ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

STUDY OF OVINE PASTEURELLOSIS IN HIGHLANDS OF SOUTH WOLLO ZONE OF THE AMHARA REGION, ETHIOPIA

By

BELAY MULATE

JUNE, 2007

DEBRE ZEIT, ETHIOPIA

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

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LIST OF ABBREVIATIONS

ACIR	Australian Center for International Research
AGID	Agar Gel Immunodiffusion
BoARD	Bureau of Agriculture and Rural Development
Bp	Base pair
CAMP	Christie Atkins Munch-Peterson
CSA	Central Statistical Authority
^{O}C	Degrees Centigrade
DNA	Deoxyribonucleic Acid
DVM	Doctor of Veterinary Medicine
Hr	Hour
I-ELISA	Indirect Enzyme Linked Immunosorbent Assay
IHA	Indirect Haemagglutination
ILCA	International Livestock Center for Africa
KDa	Kilo Dalton
Kg	Kilogram
LPS	Lipopolysaccharide
MHC	Major Histocompatability Complex
LF	Lactose Fermenter
NLF	Non- Lactose Fermenter
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PARC	Pan African Rudderpost Campaign
PBSS	Phosphate Buffer Saline Solution
RBC	Red Blood Cell
PCR	Polymerase Chain Reaction

ABSTRACT

The study of ovine pasteurellosis was conducted in highland woredas of South Wollo zone of the Amhara region to determine the prevalence rate of M*annheimia haemolytica*, *Pasteurella trehalosi* and *Pasteurella multocida* from nasal swabs (120) abattoir specimen*'s* tonsils (75) and lungs (75*),* and the serotype diversity amongst species from sheep sera (200). A total of 71 (26. 3 %) isolates of *M. haemolytica*, *P. trehalosi* and *P. multocida* were isolated from nasal swabs of apparently health and moribund sheep, pneumonic lungs and the same numbers from their corresponding tonsils samples. The *P. trehalosi* was isolated from the nasal swabs (12.5 %) and tonsils (16 %), whereas *M. haemolytica* isolates from pneumonic lungs (20 %*),* whereas *Pasteurella multocida* was the lowest among species isolated (1.8 %). The overall isolation rate of *M. haemolytica* and *P. trehalosi* was 11.1% and 13.4%, respectively. There was no significant difference ($p>0.05$) between isolates of *M. haemolytica* and *P. trehaosi* from samples from sheep. Monthly isolation rates of *Mannheimia* and P*asteurella* spp from pneumonic lungs and tonsils were conducted for a period of three months (November-January). The isolates of *Mannheimia* and *Pasteurella* species from pneumonic lungs and tonsilar tissues showed higher incidence rate of 44% and 40% in the month of December, and lower rate of 22% and 16% during the month of January, respectively. There was significant difference ($P< 0.01$) observed between the study months. A total of 200 sheep sera were examined for serotype specific antibodies using indirect haemagglutination test for 11 *M. haemolytica* serotypes. Variation in prevalence among the different serotypes was observed ($P< 0.001$). The IHA test revealed that serotype A2, A5 and A11 were the dominant serotypes with 34.5%, 33.5% and 27.5% positive by IHA whereas serotypes A9, A13 and A14 were the least positive with 8.5%, 6.5% and 5.5% respectively.

Key words: *Mannheimia haemolytica*, nasal swabs *Pasteurella trehalosi*, pneumonia, tonsils, sheep, serotypes, Indirect Haemagglutination (IHA).

1. INTRODUCTION

Ethiopia is basically an agrarian society and the 85% the population's socio-economic activities are based on farming and animal husbandry. The animal resources play a vital role in the overall economy of the country (Solomon, 1994). The population of small ruminants in the country varies greatly with the differences in altitudes. The sheep population in highlands is 75 % and whereas goats 27 %, while the low lands sheep and goat population ratio is just opposite to highlands population (Alemayehu and Fletcher, 1991).

The estimated population of sheep is 14.5 million and 13.5 million goats in the country, which significantly contribute to the national economy (CSA, 2003). They provide more than 30 % of all domestic meat consumption and generate cash income from export of meat, and skin (Alemayehu and Fletcher, 1991). Even-though small ruminant represent a substantial proportion of the Ethiopian livestock resource; the productivity per animal is very low. Small ruminant production in the country is highly affected by infectious and parasitic diseases apart from poor nutrition and under developed management (Tambely, 1998; Tekleye *et al*., 1992). Therefore a top priority in operating successful small ruminant enterprises must be taken up in providing an adequate attention to health problems and reducing reproductive losses. Losses due to mortality are an easy quantifiable economic impact, but losses due to production and morbidity are difficult to estimate.

Pneumonia is one of the most important health problems to small ruminant production and commonly caused by bacteria, viruses, or a combination of both, and metazoan parasites and various physical and chemical agents (Radostits *et a*l., 1994). Most of the bacteria that reside in the upper respiratory tract have the ability to establish themselves in the lower respiratory tract (lung) and cause disease when the defense mechanism of the host is affected (Lopez, 1995). Potential respiratory diseases of sheep that kill a significant number of animals include pasteurellosis, maedi-visna, pulmonary adenomatosis, and verminous pneumonia (Biberstein and Thomson, 1996).

Bronchopneumonia caused by *Pasteurella multocida* or *Mannheimia haemolytica* has a devastating effect on sheep and goats of all ages and cause heavy losses in sheep industries in many parts of the world (Gilmour**,** 1993)**.** In one of the studies conducted in New Zealand estimated losses in carcass weight of 1.5 kg and reduction of live weight gain 0.6 to 0.9 kg for

every 10 % of lung surface grossly affected by pneumonia (Alley, 1987). Under field condition, the severity of pneumonia varies and economic losses result from both mortality and poor productivity of chronically affected animals. Death loss in lambs is usually over 2 % but may reach as high as 20 % (Gilmour and Gilmour, 1989; Radostits *et al*., 1994).

Infectious and parasitic pneumonia in sheep comprise 38.2 % of total cases, and 80 % of the overall mortality rates in the highlands of Ethiopia (Tekleye *et al*., 1992), and it is observed that 18.6% of the morbidity rate and 10.6 % mortality were due to bronchopneumonia mainly caused by *Pasteurella* organism (ILCA, 1988).

In the highlands of Wollo, sheep are the predominant animal species that comprises of among the farm animals and is because of grazing land shortage for large animals, easy to keep sheep on hilly and mountainsides, high reproductive performance, fast growth rate and high market demand.

Pasteurellosis in sheep, locally known as "Giffaw" is widely distributed in highlands of Wollo, and is considered as one of respiratory diseases in sheep population. Despite annual vaccination of sheep against pasteurellosis with a monovalent vaccine (inactivated *P. multocida* biotype A); there are incidences of high morbidity and mortality of sheep following respiratory distress. Therefore, pasteurollosis in sheep is a high-priority issue at the South Wollo level due to the significant economic losses it causes through mortality, morbidity, and the high cost of treatment. So, the objectives of this study are:

- To isolate and characterize most prevalent *Pasteurella* and *Mannheimia* species in sheep.
- To determine the possible major serotypes of *Mannheimia haemolytica* involved in pasteurellosis of sheep in woredas of South wollo zone of the Amhara region.

2. LITERATURE REVIEW

2. 1. Pasteurella **and** *Mannheimia*

The *Mannheimia* and *Pasteurella* are small, non-motile, non-spore forming, gram-negative rods or coccobacilli and facultative anaerobic bacteria that belong to the family *Pasteurellaceae.* They are oxidase and catalase positive and reduce nitrates and attack carbohydrates fermentatively. Bipolarity of *Pasteurella* and *Mannheimia* can be seen in Giemsa-stained or Leishman-stained smears (Quinn *et. al*., 2002).

2.1. 2. Taxonomy and classification

The *Mannheimia* and *Pasteurella* are grouped taxonomically in

Superkingdom	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Pasteurellales*
Family	*Pasteurellaceae*
Genera	*Mannheimia* and *Pasteurella (NCBI,* 2004*).*

Based on number of characteristics including pathogenicity, antigenic nature and biochemical activity, *Pasteurella haemolytica* can be differentiated into two biotypes, biotype A and T. Biotype A ferments arabinose and whereas biotype T ferments trehalose; however, based on molecular, biological techniques and analysis of phenotypic data biotype T was reclassified as *P. trehalosi* and biotype A as *M. haemolytica* and M. *glucosida* (Kilian and Fredericksen, 1981). Based on quantitative evaluation of phenotypic and genomic characteristics, Angen and co-workers (1999) have classified trehalose-negative *[P]. haemolytica* complex into five new species 1. *M. haemolytica. 2. M. glucosida. 3. M. ruminalis*. 4. *M. granulomatis* 5.*M. varigena*

Based on extractable surface antigens, 17 serotypes of *M. haemolytica* and *P. trehalosi* are recognized. Serotype 3, 4, 10, and 15 are classified as *P. trehalosi.* The remaining serotypes (1, 2, 5, 6, 7, 8, 9, 12, 13, 14, 16 and 17) are classified as *Mannheimia* except serotype 11, which varied from biotype A by being CAMP test negative and fermenting the cellobiose and

salicin and is reclassified as *M. glucosida* (Kilian and Fredericksen, 1981). In addition to isolates that express these serotypes, approximately 10% of disease isolates recovered from cattle and sheep are untypeabale (Quire *et al*., 1986; Gilmour and Gilmour, 1989).

The *P. multocida* serogroups have been identified based on difference in capsular polysaccharide and are designated A, B, D, E and F. They are further subdivided into 16 somatic types based on their serological differences of their cell wall lipopolysaccharides (Carter, 1984). It is known that besides the geographical distribution these serogroups are more or less specific with regard to the host and the disease induction (Quinn *et al.,* 1994; Boyce *et al.,* 2000).

2.1. 3. Morphology and staining characteristics

Freshly isolated strains of *Pasteurella* and *Mannheimia* from the carcasses of animal appear as short ovoid rods measuring 1µm in length and 0.5-0.8 µm in width, and most of them are capsulated. After repeated culture the organism tends to form longer rods and become more pleomorphic. The organisms are Gram negative with a tendency to bipolar staining when stained with Methylene blue or Leishman's stain. Old culture usually revealed Gram-negative rods of various sizes. Cells are arranged in chains and filamentous forms are occasionally observed. The bipolarity feature is lost due to continuous culturing (Sharma and Adlakha, 1996; Carter, 1984).

