ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

MALDI TOF MS AND MOLECULAR DETECTION OF *MANNHEIMIA HAEMOLYTICA* FROM SHEEP AND GOATS IN HOLETA AND SEBETA TOWN, OROMIA SPECIAL ZONE, ETHIOPIA

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MALDI TOF MS AND MOLECULAR DETECTION OF *MANNHEIMIA HEMOLYTICA* FROM SHEEP AND GOATS IN HOLETA AND SEBETA TOWN, OROMIA SPECIAL ZONE, ETHIOPIA

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

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DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
AHI	Animal Health Institute
AST	Antimicrobial sensitivity test
BA	Blood agar
BHI	Brain-Heart Infusion
BRD	Bovine respiratory disease
BTS	Bacterial Test standard
BUG	Biolog Universal Growth
CCPP	Contagious Caprine Pleuro Pneumonia
CSA	Central Statistics Authority
CT	Cycle threshold
CVMA	College of Veterinary Medicine and Agriculture
DNA	Deoxyribo nucleic acid
FAO	Food and Agriculture Organization
FMHACA	Food, Medicine, and Health Care Administration
GDP	Gross domestic product
HCCA	α-cyano-4-hydroxycinnamic acid
IF	Inoculation fluid
IPC	Internal positive control
MALDI TOFMS	Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
MH	Mannheimia hemolytica
NCBI	National Center for Biotechnology Information

OIE	Office International des Epizooties / The world organization for animal health
PA	Peasant Association
PHI	Public Health Investigation
PCR	Polymerase Chain Reaction
PPR	Peste des Petits Ruminants
rRNA	ribosomal Ribonucleic Acid
SPP	Species
URT	Upper Respiratory Tract
USA	United States of America
VP	Voges Proskauer

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ABSTRACT

Mannhemia haemolytica is one of the most important bacteria among causative agent of pneumonic pasteurellosis in small ruminants throughout worldwide. It is one of the most economically devastating pathogen in sheep and goats in Ethiopia. A cross sectional study was carried out from November 2021 to May 2022 with the aim to identify *Mannheimia haemolytica* from sheep and goat in Sebeta and Holeta town, Oromia special zone, Ethiopia. A total of 235 samples (213 nasal swabs and 22 whole blood) were collected from sheep and goats for *Mannheimia haemolytica* identification. Sheep and goat with clinical signs suggestive of pneumonic pasteurollosis were purposively sampling. Bacterial identification was conducted using biochemical, Biolog, Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI TOF MS) and Real time PCR detection. Moreover, antimicrobials susceptibility test was also conducted on the identified bacterial isolates using disc diffusion method. The result showed that from a total of 235 samples, only two nasal swab samples were positive for *M. haemolytica* (0.85%). The two isolates were confirmed by all the tests and similar result was obtained by; biochemical, Biolog, MALDI TOF MS and real time PCR. Up on antimicrobial susceptibility testing, the two isolates were resistant to Streptomycin, Erythromycin and Clindamycin whereas they were susceptible to Tetracycline, Chloramphenicol, Trimethoprim/sulfonamides and Penicillin. Generally, this study revealed that *M. haemolytica* is among the causative agent of pneumonic pasteurellosis in sheep and goat in the study area. Although, the other remaining bacteria responsible for the disease. The research suggests that a combination of diagnostic methods such as MALDI TOF MS, Biolog, and real-time PCR should be used, as well as for a more in-depth investigation to identify the strain or serotype of *M. haemolytica* using advanced molecular sequencing and also analysis of the remaining causal agent from various species and locations in countries that are significant for disease control and prevention and also to address the present vaccination and antibiotic resistance issues.

Keywords: MALDI TOF MS, Real time PCR, *M. haemolytica*, Sebeta, Holeta, Sheep, Goat

1. INTRODUCTION

Ethiopia has the largest small ruminant population in Africa's within 40 million sheep and 51 million goats (CSA, 2021). Small ruminants have a multifaceted role in Ethiopia, as they are in other developing countries. The sector accounted for up to 40% of agricultural GDP and 20% of national foreign exchange(The World Bank, 2017). However, a combination of health problems, poor management, and climate changes make efficient use of this potential resource difficult(CSA, 2021). *Mannheimia haemolytica* is bacteria among causative agent of pneumonic pasteurellosis in sheep and goats. It is one of Sub-Saharan Africa's most serious pathogen cause significant economic loss due to morbidity and mortality(Legesse *et al*., 2018). Pneumonic pasteurellosis is a term used to describe a group of infectious diseases caused mostly by *Mannheimia haemolytica (M.haemolytica)* and *Pasteurella multocida (P.multocida)*(Disassa *et al*., 2013). *Mannheimia haemolytica* is gram-negative rods or coccobacilli that are non-motile, non-spore forming, facultative anaerobic, oxidase and catalase-positive, bipolar that are classified as belonging to the genus *Mannheimia*(Tefera & Smola, 2001). *M. haemolytica* is an opportunistic pathogen that residing in the respiratory tract of healthy and sick small ruminants. This pathogen in sheep and goats identified by an acute infection with high fever, coughing, dyspnea, muco-purulent nasal discharge, anorexia and depression and can cause infection when the body's defenses are impaired by a number of stress(Ahmed *et al.*, 2017). The Presumptive diagnosis of *M.haemolytica* was based on the presence of clinical symptoms, bacterial isolation, and identification using Biolog, MALDI TOF MS and real time PCR. Bacterial culture colonies produced by *M.haemolytica* are odorless, moist, smooth, grayish, form hemolysis on blood agar and ferment mannitol, glucose, maltose(Jesse *et al*., 2020). Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry identification is a reliable alternative method for detecting protein, particularly ribosomal protein, which is abundant in *M.haemolytica*(Driessche *et al*., 2019). *M.haemolytica* identification using cultural, and biochemical characters is a time consuming and labor intensive process. However, molecular technologies, particularly polymerase chain reaction, allow for quick detection and identification of bacteria in mixed or pure cultures(Nefedchenko *et al*., 2016).

PCR approaches have been employed in nucleic acid detection of *M. haemolytica* for a long time to address some of the limitations of biochemical and serological methods while also improving sensitivity and speed. When compared to conventional PCR, real-time PCR is faster and may detect lower DNA quantities (Ghizlane Sebbar *et al*., 2018). In most situations, *M. haemolytica* identified by culture and biochemical test but they cannot be confirmatory by MALDI TOF MS, Biolog and Real Time PCR. In Ethiopia *M. haemolytica* has been identified by(Ali *et al*., 2022) who reported (32.62% of *M.haemolytica*) from ovine pneumonic pasteurellosis case in Selected Areas of Amhara Region. However, there is a limited information that identified *M. haemolytica* in Sebeta and Holeta towns using Biolog, MALDI Biotyper and Real time PCR.

GENERAL OBJECTIVE

- To Identify *Mannheimia haemolytica* as a cause of pneumonic pasteurollosis from Sheep and Goats in Holeta and Sebeta towns, Oromia, Ethiopia.

Specific objective

- To isolate and identify *M. haemolytica* by using Biochemical, BIOLOG, MALDI TOF and Real time PCR detection from sheep and goat
- To determine antimicrobial susceptibility pattern of *M.haemolytica* isolate

2. LITERATURE REVIEW

2.1. Overview of *Mannheimia haemolytica*

Mannheimia haemolytica is an important animal pathogen associated with pneumonic pasteurellosis. *M.haemolytica* are gram-negative rods or coccobacilli, facultative anaerobic bacteria that are non-spore forming, and non-motile. They are oxidase and catalase positive. In Giemsa-stained bipolarity of *M. haemolytica* may be detected(Catry *et al*., 2005). *P.haemolytica* was first identified in 1932, while *P.multocida* was first discovered in 1878 from fowl cholera-infected birds. Louis Pasteur is the man whom *Pasteurella* named in his honor in 1880 (Shayegh *et al*., 2009). *P.haemolytica* biotype A was allocated to a new genus *Mannhaemia* in 1999 (Olsen & Bisgaard, 1999). The name *Mannhaemia* was given in tribute to the German scientist Walter Mannheim for his significant contributions in the recent taxonomy of the family *Pasteurellaceae*. This new genus now contains several species based on phenotypic and genomic characteristics (Angen *et al*., 2002). In recent years, genotypic methods, especially nucleic-acid based assays, allow the detection of microorganisms by dramatically improving the sensitivity and decreasing the time required for bacterial identification. Trehalose-negative *P.haemolytica* classified into five new species M. *haemolytica, M.glucosidal*, *M.ruminalis*, *M.granulomatis* and *M.varigena while* Trehalose positive classified as four serotype of *P.trehalosi*.

Mannhaeimia haemolytica, formerly known as *P. haemolytica,* is divided into two biotypes, A and T, based on the fermentation of arabinose and trehalose. The 17 serotypes of *M. haemolytica* and *P. trehalosi* are identified based on extractable surface antigen. Biotype A, is divided into 13 serotypes (A1, A2, A5, A6, A7, A8, A9, A11, A12, A13, A14, A16, and A17) and remaining four T Serotype 3, 4, 10, and 15 are classified as *P. trehalosi* (Ozbey *et al*., 2004). *P. multocida* based on capsule antigens, are divided into five (A-E) capsular serogroups (A, B, D, E, and F) and sixteen somatic serotypes (1-16) (OIE, 2018). There are two forms of pasteurellosis in small ruminants, namely pneumonic and septicemic. The septicemic form is caused by *B. trehalosi* and the pneumonic form by *M. haemolytica* type A and *P. multocida* type A and D (Berhe *et al*., 2017).

