multocida* and *M. haemolytica* were performed using techniques recommended by Hardy Diagnostics, Santa Maria, CA, USA (Alemneh and Tewodros, 2016). After overnight incubation, the cultured samples were agitated wisely to aid mixing. Then, for isolation a loopful of the broth culture was taken and streaked over an identified petri-plate containing blood agar base (Techno Pharmchem, India) supplemented with 6% defibrinated sterile sheep blood and immediately incubated aerobically at 37°C for 24 h.

In culture-positive plates, typical suspected colonies were subjected to Gram's staining to study staining reactions and cellular morphology under light microscope at 100x magnification. Mixed and Gram-negative, coccobacilli bacteria were again subcultured with due care, on both blood and MacConkey agar plates to get pure cultures for further analysis (Quinn *et al*., 2002b; Quinn *et al*., 2011). The growths of typical colonies on blood agar were depicted based on the general appearance of colonies (morphology, color, shape, size and consistency), the presence or absence of haemolysis, and the type of haemolysis. On MacConkey agar, the colonies were defined for the presence or absence of growth, general appearance and ability to ferment lactose (Marru *et al*., 2013; Jilo *et al*., 2020).

Pure cultures of single colony type from both blood and MacConkey agars were transferred onto nutrient agar for a series of primary and secondary biochemical tests (Yegoraw *et al*., 2017). Primary tests such as catalase, and oxidase, tests were conducted. In addition, secondary biochemical tests including indole, citrate utilization test, urease test, H_2S production test by Triple Sugar Iron Agar, fermentation of sugars such as (glucose, sucrose, lactose, mannitol, arabinose and trehalose) were performed for final identification of bacteria to species level (Quinn *et al*., 2002b; Quinn *et al*., 2011). The assay for biochemical properties of the bacterial isolates were conducted according to MacFaddin's method (Marru *et al*., 2013), and the final isolates of bacteria were stored at -20 °C in 20% glycerol in TSB for further molecular test.

Table 3.1. Biochemical characteristics of *M. haemolytica* and *P. multocida* isolates

Biochemical tests	*M. haemolytica*	*P. multocida*
Haemolysis	+	–
Growth on MacConkey agar	+	–
Catalase	+	+
Oxidase	+	+
Indole production	–	+
H_2S production	–	–
Urease	–	–
Glucose	+	+
Sucrose	+	+
Lactose	+	–
Mannitol	+	+
Arabinose	+	–
Trehalose	–	–

Note: "+" = Positive reactions; "–" = Negative reactions for the respective tests

3.6. Molecular Identification

3.6.1. DNA extraction

Presumptively identified 24-48 h growth pure cultures of *M. haemolytica* and *P. multocida* were transferred into 1.5-ml Eppendorf tubes (Legesse *et al*., 2018). Then, the genomic DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) as per the manufacturer's instructions. At the same time, the genomic DNA was also isolated from *M. haemolytica* and *P. multocida* strains maintained at NVI collection as positive controls. In addition, a negative control consisting of all components of reaction mixture except the DNA template was included for PCR reactions. The extracted DNA concentration was measured using NanoDrop UV spectrophotometer and finally, stored at -20°C until use (Singh *et al*., 2018; Tabatabaei and Abdollahi, 2018).

3.6.2. PCR amplification

Primers targeting virulence associated genes of *M. haemolytica* viz. *Rpt2*and *PHSSA* genes coding for methyltransferase and serotype specific antigen respectively were used in mPCR assay (Table 3.2) as described by (Kumar *et al*. (2015); Bahr *et al*., 2021). In detail, a final volume of 25 µL PCR amplification mixture containing 3 µL RNase free water, 2 µL (5pm/µL) of each primer pairs, 10 µL of IQ Super mix containing (DNA polymerase, dNTPs and buffer) and 4 µL DNA template. One reaction tube without the DNA template and the other with DNA template from reference *M. haemolytica* isolate from the collection (MH-NVI) were included as negative and positive controls, respectively. Then, amplified under the following conditions: a cycle of an initial denaturation at 95 °C for 3 min. followed by 35 cycles consisted of denaturation at 95 °C for 1 min, annealing at 48 °C for 1 min and elongation at 72 °C for 30s and a cycle of final elongation at 72 °C for 5 min and hold at 4°C. The PCR conditions were applied according to Deressa *et al*. (2010) for the detection of *M. haemolytica*.

Conventional polymerase chain reaction (PCR) was used for the detection of *P. multocida*. Presumptively identified as *P. multocida* and *M. haemolytica* isolates were subjected to PCR assay using specific primer pairs targeting the capsular biosynthesis gene (*capA*) (Table 3.2) of *P. multocida* as described by Legesse *et al*. (2018). PCR amplification for *P. multocida* was performed to have a final volume of 20 µL PCR reaction mixture containing 3 µL RNase free water, 2 µL (5pm/µL) of each primer (forward and reverse), 10 µL of IQ Super mix and 3 µL template DNA. Negative and positive controls were included one without DNA template and the other reaction tube with the DNA of *P. multocida* strain from NVI collection respectively. Amplification protocol for *capA* gene comprised: an initial denaturation at 95°C for 5 min, followed by 35 cycles of each denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 90 s and a cycle of final extension at 72 °C for 7 min.

Table 3.2. Primer pairs of virulence associated genes of *M. haemolytica* and capsular genes of *P. multocida* used in the current study

Target Gene	Primer sequence (5′- 3′)	Size (bp)	References
PHSSA	(F)5′ -TTC ACA TCT TCA TCC TC-3’		(Kumar *et al*., 2015)
	(R)5′ TTT TCA TCC TCT TCG TC-3	325	
Rpt2	(F)5′ - GTT TGT AAG ATA TCC CAT TT- 3’		(Bahr *et al*., 2021)
	(R)5′- CGT TTT CCA CTT GCG TGA- 3	1022	
CapA	(F)5’-TGCCAAAATCGCAGTCAG- 3’		(Legesse *et al*., 2018)
	(R)5′ -TTGCCATCATTGTCAGTG- 3’	1044	

Note: ‘F’ = Forward and ‘R’ = Reverse

3.6.3. Agarose gel electrophoresis and gel documentation

The amplified PCR products were separated in agarose gel 2% w/v and 1.5% w/v prepared from 1×TAE as running buffer (Merck KGaA, Germany) stained with GelRed for *M. haemolytica* and *P. multocida* respectively. PCR product (10µl) was mixed with loading buffer (4 µL) and load into pre-prepared gel wells. DNA ladder for *M. haemolytica* (100 bp) while *P. multocida* (1 kb plus) were also loaded into the first and last lane. Then, run at 120 volt for an hour in horizontal submarine electrophoresis apparatus (EC 2060, USA).In addition, positive control and negative controls were run and separated in parallel with the samples. Then, the PCR products were visualized under UV transilluminator and photographed in gel documentation system (UVitec, Cambridge, UK). Finally, positive samples were determined and recorded from each well based on the observed band sizes of around 1022 bp and 325 bp for *Rpt2* and *PHSSA* genes of *M. haemolytica* and 1044 bp for *capA* gene of *P. multocida* respectively.

3.7. Antimicrobial Susceptibility Test

The antimicrobial susceptibility test was conducted for *M. haemolytica* isolates using Kirby–Bauer disk diffusion method (Hudzicki, 2009) on Muller-Hinton Agar (MHA) (Himedia, India) at NVI, Debre-Zeit, Ethiopia as per Clinical and Laboratory Standards Institute (CLSI) with *Escherichia coli* ATCC 25922 as a quality control organism. The antibiotics commonly used in the treatment of the respiratory ailments were chosen, namely; Oxytetracycline (OT80

µg), Tetracycline (TE 30 µg), Amoxicillin/clavulanic acid (AMX 30µg), Chloramphenicol (C 30 µg), Ampicillin (AMP 10 µg), Erythromycin (E15 µg), Streptomycin (S 10 µg) and Gentamycin (CN 10 µg). The samples were inoculated on MHA. Disks containing the antimicrobial agents were applied within 15 minutes of inoculating the plate and the plates were incubated at 37°C for 18 to 24 hours in an incubator based on the procedure recommended by Carter (1990) and Quinn *et al*. (1999). The diameters of clear zones produced by antimicrobial inhibition of bacterial growth were measured in mm with transparent metric ruler and interpreted as susceptible, intermediate and resistant according to CLSI (2015) and CLSI (2020) as described in (Table 3.3).