2. 1. 4. Growth requirements

The organisms are aerobic or facultative anaerobic. The optimum temperature for growth is 37 ^{0}C at pH 7.2 to 7.4. Although non-enriched media support their growth, the *Pasteurella* and *Mannheimia* spp. grow best in the presence of serum or blood. Bovine blood is more suitable than the blood of sheep and horse for the demonstration of haemolysis (Carter, 1984; Biberstein and Hirsh, 1999; Quinn *et al.,* 2002). These microorganisms grow well in medium containing amino acids, a mixture of salts, vitamins, sugars like galactose and glucose (Smith and Phillips, 1990). *Mannheimia* spp requires a higher concentration of iron for production of cytotoxin than is needed for growth (Gentry *et al*, 1987; Angen *et al*., 1999).

2. 1. 5. Cultural characteristics

The colonies produced by *Mannheimia haemolytica* and *P. trehalosi* are odorless, moist, smooth, grayish, translucent measuring approximately 1-3 mm in diameter on tryptose-serum agar or blood agar plates while the colonies of *P. multocida* are round, grayish, shiny and non-haemolytic. Some colonies of pathogenic strains of P*asteurella multocida* are mucoid due to the production of think hyaluronic acid capsules. The colonies have a subtle but characteristic odor (Merchant and Packer, 1983; Quinn, *et al*., 2002).

The *Mannheimia haemolytica* and *P. trehalosi* grow on blood agar in the form of smaller colonies with slight thickening in the center. On horse, sheep or rabbit-blood agar the colonies are circular surrounded by a narrow zone of β - haemolysis, but on blood agar plates made from young lambs gives rise to double-zone of β- haemolysis- an inner complete and an outer wide partial, which increases in size at room temperature. They produce a diffusible substance that enhances the hemolytic effect of *staphylococcus aureus* β-toxin. *Mannheimia haemolytica* and *P. trehalosi* is very distinct from *Pasteurella multocida* by their growth on MacConkey agar as pink to red colonies (Smith and Phillips, 1990; Quinn *et al*., 2002).

2.1. 6. Biochemical characteristics

Although *Pasteurella* and *Mannheimia* spp. have limited fermentative ability of carbohydrates, they utilize number of carbohydrates with acid production but not gas (Table. 1). In triple sugar iron (TSI) agar slopes, yellow (acid) slant, yellow (acid) butt without H_2S production (Y/Y/ H_2S-) are typical reaction for *Pasteurella* and *Mannheimia* spp. (Quinn *et al*., 2002)

2.1. 7. Chemical and physical properties

None of the *Pasteurella* is resistant to adverse agents, and can easily be killed with common chemical and physical agents. Exposure of suspension to disinfectant such as to 0.5 % phenol for 15 minutes, to heat at 55 ^{o}C, to ultraviolet light and colonies on solid media to sun light are lethal, and are susceptible to commonly used antibiotics (Quinn *et al*., 1994)

Table 1. Comparison showing the fermentative activity between *Pasteurella* and *Mannheimia* spp

Feature	*P. multocida*	*M. haemolytica*	*M. trehalosi*	*P. pneumotropica*
Hemolysis	-	+	+	-
Growth on McConkey agar	-	+	+	v
Distinctive odor from colonies	+	-	-	-
Indole production	+	-	-	+
Catalase activity	+	+	-	+
Urease activity	-	-	-	+
Gas from carbohydrates	-	-	-	+
Ornithine decarboxylase activity	+	-	-	+
Acid production				
Lactose	-	+	-	V
Sucrose	+	+	+	+
D-Trehalose	V	-	+	+
L-Arabinose	V	-	-	-
Maltose	V	+	+	V
D-xylose	-	+	-	V

Source: Quinn *et al* (2002)

+ = Positive; - =Negative; V = Variable

2. 2. Epidemiology

2.2.1. Distribution and mode of transmission

The primary cause of pasteurellosis in sheep is *M. haemolytica* and *P. trehalosi*, while *P. multocida* (biovars A and D) is occasionally associated with the disease. *Pasteurella* and *Mannheimia* spp. are common commensals on mucus membranes of most animal species in all climatic zones. Most ruminants are asymptomatic carriers of *M. haemolytica* or *P. trehalosi* and they frequently carry strains of *P. multocida* as well (Carter, 1984; Biberstein and Thomson, 1996). *Pasteurella multocida* infections are endogenous; the organism, which is normally commensals of the upper respiratory tract, may invade the tissue of immunosuppressed animals. Exogenous transmission can occur either by direct contact,

through aerosols or consumption of contaminated feed and water. It is often a secondary invader in pneumonia of sheep and other animals.

Pasteurella trehalosi and *M. haemolytica* are normal inhabitants of sheep and colonization of the nasopharynx and tonsils occurs soon after birth, probably from the ewe and persists through adult life. Disease occurs when the defense mechanism of the host is affected. They are also frequently involved in a primary or secondary agent in pneumonia of cattle, sheep goats and swine (Merchant and Packer, 1983; Quinn *et al*., 1994).

2. 2 .2. Host range and susceptibility

Pasteurella and *Mannheimia* spp. have a wide host range and have been isolated from cattle, buffaloes, pigs, sheep, goats, bison, bighorn sheep, elk, moose, dogs, chickens, mule, mountain goats and deer associated with pneumonic lesion and septicemia (Gilmour and Gilmour, 1989). *Mannheimia haemolytica* occurs in all ages of sheep and goats but lambs and kids during the first few months of life and dams at lambing are more susceptible. Generally, *M. haemolytica* infections are the most prominent and highly pathogenic microorganism that causes very severe respiratory diseases of ruminants (Smith and Phillips, 1990). *Mannheimia glucosida* is mostly isolated from nasal cavity of sheep and causes pneumonia and different disease condition. *Mannheimia ruminalis* is isolated from rumen of cattle and sheep and its association with disease has not been reported (Bisgaard *et al*., 1986). *Mannheimia granulomatis* is mostly isolated from rabbits and hares and rarely from cattle and associated with pneumonia and skin granulomas in cattle. Whereas *M. verigena* is isolated from cattle and pigs, causes pneumonia, sepsis and other diseases (Angen *et al., 1*999). Septicemic pasteurellosis occurs more commonly in conjunction with pneumonic form of the disease in young lambs (Gilmour and Gilmour, 1989; Radostits *et al*., 1994).).

Pasteurella multocida serogroups A and D are worldwide spread serogroups which can be found in a wide range of domestic animals (e.g. from fowl to calves, pigs, sheep, goats, and rabbits) in which they cause various infections (Quinn *et al*., 1994). Serogroups B and E have been found predominantly in tropic areas where they induce hemorrhagic septicemia in cattle and wild ruminants (Townsend *et al.,* 1997). The serogroup F has been known as a causative agent of fowl cholera (Jonas *et. al.,* 2001), but in recent time, it has also been found in some mammalian species in different parts of the world. (Jaglic *et al*., 2004; Catry *et al*., 2005). Three biotypes or subspecies of *P. multocida* are recognized, namely *P. multocida* subspecies

multocida recovered from domestic animals, *P. multocida* subspecies *septica* recovered from various sources of including dogs, cats birds and man and *P. multocida* subspecies *gallicida* recovered from birds (Quinn *et al.*, 1994). Clinical infections caused by *Pasteurella* and *Mannheimia* species in domestic animals are mainly attributable to *P. multocida, M. haemolytica* and *P. trehalosi* (Table.2).

Table 2. The Principal hosts and disease conditions caused by the pathogenic *Pasteurella* and *Mannheimia* species.

Pasteurella species	Host	Disease conditions
P. multocida type A	Cattle	Bovine pneumonic pasteurellosis, enzootic pneumonia complex of calves, mastitis
	Sheep	Pneumonia; mastitis
	Pigs	Pneumonia, atrophic rhinitis
	Poultry	Fowl cholera
	Rabbits	Snuffles
	In Other animals	Pneumonia following stress
P. multocida type B	Cattle, buffaloes	Hemorrhagic septicemia in Asia
P. multocida type D	Pigs	Atrophic rhinitis, pneumonia
P. multocida type E	Cattle, buffaloes	Hemorrhagic septicemia in Africa
M. haemolytica	Cattle	Bovine pasteurellosis (shipping fever)
	Sheep	Septicemia (under 3months of age); Pneumonia; gangrenous mastitis
P. trehalosi	Sheep	Septicemia (5 to 12 months of age)

Source: Quinn *et al*. (2002)

2. 2. 3. Pathogenesis

Mannheimia and *Pasteurella* play a major role as a secondary pathogen in the final progression of severe pleuropneumonias in sheep, cattle and goats. Their pathogenesis involves many predisposing agents such as viruses (Parainfluenza virus 3, reovirus, adenovirus, and respiratory syncytial virus), *Chlamydia psittacci, Mycoplasma* species (*M. ovipneumoniae*, *M. arginini*, *M. agalactiae,* and others) and lungworms (particularly *Dictyocaulus filaria)*, environment (excessive temperatures, sudden change of feed, dust) or stress associated during weaning, dehorning and shipping (Yates *et al.,* 1983; Dungworth, 1992; Lopez, 2001;*)*. These factors seems to alter the upper respiratory tract epithelium allowing *M. haemolytica* and *P. trehalosi* to colonize, escaping clearance, and to move from

the nasopharynx to the lungs, leading to a broncho-alveolar kind of pneumonia which is accompanied by high morbidity and mortality (Vogel and Parrot, 1994; Edwards, 1996).

