

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

**EXPERIMENTAL VACCINE DEVELOPMENT AND EFFICACY TRIAL AGAINST
CAPSULAR ANTIGEN OF *MANNHEIMIA HAEMOLYTICA* SEROTYPES A2, A7 AND
THEIR COMBINATION**

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

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LIST OF ABBREVIATIONS

ACIAR	Australian Centre for International Agricultural Research
ANOVA	Analysis of variance
CFU	Colony forming unit
CIRAD/EMVT	Center for International Cooperation in Agronomic Research for Development. Department of Animal and Veterinary Medicine
CP	Capsular polysaccharide
CSA	Central Statistics Agency
DNA	Deoxyribonucleic Acid
FAO	Food and Agricultural Organization
FEMS	Federation of European Microbiological Societies
GA - SRBC	Glutaraldehyde fixed sheep red blood cells
GDP	Gross Domestic Product
GM	Geometric mean
ID	Infectious dose
I-ELISA	Indirect Enzyme Linked Immunosorbent Assay
IHA	Indirect Haemagglutination
KDa	Kilo Dalton
LKT	Leukotoxin
LPS	Lipopolysaccharide
ml	Milliliter
mm	Millimeter
NBE	National Bank of Ethiopia
NCBI	National Center for Biotechnology Information
nm	Nanometer
NVI	National Veterinary Institute
OIE	Office International des Epizootes
OMP	Outer member protein
PAMPs	Pathogen- associated molecular pattern

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SPF	Specific pathogen free
SPSS	Statistical package for social science
SSE	Sodium salicylate extract
TSI	Triple sugar iron
USDS	United States Department of State

ABSTRACT

This experimental study was undertaken from December 2011 to May 2012 with the objective of determining the efficacy of adjuvated formalin killed experimental *Mannheimia haemolytica* vaccine prepared in this study from serotypes of this organism: serotype A2, A7 and combined serotypes (A2 and A7) for ovine pneumonic pasteurellosis. The efficacy was determined by using indirect haemagglutination (IHA) titer and challenge protection test. This experiment used 47 clinically healthy male lambs and grouped in to four treatment groups, group 1 with 14 lambs receive A2 serotype vaccine, group 2 consists of 13 lambs and vaccinated by serotype A7, the combined serotype vaccinated and the control are group 3 and 4, respectively and each consist of 11 and 9 lambs, respectively. Pre vaccination titer was measured from all lambs to assess their exposure and found that all lambs showed a moderate titer. This experimental vaccine was injected subcutaneously with a single dose to the vaccinated groups. All the vaccinated groups showed a significant higher IHA $\log_{10}$ titer at the 3rd and 4th week post vaccination than the controls. At the 3rd week a relative higher titer was observed in A7 and the combined serotype vaccinated groups having a GM antibody titer of $\log_{10}^{1.65}$ and $\log_{10}^{1.66}$, respectively. Similarly, at 4th week a higher titer was observed in A7 and combined serotype vaccinated groups having GM titer of $\log_{10}^{1.58}$ and $\log_{10}^{1.57}$, respectively. There was no significant difference in serotype A2 vaccinated groups at the 3rd and 4th week of post vaccination than A7 and the combined serotype vaccinated, but the IHA test result revealed lowered GM titer ($\log_{10}^{1.60}$ and $\log_{10}^{1.45}$) for the respective weeks. Post challenge clinical investigation showed that significant higher morbidity was seen in control group expressed by 1.5°C increase in rectal temperature and respiratory signs. Following intra tracheal challenge only 3 (15%) lambs from vaccinated group were found to have a +1 lung lesion score. 100% of the lambs in unvaccinated control group were found to have lung lesion scores between +2 and +3, but no significance difference between the different vaccinated groups. This experiment also found a moderately weak correlation ($r^2=0.314$) between antibody titer and extent of lung lesion score. Therefore, the tested experimental vaccines showed sufficient efficacy especially A7 and the combined serotypes.