Pneumonic form is the most important respiratory disease affecting small ruminants and develops disease when the immune system of the animal is compromised by stress factors (Makonnen *et al*., 2015).The host and disease causing specificity and susceptibility of each serogroup are indicated in (Table.1).

Table 1: Summary of common diseases caused by *M. haemolytica* and *P. multocida* serotypes in animals

Hosts	**Name of the Disease**	**Serotypes**
Cattle	Hemorrhagic septicemia (HS) Occasionally, HS like septicemia disease Bovine pneumonic pasteurellosis	*P.multocida*serotypesB2andE2 *P.multocida* serotype B3,4 *M. haemolytica* A1; *P multocida* A
Buffalo	Hemorrhagic Septicemia (HS)	*P. multocida* serotypes B2 and E2
Shoats	Pneumonic pasteurellosis Septicemia pasteurellosis	*M. haemolytica* A *B. trehalosi*
Pigs	Sporadic outbreaks of HS Atrophic rhinitis Pneumonia	*P. multocida* serotype B2 Toxigenic strains of *P multocida* type D, occasionally, *P. multocida* type A
Poultry	Fowl cholera	*P. multocida* type A (type F in turkeys) and type D are less common.

Source: (Centre *et al*., 1993)

2.2. Taxonomy and Classification

The *Mannheimia haemolytica* and *Pasteurella multicoda* is grouped taxonomically under

Super kingdom	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Pasteurellales*
Family	*Pasteurellaceae*
Genera	*Mannheimia* and *Pasteurella*
Species	*Mannheimia hemolytica and Pasteurella multicoda*

Source: (Schoch *et al*., 2020)

2.3. Epidemiology of pasteurellosis

Pasteurellosis caused by *Mannheimia hemolytica* is one of the most economically substantial infectious diseases affecting small ruminants, with an international distribution (Table 2). However, Iceland was the first country to report it, followed by Australia, the United Kingdom, Ethiopia, Norway, South Africa, Somalia, and the United States (Berhe *et al*., 2017). The disease is reported most frequently in Asia and Africa countries where sheep or goat breeding is widespread. It is also common in USA and Canada where cattle breeding is also common. In Europe, pasteurellosis widespread in many countries where sheep and cattle are present such as the Netherlands, Germany, Italy and France (Jilo *et al*., 2020).

Mannheimia haemolytica are a common commensal bacterium found in the upper respiratory tracts of sheep and goats and they effect of all ages group (Chukwuebuka *et al*., 2017). Outbreak of pasteurellosis is often associated with climate changes and occur in spring and summer, but can happen sporadically at any time of the year(Jilo *et al*., 2020). Pasteurellosis caused by *Mannheimia haemolytica* are transmitted from sick to healthy animals by direct contact and aerosol. Particularly, when animals are closely confined in inadequately ventilated held for long periods. These infectious disease are transferred by direct contact with body fluids (such as saliva and nasal secretions, coughed up or exhaled from infected animals or recovered carriers), contaminated feeders and troughs(Wilson & Ho, 2013). The worst epidemics occur during the rainy season, in poor body condition, stress and wet conditions seem to contribute to the spread of the disease. The majority of *M. haemolytica* infections are mostly endogenous, caused by the normally resident bacteria on the upper respiratory tract, although exogenous infections can also occur by direct contact with sick animals or through infected aerosols(Miller & Clay, 2008).

Table 2: Distribution of *M. haemolytica* and *P. multicoda* in different geographical locations

Country	Region	Host species	*M.H*	*P.M*	Authors
Iraq	Baghdad	Goat	4.46	-	(Ahmed *et al*., 2017)
Morocco		Sheep	42	15	(Ghizlane Sebbar *et al*., 2018)
		Goat	100	0	
Ethiopia	Afar (Mille)	Sheep	-	-	(Shiferaw *et al*., 2006)
		Goats	-	-	
	Oromia (Hararghe)	Sheep	87.5	12.5	(Marru *et al*., 2013)
	Oromia	Sheep	12	8	(Tolera *et al*., 2019)
	Amhara	Sheep	32.62	-	(Ali *et al*., 2022)

M.H = M. haemolytica, *P.M = P. multicoda*

2.4. Diagnostic Methods of *Mannheimia haemolytica*

2.4.1. Clinical signs

Clinical signs of pneumonic pasteurellosis caused by *Mannheimia haemolytica* was range from intermittent coughing to rapid death, fast respiration, nasal discharges, inappetance, weight loss and increases temperature from 40.4°C to 42°C are common in affected animals. The majority of cases occur within two weeks after transportation, and disease progression can be fast, with death occurring without presenting any clinical indications of disease (Tolera *et al*., 2019). Stress factors cause immune suppression through the release of steroids from the adrenal cortex inhibiting the leukocyte production that lead to decrease in circulating leukocytes. Pasteurellosis caused by *Mannheimia haemolytica* is distinguished from other respiratory infection by its high mortality and rapid progression to death (Berhe *et al*., 2017). *Mycoplasma* species, contagious caprine pleuro-pneumonia (CCPP), and other respiratory disorders can be confused with pneumonic pasteurellosis (Ehrenberga *et al*., 2001). The Viral pneumonia also caused by *peste des petits ruminants* (PPR), parasite pneumonia produced by lung worms such as *Dictyocaulus filarial*, mycotic pneumonia caused by *Aspergillus* species upper respiratory tract disease (Berhe *et al*., 2017).

2.4.2. Culture and Biochemical characteristics

The colonies produced by *M.haemolytica* are odorless, moist, smooth, grayish, form beta hemolysis on blood agar plates while the colonies of *P. multocida* are round, grayish, shiny and non-haemolytic. *M. haemolytica* is very distinct from *P.multocida* by their growth on MacConkey agar as pink pin point colonies (Tabatabaei & Abdollahi, 2018). Biochemical characterization of *P.multocida* and *M.haemolytica* were indicated in (Table 3).

Table 3: Biochemical characteristics of *M. haemolytica* and *P. multocida*

Reaction	*M. haemolytica*	*P. multocida*
Haemolysis on blood agar	+	-
Motility	-	-
Indole formation	-	+
Litmus milk	Acid	Neutral
Glucose	+	+
Sucrose	+	+
Lactose	+	-
Oxidase	+	+
Catalase	+	+
MacConkey agar	+	-

+=indicates present, - =indicates not present

Source : (Rawat *et al*., 2019)

2.4.3. MALDI Biotyper identification method

MALDI TOF MS identification has recently emerged as a powerful technique for identification of bacteria and altering the workflow of well-established diagnostics laboratory (Alizadeh *et al*., 2021)**.** MALDI Biotyper is one of the most powerful proteomics technologies for detecting a wide range of microorganisms, including bacteria, fungus, and parasites, as well as providing us with precise, rapid, easy, and inexpensive in identification of *Mannheimia haemolytica*. MALDI Biotyper is a rapid technology first developed in 1987 by Franz Hillen and who honored Nobel Prize in 2002. Matrix is a small organic molecule that absorbs UV light and aids in the ionization process. Furthermore, it is an open system that may be supplemented with own reference data.

The Biotyper 3.0 database from the Bruker MALDI Biotyper system contains a few, selected representatives of the family Pasteurellaceae(Kuhnert *et al*., 2012). MALDI Biotyper is composed of three principal units. The first is the ion source that makes ionization and transfers sample molecule ions into a gas phase. The second unit is the mass analyzer that allows ion separation according to m/e. The last unit is a detection device for monitoring separated ions (Torres- sangiao *et al*., 2021). Routine laboratory diagnoses of bacteria identification are mainly based on conventional phenotypic characteristics, biochemical tests and molecular detection. These analyses require expertise and are time-consuming (Rychert *et al*., 2019). But, MALDI-TOF technology allows accurate bacterial identification of a wide variety of species in reduced time, from between 24 and 48 hours, with a small amount of bacterial biomass (Singhal *et al*., 2015).

Only a few articles have confirmed the applicability of MALDI-TOF MS for fast identification of various species of bacteria isolated from animals, including *M. haemolytica* (Kuhnert *et al*., 2012). When MALDI-TOF MS was compared to other diagnostic techniques, it was found to be extremely accurate for Pasteurellaceae (Puchalski *et al*., 2016). Furthermore, highly specialized employees are not required and acquisition of such a technology is more expensive than normal identification methods (Chen *et al*., 2021). Rapid MALDI-TOF MS identification leads to more effective therapy, which leads to a shorter disease duration(Wragg *et al*., 2014).). Database improvements will improve MALDI-TOF MS performance in terms of MALDI-TOF MS identification (Kuhnert *et al*., 2012). Databases are being improved across the world by manufacturers, research institutes, and interest groups. Most closely related species may be effectively identified using MALDI-TOF MS. However, there are some exceptions. The inability to discriminate between related species can be due to the inherent similarity of the bacteria themselves. For instance, MALDI-TOF MS is currently unable to distinguish E. coli from Shigella(Rychert *et al*., 2019). This is most likely due to the possibility that these two species are actually one, as taxonomists have indicated. The inclusion of proteomic based techniques to the standard MALDITOF MS technology may enhance discriminating in the future. Another reason similar species may be incorrectly identified is a lack of sufficient spectra in the database (Almuzara *et al*., 2019). Two sample preparation procedures were used for MALDI TOF MS identification: direct transfer method and extended direct transfer method.