2. 2. 4. Virulence mechanisms

Pathogenic bacteria produce virulence factors that enhance their ability to escape host defense mechanisms and increase the ability of the organisms to colonize and invade deeper tissues. Members of *Mannheimia* and *Pasteurella* spp produce a number of substances that are associated with the pathogenicity of these groups of microorganism. These include the capsule that plays a great role in adherence and invasion, outer membrane proteins that are important in eliciting the protective immune response, adhesins implicated in colonization, the neuraminidase that reduces the viscosity of respiratory mucus and allows closer bacterial apposition to the cell surface (Biberstein and Hirsh, 1999), the lipopolysaccaride causes immune-mediated hypersensitivity that can exacerbate inflammation and tissue damage (Laurence *et al*., 1990), and the Leukotoxin that produces a lot of biological effects: at higher concentrations, the toxin creates pores in the cell membrane that leads to swelling and lyses. At sub-lytic concentration the toxin activates neutrophils inducing inflammatory cytokine production, invokes cytoskeleton changes and causes apoptosis and the down regulation of MHC II proteins on the surface of macrophages affecting their ability to present antigen (Laurence *et al*., 1990; Biberstein and Hirsh, 1999; Highlander, 2001).

2. 3. Pasteurellosis in sheep

Pneumonia in older sheep and septicaemia in lambs associated with *M. haemolytica*, *P. trehalosi* have been extensively recorded as primary diseases elsewhere (Shewan, 1986; Gilmour, 1993). Ovine pasteurellosis occurs in two principal forms: a pneumonic form commonly caused by *M. haemolytica* and rarely by *P. multocida* (Biotype A and D) and a systemic form often due to *P. trehalosi* (Biberstein and Hirsh, 1999).

2. 3. 1. Pneumonic pasteurellosis

Clinical symptoms

Pneumonic pasteurellosis in sheep is stress induced respiratory disease. Outbreaks of acute pneumonic pasteurellosis often commence with sudden death before clinical signs are

observed. As outbreak proceeds respiratory signs become more apparent particularly in older sheep rather than in lambs. Signs include dullness, anorexia, and fever as high as-42.6^{o}C; increase heart rate, a substantial weight loose and acute bronchopneumonia, dyspnea. Increased lacrimation and a cough are often present. On auscultation, respiratory sounds are loud and prolonged. Affected sheep shows frothy saliva in the mouth, and a serous nasal discharge. In acute cases, death occurs in 1 to 3 days (Gilmour and Gilmour, 1989; Radostits *et al.*, 1994)

Lesions

Generally a case from pneumonic pasteurellosis may present petechial and ecchymotic hemorrhages throughout the body. However, the most predominant characteristic changes occur in the thoracic cavity (Gilmour and Gilmour, 1989). In sheep dying from peracute cases, there are green gelatinous exudates on the pericardium and large quantities of straw colored pleural exudates. The lungs are enlarged, heavy and appear red to dark purple in color with frothy fluid from the cut surface (Radostits *et al.*, 1994). In acute and subacute cases, consolidation involving large area of the lung, often in the apical and dorsal diaphragmatic lobes as well as regular areas of lung necrosis with softening and cavitations in some cases are evident. The pleurae may be thickened and opaque or clearly edematous and adhesion may be present in lung lobes (Gilmour and Gilmour, 1989). In protracted infection of pneumonic pasteurellosis, normal lung tissues are often demonstrated from areas of dark red consolidation, particularly when cut, and may contain pus filled nodules. An organized pleurisy with or without adhesion to the lobes is also found. Histologically, characteristic cellular alteration including, alveolar necrosis with alveolar spaces filled with clusters of spindle shaped macrophages (Gilmour, 1993; Radostits *et al*., 1994).

2. 3. 2. Systemic pasteurellosis

Clinical symptoms

Most cases are acute or Peracute, resulting in death within 8-24 hr after the onset infection. Because the course is very short, clinical signs may be easily overlooked. Animals first show dullness, then reluctance to move, fever, salivation, and serous nasal discharge. Edematous swelling is frequently seen, beginning in the throat region and spreading to the parotid region,

neck and brisket. Mucous membranes are congested. There is respiratory distress, and usually the animal goes down and dies within hours. Occasional cases linger for several days. Recovery is rare with no evidence of chronic form (Kopecha, 1997).

Lesions

The most obvious changes in affected animals are the edema, widely distributed hemorrhages, and general hyperemia. In most cases, there is an edematous swelling of the head, neck, and brisket region. Incision of the swellings reveals a clear or straw-colored serous fluid. The edema is also found in the musculature, and the sub serous petechial hemorrhages, which are found throughout the animal, are particularly characteristic. Blood-tinged fluid is often found in the pericardial sac and in the thoracic and abdominal cavities. Petechial hemorrhages are particularly prominent in the pharyngeal and cervical lymph node (Kopecha, 1997). Histologically, the characteristic lung lesion is acute inflammation and emboli in small arterioles and capillaries (Gilmour, 1993; Black *et al.*, 1997).

2. 4. Diagnosis

2. 4. 1. Field diagnosis

Tentative field diagnosis can be made on the bases of the history, clinical signs, post-mortem gross pathological findings and epidemiological features, including species, age group season associated with management factors (OIE, 2004). Clinical symptoms including dyspnea, frothing in the mouth, occulo-nasal discharge, cough and fever ranging from 40.6-42.6^{o}C are the most prominent symptoms. (Gilmour and Gilmour, 1989)

2. 4. 2. Bacteriological diagnosis

In pneumonic pasteurellosis the organism is present in large numbers in lung lesion and exudates. In the acute and generalized (septicemic) form, it can be recovered from the liver, spleen, kidney, lymph nodes and heart blood. In sub acute cases, the organism is often confined to the lung lesions, portion of pneumonic lung from the edge of the lesion, and in generalized (septicemic) forms pieces of liver, spleen, kidney and lymph nodes could be

submitted for isolation. Specimens from live animals may include pus, exudates, nasal swab and bronchial lavages (Quinn *et al*., 1994; Radostits *et al*., 1994).

2. 4.3. Serological diagnosis

The identification of the specific serotype of *Pasteurella* / *Mannheimia* is carried out using different serological methods. These consist of a rapid slide agglutination test, an IHA test for capsular typing, the AGID test, indirect enzyme linked immunosorbent assay (I-ELISA) and Polymerase chain reaction (PCR) assay (OIE, 2004; Rimler, 1993).

In rapid slide agglutination test, a single colony of *Pasteurella* is mixed with a drop of saline on a slide, a drop of antiserum is added, and is mixed thoroughly after warming the slide gently. A coarse, floccular agglutination appearing within 30 seconds is considered as positive. Old cultures give a fine, granular agglutination, which takes longer time to appear (De Alwis, 1993; OIE, 2004).

Indirect haemagglutination test has been widely used over a long period of time for capsular serotyping of *Mannheimia* and *Pasteurella* organisms and was able to identify 17 serotypes of *Mannheimia haemolytica*, *Pasteurella trehalosi*, (Fodor and Varga, 1988) and 5 biotypes of *P. multocida*, and to detect serum antibody responses to individual serotype for vaccine evaluation and in sero-epidemiological investigation of pasteurellosis (Hussein and Mohammed, 1984).

In serological test cross-reaction between serotypes could occur at lower dilution by IHA test (Hussein and Mohammed, 1984). Two serotypes (A2 and A8) with rough LPS lacks the o-polysaccharides, while most of the *M. haemolytica* serotypes with smooth LPS share common o-chain epitopes. The o-chain epitopes of serotypes T3 and T15 and of serotypes T4 and T10 are identical and responsible for cross-reaction (Lactoriox *et al*., 1993) Table 3

Table 3. Comparison showing the percentage of cross-reaction among serotypes of *M. haemolytica* with hyper immune serum

SMH	Hyper immune serum of *M haemolytica*												
	A1	A2	A5	A6	A7	A8	A9	A11	A12	A13	A14	A16	A17
A1	100												
A2		100											
A5	25		100	90		75	75			90	50		
A6				100							100		
A7		100			100								
A8						100		100					
A9				trace	trace	25	100						
A11								100	100	100			
A12									100	100			
A13										100			
A14				90			100				100		25
A16							25	90	trace			100	
A17													100

Source: Mekonen, (2000)   SMH = Serotypes of *M. haemolytica*

Agar gel immunodiffusion test is used for 'capsular' as well as 'somatic' typing, depending on the antigens and anti sera used. The double-diffusion is commonly employed technique (OIE, 2004).

Indirect enzyme linked immunosorbent assay has been found superior to microtitre agglutination for quantifying antibody response in animals against P*asteurella* and *Mannheimia*. The test is reported to be highly sensitive and specific; presumably, the specificity is due to antibodies recognizing lipopolysaccarides (LPS) or LPS protein complexes (OIE, 2004; Rimler, 1993).

2. 4. 4. Molecular diagnostic techniques

Molecular biology has produced techniques that have significantly increased understanding of the epidemiology of pasteurellosis. The techniques includes, DNA fingerprinting and ribotyping. They are close on unique properties of the bacterial chromosomal DNA, rather

than phenotypic expression of the bacteria (Rimler, 1993). A *P. multocida*-specific PCR identified all subspecies of *P. multocida* through specific amplification of a ~ 460 bp DNA fragment whereas in *M. heamolytica*-specific PCR identified through specific amplification of ~ 921 bp DNA fragment within the pomA gene (Zeng *et al*., 1999).

2. 4. 5. Differential diagnosis

Parasitic pneumonia and Maedi-visna are common in sheep, but should not be confused with pasteurellosis because of their longer course. Death without prior illness in lambs should be differentiated from septicemia caused by *Haemophilus Agni,* but the rate of spread in the flock and flock mortality rate are much lower in pasteurellosis (Radostits *et al*., 1994). Furthermore, pneumonic pasteurellosis present a variety of appearance, many of which resemble and can be confused with other conditions; thus, further re-examination should be carried out for confirmation (Gilmour, 1993).

2. 5. Control and prevention

2. 5. 1. Management

Pasteurellosis represents diseases, amongst which is influenced by a wide variety of environmental and management risk factors. Thus, the reduction or even elimination of such predisposing factors is of major importance (Gilmour and Gilmour, 1989). Most cases are acute or per acute, resulting in death within 8-24 hr after the onset of disease. Management Factors such as avoiding crowding, mixing of different flocks, and deprivation of feed and water, exposure to aerosol infection from other sheep and providing shelter especially during extreme weather condition reduce the outbreaks of the disease (Gilmour and Gilmour, 1989; Kehrenberg *et al*., 2001).