Table 3.3. Inhibition zone interpretive charts for antimicrobials

Antimicrobial Agent	Disc Potency (µg)	Inhibition zone (mm)		
		Susceptible (≥)	Intermediate	Resistance (≤)
Ampicillin	10 µg	22	19-21	18
Amoxicillin-clavulanate	20 µg	20	_	19
Chloramphenicol	30 µg	29	26-28	25
Erythromycin	15 µg	27	25-26	24
Gentamycin	10 µg	15	13-14	12
Oxytetracycline	80µg	23	20-22	19
Streptomycin	10 µg	15	12-14	11
Tetracycline	30 µg	29	26-28	25

3.8. Data Management and Analysis

Raw data collected were recorded in the format developed for this purpose and later on entered into a Microsoft Excel spreadsheet (Microsoft Excel 2010, Microsoft Corporate, USA) coded, and then imported into STATA V. 14 software (StataCorp, Texas, USA) for statistical analysis. Descriptive statistics involving frequency and percentage were computed to illustrate the rate of isolation and identification, molecular detection and sensitivity pattern differences.

Chapter 4. RESULTS

4.1. Isolation and Identification of *P. multocida* and *M. haemolytica*

Identification of the bacterial species was made by observation of their colonial morphology, Gram staining reaction, and biochemical characteristics. In the present study, the frequency of *M. haemolytica* and *P. multocida* isolation and identification by bacteriological tests were 41/154 (26.62 %) and 6/154 (3.90 %) respectively and overall species 47 (30.52%) (Fig. 4.1).

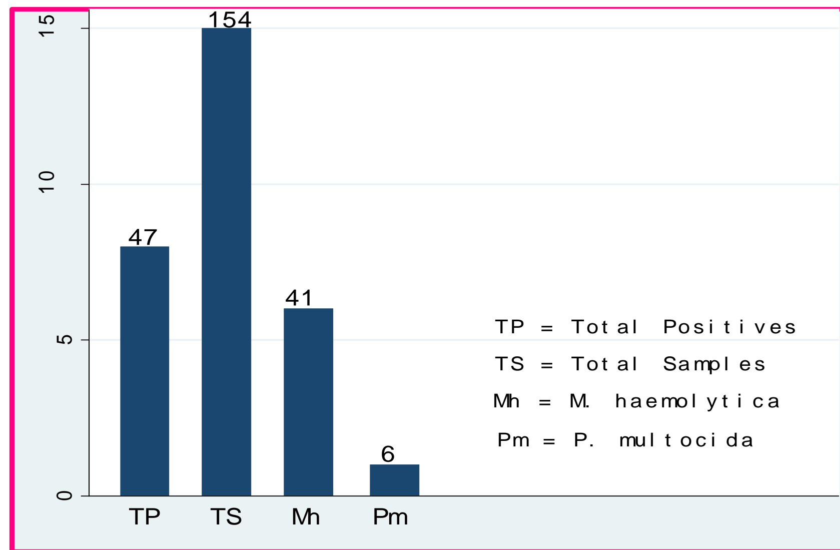


Figure 4. 1. A bar graph showing frequency and recovery rates of isolates with respect to each species

Among 41 isolates of *M. haemolytica*, 15 (36.59%), 12 (29.27%), 8 (19.51%) and 6 (14.63%) isolates were from Kuta Ber, Kombolcha, Dessie Zuria and Haik respectively. Out of 6 isolates of *P. multocida*, 3 (50%), 2 (33.33%) and 1 (16.67%) isolates were identified from Kuta Ber, Dessie Zuria and Kombolcha districts (Table 4.1). On the basis of these results, *M. haemolytica* was mainly circulated in the study districts and it's found to be the most common cause of ovine pneumonic pasteurellosis.

Table 4.1. Frequency and recovery rates of isolates with respect to study districts

Study Sites	Frequency	Species of bacterial isolates		Total (%)
		M. haemolytica (%)	*P. multocida* (%)	
Dessie Zuria	38	8 (21.05%)	2 (5.26%)	10 (26.32%)
Kuta Ber	53	15 (28.30%)	3 (5.66%)	18 (33.96%)
Haik	33	6 (18.18%)	Nil (0%)	6 (18.18%)
Kombolcha	30	12 (40%)	1 (3.33%)	13 (43.33%)
Total	154	41 (26.62%)	6 (3.90%)	47 (30.52%)

4.2. Multiplex PCR Assay for Detection of *M. haemolytica*

Molecular analyses of 41 culture positive nasal swab isolates using primers targeting *PHSSA* (325 bp) and *Rpt2* (~1022 bp) genes of *M. haemolytica* in a mPCR assay revealed that seven isolates (17.07%) and nil (0%) were detected respectively for each gene (Fig.4.2). Rather than the expected value of *Rpt2* (~1022 bp) gene, there were non-specific band around 700 bp. In addition, there were no co-detection of both *PHSSA* and *Rpt2* genes for *M. haemolytica*.

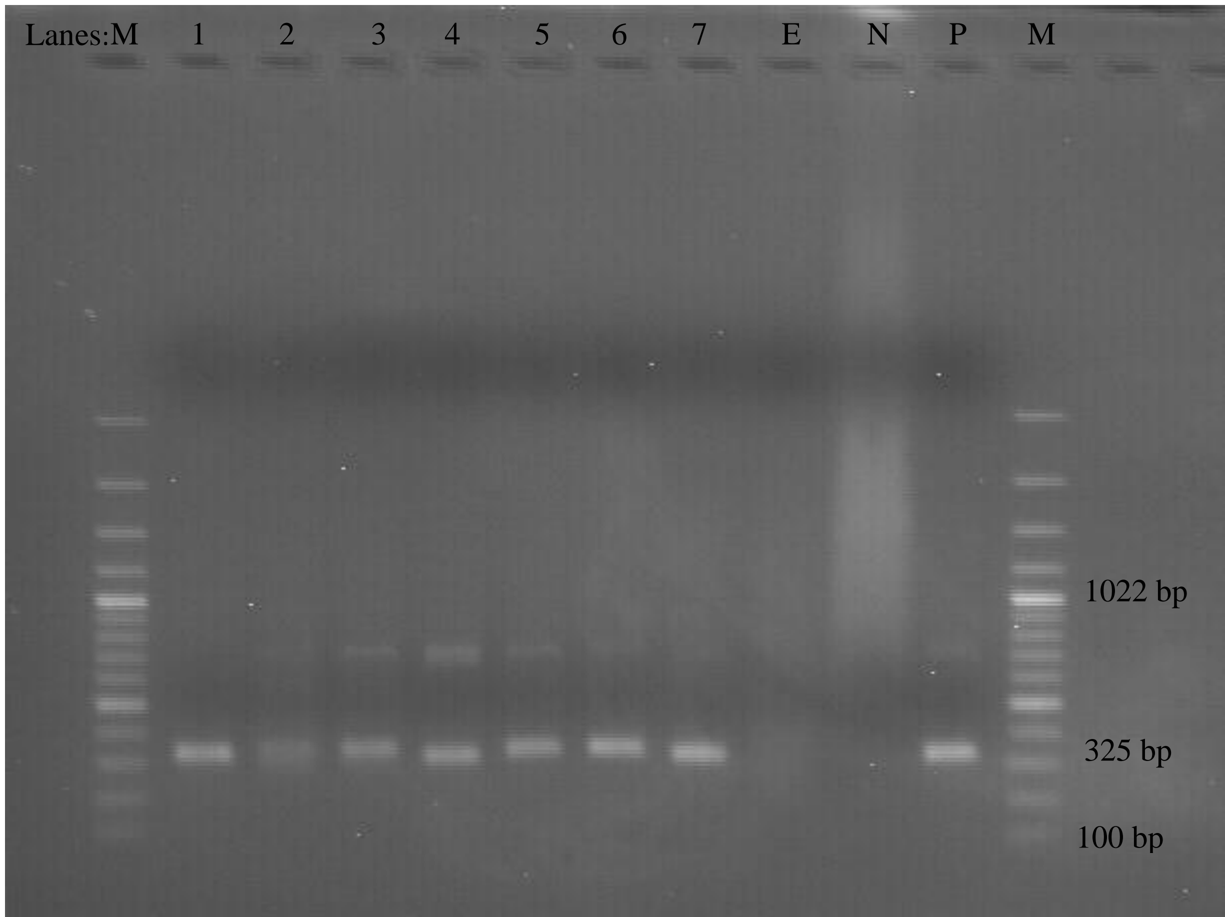


Figure 4. 2. mPCR amplification of *PHSSA* (325 bp) and *Rpt2* (~ 1022 bp) genes of *M. haemolytica*