Direct transfer sample preparation for MALDI Biotyper: Bacterial single colonies on BHI agar were picked and smear directly onto a MALDI target sample position using an applicator then added 1 µl of BTS onto positions and dried at room temperature for 5 minutes and overlaid with 1 µl of alpha-cyano-4-hydroxycinnamic acid matrix solution. Target plate was placed in the MALDI- Biotyper (Bruker, Germany) for bacterial identification. If the result reads no peaks or no identification, extended direct transfer sample preparation would be followed.

Extended Direct Method for MALDI-TOF MS: Bacterial colonies on BHIA were picked and smear onto a sample position using an applicator. Samples were then overlaid with 1 µl of BTS onto target plate dried and deposit 1 µL of 70% formic acid air-dry at room temperature then add alpha-cyano- 4-hydroxycinnamic acid matrix solution (Bruker Daltonics, Germany). Target plate was placed in the MALDI Biotyper for bacterial identification(Zhou *et al*., 2020). The identification criteria of MALDI Biotyper ™ system log (score values) higher than 2.0 indicates species identification, while log (score values) between 1.7 and 2.0 are sufficient for identification of the genus. For values below 1.7, no isolate identification is possible (Almuzara *et al*., 2019).

2.4.4. BIOLOG identification method

The Biolog GEN III carbon source and chemical sensitivity utilized technology produces a characteristic pattern or "metabolic fingerprint" from discrete test reactions performed in a 96 well of microplate. It is quick and easy system to identify   more than 2600 species of Gram-positive and Gram-negative bacteria, yeast, and filamentous fungi (Wragg *et al*., 2014). In 1926, Dutch scientist L.E. Den Dooren de Jong demonstrated bacteria that could be identified depending on how they used various carbon sources. Phenotypic features were the basis for all of the early work in bacterial taxonomy(Park *et al*., 2006). Biolog's newest generation redox chemistry allows microorganisms to be tested without the need of gram stain or other pre-tests by utilizing the Biolog GEN III Microplate. Biolog has identifies 94 phenotypic tests, 71 of which are biochemical and 23 of which are chemical sensitivity tests. The Omni Log Systems software is used to identify microorganisms based on GEN III Microplate (Biolog Inc, 2011). All required nutrients and biochemical are prefilled and dried in 94 wells of microplate. Tetrazolium redox dyes are used to colorimetrically to identify carbon source utilization or inhibitory chemical resistance. Biolog semi or fully-automated systems employ a GEN III coated microplate with an inoculation fluid protocol to phenotypically identify bacterial species. Using a turbidimeter, a single colony grown on agar medium was selected, emulsified into 'inoculating fluid', and the bacterial inoculum's cell density was measured and adjusted for a given transmittance (90 to 98 %) (Holmes *et al*., 1994). The cell suspension is then inoculated into the GEN III Microplate, 100 μl per well, and incubated aerobically for 22 hours at 33^{0}C.

During incubation, increased respiration in the wells causes the tetrazolium redox dye to be reduced, resulting in a purple tint (Strejcek *et al*., 2018). Negative control should be colorless at all times (A-1). A positive control well (A-10) is also included as a reference for the chemical sensitivity tests (Van Heerden *et al*., 2001). The purple color microplate's phenotypic fingerprint was read in the Biolog Micro Station reader after 22 hours of incubation, and the findings were interpreted as genus/species/sub-species using the Omni log Microbial identification system's software (GEN III 2.7.1.40.15G).

2.4.5. Molecular Detection

The polymerase chain reaction (PCR) is a potent method that has quickly become one of molecular biology's most extensively utilized tools. PCR revolutionary nucleic acid amplification in modern biology. PCR was invented by the American biochemist, Kary Mullis in 1984. However the basic principle of replicating a piece of DNA using pairs of primers had already been described by Gobind Khorana in 1971. The technique involves amplification of genetic material to billions of copies from minute amounts and even when that source DNA is of relatively poor quality. The usage of PCR in molecular diagnostics has grown to the point that it is now considered the gold standard for detecting nucleic acids. Real-time PCR has improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination (Kralik & Ricchi, 2017). The real-time PCR method is used to detect and quantify an amplified PCR product while the reaction proceeds in real time.

Real-time PCR is based on the incorporation of a fluorescent dye where the increase in fluorescence signal, generated during PCR, is in direct proportion to the amount of the PCR product. The fluorescent molecules added to the PCR mixture create fluorescent signals that are detected at the same time as the amplification progresses. PCR is useful in the investigation and diagnosis of a number of diseases. Use of a closed system, reduced turnaround time, dynamic range of target detection, and feasibility for quantitation are a few of the advantages of this method(Joshi & Deshpande, 2011). There are three major steps involved in the PCR amplication: denaturation, annealing, and extension (Garibyan & Avashia, 2013). BactoReal Kit *Mannheimia haemolytica* is rapid and sensitive to detect the 16S rDNA gene of *M. haemolytica.* In PCR process first DNA was extracted according to manufacturer instruction, Master mix were prepared and Real-time PCR was amplify and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye (Giri Putra *et al*., 2020). PCR analysis based on *M.haemolytica* BactoReal Kit manual.

Table 4: Strengths and limitations of different diagnostic techniques used to identify *M. haemolytica*

Test	Application	Strengths	Limitations
Bacteriological Isolation	Detect bacteria	-Cheap -Indication of live bacteria -Quantification Possible	-More time consuming -Lower sensitivity -Many media need
Biolog	Phenotypic detection	-Live organism required -Various species identification -Rapid response -Amples of biochemical test	- Specific Inoculation fluid - Specific media used - Consumables cost expensive -Data base limit
MALDI TOF MS	Protein detection	-Many species identification - Fast, simple and cost saving -Sample preparation is simple -Sample requirement is minimal	-Similar species may be incorrectly identified -Cutoffs value vary between studies -Incomplete databases -Initial cost of the instrument is high
Real time PCR	DNA detection	-No live organisms required -Pooling of samples possible -fast, specific and sensitive	Depend on the specific designed primer - expensive

Source: (Pardon *et al*., 2020)

2.5. Control and prevention

2.5.1. Management

Pasteurellosis caused by *M.haemolytica* is complex multifactorial disease and difficult to control but good management and prevention methods are desirable. Infections from these disease are associated with poor management, environmental/ climatic changes and health services (Legesse *et al*., 2018). Management factors such as overcrowding, transportation, deprivation of feed and water, exposure to aerosol infection and sudden Climatic changes. Such, stressful conditions increase sheep and goats' susceptibility to pasteurellosis (Alhamami *et al*., 2021). Most of the cases are acute or per acute, resulting in death after onset of the disease. Reduction or even elimination of such predisposing factors is major important in disease prevention and control (Clawson *et al*., 2016).

2.5.2. Treatment

The treatment used against pasteurellosis caused by *M.haemolytica* is directed towards saving the lives of animals, depends on early detection of the disease and proper administration of susceptibility drug. The use of broad spectrum antibiotics in the face of an outbreak is common approach to control pasteurellosis in small ruminants (Cuevas *et al*., 2020). Antibiotic susceptibility tests are important, because there is a resistance to antibiotics for *M.haemolytica*. The antibiotics such as Penicillin G, Ceftiofur, trimethoprim/sulfonamides, florfenicol, enrofloxacin, tilmicosin tetracycline are the most commonly susceptible antibiotics to *M.haemolytica* (Kehrenberg *et al*., 2001). However, the bacteria has been found resistance to some of antibiotics such as: Cephalosporins, streptomycin, macrolides, erythromycin, fluoroquinolones. So, isolates should be tested and a suitable antibiotic to be selected based on the in *vitro* sensitivity test (Mcallister *et al*., 2021). In several European countries like Belgium, Germany and Netherlands, there is guidelines and legislation requires sampling and susceptibility testing of animal pathogens before specific antimicrobial agents can be used (Schönecker *et al*., 2020).

2.5.3. Vaccines

The development of effective vaccine used for the *M. haemolytica* are very important in prevention and control strategy. To reduce the incidence, burden of the disease and minimize antimicrobial use. Currently, several vaccine types exist against pasteurellosis globally. Problems with vaccination arise where there is more than one serotype circulating, due to the lack of cross-protection (Abed *et al*., 2020). At present, only a monovalent *P. multocida* serotype A vaccine is commercially available in Ethiopia, although *P. multocida* serotypes A and D and 11 serotypes belonging to *M. haemolytica* have long been detected (Berhe *et al.*, 2017). Consequently, repeated outbreaks are reported in Ethiopia even among vaccinated sheep and goats, which practitioners and communities ascribe to vaccine failure. In Ethiopia, a variety of serotypes of *P. multocida* and *M. haemolytica* are endemic and posing a major threat of small ruminant productions (Berhe *et al*., 2017).

2.6. Antimicrobial Sensitivity Test

Antimicrobials are substances that eliminate or stop the development of bacteria, particularly pathogenic bacteria. Antibiotics are chemicals generated by fungus and other microbes that either kill or inhibit bacterial growth (FMHACA, 2018). The improper use of antibiotics hinders efforts throughout the world to combat infectious illnesses by fostering the growth and spread of antibiotic resistance (Berhe *et al*., 2021). Infectious bacterial infections in humans and animals are treated, controlled, and prevented with the use of antibiotics. Antibiotics lose their efficacy if bacteria develop antimicrobial resistance. The capacity of a microbe to inhibit an antibiotic from working against it is known as antimicrobial resistance (Ibrahim *et al*., 2019).