2. 5. 2. Chemotherapy and chemoprophylaxis

Antimicrobials are still the tools of choice for prevention and control of infections due to *Pasteurella* and *Mannheimia* spp. *(*Kehrenberg *et al.*, 2001). The first priority of treatment against pasteurellosis is directed towards saving the lives of animals and depends on early detection of the disease and administration of proper sensitive antibiotics. The antibiotics such

as Oxytetracycline, trimethoprim, sulfonamides, penicillin, timilcosin, streptomycin and florfenicol are the most commonly used drugs to treat ovine pasteurellosis (Kehrenberg, *et a*l., 2001). It is well known that theses organisms are having the tendency of becoming resistance to antibiotics the antimicrobial sensitivity of the *Pasteurella* and *Mannheimia* isolates should be tested and a suitable antibiotic should be chosen on the basis of the *in vitro* sensitivity test (Radostits *et al.*, 1994; Biberstein and Hirsh, 1999; Kehrenberg *et al.*, 2001). The use of long-acting antibiotics in the face of an outbreak is common approach to prevent pasteurellosis in small ruminants. Animals at risk of pasteurellosis are given long acting oxytetracycline intramuscularly at dose rate of 20mg/kg body weight and feeding of broad-spectrum antibiotics, especially tetracycline, to lambs in feedlot, can be used for preventing pneumonia in recently weaned lambs (Gilmour and Appleyard, 1989; Radostits *et al.*, 1994).

2. 5. 3. Immunoprophylaxis

There has been considerable activity in the development of effective vaccine for the control of pasteurellosis for sheep. Two different approaches have been exploited: First is the use of *pasteurella* vaccine containing the serotypes most commonly seen in a given locality, the second other approach is to control by vaccination of lambs with parainflueza-3 virus vaccine (Radostits *et al*., 1994). Killed vaccines; from locally isolated *P. multocida* types A and D and *M. haemolytica* and *P. trehalosi* in oil adjuvant are widely used for prevention of pneumonic pasteurellosis of ruminants. Such vaccines were found to be effective in prevention of the natural disease caused by homologous strains (Mosier, 1993). Currently in Ethiopia, inactivated monovalent killed *P. multocida* type A vaccine is being used for the control and prevention of pasteurellosis in small ruminants (Gelagay *et al*., 2004).

2. 5. 4. Immunity

Colostral immunity in lambs appears to be fairly lasting for approximately 4-5 weeks (Gilmour and Gilmour, 1989). However, the duration of immunity following natural infections has not been investigated adequately (Mosier, 1993). Immunity to *Pasteurella* and *Mannheimia* organisms is thought to be predominantly humoral and may be present up to six months (Carter and Chengappa, 1991).

2. 6. Status of sheep pasteurellosis in Ethiopia

In Ethiopia, a number of attempts have been made so far to identify the different serotypes of both *Mannheimia* and *Pasteurella* spp. in small ruminants. Recent work on pasteurellosis of small ruminants has demonstrated different species and serotypes of, *M. haemolytica* and *P. trehalosi* such as A1, A2, A5, A6, A7, A8, A9, A12, A11, A13, A14 and T3, T4, T10 and T15. Of which A1, A2, A8, A7, T3 and T4 are the dominant serotypes (Gelagay, 1996; Tesfaye, 1997; Aschalew, 1998; Mekonen, 2000; Mesele, 2005). The list of serotypes/ species of *Pasteurella* and *Mannheimia* and their distribution are given in table 4. Gelagay (1996) reported prevalence of *M. haemolytica* serotype A2 (36%) and A8 (35%) in North Shoa zone of Amhara region. Mesele (2005) reported 1.1%, 5.6% and 25% prevalence of *P. trehalosi*, *M. haemolytica* and *P. multocida,* respectively from sheep slaughtered at Debre Zeit ELFORA export abattoir. In Arsi, Mekonen (2000) reported 85%, 12% and 3% prevalence of *M. haemolytica*, *P. trehalosi* and *P. multocida,* respectively.

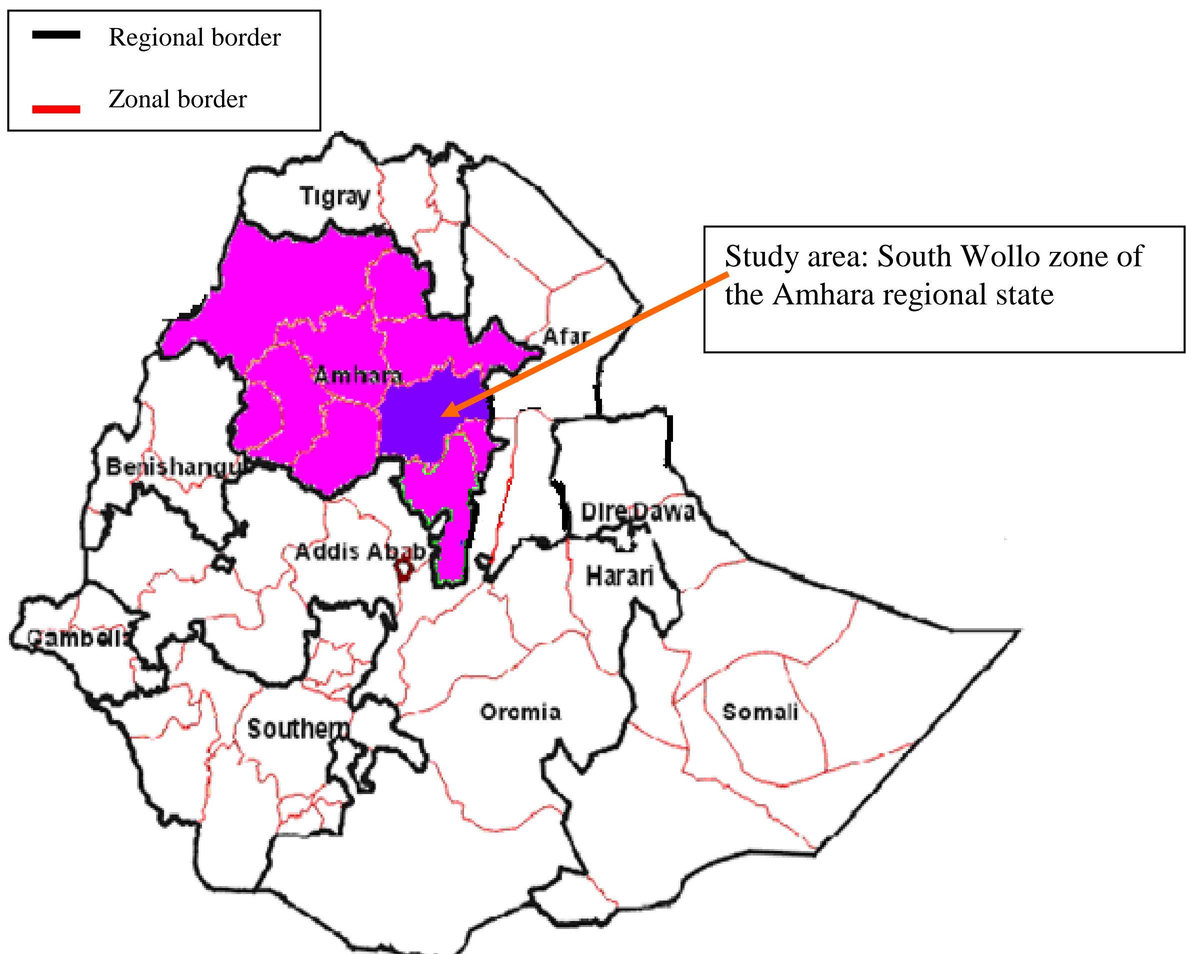
Table 4. Showing the isolations and serotypes of *Mannheimia* and P*asteurella* serotypes made in different regions of Ethiopia in small ruminants

Isolated species	Serotypes	Place	Authors
M. haemolytica	A1, A2, A5, A6, A7, A8, A11, A13 and A14	N. Shoa	Gelagay (1996)
M. haemolytica *P. trehalosi*	A1, A2, A5, A6, A7, A8, A11 and A12 T3, T4 and T10	Wollo	Tesfaye (1997)
P. haemolytica *P. trehalosi*	A1, A2, A5, A6, A7, A8 And A12. T3, T4 and T10	N. Shoa	Aschalew (1998)
M. haemolytica *M. trehalosi*	A1, A2, A5, A7, A8, A9, A11, A12 and A13 T15	Arsi	Mekonen (2000)
M. haemolytica *M. trehalosi*	A1, A2, A5, A6, A7, A8 and A12. T3, T4, T10 and T15	Debre Ziet ELFORA (Abattoir)	Mesele (2005)

3. MATERIALS AND METHODS

3. 1. Study area

The present study was conducted in sheep rearing highland woredas of South Wollo zone of the Amhara National Regional State (Fig.1). The altitude of the area ranges from 2400 to 2600 meter above sea level and experiences a bimodal rainfall pattern with a short rainy season from February to March and the long rainy season, which starts at the end of June and ends at the end of September. The annual rainfall of the area is ranged from 850 to 1100 mm and the mean annual minimum and maximum temperature are 7.8 ^{o}C and 21 ^{o}C, respectively (NMSA, 2005). The main farming system is mixed farming and sheep are the predominant animal species kept in the area.



Source: CSA, (2003)

Figure 1. Political map of Ethiopia showing Administrative Regions and zones

3.2. Study animals

The study animals were indigenous (local) highland Wollo fat-tailed sheep of all age and sex groups kept under extensive management system. The sheep population of the study woredas showed in table 5

Table 5**.** Showing the sheep population from different woredas of South Wollo zone

Woredas	Sheep population
Dessie Zuria	2,27,943
Kutaber	72,077
Tehuledere	29,881
Kalu	43,125
Legambo	2,02,617
Total	5,75,643

Source BoARD, (2004)

3. 3. Study design

The type of the study was cross-sectional, with simple random sampling technique, which was conducted from September 2006 to April 2007 to establish the prevalence of *Pasteurella trehalosi*, *Pasteurella multocida* and *Mannheimia haemolytica* isolated from the respiratory tract of sheep in major sheep rearing woredas of South Wollo zone of the Amhara National Regional State and at Dessie municipal abattoir, and to determined the serotype prevalence of *Mannheimia haemolytica* from serum samples of sheep collected in the study woredas (Dessie Zuria, Kutaber, Tehuledere, kalu and Legambo). The desired sample size for the study was calculated using the following formula given by Thrusfield (2005).

$$n = \frac{1.96^2 . P_{ex} (1 - P_{ex})}{d^2}$$

Where: n = required sample size

P_{ex} = expected prevalence

D = desired absolute precision

1.96 = z-value for the 95% confidence level

The prevalence of *Mannheimia* and *Pasteurella* serotypes in sheep in Northeast Ethiopia was reported to be 15%. Therefore, at 95% confidence level, 5% precision and 15% expected prevalence, 195 animals were examined clinically, at postmortem and samples collected.