Lane M: 100 bp DNA Ladder; Lanes 1-7: Test positive; Lane E: Extraction control; Lane N: Negative control and Lane P: Positive control

4.3. PCR for Detection of *P. multocida*

Isolates presumptively identified as *P. multocida* and *M. haemolytica* isolates were analyzed using primers specific for capsular biosynthesis (*CapA*) gene, all *P. multocida* isolates showed negative and *M. haemolytica* isolates included were also negative rather than 4/7 (57.14%) *PHSSA* gene positive isolates resulting non-specific band size of about 650 bp different from the expected 1044 bp (Fig. 4.3).

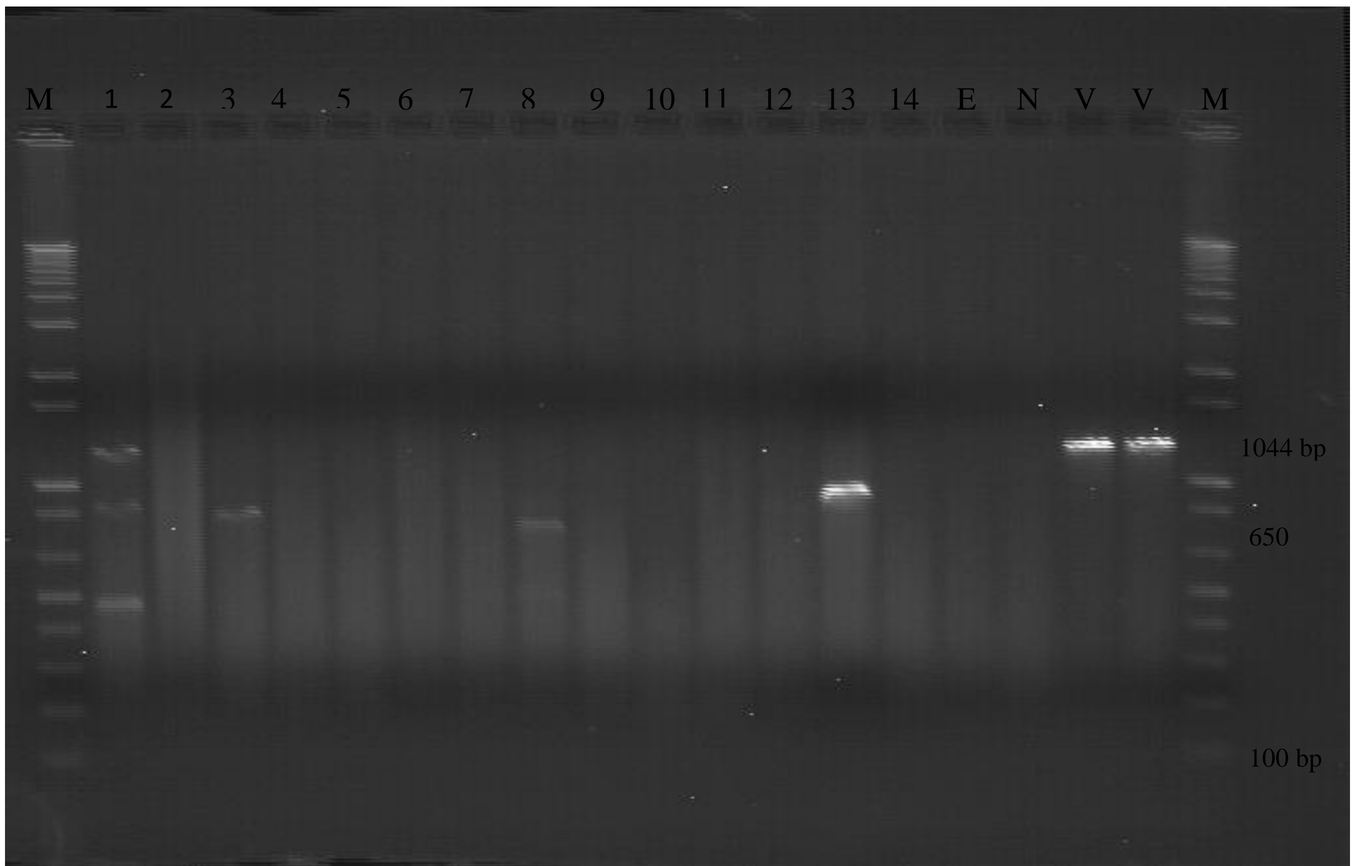


Figure 4. 3. Agarose gel electrophoresis showing PCR products (~1044 bp) using *cap-A* gene of *P. multocida*

Lane: M = 1 kb plus DNA molecular marker, E: Extraction control, Lanes 1, 3, 8 and 13: *M. haemolytica* positive isolates (~650), N: Negative control, V: NVI vaccine strain (*P. multocida* type A) as positive control around 1044 bp.

4.4. *In vitro* Antimicrobial Susceptibility Testing

Seven isolates of PCR positive *M. haemolytica* isolated from nasal swabs of clinical cases during the study period were subjected to a panel of eight antimicrobials by disc diffusion technique. Among eight different antibiotics tested, the isolates were susceptible for ampicillin and gentamycin (100%), oxytetracycline and tetracycline (85.71%) and these drugs were found most effective drugs against *M. haemolytica* isolates and followed by chloramphenicol (71.43%). On the other hand, high resistance was recorded against amoxicillin/clavulanic acid and erythromycin (100%), and followed by streptomycin (71.43%) as depicted in (Table 4.2).

Table 4.2. Percentage of antimicrobial susceptibility test result of PCR positive *M. haemolytica* isolates

Antimicrobials tested	Disc Potency (μg)	*M. haemolytica*		
		Susceptible	Intermediate	Resistant
Amoxicillin	20	_	_	7 (100%)
Ampicillin	10	7 (100%)	_	_
Chloramphenicol	30	5 (71.43%)	2 (28.57%)	_
Erythromycin	15	_	_	7 (100%)
Gentamycin	10	7 (100%)	_	_
Oxytetracycline	80	6 (85.71%)	_	1 (14.29%)
Streptomycin	10	2 (28.57%)	_	5 (71.43%)
Tetracycline	30	6 (85.71%)	1 (14.29%)	_

Chapter 5. DISCUSSION

Bacteriological and biochemical tests were conducted and the result revealed that *M. haemolytica* was associated with one fourth of suspected pneumonic cases of sheep in South Wollo. The finding was nearly agrees with Legesse *et al*. (2018), who reported *M. haemolytica* was associated with one third of pneumonic cases of sheep in central Ethiopia.