3. MATERIALS AND METHODS

3.1. Description of the Study area

The study was conducted from November 2021 to June 2022 in Sebeta and Holeta towns of Oromia special zone Ethiopia (Figure 1). Sebeta town is located at 25 km south west from Addis Ababa at an altitude of 1800–3385m above sea level and at 8°55–8.917°N and 38°37–38.617°E, latitude and longitude respectively. It receives an average annual rainfall of 1073 ml and annual temperature that ranges from 11.3°C to 28°C. It has a total area of 102,758km^2(Performance & Regions, 2021).

Holeta town is located in the central part of the country, 31 km west of Addis Ababa in Oromia Special Zone, Ethiopia. Surrounded between latitude 8°53′ to 9°14′ N and longitude 38°21′ to 38°36′ E. Holeta town has an area of 5550 hectares and 2449m above sea level. Annual mean temperatures are 25.9 and 7.2°C, respectively. The minimum and maximum annual rainfall of the area ranges from 834mm and 1300mm and with an average 1067mm (Performance & Regions, 2021). Mixed farming where a main farming system in the area. Traditional housing and natural grazing pasture were the largest husbandry practice.

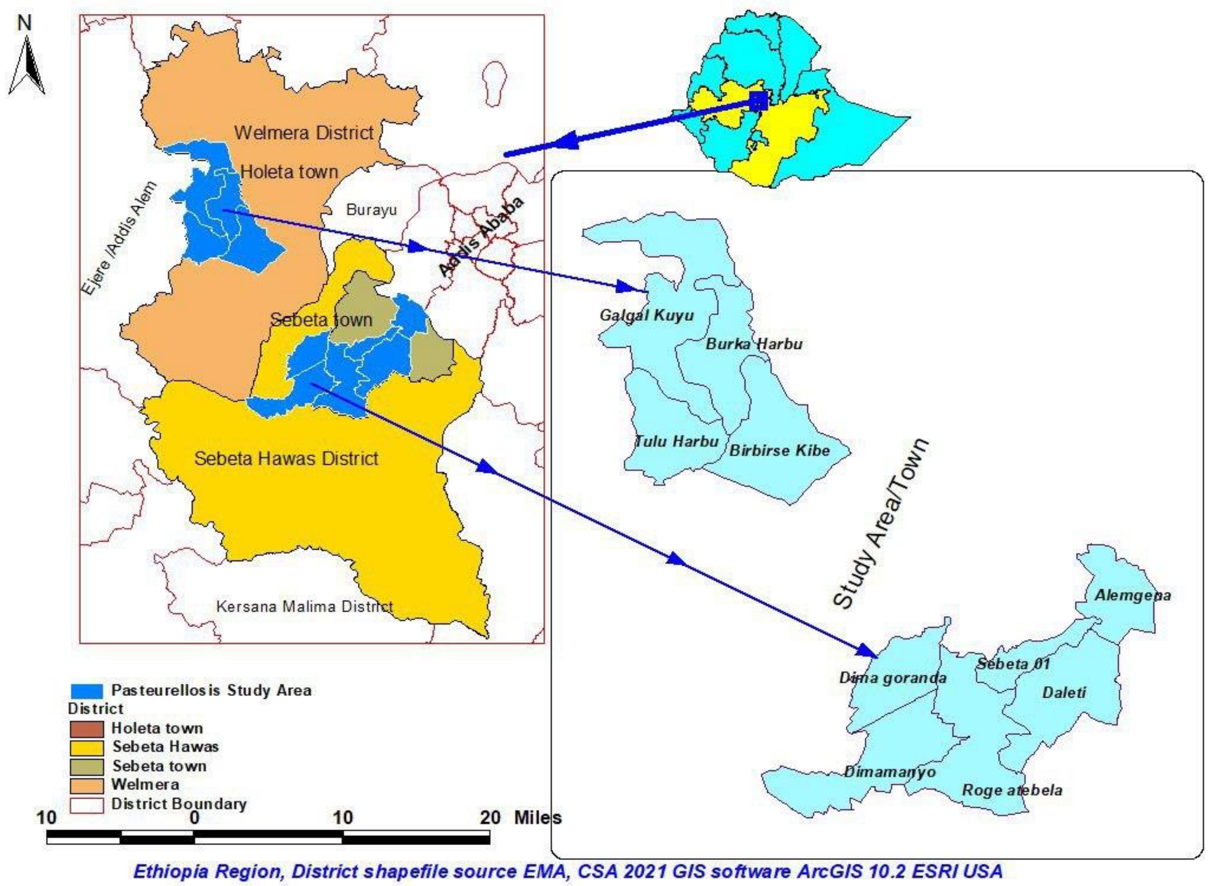

Figure 1: Map of the study areas

3.2. Study Population

The study populations were clinical suspected sheep and goats suggestive of pneumonic pasteurollosis or other respiratory problems that brought to veterinary clinics for treatment. Sheep and goat with clinical sign of nasal discharge, respiratory difficulty, fast respirations, salivation, and coughing with regardless of age and sex were considered for sampling.

3.3. Study design

Cross sectional study was carried out in Sebeta and Holeta towns of the Oromia special zone from November 2021 to May 2022 with the aim of *M.haemolytica* identification and molecular detection. Nasal swab and whole blood samples were collected from clinically suspected sheep and goat. Clinical information of sheep and goats were collected including the name of the clinic, date of sampling, type of sample, age, sex, and animals' identification number were recorded for each animal on the sample collection format (Annex 7).

3.4. Sampling method

Before the beginning of the study, primary data were obtained from veterinary clinics about the disease. Individual animals were selected purposively based on the suggestive clinical signs of respiratory infection of sheep and goat that brought to veterinary clinics. Study site were selected based on presence of veterinary clinic. Clinically suspected animals nasal swab and whole blood were sampled for isolation, identification and detection of *M. haemolytica.*

3.5. Sample Size Determination

Purposive sampling techniques were used based on the presence of clinical signs of pneumonia suggestive of pneumonic pasteurellosis in sheep and goats brought to the respective veterinary clinics of Sebeta and Holeta towns.

3.6. Sample collection and transportation

Nasal swab samples were collected from Sebeta and Holeta veterinary clinic after disinfecting external part of the nose with 70% alcohol. Samples were collected from clinically pneumonic sheep and goat with respiratory signs such as an irregular breathing, coughing and nasal discharge (Marru *et al*., 2013)**.** Sterile cotton tipped swabs moistened in Stuart transport medium (Oxoid, England) and the swabs are carefully inserted into nostril and rotated against the wall of the nasal cavity. Then swab were placed in labeled sterile screw capped test tube that contains 5 ml of Stuart transport medium (Tabatabaei & Abdollahi, 2018). Also, whole blood samples were collected from Jugular vein of sheep and goat by aseptic procedure in anticoagulant green stopper vacutainer tube. Then, kept in ice box and transported to Animal Health Institute (AHI), Sebeta for bacteriological investigation (Annex 1).

3.7. Isolation, identification and molecular detection

3.7.1. Clinical Investigation

Clinical investigation was conducted at the veterinary clinics of Sebeta and Holeta to collect samples from sheep and goats that with difficulty breathing, depression, high fever, fast respirations, salivation, coughing, and nasal discharge.

3.7.2. Isolation and Biochemically methods

Nasal swabs were enriched in Brain heart infusion broth (CRITERION, USA) and incubated at 37˚c for 24 hours. Then, a loop full of broth cultures were streaked on to blood agar (TY MEDIA, India) (Annex 2) supplemented with 7% sheep blood and incubated at 37ºC for 24 to 48 hours and the colony of *M. haemolytica* checked for β-hemolytic, white-grayish, medium-sized, round, and mucoid. Typical colonies were sub cultured on MacConkey agar (CRITERION, USA) and subsequently subcultured on BHIA for further testing. A single colony from BHIA was tested for Gram reaction, Geimsa stain, and pin point colony on Mac Conkey agar, Motility, oxidase, MALDI TOF MS , Biolog, and Real time PCR confirmation (Annex 8) (Alemneh *et al*., 2019).

Gram's staining was used to figure out the size, shape, and arrangement of bacteria. Gram negative *M.haemolytica* isolates were small, rod or cocci bacilli, single or pairs chain, and pink hue (Tadesse & Alem, 2006) and Giemsa staining was used to reveal *M. haemolytica's* bipolarity. Smears were transferred from a colony on Brain heart infusion agar to a microscope slide, dried, and then fixed for 3 minutes with absolute methanol. Giemsa was applied to the smear for 60 minutes, and then the stain was washed away with tap water and allowed to dry. The slides were viewed at 100x with oil immersion. Following staining, a bipolar appearance of coccobacilli arranged in pairs with purple color was found (OIE, 2018).

Biochemical Tests: All of the retrieved isolates were identified using the techniques proposed by (Sehgal *et al*., 2018). *M.haemolytica* was tested for oxidase test to detect cytochrome oxidase activity on the (OxiStrips; Hardy Diagnostics, Santa Maria, CA 93455) (Shields & Cathcart, 2013). Catalase test to determine bacteria produce the enzyme catalase according to (Reiner *et al*., 2013) and for motility test *M.haemolytica* suspicious isolates were stab inoculated using a straight wire down the center of motility medium. Organisms were not migrated away from the stab line and grew only along the stab line, leaving the surrounding medium reasonably clear. Indole, urease, TSI, Simmon's citrate tests (ANNEX 4) and tested for sugar fermentation such as arabinose, maltose, sucrose, glucose, lactose and trehalose (Rasheed & Jwher, 2022).