3. 4. Sample collection

3. 4. 1. Nasal swabs collection

Nasal swabs were collected from randomly selected sheep. Before collecting the swabs, the nostrils of the animals were well cleaned with cotton wool soaked in 70 % ethyl alcohol. Then sterile cotton-tipped swabs in screw-capped test tube moistened with tryptose Soya broth (Oxoid, Hampshire, England) were inserted into the nostrils of each sheep, and the mucosa surface rubbed by rotating the swabs. The swabs were then placed back into 3 ml of sterile tryptose Soya broth (Oxoid, Hampshire, England) in universal tubes and transported packed in ice to the laboratory after collection.

3. 4. 2. Tissue samples collection

Pneumonic lungs and tonsils were collected from randomly selected slaughtered sheep at Dessie municipal slaughterhouse in the months of November, December and January. Piece of affected part of lung, and simultaneously tonsils from the corresponding animals were taken after close inspection and put into separate sterile containers and transported to the laboratory in cool box (Sisay and Zerihun, 2003).

3. 4. 3. Serum samples collection

Blood samples for serum extraction were collected directly from jugular vein using sterile needles and plain vaccutainer tubes from randomly selected sheep flock. Up to 5-8 ml of blood was withdrawn and the tubes left to stand in inclined position over night at an ambient temperature to allow clotting, and the sera were collected using sterile Pasture pipette and transferred to sterile testes tubes, labeled and stored at -20 ^oC in deep freezer until they were processed.

3. 5. Bacteriological examination

In the laboratory nasal swabs were incubated immediately at 37 ^oC for 24 hours. Where as lung and tonsil samples were disinfected with 70 % alcohol and dried, and the samples were cut into pieces with sterile scissors assisted by tongue forceps and put into 3 ml of tryptose Soya broth (Oxoid, Hampshire, England) in universal tubes. The universal tubes were loose capped and incubated at 37 ^0C for 24 hours. After 24 hours incubation of the nasal swabs, lung tissues and tonsil samples in tryptose Soya broth (Oxoid, Hampshire, England), a loop full of culture was transferred onto blood agar (Titan Biotech Limited, Bhiwadi) containing 7% difibrinated sheep blood and MacConkey's agar (Titan Biotech Limited, Bhiwadi), and then streaked by inoculating loop. The plates were incubated at 37 ^0C for 24 hours, and after 24 hours incubation, blood agar plates examined for the presence and type or absence of haemolysis and general appearance of the colonies including colour, shape, size and contour. The colonies suggestive of *Pasteurella* and *Mannheimia* were selected and smears were stained with Gram's staining and microscopically examined under oil immersion. Gram-negative, coccobacilli, short rods with or without bipolar staining were subjected for oxidase, urease, catalase, indole and H_2S production tests. Colonies, which were oxidase positive, urease negative, indole negative/positive, catalase positive/negative and yellow (acid) slant, yellow (acid) butt. , H_2S negative in TSI agar were subjected for further biochemical tests.

The growth on MacConkey's agar (Titan Biotech Limited, Bhiwadi) was examined for the presence or absence of growth and lactose fermentation as indicated by pink coloured colonies and non-lactose fermentation by absence of pink colour and general appearance of colonies. Accordingly colonies were grouped as lactose fermenters (LF) and non-lactose

fermenters (NLF). Plates with *Pasteurella* and *Mannheimia* like colonies were kept for further biochemical testing whereas mixed colonies were further sub-cultured.

3. 6. Biochemical characterization

After primary characterization and oxidase, urease, indole, catalase and H_2S test of the isolates, biochemical characterization of the organisms was preformed based on Glucose, lactose, maltose, salicin, sucrose, trehalose and xylose and nitrate reduction tests. *Mannheimia haemolytica* isolates were selected on the basis of xylose and lactose fermentation and lack of fermentation of trehalose and salicin and were catalase positive. Where as, isolates of *Pasteurella trehalosi* utilised only trehalose and salicin and were catalase negative. *Pasteurella multocida* were selected on the basis of indole production, nitrate reduction, characteristics sweetish odour and absence of growth and haemolysis on MacConkey and blood agar, respectively.

3.7. Serotyping

Sera were serotyped for *M. haemolytica* using the indirect haemagglutination (IHA) test introduces by Biberstein (1978) for serotyping *Mannheimia haemolytica.* Serotping was conducted using capsular extract antigen. Briefly, Capsular antigen was extracted from a 24 hr culture of bacteria of known serotypes in tryptose Soya broth, which was inactivated in a water bath at 60 ^{0}C for 30 minutes, and centrifuged at 3000 rpm for 30 minutes; the clear supernatant was collected into sterile test tubes to be used as capsular extract antigen. Fresh sheep blood was collected in Alsever's solution at proportion of 3:5. The suspension was centrifuged at 2500 rpm for 5 minutes, washed twice with phosphate buffer saline solution (PBSS), and again centrifuged at 2500 rpm for 5 minutes. For sensitisation of the sheep red blood cells (RBC), 50µl of packed RBC were added to 5 ml of capsular extract antigen, and then 50 µl of glutaraldehyde was added and homogenized with gentle shaking, incubated for 1 hr at 37 ^{O}C. After incubation the suspension was centrifuged and washed twice with PBSS. Finally, the pellet was adjusted with PBSS to give a1% suspension of RBC. In v bottomed micro-plates 50 µl of PBSS were added to all wells and 50µl of test sera to the first column and serially diluted by pipetting 50µl up to column 12. Fifty microliters of sensitised RBC were added to each well and incubated for one hour at 37 ^{O}C. Results were recorded based on

complete or more than 50% an agglutination seen in each well. The titre showing 1/40 dilution and above were taken as positive.

3. 8. Data analysis

The collected data were organized and arranged using the Microsoft Excel spreadsheet computer programme and analysed using SPSS software version11.5. Prevalence of *Pasteurella* and *Mannheimia* isolates was analysed using percentages and serotype distribution compared using percentages and mean percentages. The relation between incidence of pneumonic lungs and *Pasteurella* and *Mannheimia* isolates was analysed by coefficient of correlation**.**

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4. RESULTS

4. 1. Morphology

Pasteurella and *Mannheimia* isolates were revealed different morphological features on smear made from fresh isolate cultures. The isolates were Gram-negative, short ovoid rods with an occasional tendency to bipolar staining. Cells arranged in chain were also observed.

4. 2. Cultural characteristics

The *Mannheimia haemolytica* and *pasteurella trehalosi* revealed moist, smooth greyish, odourless and haemolytic colonies on blood agar, while *P. multocida* revealed round, greyish, non-haemolytic occasionally mucoid colonies. All the isolates of *Mannheimia haemolytica* and *pasteurella trehalosi* were grown on MacConkey's agar and showed pink to red small pinpoint colonies. None of P. *multocida* isolates showed growth on MacConkey agar.

4.3. Biochemical test Characterization

A total of 71 isolates from nasal swabs tonsils and pneumonic lungs were identified using biochemical tests. All the isolates were positive for oxidase and negative for urease indole and H_2S production but the *P. multocida* isolates were positive for indole production. All the isolates were able to utilise glucose fermentatively. Bacteriological and biochemical test results are shown in table 6.

Table 6. Showing the summary of biochemical tests for *M. haemolytica*, *P trehalosi* and *P multocida.*

Type of test	Type of species			Total positive
	M. haemolytica	*P. trehalosi*	*P. multocida*	
Haemolysis on blood agar*	30	36	-	66
Growth on MacConkey agar	30	36	-	66
Distinct odour	-	-	5	5
Oxidase	30	36	5	71
Catalase activity	30	-	5	35
Indole production	-	-	5	5
Urease activity	-	-	-	-
H_2S production in TSI Slant	-	-	-	-
Nitrate reduction	30	36	5	71
Glucose	30	36	5	71
Sucrose	30	36	5	71
Maltose	30	36	3	69
Lactose	30	-	-	30
Salicin	-	36	-	36
Xylose	30	-	-	30
Trehalose	-	36	-	36

* n = 71 for each test

4.4. Bacteriological findings

A total of 71 samples out of 270 were positive for *M. haemolytica*, *P. trehalosi* and *P. multocida.* (Table 7). The percentage rate of recovery of *P. trehalosi* varied from the nasal swabs (12.5 %) and tonsils (16 %), whereas 20 % of *M. haemolytica* was isolated from pneumonic lungs, while *Pasteurella multocida* was the least isolated species (1.8 %) from both nasal swabs and abattoir specimens (lungs and tonsils). The overall isolation rate of *M. haemolytica* and *P. trehalosi* from nasal swabs and abattoir specimens were constituted 11.1% and 13.4%, respectively. There was no significant deference ($P > 0.05$) between isolates of *M. haemolytica* and *P. trehalosi* from sampled sheep. Both species were isolated almost equally in the study area.

Table 7. Samples showing positive for isolation of M. *haemolytica*, *P. trehalosi* and *P. multocida* from the respiratory tract of sheep.

Type of samples	No samples processed	Positive	Species identified		
			M. haemolytica	*P. trehalosi*	*P. multocida*
Nasal swab	120	27	9 (7.5 %)	15(12.5%)	3 (2.5%)
Tonsil	75	19	6 (8 %)	12 (16 %)	3 (1.5 %)
Lung	75	25	15 (20 %)	9 (12 %)	1 (1.5 %)
Total	270	71	30 (11.1%)	36 (13.4%)	5 (1.8 %)

From the total isolates *Mannheimia haemolytica, P. trehalosi* and *P. multocida* showed 9 (33.3%), 15 (55.5%) and 3 (11.1%) positive in nasal swabs respectively. Out of these isolates, 9 (33.3 %) and 18 (66.7 %) were isolated from apparently healthy and moribund sheep with respiratory syndrome, respectively (Table 8.). There was high significant difference ($P<0.001$) between isolates from nasal swabs of healthy and moribund sheep with respiratory syndrome.