The isolation of overall *Pasteurella* species recovered from deep nasal swab samples were 47 (30.52%), of which, 26.62% and 3.90% were tentatively identified as *M. haemolytica* and *P. multocida* respectively (Table 4.1). The clinical findings of the present study were almost comparable with the previous findings of Marru *et al*. (2013) in Haramaya District, Eastern Hararghe, Ethiopia (31.33%), of which 21.69% *M. haemolytica* and 9.64% of *P. multocida*, Akane *et al*. (2022) in Northeast Amhara (32.62%), Tewodros and Annania (2016) in Fogera woreda, Ethiopia, who reported as 25.91% and 6.68% were *M. haemolytica* and *P. multocida* respectively and an overall *Pasteurella* species (32.6%). The result indicates that *M. haemolytica*, the most frequently isolated bacterial pathogen from the nostril, is considered as the main causative agent involved in ovine pneumonic pasteurellosis. While, the clinical finding of *P. multocida* in the current study was lower than the findings of Marru *et al*. (2013), and Tewodros and Annania (2016). This implies that the possible role of *P. multocida* in the etiology and pathogenesis of ovine pneumonia should not be under estimated, but the load of the bacteria might be lessened by the annual vaccination scheme of the country. However, due to poor management practices and the monovalent vaccine produced by NVI, Ethiopia which does not cross-protect against different serotypes of *M. haemolytica* makes it major causative bacteria for pneumonic pasteurellosis.

Although in the present study *B. trehalosi* was not recovered, the findings indicates lower isolation rate than the work done by Legesse *et al*. (2018) in central Ethiopia, who described (36.84%), of which *M. haemolytica* (34.21%) and *B. trehalosi* (2.63%) and Yegoraw *et al*. (2017) reported in the same area, central Ethiopia as *M. haemolytica* (29.71%), *B. trehalosi* (6.69%) and overall species (36.6%). The difference might be arising due to season of sample collection, type of sample taken, different sample size, geographical variation and sensitivity of tests used. In addition, the current finding was much lower than Sisay and Zerihun (2003) reports which was conducted in the same area, Wollo (39.04%), but higher than *M.*

haemolytica recovery rate (20.5%) and *P. multocida* (0.98%). Sisay and Zerihun (2003) also found *B. trehalosi* (16.1%) and *M. glucosida* (1.46%) which were inconsistent with the present study. This indicates that management practices were somewhat improved than the previous and vaccination against *P. multocida* unlike *M. haemolytica* and *B. trehalosi* infections.

In contrast to the current result, the findings of Hawari *et al*. (2008), Khalili *et al*. (2016) and Bahr *et al*. (2021) revealed much higher *P. multocida* isolation; 25.51%, 1.56% and 15.48%, and lower *M. haemolytica* isolation; 2.55%, 0% and 7.74%; with overall species of (28.06%), (1.56%) and (23.22%) respectively. According to those scholars' finding, *P. multocida* was the main causative agent involved in ovine pneumonic pasteurellosis and *M. haemolytica* has limited role for the disease in Jordan (Middle and Northern), Iran (East Azerbaijan province) and Egypt; and the reverse is true in Ethiopia.

Further molecular detection was performed through mPCR assay by concurrently amplifying virulence associated genes of *M. haemolytica* specific to *Pasteurella haemolytica* serotype specific antigen (*PHSSA*) and *Rpt2* gene loci coding for methyltransferase (Table 3.2). Out of 41 isolates of previously identified as *M. haemolytica* by standard microbiological techniques (Table 4.1), 7/41 (17.07%) isolates were confirmed to harbor *PHSSA* (325 bp) gene, thus belonging to serotype A1 and all culture positive isolates of *M. haemolytica* were remain negative for *Rpt2* (1022 bp) gene (Fig. 4.2). The PCR detection of the present study was higher than the previous findings of Akane *et al*. (2022) who reported *PHSSA* (10.87%) and *Rpt2* (10.87%) genes. This observation and finding was contrary to the findings of Legesse *et al*. (2018) who detected *PHSSA* (80.77%) and *Rpt2* (19.23%) in central Ethiopia, Kumar *et al*. (2015) in India and Hawari *et al*. (2008) in Jordan; in which those scholars' reported that all isolates identified to be *M. haemolytica* through microbiological and biochemical tests were all found to be PCR positive.

Thus, in earlier studies of Legesse *et al*. (2018) and Akane *et al*. (2022), *PHSSA* and *Rpt2* genes in *M. haemolytica* make them a suitable molecular diagnostic target in contrast to the present study in which only *PHSSA* gene found to be diagnostic importance unlike species-specific *Rpt2* locus, which modulates type III restriction-modification system. The serotype specific antigen, *PHSSA*, is reported to play a role in conversion of commensal microbes into

pathogenic in stressful situations indicating its involvement in pathogenesis of pneumonic pasteurellosis due to *M. haemolytica*.

On the other hand, when analyzed *M. haemolytica* isolates in a conventional PCR assay using primers specific for capsular biosynthesis (*cap-A*) gene, resulting in 8.51% non-specific PCR product size of about 650 bp different from the expected 1044 bp (Fig. 4.3). This result agreed with the reports of Legesse *et al*. (2018) in which the non-specific PCR band obtained in PCR assay targeting capsular biosynthesis gene of *P. multocida* when applied to *M. haemolytica* isolates may show that *P. multocida* and *M. haemolytica* share common sequences in their capsular gene(s) at the primer binding site but at different positions.

Changes in susceptibility to antimicrobial agents for both *P. multocida* and *M. haemolytica* isolates have been reported (Alarawi and Saeed, 2021). Therefore, the use of research outcomes conducted on antibiotics susceptibility tests remain necessary. According to antibiotics susceptibility test results, *M. haemolytica* isolates were susceptible to ampicillin and gentamycin (100%), oxytetracycline and tetracycline (85.71%) were found to be the most effective antibiotics on *M. haemolytica* isolates, followed by chloramphenicol (71.43%). In contrary, streptomycin (28.57%) was inefficient drug against the isolates (Table 4.2).

The present finding in lines with Seker *et al*. (2009), Marru *et al*. (2013), Musteria *et al*. (2017) and Sahay *et al*. (2020) who were reported chloramphenicol and tetracycline were effective drugs. Ampicillin and gentamycin were the drugs of choice for the treatment of ovine pneumonic pasteurellosis as the findings of Sahay *et al*. (2020) and agreed with the present result. Very close efficacy of ampicillin (80%) with the present study was also reported previously by Önat *et al*. (2010). Moreover, the present finding was consistent with Seker *et al*. (2009) who reported that gentamycin, chloramphenicol and tetracycline were effective drugs against *M. haemolytica* isolates. However, the result contradicts the findings of Alarawi and Saeed (2021) who reported as ampicillin and gentamycin were intermediately effective; Marru *et al*. (2013) and Musteria *et al*. (2017) also stated as only ampicillin was intermediate drug, but in the current finding, chloramphenicol and tetracycline were 28.57% and 14.29% intermediately sensitive respectively.

On the other hand, the interesting findings of the present study demonstrates that the highest resistance of *M. haemolytica* isolates against amoxicillin and erythromycin (100%) which were totally inactive, followed by streptomycin (71.43%) as shown in (Table 4.2). The findings were consistent with the previous outcomes of Alarawi and Saeed (2021) in the case of streptomycin and amoxicillin/clavulanic acid, Seker *et al*. (2009) reported that streptomycin (75%) and erythromycin (82.5%) were resistant. The present result also coincides with Musteria *et al*. (2017) who found to be streptomycin (72.3%) resistant. However, this finding was disagreed with the work done by Abera *et al*. (2014) and Alarawi and Saeed (2021) who were reported as *M. haemolytica* was intermediately sensitive against erythromycin. The variation might be due to difference in the strain of the isolate that may cause pasteurellosis in different species of animals or due to the existence of host factors that may affect the action of drug in sheep or resistance might be occurred due to chromosomal mutation and accusation of transferable genetic material.

Chapter 6. CONCLUSION AND RECOMMENDATIONS

Sheep are the most dominant animal species kept extensively in the study districts. These small ruminants play an important role as a principal source of domestic consumption for farmers. However, the benefits remain minimal due to prevailing of diseases such as pneumonic pasteurellosis. The current findings of both biochemical and molecular detection confirmed that *M. haemolytica* was considered as a principal causative agent for ovine pneumonic pasteurellosis whereas *P. multocida* had limited role for the cause of ovine pasteurellosis in study districts of South Wollo. Moreover, further serotyping and molecular techniques are needed to identify the isolate to the strain level. According to antimicrobial susceptibility test results, isolates of *M. haemolytica* were completely susceptible to ampicillin and gentamycin, hence these drugs were most effective however, and the isolates were totally resistant to amoxicillin and erythromycin.