Key words: *Mannheimia haemolytica*, lambs, challenge protection, indirect haemagglutination.

1. INTRODUCTION

The economy of Ethiopia is based on agricultural sector which contributes 40-50% of the Gross Domestic Product (GDP), over 90% of the foreign exchange earnings, and about 85-90% of the employment opportunities in the country (USDS, 2010). The majority of agricultural output is generated from crop and livestock integrated production systems. The livestock sub-sector alone contributes 12% of the total and over 45% of the agricultural GDPs (NBE, 2008). The country has one of the largest livestock inventories in Africa with livestock ownership currently supporting and sustaining the livelihoods of an estimated 80% of the rural poor (FAO, 2004). According to FAO (2004), the total annual meat production comes from cattle (63%), sheep (25%) and goats (12%). At the national level, sheep and goats accounts for about 90% of live animal/meat and 92% of skin and hide export trade value. However, the country has benefited little from this enormous resource and the real contribution of the sub-sector to the national economy is far below expectation (Abebe *et al.*, 2002). Owing to a multitude of problems disease being one of the most important and alone accounts for 30% and 20% mortalities in lamb and adults, respectively (Kassahun *et al.*, 1989).

Disease is a major constraint to improved productivity; small ruminants are particularly susceptible to respiratory infections mainly, by *Mannheimia haemolytica* (Gilmour and Gilmour, 1989; Brogden *et al.*, 1998). Enzootic pneumonia caused by *M. haemolytica* occurs worldwide in temperate, subtropical, and tropical climates (Gilmour and Gilmour, 1989).

Respiratory diseases are a great economic concern for sheep producers in different parts of Ethiopia. Outbreaks of respiratory diseases occur frequently, killing significant numbers of sheep. One of the potential respiratory diseases of sheep is pasteurellosis (Blood and Radostits, 1994; Biberstein and Thomson, 1996). Pasteurellosis is a complex disease that develops when the immune system of the animal is compromised by stress factors such as crowding, transportation, draught, and inclement weather. Concurrent respiratory infections and/ or infestations by *Chlamydia psittaci*, viruses (parainfluenza-3, reovirus, adenovirus, and respiratory syncytial virus), *Mycoplasma* species (*M. ovipneumoniae*, *M. arginini*, *M. agalactiae*, and others) and

lungworms (particularly *Dictyocaulus filaria*) can also suppress the animal's immune system, allowing opportunistic microorganisms such as *Mannheimia haemolytica* and rarely *P. multocida* A and D to colonize the lung and cause pasteurellosis (Gilmour and Gilmour, 1989; Blood and Radostits, 1994).

Due to the pathogenic nature, sporadic occurrence and widespread distribution of *Mannheimia* organisms control of pasteurellosis is particularly difficult and it is generally accepted that vaccination is the desired approach to prevention. However available vaccines in the early 1980s' were not providing effective protection against the range of different types of *M. haemolytica* or *Bibersteinia trehalosi* found in field cases (Donachie, 2003).

A reliable supply of pure, safe, potent, and effective vaccines is essential for maintenance of animal health and the successful operation of animal health programs. Immunization of animals with high quality vaccines is the primary means of control for many animal diseases. In other cases, vaccines are used in conjunction with national disease control or eradication programs. The approach to ensuring the purity, safety, potency, and efficacy of veterinary vaccines may vary from country to country depending on local needs. However, proper standards and production controls are essential to ensure the availability of consistent, high quality products for use in animal health programs. There is also growing public interest in the beneficial implications for animal welfare of the use of veterinary vaccines as a means of disease control. In any case, if vaccines are used, successful performance requires that should be produced in a manner that ensures a uniform and consistent product of high quality vaccine (OIE, 2008).