Table 8. The percentage of positives from nasal swabs for *M. haemolytica*, *P. trehalosi* and *P. multocida* from the healthy and moribund sheep.

Status of sheep	*No of animal sampled*	*Isolated species*		
		M. haemolytica	*P.trehalosi*	*P. multocida*
Healthy sheep	65	3 (33.3%)	4 (44.5%	2 (22.2%)
Sick sheep	55	6 (33.3%)	11 (61.1%	1 (5.6%)
Total	120	9 (33.3%)	15 (55.5%	3 (11.21%)

For the purpose of *Mannheimia* and *Pasteurella* isolation, pneumonic lungs and the same number of corresponding tonsils were examined from slaughtered sheep at Dessie municipal abattoir for three months, November, December and January. Twenty-five pneumonic lungs and corresponding tonsils were sampled each month. Monthly isolation rates of *Mannheimia* and *pasteurella* spp from pneumonic lungs and corresponding tonsils are presented in table 9 and 10. It was observed that the isolation rates of *Mannheimia* and *Pasteurella* species from pneumonic lungs and tonsilar tissue were higher (44 %) and (40 %) in the month of December, and lower (22 %) and (16 %) in the month of January, respectively. There was significant difference ($P < 0.01$) between isolation rates of *Mannheimia* and *Pasteurella* species in the study months.

Table 9. Monthly wise distributions of pneumonic lungs showing positive for *Mannheimia* and *Pasteurella* collected from slaughter house, Dessie abattoir.

Month of sampling	Pneumonic lungs sampled	Isolated species			No of total isolates
		M. haemolytica	*P. trehalosi*	*P .multocida*	
November	25	4	4	0	8 (32)
December	25	7	3	1	11 (44)
January	25	4	2	0	5 (22)
Total	75	15	9	1	25 (33.3)

Table 10. .Monthly wise distributions of tonsilar tissue of sheep showing positive for *Mannheimia* and *Pasteurella* collected from slaughter house, Dessie abattoir

Month of sampling	*Tonsils*	*Isolated species*			*No of total isolates*
		M. haemolytica	*P. trehalosi*	*P. multocida*	
November	25	1	4	0	5 (20 %)
December	25	3	6	1	1 0 (40%)
January	25	2	2	0	4 (16 %)
Total	75	6	12	1	19 (25.3 %)

4. 5. Seroptyping

Sheep Sera collected from highland woredas of South Wollo zone of the Amhara regional state, namely, Dessie Zuria, Tehuledere, Kutaber, Kalu, and Legambo were serotyped using the indirect haemoagglutination (IHA) test as per Biberstein (1978) for serotyping of *M. haemolytica*. A total of 200 serum samples, forty sera from each woreda were examined for specific antibodies. The *M. haemolytica* serotype positives have been computed for each woreda (Table 11). Variation in prevalence among the different serotypes was observed (P<. 0.001). In dessie Zuria, serotype A2 (62,5%) followed by A5 (50%) and A6 (45%), in Tehuledere A11 (25.5%), A2 and A5 (22.2 %) each, in Kutaber A2 (50%), A6 (47.5 %) and A5 (40%), in Kalu A11 (47%), A2 (25%) and A5 (22.5%) and in Legambo, A2 (37.5%), A11 (32.5%) and A5 (22.5%) were the dominant serotypes detected. On the other hand, A13, A14 and A9 were the least identified serotypes in most of the study woredas.

Table 11. Prervalence distribution of *M. haemolytica* serotypes in sheep sera in different woredas of South Wollo Zone

M. haemolytica *serotypes*	*Seropositive (%) at dilution ≥1/40*				
	Dossier Zuria (n = 40)	Tehuledere (n = 40)	Kutaber (n = 40)	Kalu (n = 40)	Legambo (n = 40)
A1	7 (17.5)	3 (10)	14 (35)	9 (22.5)	2 (5)
A2	25 (62.5)	9 (22.5)	20 (50)	10(25)	15 (7.5)
A5	20 (50)	9 (22.5)	16 (40)	9 (22.5)	13 (32.5)
A6	18 (45)	1 (2.5)	19 (47.5)	6 (15)	10 (25)
A7	6 (15)	5 (12.5)	12 (30)	8 (20)	9 (22.5)
A8	7 (17.5)	6 (15)	4 (10)	6 (15)	7 (17.5)
A9	4 (10)	1 (2.5)	3 (7.5)	3 (7.5)	9 (22.5)
A11	7 (17.5)	10 (25)	6 (15)	19 (47.5)	13 (32.5)
A12	4 (10)	9 (22.5)	7 (17.5)	5 (12.5)	9 (22.5)
A13	3 (7.5)	4 (10)	3 (7.5)	1 (2.5)	2 (5)
A14	5 (12.5)	1 (2.5)	3 (7.5)	1 (2. 5)	1 (2.5)

The overall prevalence rates and proportionate of each of *M. haemolytica* serotype in highlands of South Wollo zone of the Amhara region were computed and the results are shown in table 12. All tested *M. haemolytica* serotypes have shown positive agglutination reaction with numbers of sheep sera, but variations in prevalence among the different serotypes were observed (P<0.001). Serotype A2 (34.5 %), followed by A5 (33.5 %), A11 (27.5%) and A6 (27%) were the dominant serotypes identified, while A9 (8.5 %), A13 (6.5 %) and A14 (5.5 %) were the least among the eleven serotypes examined.

Table 12. Percentage prevalence and distribution of *M. haemolytica* serotypes in highland Wollo sheep

M. haemolytica *serotypes*	*Positive at dilution (n=200)*			*Total positive at≥1:40*	*Percent (%) positive at dilution*		*Geometric mean positive*	
	1/20	1/40	1/80		1/20	1/40	1/20	≥1/40
A1	39	23	13	36	37.5	18	31	50
A2	47	39	30	69	58	34.5	36	54
A5	40	33	34	67	53.3	33.5	38	57
A6	41	25	29	54	45.5	27	37	58
A7	38	23	23	46	42	23	35	56
A8	26	19	11	30	28	15	33	51
A9	10	8	9	17	13.5	8.5	39	57
A11	8	36	19	55	31.5	27.5	45	51
A12	42	20	11	31	36.5	15.5	29	51
A13	16	7	6	13	14.5	6.5	31	55
A14	11	8	3	11	11	5.5	31	48

Among the eleven *M. haemolytica* serotypes identified, the proportion of dominant serotypes, A2, A5, A11 and A6 accounted for 66.6 % of the total positive sera (Fig. 2)

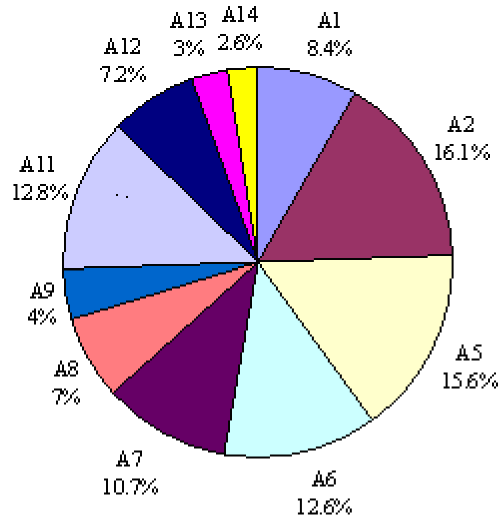

Figure 2. Proportionate percentage isolation and distribution of *M. haemolytica* serotypes in sheep in woredas of South wollo

Out of eleven *M. haemolytica* serotypes identified from sheep sera in highland woredas of South Wollo zone of the Amhara region, serotype A6 (1/58), followed by A5 and A9 (each 1/57) had shown the highest mean antibody titre. Whereas, serotype A14 with mean positive antibody titre1/48 was the least. Serotype A11 with 1/51 means positive antibody titre was the least among the dominant serotypes (Fig 3).

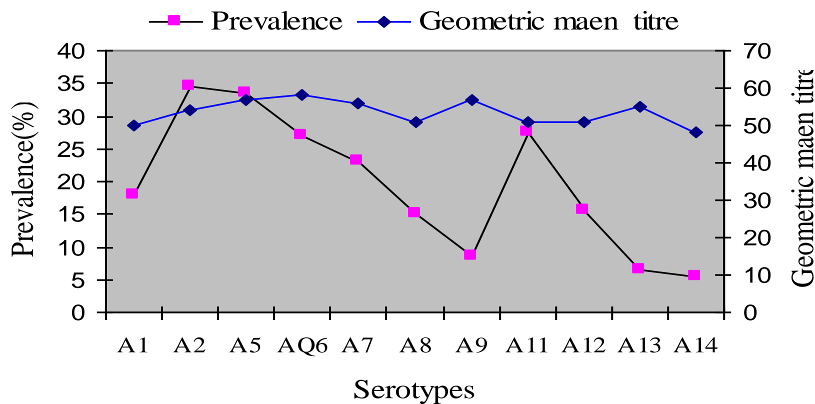

Figure 3.Prevalence and geometric mean antibody titres against serotypes of *M. haemolytica* in sheep sera

5. DISCUSSION

Pasteurellosis in sheep caused by *M. haemolytica* and *P. trehalosi* have posed health problem in most part of sheep breeding and rearing regions of Ethiopia due to the significant economic losses they causes through mortality, morbidity, and the high cost of treatment (Gelagay, *et al*., 2004).

In this study, an attempt was made to differentiate between *M. haemolytica*, *P. trehalosi* and *P. multocida* based on their growth on MacConkey agar, haemolytic pattern, Oxidase and Catalase activity, Indole and H_2S production, colony morphology and fermentation of different sugars with acid production (Quinn *et al.,* 2002).