On the basis of the current findings, the following points are recommended**:**

- The development of new polyvalent vaccine including *M. haemolytica* or its antigenic determinants has paramount importance
- Direct molecular detection was found efficient; therefore, we recommend using it for a routine examination
- Continuous monitoring of antimicrobial resistance should be conducted before treating except for critical ones in the appropriate selection of antimicrobial agents

Chapter 7. REFERENCES

ABBOTT, K. 2018. *The Practice of Sheep Veterinary Medicine,* South Australia, University of Adelaide Press.

Abera, D., Sisay, T. & Birhanu, T. 2014. Isolation and identification of Mannhemia and Pasturella species from pneumonic and apparently healthy cattle and their antibiogram susceptibility pattern in Bedelle District, Western Ethiopia. *Afr. J. Bacteriol. Res.,* **6,** 32-41.

Agidew, A.-M. A. & Singh, K. 2018. Determinants of food insecurity in the rural farm households in South Wollo Zone of Ethiopia: the case of the Teleyayen sub-watershed. *Agric. food Econ.,* **6,** 1-23.

Aitken, I. D. 2007. Diseases of Sheep. 4th ed. Edinburgh: Blackwell Publishing.

Akane, A. E., Alemu, G., Tesfaye, K., Ali, D. A., Abayneh, T., Kenubih, A., Ejo, M., Abat, A. S., Admassu, B. & Ibrahim, S. M. 2022. Isolation and Molecular Detection of Pasteurellosis from Pneumonic Sheep in Selected Areas of Amhara Region, Ethiopia: An Implication for Designing Effective Ovine Pasteurellosis Vaccine. *Vet. Med. Res. Rep.,* **13,** 75.

Alarawi, F. A. & Saeed, E. M. 2021. Isolation, Antibiogram and Molecular Detection of Mannheimia and Pasteurella Associated with Pneumonia in Sheep in Al-Madinah Region, Saudi Arabia. *Int. J. Vet. Sci.,* **10,** 135-140.

Alemneh, T. & Tewodros, A. 2016. Sheep and goats pasteurellosis: Isolation, identification, biochemical characterization and prevalence determination in Fogera Woreda, Ethiopia. *J. Cell Anim. Biol.,* **10,** 22-29.

Assefa, Y., Van Den Berg, J. & Conlong, D. E. 2008. Farmers' perceptions of sugarcane stem borers and farm management practices in the Amhara region of Ethiopia. *Int. J. Pest Manag.,* **54,** 219-226.

Ayelet, G., Yigezu, L., Gelagay, E., Tariku, S., And& Asmare, K. 2004. Epidemiologic and serologic investigation of multifactorial respiratory disease of sheep in the Central Highland of Ethiopia. *Int. J. Appl. Res. Vet. Med.,* **2,** 274-278.

Babu, R., Balasubramaniam, G., Murthy, T. G. K., Sivaseelan, S. & Udhayavel, S. 2019. Isolation and identification of bacterial species from small ruminants affected with Pneumonia and their antibiogram pattern.

Bahr, A., Salib, F., Soliman, Y. & Amin, M. 2021. Multi-drug resistant Pasteurella multocida and Mannheimia haemolytica strains isolated from different hosts affected by pneumonic pasteurellosis in Egypt. *Adv. Anim. Vet. Sci.,* **9,** 356-364.

Berhe, K., Weldeselassie, G., Bettridge, J., Christley, R. M. A. & Abdi, R. D. 2017. Small ruminant pasteurellosis in Tigray region, Ethiopia: marked serotype diversity may affect vaccine efficacy. *Epidemiol.& Infect.,* **145,** 1326-1338.

Blackall, P., Bojesen, A. M., Christensen, H. & Bisgaard, M. 2007. Reclassification of [Pasteurella] trehalosi as Bibersteinia trehalosi gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.,* **57,** 666-674.

Bote, Y., Legesse, K., Tassew, A. & Zeit, E. 2017. Isolation, identification and antimicrobial susceptibility of Pasteurella multocida from cattle with hemorrhagic septicemia in Assosa and Bambasi districts, Benishangul Gumuz Regional state, Ethiopia. *Int. J. Anim. Res.,* **1,** 67-75.

Cafer, A. & Rikoon, S. 2017. Coerced agricultural modernization: a political ecology perspective of agricultural input packages in South Wollo, Ethiopia. *J. Rural Soc. Sci.,* **32,** 6.

Carter, G. 1990. Isolation and identification of bacteria from clinical specimens. *Diagnostic proced.Vet.Bacteriol. Myco.* Elsevier.

Chen, H. I., Hulten, K. & Clarridge III, J. E. 2002. Taxonomic subgroups of Pasteurella multocida correlate with clinical presentation. *J. Clin. Microbiol.,* **40,** 3438-3441.

Christensen, H., Kuhnert, P., Nørskov-Lauritsen, N., Planet, P. J. & Bisgaard, M. 2014. The Family Pasteurellaceae. 535-564.

CLSI 2015. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. 3rd ed. CLSI guideline M45. *Wayne, PA: CLSI.,* **35,** 50-51.

CLSI 2020. Performance standards for antimicrobial susceptibility testing. 30th ed. CLSI supplement M100. *Wayne, PA: CLSI.,* **40,** 74-77.

Deressa, A., Asfaw, Y., Lubke, B., Kyule, M., Tefera, G. & Zessin, K. 2010. Molecular detection of Pasteurella multocida and Mannheimia haemolytica in sheep respiratory infections in Ethiopia. *J. Appl. Res. Vet. Med. ,***8,** 101.

EthioGIS 2007. Political map of Ethiopia showing Administrative Regions and zones.

Ferede, Y., Mekuriaw, S., Mazengia, H. & Amane, A. 2013. Sero-Typing and Evaluation of The Level of Protective Antibody Titer in Northwest Ethiopian Sheep Before And After Ovine Pasteurollosis Vaccination. *Int. J. Pharm. Med. Bio. Sc.,* **2**.

Fthenakis, G. C. 2007. Diseases of Sheep, ID Aitken (Ed.), Blackwell Publishing, Oxford, UK (2007), p. 610, ISBN-13: 978-14051-3414-9. Elsevier.

Giadinis, N. D. & Petridou, E. J. 2008. Pasteurellosis of Small Ruminant in Greece. *Vet. J. Repub. Srpska,* **8,** 99-101.

Haile, A., Getachew, T., Mirkena, T., Duguma, G., Gizaw, S., Wurzinger, M., Soelkner, J., Mwai, O., Dessie, T. & Abebe, A. 2020. Community-based sheep breeding programs generated substantial genetic gains and socioeconomic benefits. *Anim.,* **14,** 1362-1370.

Hailu, S. M., Kitila, D. B., Gemeda, A. E. & Tarekegn, M. 2017. Pasteurella organism: Its isolation and identification from pneumonic lungs of goats in Ethiopia. *J. Adv. Vet. Anim. Res.,* **4,** 147-154.

Hawari, A., Hassawi, D. & Sweiss, M. 2008. Isolation and identification of Mannheimia haemolytica and Pasteurella multocida in sheep and goats using biochemical tests and random amplified polymorphic DNA (RAPD) analysis. *J. Biol. Sci.,* **8,** 1251-1254.

Hudzicki, J. 2009. Kirby-Bauer disk diffusion susceptibility test protocol. *Am. Soc.Microbiol.,* **15,** 55-63.

Ibrahim, S. M., Getnet, K., Abera, B., Getie, H., Molla, W., Alemu, S., Admassu, B., Abat, A. S., Dejene, H. & Birhan, M. 2022. Serotyping and Seroprevalence of Mannheimia haemolytica, Pasteurella multocida, and Bibersteinia trehalose and Assessment of Determinants of Ovine Pasteurellosis in West Amhara sub-region, Ethiopia. *Front. Vet. Sci.***,** 484.