3.7.3. MALDI TOF MS identification system

Direct sample preparation methods was performed by using young and pure singe colony sub cultured on BHI agar (CRITERION, USA) and incubated at 37°C for 24 hours. A single colony was deposited directly on an assigned position of an MSP 96 target plate, followed by drying at ambient temperature. The dried sample was overlaid with 1 µl of HCCA matrix solution (α-cyano-4-hydroxycinnamic acid, Bruker Daltonik GmbH, Lot number- 7020120107), followed by air-drying at ambient temperature for crystallizing (Annex 9). Then, samples were analyzed in an MALDI-Biotyper (Bruker Daltonik, Germany) and spectra were calibrated before analysis using a standard calibration mixture including peptides and proteins obtained from E.coli Bacterial test standard(BTS) (Bruker Daltonik, Lot number:- 7030220010)(Almuzara *et al*., 2019). Then, interpreted *M. haemolytica* organism with score value and National Center for Biotechnology Information (NCBI) (ANNEX 11). Isolated *M. haemolytica* was further tested by Real time PCR.

3.7.4. Biolog identification system

Biolog bacterial identification was performed by using sinlge colony cultured on BHI agar (CRITERION, USA) at 37°C for 24 they were sub cultured on Biolog Universal growth (BUG) media (BIOLOG, USA) incubated at 37^{o}C for 24hrs. A pure colony was selected from BUG and emulsified into‘inoculating fluid A’ (IF-A (Catalog No. 72401) for subsequent inoculation on to the GEN III Microplate (Cat. No 1030). Prepared inoculum was measured to a specified transmittance using a turbidometer at (90-98%). For each isolate, 100 µl of the bacterial cell suspension was inoculated into each well of the Microplate, using automatic multichannel pipette and incubated at 33°C for 22 hr (Wragg *et al*., 2014). Microplate was read in the Micro Station reader after 22 h and results interpreted by the Biolog identification data base system software (GEN III_2.7.1.40.15G) (ANNEX 10).

3.7.5. Real Time PCR

Deoxyribose nucleic acid (DNA) extraction

The isolate inoculated in to the Brain-Heart Infusion (BHI) broth (CRITERION, USA) incubated overnight at 37°C were used to DNA extraction. The bacterial genomic DNA was extracted using QIAamp DNA Mini kit (QIAGEN, Germany) (Lot: 166034227) according to manufacturer’s guidelines (Annex 3). The extracted DNA samples were stored at -20°C until PCR amplification was performed. The primers and probes from the BactoReal® Kit were used to detect specific genes 16S rDNA of *M. haemolytica* in real-time PCR (ingenetix, GmbH, Arsenalstra 11, A-1030 Vienna, Austria). The amplification of *M. haemolytica* specific DNA was revealed by a probe-specific amplification curve (FAM channel) (BactoReal® *Kit- M. haemolytica* Manuals). In this study, primers targeting 16s rDNA gene of *M. haemolytica* were used, as they have been used in previous studies (Arabia *et al*., 2021)**.**

The PCR was carried out in final volume of 20 µl reaction mixture containing 1µl of each primer and CR-1 Assay Mix of *M.haemolytica*, 10µl of DNA Reaction Mix, 3µl PCR grade water, and 5µl extracted DNA from samples. A total volume of 15 µl of master mix was added into wells of micro plate and 5 µl of extracted DNA. For negative and positive controls, nuclease free water and positive Control-DNA were used from the BactoReal® kit, respectively. The micro plate was sealed tightly by optical adhesive film. PCR was conducted on AB Applied Biosystems (7500 Fast system) using cycling conditions. The PCR cycling conditions used to amplify 16s rDNA gene of *M. haemolytica* included (Annex 4) an initial denaturation at 50^0c for 2minutes and at 95°C for 20sec, followed by 45 cycles of denaturation at 95°C for 5 sec, annealing and extension at 60°C for 1 min and the data was analyzed based on manual in the BactoReal® kit of *M. haemolytica.*

3.7.6. Antimicrobial sensitivity testing

Antimicrobial susceptibility testing of *M. haemolytica* isolates was performed according to (Testing, 2015). The bacterial isolates were tested for antibiotic susceptibility test using the Kirby-Bauer disk diffusion method (Annex 6). Three to five isolated colonies were transferred to 5 ml of 0.85% sterile saline water and turbidity was measured using densitometer (Annex 5) and adjusted to 0.5 standards McFarland. After measuring the turbidity, a sterile cotton swab was dipped into the suspension and then bacteria were swabbed uniformly over the surface of Mueller-Hinton agar plate (CRITERION, USA) by rotating 60° and allowed to stand for 3-5 minutes to observe any excess moisture from the medium. Antimicrobial discs (Oxoid, England) were applied to the media using a disc dispenser (ANNEX 5) gently pressed with the point of forceps to ensure complete contact with the agar surface and then incubated at 37^0C for 16–18 hrs. Zone of inhibition was measure by using a digital caliper. 10 antimicrobials were selected according to the national AMR surveillance strategic document of Ethiopia (Ibrahim et al., 2019).

3.8. Data analysis

The information gathered was coded and then entered into a Microsoft Excel spreadsheet. The frequency and percentages were analyzed using descriptive statistics.

3.9. Ethical clearance

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

4. RESULTS

4.1. Clinical investigation

Nasal discharge, fever, irregular breathing patterns, fast respirations, salivation, and coughing were all observed sign in the study animals (Fig. 2).

Figure 2: Clinical sign of pneumonia pasteurellosis caused by *M. haemolytica* in a goat include nasal discharges and foam on the mouth.

4.2. Laboratory analysis

Two *M.haemolytica* positive isolate from nasal swab were found a total of 235 animals (213 nasal swabs and 22 whole blood samples) with clinical manifestation of respiratory problems. Collected samples 154 and 81 were from Sebeta and Holeta town, respectively.

4.2.1. Isolation and biochemical test results

The putative colonies of two *M. haemolytica* positive isolates were identified as beta -hemolysis, white to gray, medium-sized, round, and mucoid colonies on blood agar and BHI agar growth, as well as lactose fermentation and pin point pink colonies on MacConkey agar (Fig.3). Isolates phenotypically identified as *M. hemolytica* were gram-negative, cocci bacilli arranged in chains with an occasional tendency of bipolar on Giemsa staining (Fig. 4).

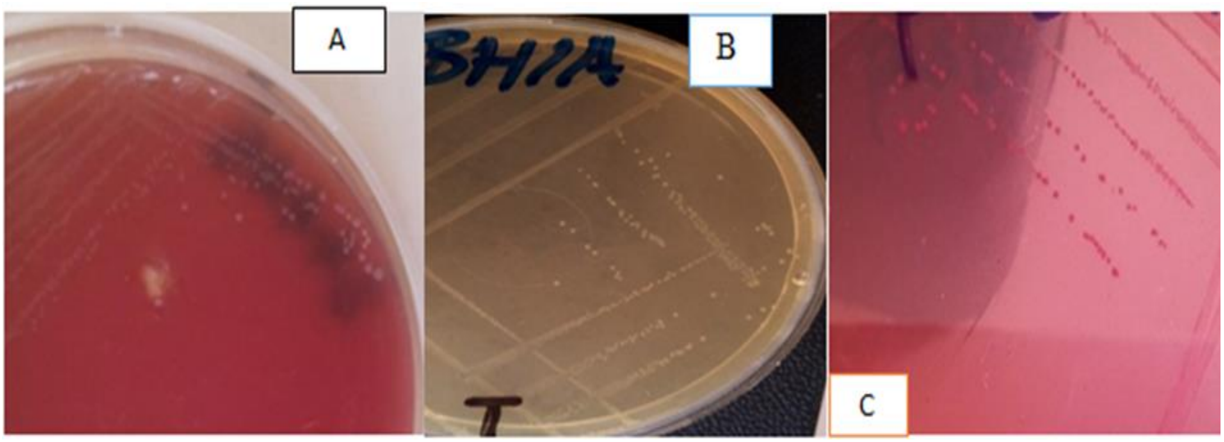

Figure 3: Colony morphology of *M. haemolytica on Blood agar (A), BHIA (B) and* MacConkey agar*(C).*

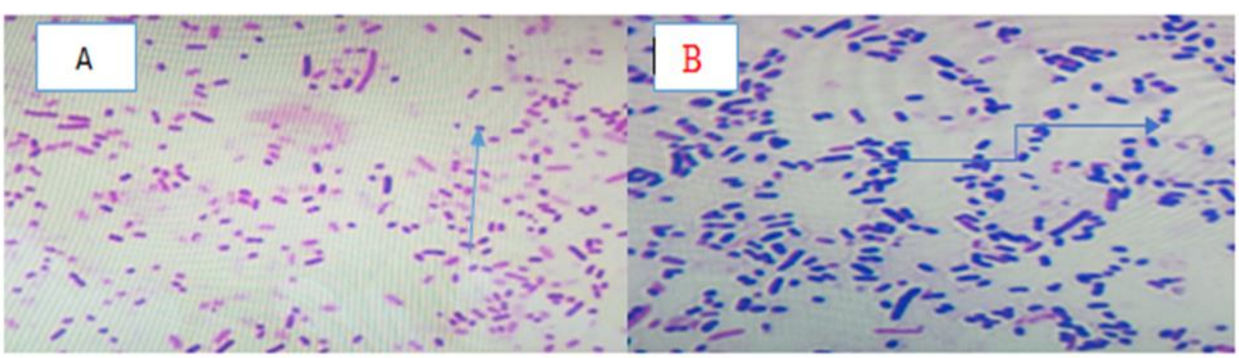

Figure 4: Microscopic appearance of *M. haemolytica:* - Gram-negative and rod shaped (A) and bipolar appearance by Geimsa staining (B)

Biochemically, the isolates were positive for catalase bubbles formation, oxidase positive showing purple color, non-motile and negative for indole, urease, Simmon's citrate tests, and yellow slant and yellow butt on TSI tests. (Fig .5).Sugar fermentation profile indicated that the isolates were able to metabolize different sugars such as arabinose, sucrose, glucose and lactose but not trehalose (Table 5).