The aetiological agent of pasteurellosis in sheep was found to be wide spreading in the study area. Though there were little variation in biochemical characteristics, the results of morphological, staining, colony, cultural and biochemical activities were in total agreement with those documented by Merchant and packer (1983), Carter and Chengappa (1991), Carter (1994) and Quinn *et al* (2002). The haemolytic activity of *M haemolytica* and *P. trehalosi* isolates were lost after subsequent subcultures and were in agreement as reported by Carter and Chengappa (1991), Quinn *et al.* (2002). Two distinct colony types were observed on MacConkey agar, lactose fermenter (*M. haemolytica*) with pink and non-lactose fermenter *(P. trehalosi*) other than pink colour. Whereas the *P. multocida* isolates neither grew on MacConkey agar nor haemolysed sheep red blood cells as observed by Carter and Chengappa (1991) and Quinn *et al* (2002).

The isolates of *M haemolytica, P. trehalosi* and *P. multocida* were positive for oxidase test and negative for urease, H_2S production in TSI agar slant, and Indole test. Further they utilized glucose fermentatively, but the *P. multocida* isolates were indole positive which coincided with those described by Quinn *et al* (2002*).* The *Mannheimia haemolytica* isolates fermented xylose and lactose, but failed to ferment trehalose and salicin, and were catalase positive, whereas isolates of *P. trehalosi* were fermented trehalose and salicin, and were catalase negative the results are in agreement with Carter (1984) and Quinn *et al* (2002)

In the present study, *M. haemolytica and P. trehalosi* were isolated at the rate of 7.5 % and 12.5% from nasal swabs, 8 % and 16 % from tonsils and 20 % and 12 % from pneumonic

lungs, respectively. Isolation of *M. haemolytica* and *P. trehalosi* in pneumonic lungs may indicate that these species are important species in the induction of pneumonic pasteurellosis in the study area. The low percent of (1.8%) P. *multocida* isolates might indicate the occasional involvement of this species in the pneumonic pasteurellosis and similar reports have been made by Merchant and Packer (1983) Carter and Chengappa (1991), Aschalew (1998), Mekonen (2000), Sisay and Zerihun (2003). In contrary to our observation Assefa *et al* (2004) and Mesele (2005) reported high incidence rates of 15.4% and 25% of P. *multocida* from sheep slaughtered at Jijiga and ELFORA abattoir respectively. This might be due to the time of sampling and geographical variation, where sampling was conducted in June and sampled sheep were from Somali lowlands where very poor Veterinary infrastructure and vaccination against pasteurollosis (*P. multocida* biotype A) was not conducted for considerable number of years, and animals were transported long distance before being slaughtered at both abattoir, whereas, our study was conducted in highlands of Wollo, where regular vaccination was done in sheep with monovalent *P. multocida* vaccine which is attributed to the low incidence of infection.

The difference in the predominance between *P. trehalosi* and *M. haemolytica* isolates from tonsils and lungs was observed. The difference in isolation rates of *P. trehalosi* and *M. haemolytica* 16% and 8% from tonsils and 12 % and 20% from lungs, respectively may indicate that a constant competition which coexists between these species, influenced by strain, serotype and environmental factors(Lopez,2001)

From total isolates of nasal swabs of moribund sheep with respiratory syndrome, *M. haemolytica* and *P. trehalosi* constituted 33 % and 55.5% in their proportion, respectively. This result is in agreement with the findings of Tesfaye (1997) for the same area and Aschalew (1998) in highlands of central Ethiopia, where they reported 28.8 % and 35 % for *M. haemolytica* and 42 % and 58.3 % for *P. trehalosi*, respectively from nasal swabs of sheep with pneumonic symptoms. The high proportion of *Mannheimia* and *Psteurella* spp in nasal swabs of moribund sheep could be due to these organisms are normal inhabitants of the upper respiratory tract of sheep and invasion to the lower tissue (lung) of moribund sheep when the immune system of the animals is compromised by different factors (Lopez, 1995) and pneumonia associated with these bacteria species recorded as primary diseases in sheep elsewhere (Shewan, 1986; Gilmour, 1993).

Monthly isolation rate of *Mannheimia* and *Pasteurella* species from pneumonic lungs and tonsilar tissues in slaughtered sheep at Dessie abattoir varied from month to month. The incident of pneumonic pasteurellosis caused by *M. haemolytica, P. trehalosi* and *P. multocida* was higher (44%) in the month of December and lower (22%) in the month of January. There was significant difference (P< 0.01) between the study months. High prevalence rate of pneumonic pasteurellosis in slaughtered sheep in the month of December may be due to high stress full conditions on sheep population (Yates *et al*., 1983; Dungworth, 1992; Radostits *et al*., 1994; Kehrenberg *et al*., 2001; Lopez, 2001) were night temperature fall bellow 7 ^{O}C, chilly and cool in month of December. On the other hand the prevalence of pneumonic pasteurellosis in slaughtered sheep in the study months was lower (33.3%) than the values (58.8%) and (56 %) previously reported by Tesfaye (1997) and Mekonen (2000) in highlands of Northeast Ethiopia and Arsi, respectively. This may be due to expansion of animal health extension and veterinary services and the intensification of near by private veterinary drug shop, which enable the farmers (animal owners) to have an access to control the most prevalent animal diseases in general and particularly the ovine pasteurellosis.

The serological results showed that the predominance distribution pattern of *M. haemolytica* serotypes between the woredas were almost similar. This might be due to similar agro climatic and animal management systems and all woredas are located between 2400 and 2600 metre above sea level and practice crop- livestock farming, where sheep share grazing areas freely with other animal species.

The results of IHA test were tabulated to know the overall prevalence and distribution of *M, haemolytica* serotypes in the highlands of South Wollo zone. The prevalence of each serotype was varied (P<0.001). Of 200 sheep sera tested, A2 (34, 5 %), A5 (33.5%), A11 (27.5%) and A6 (27%) were the dominant serotypes and A8 (15%), A9 (8.5 %), A13 (6.5 %) and A14 (5.5 %) were the least detected. The result of this study is in agreement with the reports of Pegram *et al* (1979) in Ethiopia the most prevalent serotype was A2, while A14 and A9 were the least identified. Hussein and Mohammed (1984) in Sudan identified the predominant A2 and A6 serotypes and A13 and A14 were among the lowest identified serotypes. Frank (1982) in the United States, Fodor and Varga (1988) In Hungary, Prince *et al* (1985) and Gilmour and Gilmour (1989) in New Zealand identified A2 as the most prevalent serotype. Aschalew (1998) in central highland of Ethiopia A2, A6 and were the most dominant ones, while A8 was the least screened serotype and Kirkan and Kaya (2005) and Lhan and Keles (2007) in

Turkey identified A2 and A6 as most prevalent and A8 was the least identified. On the other hand, the present work differs from that of Bekele (1996) and Sisay and Zerihun (2003) who reported that 29 % and 16.9 % prevalence for A1, in East and Northeast Ethiopia, respectively, which was the most prevalent serotype and the vast deference might be due to geographical location, the time of sampling, serotypes involved in pasteurellosis which varied from year to year, area to area and flock to flock (Gilmour and Gilmour, 1989).

6. CONCLUSIONS AND RECOMMENDATIONS

In the highlands of South Wollo zone, sheep are the most kept animal than other species and plays an important role in the economy and livelihood of farmers. These small ruminants serve as a principal source of cash income for household expense as well as domestic consumption. However, efficient utilization of this resource is impaired by different factors, such as health problem, poor management and shortage of feed.

Pasteurellosis in sheep caused by *M. haemolytica* and *P. trehalosi* has become an important health problem in sheep in the present study area.

Both bacterial and serological results of this study showed that the causal agents of pasteurellosis are prevalent in the area, and serotypes A2, A5, A6 and A11 were dominant over the other serotypes.

The current pasteurellosis vaccine, which is used for the control and prevention of pneumonic pasteurellosis, has not included causative agents in the field apart from the strains of *M. haemolytica* which are incriminated in causing pasteurelosis. On the basis of the results obtained the following are recommended**.**

- The epidemiological feature of pasteurellosis should be studied for better understanding of the problem.
- Further organized investigation should be taken for the determination of the magnitude and the distribution of the various serotypes of *Mannheimia* and *Pasteurella* in different parts of the country.
- Developing a polyvalent vaccine consisting of the most predominant serotypes of *Mannheimia haemolytica* which are circulating in sheep in the study area is of paramount importance.

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8. ANNEXS

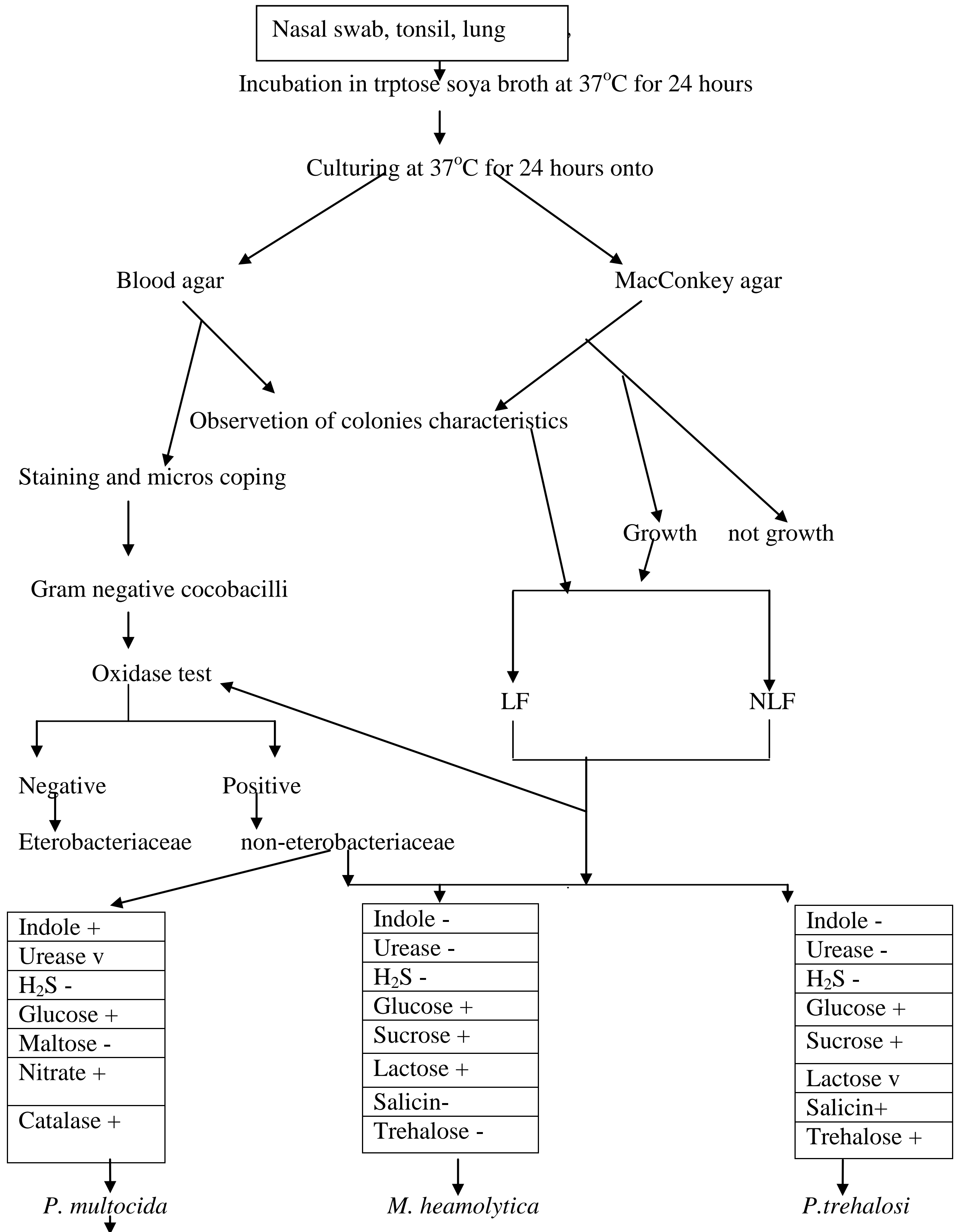
8.1. Sample collection format.