Jesse, F., Boorei, M., Chung, E., Wan-Nor, F., Lila, M. M., Norsidin, M., Isa, K., Amira, N., Maqbool, A. & Odhah, M. 2020. A review on the potential effects of Mannheimia haemolytica and its immunogens on the female reproductive physiology and performance of small ruminants. *J. Anim. Health Prod.*

Jilo, K., Belachew, T., Birhanu, W., Habte, D., Yadete, W. & Giro, A. 2020. Pasteurellosis status in Ethiopia: A comprehensive review. *J. Trop. Dis.,* **8,** 351.

Kabeta, T., Fikadu, T., Zenebe, T. & Kebede, G. 2015. Review on the pneumonic pasteurellosis of cattle. *Acad. J. Anim. Dis.,* **4,** 177-184.

Karimkhani, H., Zahraie, S. T., Sadeghi, Z. M., Karimkhani, M. & Lameyi, R. 2011. Isolation of pasteurella multocida from cows and buffaloes in Urmia's slaughter house.

Kehrenberg, C., Schulze-Tanzil, G., Martel, J.-L., Chaslus-Dancla, E. & Schwarz, S. 2001. Antimicrobial resistance in Pasteurella and Mannheimia: epidemiology and genetic basis. *Vet. Res.,* **32,** 323-339.

Khalili, I., Ghadimipour, R., Ghaderi, R., Shokri, G., Jabbari, A., Razmaraii, N. & Ebrahimi, M. 2016. Isolation, identification, and monitoring of antibiotic resistance in Pasteurella multocida and Mannheimia haemolytica isolated from sheep in East Azerbaijan province, Iran. *Arch. Razi Inst.,* **71,** 153-160.

Kumar, J., Dixit, S. K. & Kumar, R. 2015. Rapid detection of Mannheimia haemolytica in lung tissues of sheep and from bacterial culture. *Vet. World,* **8,** 1073.

Kutzer, P., Szentiks, C. A., Bock, S., Fritsch, G., Magyar, T., Schulze, C., Semmler, T. & Ewers, C. 2021. Re-Emergence and Spread of Haemorrhagic Septicaemia in Germany: The Wolf as a Vector? *Microorg.,* **9,** 1999.

Laishevtsev, A. Mannheimiosis of cattle, sheep and goats. IOP Conference Series: Earth and Environmental Science, 2020 Moscow, Russia. IOP Publishing, 072038.

Legesse, A., Abayneh, T., Mamo, G., Gelaye, E., Tesfaw, L., Yami, M. & Belay, A. 2018. Molecular characterization of Mannheimia haemolytica isolates associated with pneumonic cases of sheep in selected areas of Central Ethiopia. *BMC Microbiol.,* **18,** 1-10.

Maleb, A., Elmalki, J., Bouayadi, O., Lahlou, Y. B., Frikh, M., Abdeljaouad, N., Lemnouer, A., Yacoubi, H. & Elouennass, M. 2018. Serious phlegmonous lesion of the hand following an injury by vegetal thorn: Never forget Pasteurella multocida! *Trauma Case Rep.,* **13,** 18-21.

Marru, H. D., Anijajo, T. T. & Hassen, A. A. 2013. A study on Ovine pneumonic pasteurellosis: Isolation and Identification of Pasteurellae and their antibiogram

susceptibility pattern in Haramaya District, Eastern Hararghe, Ethiopia. *BMC Vet. Res.,* **9,** 1-8.

Martinez, J. L. 2014. General principles of antibiotic resistance in bacteria. *Drug Disc. Today: Techno.,* **11,** 33-39.

Mcrae, K. M., Baird, H. J., Dodds, K. G., Bixley, M. J. & Clarke, S. M. 2016. Incidence and heritability of ovine pneumonia, and the relationship with production traits in New Zealand sheep. *Small Rumin. Res.,* **145,** 136-141.

Mengstie, F. 2015. Serotyping and identification of predisposing factors for the occurrence and distribution of ovine pasteurellosis in Bonga sheep breed. *Asian J. Med. Biol. Res.,* **1,** 175-181.

Michael, G. B., Bosse, J. T. & Schwarz, S. 2018. Antimicrobial resistance in Pasteurellaceae of veterinary origin. *Microbiol. Spectrum,* **6,** 6.3. 02.

Miller, M. W., Knowles, D. P., Bulgin, M. S., Clay, B. R., Cook, W. E., Srikumaran, S., Scarfe, A. D. & Layfield, D. 2008. Pasteurellosis transmission risks between domestic and wild sheep.

Mohamed, R. & Abdelsalam, E. 2008. A review on pneumonic pasteurellosis (respiratory mannheimiosis) with emphasis on pathogenesis, virulence mechanisms and predisposing factors. *Bulg. J. Vet. Med.,* 11**,** 139-160.

Mohammed, Y., Yimer, F., Tadesse, M. & Tesfaye, K. 2018. Variability and trends of rainfall extreme events in north east highlands of Ethiopia. *Int. J. Hydro.,* **2,** 594-605.

Mukh, S., Devita, T., Darlina, D. & Siti, N. 2011. The feasibility of gamma irradiation for developing malaria vaccine. *At. Indones.,* **37,** 91-101.

Musteria, M., Getnet, F., Anteneh, W. & Yehualashet, B. 2017. Isolation of Pasteurella multocida and Mannheimia hemolytica from Pneumonic Calves and Their Antibiotic Susceptibility in Harar and Haramaya, Eastern Ethiopia. *Glob. Vet.,* **19,** 627-632.

Navarro, T., Ramos, J., De Arcaute, M. R. & González, J. 2019. Predisposing factors inducing ovine respiratory complex in intensive-reared lambs. *Small Rumin. Res.,* **180,** 106-111.

Nicholas, R., Ayling, R. & Loria, G. 2008. Ovine mycoplasmal infections. *Small Rumin. Res.,* **76,** 92-98.

Önat, K., Kahya, S. & Çarli, K. T. 2010. Frequency and antibiotic susceptibility of Pasteurella multocida and Mannheimia haemolytica isolates from nasal cavities of cattle. *Turkish J. Vet. Anim. Sci.,* **34,** 91-94.

Quinn, P., Carter, M., Markey, B. & Carter, G. 1999. Clin. Vet. Microbiol. London: Mosby publish ring.

Quinn, P., Markey, B. K., Carter, M., Donnelly, W. & Leonard, F. 2002a. *Vet. Microbiol. Microbial Dis.*, Blackwell sci.

Quinn, P., Morkey, B., Carter, M., Donnelly, W., Lenard, F. & Maguire, D. 2002b. Pasteurella species and Mannheimia haemolytica. *Vet. Microbiol. Microbial Dis., 1st ed. London: Black Well Sci. Ltd***,** 137-42.

Quinn, P. J., Markey, B. K., Leonard, F. C., Hartigan, P., Fanning, S. & Fitzpatrick, E. 2011. *Vet. Microbiol.Microbial Dis.*, John Wiley & Sons.

Rawat, N., Gilhare, V. R., Kushwaha, K. K., Hattimare, D. D., Khan, F. F., Shende, R. K. & Jolhe, D. K. 2019. Isolation and molecular characterization of Mannheimia haemolytica and Pasteurella multocida associated with pneumonia of goats in Chhattisgarh. *Vet. World,* **12,** 331.

Register, K. B. & Brockmeier, S. L. 2019. Pasteurellosis. *Diseases of swine***,** 884-897.

Rosell, S. & Holmer, B. 2007. Rainfall change and its implications for Belg harvest in South Wollo, Ethiopia. *Geografiska Annaler: Series A, Phys. Geog.,* **89,** 287-299.

Rosell, S. & Holmer, B. 2015. Erratic rainfall and its consequences for the cultivation of teff in two adjacent areas in South Wollo, Ethiopia. *Norsk Geografisk Tidsskrift-Norwegian J. Geog.,* **69,** 38-46.