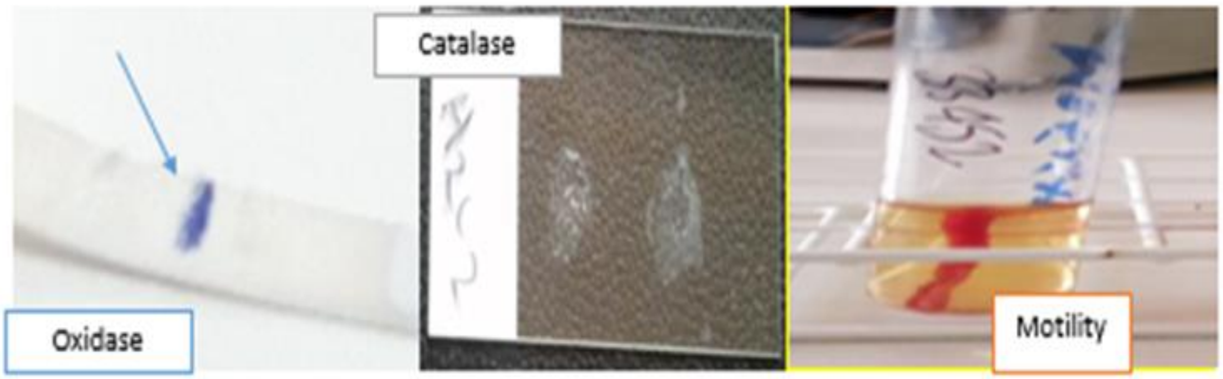

Figure 5: Isolated *M. haemolytica* showing oxidase positive, catalase positive and none motile left to right.

Table 5: Summary of *M. haemolytica* biochemical test results

Biochemical	***M. haemolytica***
Haemolysis on Blood agar	+ **(**beta hemolysis)
MacConkey agar	Pink Pin point
Gram stain	G-ve (Pink color)
Giemsa stain	Bipolar
Oxidase	+ (Purple color)
Indole	-
Motility	Non motile
Glucose	+
Trehalose	-
Arabinose	+

4.2.2. MALDI Biotyper identification results

The two *Mannheimia haemolytica* presumptive isolates were confirmed by MALDI Biotyper from a total of 213 nasal swab samples tested by culture and biochemical tests (Fig. 6) and (Table 6)

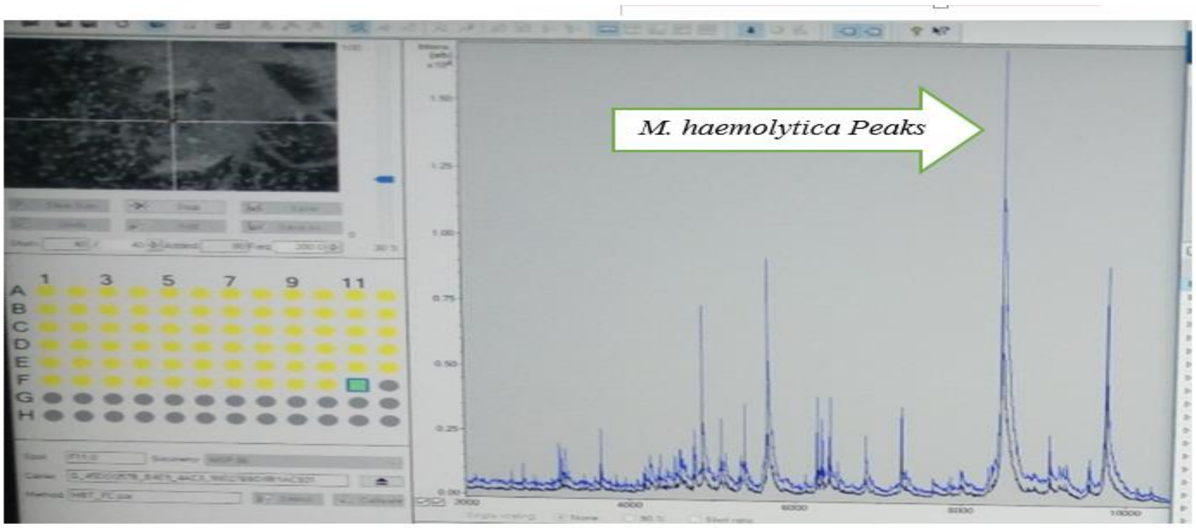

Figure 6: Spectrum Peaks of *Mannheimia haemolytica* detected on MALDI TOF MS

Table 6: Isolates confirmed by MALDI TOF MS

Town	Typeof samples	Score value	Matched Pattern	NCBI Code
Sebeta	Nasal swab	2.05	*Mannheimia haemolytica* ISU 29772 ISUV	75985
Sebeta	Nasal swab	1.83	*Mannheimia haemolytica* DSM 10531T DSM	75985

4.2.3. Biolog identification results

Biolog confirmed phenotypically two *Mannheimia haemolytica* isolates that had been confirmed by MALDI TOF MS too (Fig. 7) and (Table 7).

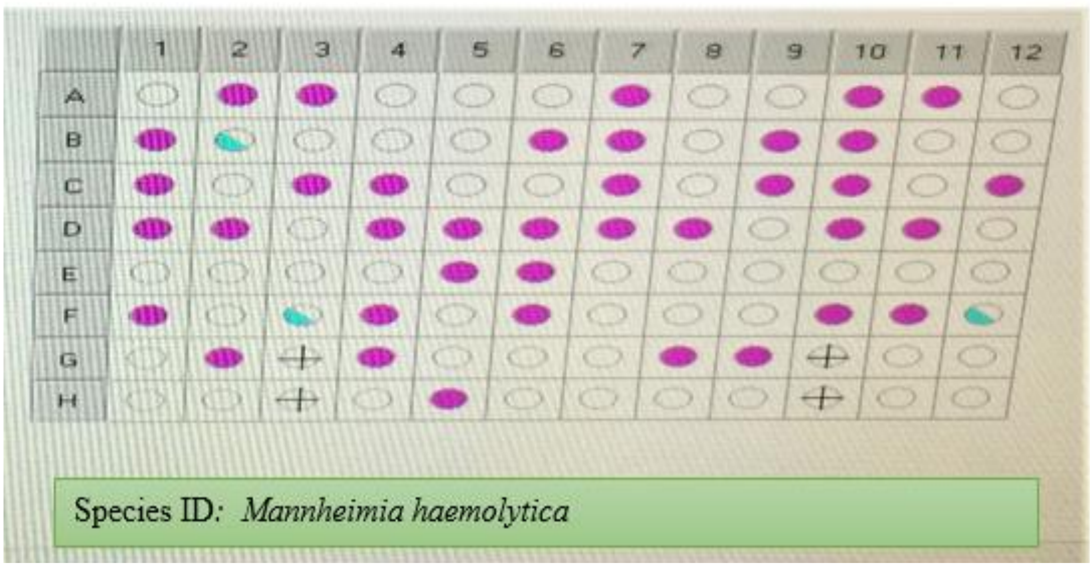

Figure 7*:* Shows *Mannheimia haemolytica* identified by using GEN III miroplate

Table 7: Isolates confirmed phenotypically by Biolog

Town	SPP	Age	Sex	Type of samples	ID %	species ID
Sebeta	Caprine	Young	M	Nasal swab	81	*Mannheimia haemolytica*
Sebeta	Ovine	Adult	M	Nasal swab	78	*Mannheimia haemolytica*

4.2.4. Real time PCR results

Out of 213 nasal swab samples, two bacterial isolates confirmed by MALDI Biotyper were found positive in real-time PCR for *M.haemolytica* specific 16S rDNA genes (Figure 8) and (Table 8).

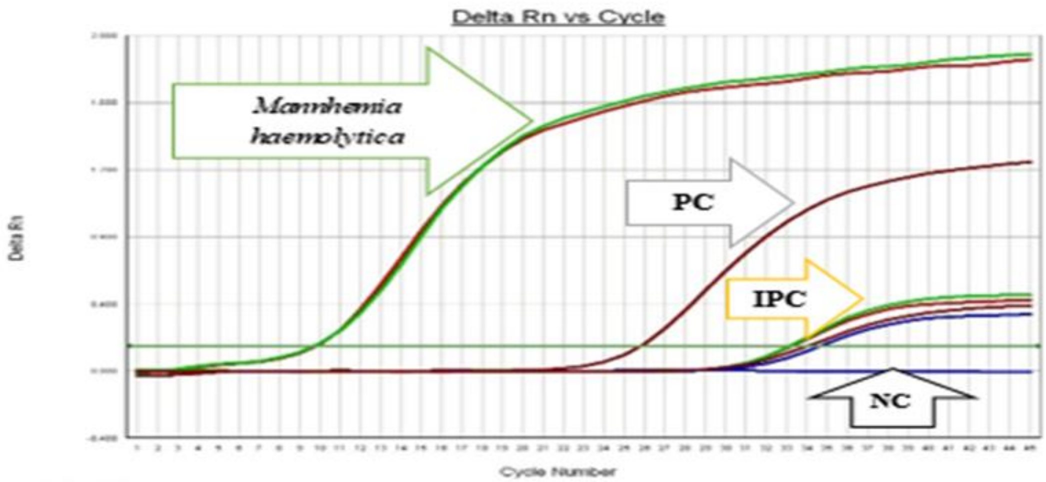

Figure 8: Detection of the 16S rDNA specific gene to *M. haemolytica* using real-time PCR amplification

Table *8:* Real time PCR detection of *M. haemolytica* and CT value

Kit used	**Gene detected**	**Type of samples**	**Ct Value**		**Result**
			IPC	Sample	
Bactorealkit@ *Mannheimia haemolytica*	16S rDNA gene of *M. haemolytica*	Nasal swab	33.29	9.82	*Mannhemia haemolytica*
		Nasal swab	33.17	9.71	*Mannhemia haemolytica*
		NTC	34.67	Undet	Negative
		Positive control	34.07	25.81	Positive

4.2.5. Antimicrobial susceptibility testing results

The antibiotic susceptibility testing of *M. haemolytica* isolates were subjected to a panel of 10 antimicrobials(ANNEX 6).The isolates' antimicrobial susceptibility pattern shown on (Table 9) and (Fig. 9).