No	Sampling date	Sampling site(woredas)	Animal species	Sex	age	Health status	Type of sample

8.2. Bacteriological and biochemical diagnosis results format

No	Type of samples	Growth on McConkey agar	Haemolysis on blood agar	Odour	Oxidase test	Indole test	Catalase activity	Urease test	H_2S production.	Nitrate reduction	Xylose fermentation	Maltose fermentation	Sucrose fermentation	Glucose fermentation	Lactose fermentation	Salicin fermentation.	Trehalose fermentation.	Species

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



8. 4. Gram's stain Procedure

Make a thin smear or film

Allow the film to dry in air

Fix the film by passing through the Bunsen flame several times

Flood the slide with crystal violet for 60 seconds

Pour of the stain and wash in water add gram's iodine solution allow it act for 60 seconds

Wash off the iodine and wash with water

Decolorize with acetone alcohol till the primary stain is removed.

Counter stain with Safranin for 30 seconds and wash with water

Air dry observe under oil immersion

Interpretation: Gram-positive bacteria appear as violet, while Gram negative appears as pink.

8. 5. Deferent biochemical tests used to identified *Pasteurella* and *Mannheima* Species

8. 5.1. Catalase test

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

Procedure: A loop full of the bacterial growth is taken from the colonies of nutrient agar medium. The bacterial cells are placed on a clean microscope slide and a drop of 3 % H_2O_2 is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.

8.5.6. Oxidase test

Principle: The cytochrome oxidase enzyme is able to oxidize the substrate tetramethyl-p-phenylenediamine dihydrochloride, forming a colored end product, indophenol.

Procedure: Prepare a solution of 1 % tetramethyl-p-phenylenediamine hydrochloride, then a piece of filter paper is moistened in a Petri dish with fresh reagent and the test bacterium is streaked firmly across the filter paper with a glass rod. A dark purple colour along the streak line with in 10 seconds indicates a positive reaction.

8. 5.7. Indole test

Principle: Indole positive bacteria possess an enzyme tryptophanase which converts tryptophan to indole.

Procedure: Stab inoculate SIM medium with test bacterium and incubate at 37 ^{O}C for 18 to 24 hours. Then add Kovac's reagent (0.2 ml) to tube and stand for 10 minutes.
Interpretation: The formation of dark red ring indicates positive reaction while in negative reaction a yellow ring is formed.

8.5.8. Nitrate reduction test

Principle: Nitrate reducing bacteria possess an enzyme nitrate reductase which reduces nitrate to nitrite.
Procedure: The pure culture of organism to be tested is inoculated in to nitrate broth and incubate at 37 ^{O}C for 12-24 hours. Then add several drops of reagent "A" and "B" (A=sulfanilic acid, B= naphthylamine).
Interpretation: The formation of red color after addition of the reagent A an B indicates positive reaction while in negative reaction there is no colour change.

8. 5. 9. Urease test

Principle: Urease is an enzyme possessed by many species of microorganism that can hydrolyze urea with the formation of ammonia (alkaline).
Procedure: The surface of the agar slant is streaked with the test organism and incubated at 37 ^{O}C for 18 to 24 hours.
Interpretation: Organisms that hydrolyze urea rapidly may produce positive reaction with in 1 or 2 hours. Red (pink) colour through out medium indicates positive reaction.

8. 6. Media used for isolation and identification of *Mannheimia* and p*asteurella* spp**.**

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

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

Preparation: Dissolve 30.0g in one liter of distilled water and distributed into final containers, sterilize by autoclaving at 121 ^{O}C for 15 minutes.

8. 6. 2. Blood Agar Base (Titan Biotech Limited, Bhiwadi)

Composition (g/950 ml): Heart muscle solids from infusion 2.0; Agar 15.0; Yeast extract 5.0; pancreatic digest of casein 13.0; sodium chloride 5.0:
Preparation: Add 40.0 g of the powder in 950 liter of distilled water. Dissolve by stringing the powder completely. Sterilize by autoclaving for 15 minutes at 121 ^{o} C. Cool the base to 45 to 50 ^{O}C and add 50 ml of sterile defibrinated blood and mix properly in to pour Petri dishes.

8. 6. 3. MacConkey Agar (Titan Biotech Limited, Bhiwadi)

Composition (g/l): Peptone 17.0; protease Peptone 3.0; lactose 10.0; bile salts 1.5; sodium chloride 5.0; neutral red 0.03; agar 15.0.
Preparation: Suspend 51.5g in 1 liter of distilled water. Dissolve by stringing the powder completely. Sterilize by autoclaving at 121 ^{O}C for 15 minutes. Cool to room temperature before use.

8. 6. 4. Triple sugar iron agar (Titan Biotech Limited, Bhiwadi)

Composition (g/l): Agar 12.0; peptone 10,0; tryptone 10.0; lactose 10.0; sucrose 3.0; beef extract 5.0; sodium chloride 1.0; dextrose 0.2; ferrous sulphate 0.3; phenol red 0.25. Preparation: Suspend 65g in 1 liter of distilled water and dissolve by stringing completely. Sterilize by autoclaving for 15 minutes at 121 ^{0}C. Dispense in to tubes and allow solidifying to give agar slants.

8. 6.5. Urease agar base (BBL®, Becton Dickinson, USA)

Composition (g/l): pancreatic digest of gelatin1.0; dextrose1.0; sodium chloride 5.0; potassium phosphate 2.0 urea 20.0 phenol red0.012.
Preparation: suspend 29g of powder in 100 ml of distilled water. Mix thoroughly and sterilize by filtration. Suspend 15g of agar 900 ml distilled water, autoclave at 121°C for 15 minutes, cool to 50 ^{O}C and add 100 ml of filter sterilized urea agar base. Mix thoroughly and dispense

aseptically in sterile tubes. Cool tubed medium in slanted position so that deep buts are formed.

8. 6. 6. Nutrient agar (Oxoid, Hampshire, England)

Composition (g/l): "Lab-Lemco" powder 1.0; yeast extract 2.0; peptone 5.0; sodium Chloride 5.0; agar; 15.0.
Preparation: Suspend 28g in 1 liter of distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121 °C for 15 minutes.

8. 6. 7. Brain Heart Infusion (Difco, Becton Dickinson, USA)

Composition (g/l): Calf brains, infusion 200.0; beef heart infusion 250.0; proteose Peptone 10.0; dextrose 2.0; sodium chloride 5.0; disodium phosphate 2.5
Preparation: Dissolve 37.0g of the powder in 1liter of distilled water, Autoclave at 121°C for 15 minutes.

8.7. Reagents

8. 7. 1. Phasphate Buffered saline solution

NaCl	8.0g;
KCl	0.2g
$NaHPO_42H_2$	1.13g;
CaC	l 0.1g;
MgSO47H2O	0.76g
Distilled water	1000ml.

Adjust pH to 7.4 and autoclave at 121 °C for 15 minutes

2. Alsever's solution:

Dextrose	18.66g
NaCl	4.18g
Sodium citrate	8.0g
Distilled water	1000ml

Sterilized by steaming

9. CURRICULUM VITAE

A. Biographical Data:

Name	Belay Mulate
Date of birth	January 25 1963
Place of birth	Woldia, North Woll
Marital status	Married
Nationality	Ethiopian
Profession	Veterinarian
Occupation	Research officer in regional laboratory, Kombolca Laboratory

B. Educational background

1972/72-1976/77	Elementary School Etege Tayitu Elementary School, Woldia
1977/8-1981/82	Woldia Compressive Secondary High School Achievement: Ethiopian School Leaving Certificate Examination
1983/84-1988/89	University/Under graduate program Kharkov Zoo- Veterinary Institute Named after Borisenko, USSR Achievement: Doctor of Veterinary Medicine, DVM Degree

C. Work Experience

September, 1990-July, 2002	Wereda Veterinary Officer Woreilu, Tehuledere,Ambasel, Dessie Zuria (South wollo zone)
August, 2002-September, 2005	Department head, Kombolcha Regional Veterinary Laboratory

D. Research out put/Technical paper

a. Preliminary study on helminthosis of equine in South and North Wollo zones (2003)

E. Membership Member of the Ethiopian Veterinary Association

F. Language

Amharic	Mother Tongue
English	Writing and speaking
Russian Language	Writing and speaking

10. DECLARATION SHEET

The thesis my original work, has not been presented for a degree in any other university and that all sources of material used for the thesis have been duly acknowledged.

Name ___________________________________

Signature ___________________________________

Date of submission _____________________________

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

Prof. L. Muniyappa (BVSc., MVSc., PhD)

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