Sahay, S., Natesan, K., Prajapati, A., Kalleshmurthy, T., Shome, B. R., Rahman, H. & Shome, R. 2020. Prevalence and antibiotic susceptibility of Mannheimia haemolytica and Pasteurella multocida isolated from ovine respiratory infection: A study from Karnataka, Southern India. *Vet. World,* **13,** 1947.

Scott, P. R. 2011. Treatment and control of respiratory disease in sheep. *Veterinary Clinics: Food Anim. Pract.,* **27,** 175-186.

Sebbar, G., Zro, K., Kichou, F., Maltouf, A. F. & Belkadi, B. 2018. Isolation and identification of Mannheimia haemolytica and Pasteurella multocida species from ruminants in six different regions in Morocco. *J. Agric. Sci. Technol. A,* **8,** 387-394.

Seker, E., Kuyucuoglu, Y. & Konak, S. 2009. Bacterial examinations in the nasal cavity of apparently healthy and unhealthy Holstein cattle. *J. Anim. Vet. Adv.,* **8,** 2355-2359.

Singh, F., Sonawane, G. G. & Meena, R. K. 2018. Molecular detection of virulent Mannheimia haemolytica and Pasteurella multocida in lung tissues of pneumonic sheep from semiarid tropics, Rajasthan, India. *Turkish J. Vet. Anim. Sci.,* **42,** 556-561.

Sisay, T. & Zerihun, A. 2003. Diversity of Mannheimia haemolytica and Pasteurella trehalosi serotypes from apparently healthy sheep and abattoir specimens in the highlands of Wollo, North East Ethiopia. *Vet. Res. Commun.,* **27,** 3-14.

Tabatabaei, M. & Abdollahi, F. 2018. Isolation and identification of Mannheimia haemolytica by culture and polymerase chain reaction from sheep's pulmonary samples in Shiraz, Iran. *Vet. World,* **11,** 636.

Tadesse, B., Alamirew, K., Ketema, A., Kiflie, W. & Endashaw, M. 2017. Ruminant pneumonic pasteurellosis: Review on epidemiology, pathogenesis and virulence mechanism. *Acad. J. Anim. Dis.,* **6,** 30-39.

Taye, M., Asefa, M., Mohammed, N., And& Desa, G. 2019. Ovine Pneumonic Pasteurellosis: A Review. *American-Eurasian J. Sci. Res.,* **14,** 64-76.

Tewodros, A. & Annania, T. 2016. Sheep and goats pasteurellosis: Isolation, identification, biochemical characterization and prevalence determination in Fogera Woreda, Ethiopia. *J. Cell Animal Biol.,* **10,** 22-29.

Wilkie, I. W., Harper, M., Boyce, J. D. & Adler, B. 2012. Pasteurella multocida: diseases and pathogenesis. *P. multocida***,** 1-22.

Wilson, B. A. & Ho, M. 2013. Pasteurella multocida: from zoonosis to cellular microbiology. *Clin. Microbiol. reviews,* **26,** 631-655.

Yegoraw, A. A., Gebremeskel, A. K., Tesema, T. S. & Birhanu, B. T. 2017. Aerobic and anaerobic bacterial isolates from the respiratory tract of sheep slaughtered at Addis Ababa Abattoirs Enterprises, Central Ethiopia. *J. Vet. Med. Anim. Health,* **9,** 284-289.

Yimer, N. & Asseged, B. 2007. Aerobic bacterial flora of the respiratory tract of healthy sheep slaughtered in Dessie municipal abattoir, northeastern Ethiopia. *Review onVét. Méd.,* **158,** 473.

Zewdie, G., Derese, G., Getachew, B., Belay, H. & Akalu, M. 2021. Review of sheep and goat pox disease: current updates on epidemiology, diagnosis, prevention and control measures in Ethiopia. *Anim. Dis.,* **1,** 1-10.

Chapter 8. APPENDICES

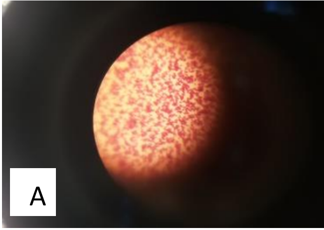
Appendix 1. Media preparation

- Measure the powder media by sensitive balance
- Add into sterile flask
- Measure the distilled water with graduate cylinder, add into the flask, and cover with aluminum foil
- Mix with temperature mixer
- Adjust and clean the number of Petri plates required
- Enter the media into the autoclave at 121 ^{0}c for 15 minutes and petri plates into hot air oven for sterilization
- Put the flask media into the water bath to reduce the temperature to 45-50°c
- Then, dispatch the media into the sterilized Petri plates in biosafety cabinet to avoid contamination
- Incubate overnight to check media contamination
- After checking the contamination, inoculate the sample onto the prepared media or put the media in refrigerator up to two weeks

Appendix 2. Gram stain

- Prepare a smear on a clean slide from colonies of fresh culture and heat gently to fix by passing the slide above the flame of the Bunsen burner
- First, fixed cells are stained with the basic dye crystal violet (primary dye) for 30-60 seconds
- Tilt the slide, and rinse the slide gently with tap water to remove excess stain
- Next, Lugol's iodine (mordant) is added and retain for 1 minute. Gram's Iodine combines with crystal violet to form di-iodine complex
- Again tilt the slide and wash off the iodine with tap water
- Third, cells are decolorized with 95 % ethanol or acetone until colour ceases to run out of the smear and excess ethanol should wash off with tap water
- Fourth, cells are covered by counter stain, Safranin for 30-45 seconds and wash briefly with water then air dry

- Finally, view under the microscope with oil immersion objective lens to observe cell morphology and gram reaction