Table 9: Antimicrobial sensitivity test results

Antimicrobial agent	**Disk Potency (µ g)**	**Number of isolates**		
		S	I	R
Amoxicillin/clavulanate	AMC-25 µ g	2	-	-
Ampicillin	AMP-10 µ g	2	-	-
Penicillin	P-10 units	2	-	-
Ceftriaxone	DA-30 µg	2	-	-
Tetracycline	TE-30 µg	2	-	-
Erythromycin	E-15 µg	-	-	2
Chloramphenicol	C-30 µg	2	-	-
Trimethoprim/ Sulfamethoxazole	SXT-25µg	2	-	-
Streptomycin	S-10 µg	-	-	2
Clindamycin	DA-10 µg	-	-	2

S=Susceptible, I=Intermediate, R=Resistance

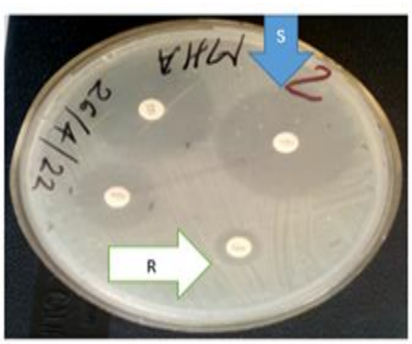

Figure 9: Antimicrobial susceptibility test result of *M. haemolytica*

5. DISCUSSION

Mannheimia haemolytica is one of the most economically significant etiological agents troubling sheep and goats globally, and it is the primary causal bacterium associated to pneumonic pasteurollosis (Abdelsalam *et al*, 2014). In this study, *M. haemolytica* was identified from sheep and goats in Sebeta and Holeta town using culture, biochemical, and Biolog, MALDI TOF MS and Real time PCR detection. Clinical finding of pneumonic pasteurollosis caused by *Mannheimia haemolytica* agent reveals are nasal discharge, fever, respiratory distress, fast respirations, salivation, in appetence and coughing. These clinical manifestations resembled to those described by (Diagnosis, 2019). The identification of *M. haemolytica* with biochemical methods is often challenging in some conditions due to antibiotic treatment, vaccination, transportation and storages (Sarker *et al*., 2016). Of the 213 cultures two (2) nasal swabs were tentatively identified as *M. haemolytica*. This finding is lower than the findings of (Singh *et al*., 2018), (Haji *et al*, 2016), (Kaoud *et al*., 2010) whose reports were 2.22% in sheep and goat, 11.2% in sheep and 14.10% and 11.80% in sheep and goats respectively.

According to the bacteriological feature *M. haemolytica* in present study discovered on blood agar as, white to grey colonies, smooth round and β-type haemolysis and on MacConkey agar appeared as pink pin point colonies as described by (Kaoud *et al*., 2010) and also in line with methods of (Gilmour *et al*., 2011). The isolate of *M.haemolytica* was found to be Gram-negative, coccobacilli, non-motile, bipolar, non-spore-forming, catalase and oxidase positive. These findings were consistent with (Ahmed *et al*., 2017). Biochemically isolates fermented mannitol, arabinose and glucose while negative for indole, citrate, urease, and, trehalose. All of these observations agreed with (Legesse *et al*., 2018) who identify *M. haemolytica* from pneumonic sheep in central Ethiopia and concluded that standard phenotypic methods can provide good diagnosis in the absence of molecular approaches.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) has improved the way veterinary bacteriology diagnosis. This method uses ribosomal proteins, which are commonly found in proteins. MALDI TOF MS is faster, reduced labor and reagent expenses for confirming *M. haemolytica* (Urban-chmiel *et al*., 2016). In present study two (2) *M. haemolytica* isolate were tentatively identified by biochemical and confirmed by MALDI TOF MS similar results were obtained by those two methods. This study supports the agreement between biochemical and MALDI TOF (Urban-chmiel *et al*., 2016) who state that degree of agreement between the two methods for identifying *M. haemolytica* was found 100%. In addition to biochemical and MALDI TOF MS similar results of *M. haemolytica* were found by Biolog in recent study. Association of this method consistent with those of (Wragg *et al*., 2014) who used MALDI-TOF MS and Biolog GEN III for 100 different reference strains, including nine *M. haemolytica* strains. Suggest that the performance of these two different systems for identifying *M.haemolytica* to species was found to be similar. The effectiveness of these methods in identifying bacteria to species was found to be similar with(Juri *et al*., 2011). Also, MALDI-TOF MS has the possiblity to replace these time-consuming (Puchalski *et al.*, 2016).

Polymerase Chain Reaction is one of the most rapid and sensitive molecular tools for bacterial identification. PCR-based identifications rely on the amplification of nucleic acid(Klima *et al*., 2017). The PCR results revealed that only two (2) *M.haemolytica* isolate were positive, which was consistent with the findings by (Deressa *et al*., 2010) who detect two *M. haemolytica* directly from nasopharyngeal swabs of sheep from Debre Birhan, Harshin, and Jijiga. The current detection rate is lower than previously study by (Tabatabaei & Abdollahi, 2018),(Hawari *et al*.,2008), (Arabia *et al*, 2021) and (Legesse *et al*.,2018) whose report 9.09 %, 20.2 %, 6% and 34.21 % respectively. To our knowledge, this is the first study in Holeta and Sebeta town described by multi approaches such as biochemical, MALDI Biotyper, Biolog, and Real time PCR detection of *M.haemolytica*.

Several factors, including identification methods, misidentification, seasonal variation and veterinary service, are likely to be responsible for the disparity of the result (Kaoud *et al*., 2010). Holeta and Sebeta towns are in the same agro ecology zone. However, result from Holeta town were the negative for *M. haemolytica 0* (0%). Finding of this study is in agreement with (Ahmed *et al*., 2017) who found results of one hundred nasopharyngeal swabs of apparently healthy field goats (0.00%) were negative for *M. haemolytica* and stated that seasonal variation is one factor for the agent, in Baghdad, Iraq. Predisposing factors such as stress in the region's current climatic conditions and changing weather patterns may have led to stress formation in the form of natural incidences of pneumonic pasteurollosis caused by *M.haemolytica* (Stephen & Soos, 2021). They also coincide with (Gilmour *et al*., 2011) who discovered a higher prevalence of *M. haemolytica* in the spring and early summer. This corresponds to prior reports of (Marru *et al*., 2013) who claimed that *M.haemolytica,* is typical flora of the upper respiratory tract, may play a secondary role after the primary starting agent has suppressed the host defense mechanism, allowing *M.haemolytica* to multiply.

Antibiotic therapy is now the most cost-effective method of controlling pasteurellosis caused by *M.haemolytica*. To avoid the emergence of antibiotic resistance, regular monitoring and rational antibiotic use are required. Antimicrobial susceptibility testing of *M.haemolytica* is necessary to determine resistance development. Antibiotic resistance has been documented in *M. haemolytica* isolates (Kehrenberg *et al*., 2001). The most effective drugs in our investigation were Amoxicillin/clavulanicacid,Chloramphenicol,Oxytetracycline,PenicillinG,Trimethoprim/sulfamethoxazole, Ceftriaxone, Tetracycline, and Ampicillin, based on antimicrobial susceptibility test results. The current findings contrasted with those of (Borg *et al*, 2021) who found 100% resistance to ampicillin, penicillin-G, 83.3 % resistance to tetracycline, 58.3 % to cefotaxime, and chloramphenicol, respectively. Furthermore, *M. haemolytica* isolates have been found to be extremely resistant to tetracycline according to (Stanford *et al*., 2020). This supports the results of (Alhamami *et al*., 2021), who reported that tetracycline was ineffective. This study was also in line with the findings of (Muktar *et al*., 2017), who claimed that chloramphenicol is 66 % effective and well tolerated against a broad range of bacteria. In addition, (Borg *et al*, 2021), found that trimethoprim-sulfamethoxazole was 83.3 % effective against *M. haemolytica* isolates.

Another obtained finding indicates that, *M. haemolytica* isolates were resistant to streptomycin, erythromycin, and clindamycin. This antimicrobial resistance to streptomycin, erythromycin, and clindamycin may be linked to the widespread use of these drugs for disease prevention and treatment (Alhamami *et al*., 2021). This finding agrees with that of (Borg *et al.*, 2021), who discovered that *M. haemolytica* isolates were 75% resistant to streptomycin. Furthermore, this outcome was consistent with a previous study from a Chinese veterinary clinic, in which the sick goat was given streptomycin on a regular basis, resulting in significant bacterial resistance. However, studies from Australia imply that streptomycin which are not available to food-producing animals, are responsible for the sensitivity

6. CONCLUSION AND RECOMMENDATIONS

M. haemolytica is one among the most significant etiological agents of pneumonic pasteurellosis in sheep and goats all over the world, which causes financial losses due to high mortality, delayed marketing and treatment costs. *M. haemolytica* pathogen exists in the upper respiratory tracts of small ruminants. It can infect and predispose in animals with compromised immune systems. In this study, *M. haemolytica* was identified from pneumonic pasteurellosis cases using biochemical, MALDI TOF MS, Biolog, and real-time PCR. Isolation and identification of *M. haemolytica* through biochemical test is more time-consuming. However, Biolog, MALDI TOF MS and Real time PCR has been more successful in the identification of the *M. haemolytica* since it requires less time and cost. Regarding to antimicrobial susceptibility test some isolates were susceptible such as: chloramphenicol, tetracycline, amoxicillin/clavulanic acid, respectively. However, others isolates were resistant to streptomycin, Clindamycin, and Erythromycin. The occurrence of *M. haemolytica* in the current investigation was found to be very low when compared to the previous finding. However this big difference was due to specific diagnostic methods used with very specific protein detection, season of study, veterinary service and animal management systems.

Based on the above information, the following points were forwarded as recommendation:

- The MALDI TOF MS, Biolog, and real-time PCR are proposed as preferable methods for rapid and easy analysis of *M. haemolytica*
- Antibiotic usage should be regulated and guided at the national level.
- Before treating the animals, an antimicrobial susceptibility test should be performed.
- To determine the strain of *M. haemolytica* more research should be conducted utilizing sophisticated molecular characterization or DNA sequencing
- It is important to isolate the *M. haemolytica* pathogen with the rest ones from different animal species and geographical areas by considering multivalent serotype vaccine development and antibiotic resistant.

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8. ANNEXES

Annex1.: General description of collected data

		Frequency	Percent
Town	Holeta	81	34.5
	Sebeta	154	65.5
Species	Caprine	50	21.3
	Ovine	185	78.7
Age	Adult	155	66.0
	Young	80	34.0
Sex	Female	100	42.6
	Male	135	57.4
Type of samples	Nasal Swab	213	90.6
	Whole blood	22	9.4
Clinical sign	Yes	235	100
Result	Negative	233	99.1
	Positive	2	.9
Total		235	100.0

Annex 2: *Blood agar base(TM MEDIA, India) Preparation*

Blood Agar Base has been used as a base for preparation of blood agar and used to isolate fastidious pathogenic microorganisms.

Dissolved 40.0gms blood agar base in 950ml distilled water. Gently heat to boiling, stirring constantly, until the medium is entirely dissolved. Autoclave at (121°C) for 15 minutes to sterilize. Cool to 45-50°C and add 7% v/v sterile defibrinated blood aseptically. Mix thoroughly and dispense onto sterile Petri plates. After dispensing, place upside down in incubator for up to 48 hours for sterility test and then put it in the refrigerator (2 - 8° C) for consequent activities

Annex 3: DNA extraction

The bacterial genomic DNA was extracted using Qiagen DNA Mini kit according to manufacturer's guidelines. About 200 µl of *M. haemolytica* in BHI broth were transferred into 1.5 ml eppindorf tubes for each samples. 20µl proteinase K and 200µl lysis buffer AL was added to tube and mixed by vortexing and incubated at 56ºC for 10 minutes. 200µl ethanol was added per tube and mixed gently by vortexing. The mixture was transferred to a labeled mini spin column placed in a 2ml collection tubes and centrifuged for 1 minute at 8000 rpm. The collection tubes were changed and 500µl wash buffer -1 was added into each spin column and centrifuged for 1 minute at 8000 rpm. The collection tubes was again changed and 500µl wash buffer -2 was added to each spin column and centrifuged for 3 minutes at 14,000 rpm. Again collection tubes were changed and centrifuged for 1 minute at 14,000rpm.

Finally, the spin columns were transferred into a labeled 1.5ml eppindorf tubes and 100µl of elution buffer was added to each spin column and incubate at room tempreture for 3 minutes and centrifuge for 1 minute at 8000rpm, then, extracted DNA samples were stored at -20°C until amplification was performed.

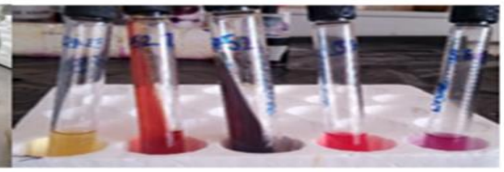
Annex 4: Real time PCR Thermal cycle Amplification

Step	Step1	Step2	Step3	
Cycles	1	1	45	
Temperature	50	95	95	60
Time[HH:MM:SS]	0:02:00	0:00:20	00:00:05	0:01:00

Annex 5: Sample collection and Laboratory sample processing


Sample collection


Oxidase test


Biochemical test


AMR Test


MALDI TOF MS


PCR sample Praparation

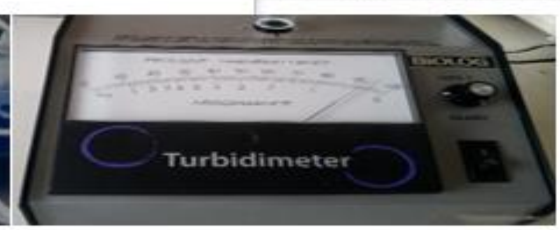
Densitometer


MALDI sample Praparation


culture Media

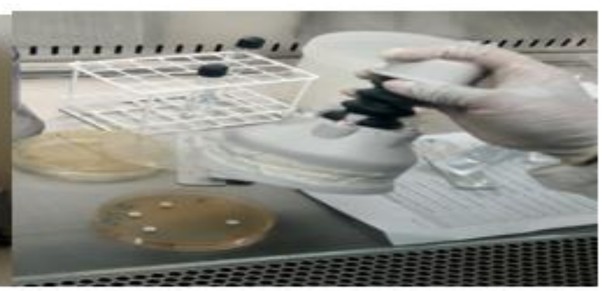

PCR master mix


Biolog sample preparation



Turbidimeter

Micro station reader, Omni Log machine


Disc Dispenser

Annex 6: Antimicrobials interpretation chart

Antimicrobial disc	Disk Potency (µ g)	S	I	R	Remark
Amoxicillin/clavulanate	AMC-25 µ g	>=27	-	-	
Ampicillin	AMP-10 µ g	>=27	-	-	
Penicillin	P-10 units	>=25	-	-	
Ceftriaxone	DA-30 µg	>=34	-	-	
Tetracycline	TE-30	>=23	-	-	
Erythromycin	E-15 µg	>=27	25–26	>=24	
Chloramphenicol	C-30 µg	>=28	-	-	
Trimethoprim/sulfamethoxazole	SXT-25µg	>=24	-	-	

S=Susceptible, I=Intermediate, R=Resistance

Annex 7 : Sample collection recording format

Date______ Region _______Zone _______ City/town /District________ Clinic _________

S/N	Clinic	Animal owner	Species	Age	Sex	Type of samples	Transport media	Clinical sign	Origin
1									
2									

Annex 8: Laboratory Diagnosis recording format

Date_________ Region ______ Zone ___ City/town /District_______ sample type_______

Lab code	BHIB/A	Blood agar	MacConkey Agar	Shape	Color	Gram stain	Oxidase	Catalase	Trehalose	Urease	Lactose	TSI	Motility	Indole	MALDI TOF	BIOLOG	AST	PCR	Genus/SPP

Annex 9: MALDI Target plate Format

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
….												
H												

Annex 10: *M.haemolytica* test result print out from Biolog

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	Abdi mannhemia haemolytica.D5E
User	Admin
Instrument	MicroStation 2 Reader
Instrument S/N	0
Incubation Hours	32.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID 33452	33452
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	Abdi
Field 7	
Field 8	
Field 9	
Field 10	
Date & Time of Read	Jun 05 2022 6:33 PM
Biolog ID DB	GEN-III_2.7.1.40.I5G
Result	Species ID: Mannheimia haemolytica
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.811	0.589	3.862	GN-Nent	Mannheimia haemolytica
2	0.118	0.072	5.715	GN-Nent	Mannheimia ruminalis
3	0.040	0.022	6.761	GN-Nent	Pasteurella multocida ss gallicida
4	0.031	0.016	7.014	GN-Nent	Mannheimia glucosida

Annex 11: *M.haemolytica* test result print out from MALDI TOF MS

Sample Name:	D2
Sample Description:	
Sample ID:	33452
Sample Creation Date/Time:	2021-12-16T11:56:00.178
Sample Type:	Standard
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
AutoXecute Method:	MBT_AutoX
Consistency Category (based on 2 best matches):	A
Applied MSP Library(ies):	BDAL / contains 8468 MSPs / dee78ccc-c113-4ede-b02e-392d0cde98c1 / 2019-04-12T11:52:02.118

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	Mannheimia haemolytica ISU 29778_2 ISUV	2.05	75985
2 (+++)	Mannheimia haemolytica ISU 29359 ISUV	2.05	75985
3 (+++)	Mannheimia haemolytica ISU 29322 ISUV	2.02	75985
4 (+++)	Mannheimia haemolytica 16994 SHDU	2.02	75985
5 (+++)	Mannheimia haemolytica DSM 10531T BRB	2.00	75985