(A) Gram reaction of *pasteurella* and *Mannheimia* species under the microscope

Appendix 3. Primary biochemical tests

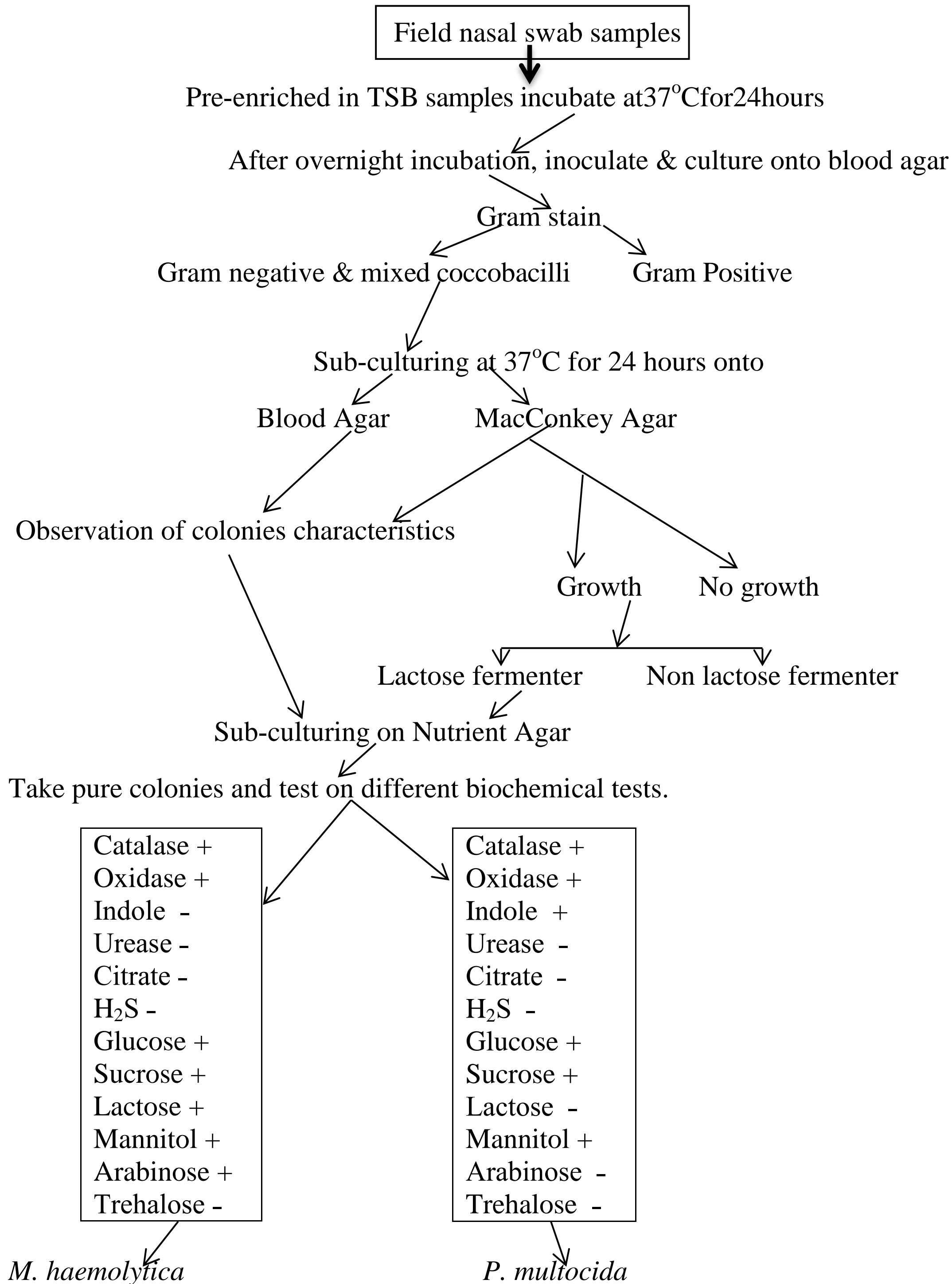
Oxidase Test

- Place a filter paper into a Petri dish
- Add 2-3 drops of Tetramethyl-P-phenylenediamine (TMPD) reagent by micropipette onto the filter paper
- Take a loop of pure bacterial colony, drop into the reagent and make smear
- Appreciate oxidization of the substrate in less than 1 min, dark purple/blue shows positive result

Catalase Test

- In a sterile zone, take a loop of pure bacterial culture, place on the clean and sterile slide
- Add 2-3 drops of hydrogen per oxide (3% H_2O_2) solution by micropipette
- Observe for immediate bubbling

Appendix 4. Flow chart for isolation and identification of *Mannheimia* and *Pasteurella spp*



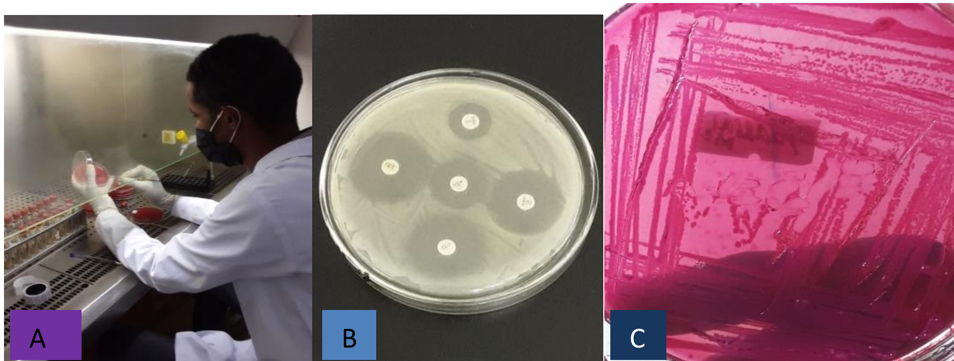
Appendix 5. DNA Extractions by Qiagen DNeasy Blood and Tissue kit

1. Appropriately label a 1.5 ml micro centrifuge tube for each sample
2. Pipette 20 µl of QIAGEN protease (Proteinase K) into the bottom of the tube to digest contaminating proteins present and degrades nucleases that may be present in DNA to protect the nucleic acids from nuclease attack
3. Add 200 µl of samples to each labeled micro centrifuge tube
4. Add 200 µl buffer AL to the sample; mix by pulse-vortexing for 15 seconds to promote lysis of the cell membrane, denaturation of proteins, DNA and other macromolecules
5. Incubate at 56 ^{0}C for 10 minutes, thenbriefly centrifuge to remove drops from the inside of the lid
6. Add 200 µl of ethanol (96-100%) to eliminate the solvents that surround the DNA, thus allowing the DNA to precipitate in pellet form,
7. Vortexing and then briefly centrifuge to remove drops from the inside of the lid
8. Using a micropipette, transfer entire contents (~600 µl) tolabeled QIAamp spin column, and centrifuge at 6000 xg (8000 rpm) for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate
9. Add 500 µl buffer AW1 to denatured proteins, centrifuge at 6000 xg (8000 rpm) for 1 min. Place QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate
10. Add 500 µl buffer AW2 to wash the salts out, centrifuge at full speed (20,000 xg; 14,000 rpm) for 3 min.
11. Place the QIAamp spin column in a clean 1.5 ml microfuge tube (not provided), and discard the collection tube containing the filtrate
12. Add 200 µl of elution buffer AE or distilled water to elute DNA from the membrane and allows stable storage of DNA, Let column stand at room temperature for 5 minutes and then centrifuge at 6000 xg (8000 rpm) for 1 min.
13. Discard the column and storethe DNA appropriately (4° C for short term, -20° C for long term)

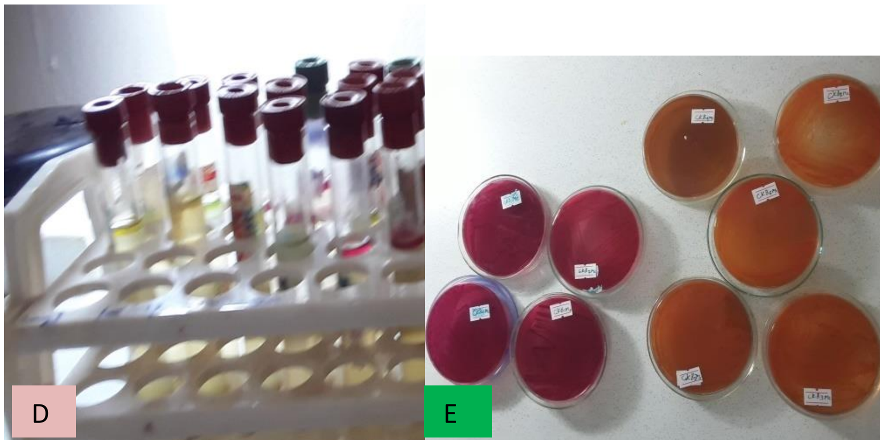
Appendix 6. Antimicrobial susceptibility tests by disc diffusion technique

1. Sterilize the area with disinfectant and open burner before performing the test
2. Prepare the pure culture colony suspension using sterile physiological saline and adjusted to 0.5 McFarland turbidity standard
3. A sterile cotton swab is dipped into the suspension, remove excess medium by pressing the swab firmly against the side of the tube above the level of the liquid
4. Spread the suspension to MHA using sterile cotton swab and allowed to stand for 3 to 5 minutes to observe any excess moisture and make it dry
5. Picking up antimicrobial-impregnated disks using sterile forceps and placed on the plate surface at a distance of 24mm
6. Gently pressed each disc with the point of forceps to ensure complete contact to avoid misplacement
7. Then, left for 30 minutes for diffusion of the antibiotics in the disc
8. The plates are inverted upside down and incubated at 37°C for 18 to 24 hours
9. After overnight incubation, the diameter of inhibition zone should be measured using a metric ruler or measuring caliper and recorded in mm
10. The result is reported as susceptible (S), intermediate (I) and resistant (R) according to CLSI guidelines

Appendix 7. Different photos during laboratory work



(A) Field samples inoculation onto blood agar, (B) Disc diffusion test for *M. haemolytica* isolates (C) *M. haemolytica* on MAC (Pin point red colonies).



(D) Indole Positive (red ring), (E) Lactose fermenter and non-lactose fermenter left to right on MacConkey Agar