Vaccines containing different serotypes are used in the control of sheep pasteurellosis. For the vaccination of sheep, bacterins, live attenuated, leukotoxin (LKT), capsule, lipopolysaccharide (LPS), subunit vaccines comprised of different surface antigens, sodium salicilate extract and potassium thiocyanate extract vaccines were used (Donachie *et al.*, 1986; Gilmour *et al.*, 1991; Diker *et al.*, 2000). However, there are some differences between these vaccines concerning their protective efficacies. Researchers found that vaccines provide a good protection against homologous strains while they were insufficiently protective against heterologous ones (Chasdrasekaran *et al.*, 1991; Diker *et al.*, 2000). Several studies performed on efficacy of

commercial serotype A1 vaccines in sheep and cows and differences were obtained concerning protection levels in these studies (Donachie *et al.*, 1986).

Since *M. haemolytica* A2 is the most common isolate from pneumonic lungs of sheep and goats throughout the world. Most research activities on the pasteurella vaccine have focused on incorporating either a suitable isolate of *M. haemolytica* A2 or suitable antigens extracted from the serotype (Bahaman *et al.*, 1991; Mosier, 1993). However, many of the available pasteurella vaccines, which contained *M. haemolytica* A2 failed to meet the efficacy requirements in the field. One of the reasons is poor immunogenicity of some isolates of *M. haemolytica* A2 due to the absence of prominent antigens (Gilmour *et al.*, 1979, 1983; Adlam, 1989; Zamri-Saad *et al.*, 1994). *M. haemolytica* A2 was found to possess no cross-reactivity with *M. haemolytica* A7 and A9 (Zamri-Saad *et al.*, 1994) or with *M. haemolytica* A1 and A6 (Purdy *et al.*, 1998). In addition, vaccination of goats using a pasteurella vaccine containing whole cell *M. haemolytica* A2 failed to cross-protect against challenge with live *M. haemolytica* A7 and A9 (Purdy *et al.*, 1998; Zamirah, 1998). Thus, most pasteurella vaccines incorporate various important serotypes of *M. haemolytica* in order to provide coverage against infections by the various common serotypes (Mosier, 1993). Identification of a suitable immunogen, which provides cross-protection against several common serotypes of *M. haemolytica*, is the focus of various researchers throughout the world. The outer membrane proteins (OMPs) of *M. haemolytica* have been shown to be immunogenic and could be used in vaccine preparations (Gatewood *et al.*, 1994; Pati *et al.*, 1996).

The different serotypes of *M. haemolytica* exhibit significant variation in their association with disease in cattle and sheep. Whereas A1 is the predominant serotype associated with bovine pneumonic pasteurellosis, other serotypes are recovered infrequently from diseased cattle, although serotype A2 is often associated with healthy animals (Frank, 1989). In contrast, A2 is the most commonly isolated serotype from cases of ovine pneumonic pasteurellosis, followed by serotypes A7 and A9. Low numbers of serotype A6 isolates are recovered from cases of both bovine and ovine pneumonic pasteurellosis. The frequent recovery of serotype A1 and A2 isolates from natural cases of disease in cattle and sheep, respectively, and the less frequent isolation of other serotypes from natural cases of disease in both species, suggests important

differences between the various serotypes which are reflected by differences in host specificity and virulence. However, very little is known about diversity within and relationships between the different *M. haemolytica* serotypes and, in particular, about variation of cell-surface components likely to be involved in virulence, including LPS, capsular polysaccharide and OMPs (Davies and Donachie, 1996).

Currently in Ethiopia there is no effective vaccine that protects sheep from serotypes of *M. haemolytica* or serotypes derived from different geographical origins and the available vaccine to protect sheep from pneumonic pasteurellosis derived from *P. multocida* type A. However, given the significant economic losses due to sheep pneumonia that is caused by these two bacteria, it is necessary to develop such a vaccine for the benefits of sheep industry.

Therefore, the objectives of this study were:

- ❖ To develop experimental vaccines against *Mannheimia haemolytica* serotypes A2, A7 and their combination
- ❖ *In vivo* evaluation of their efficacy by measuring the antibody titer and through challenge protection trial

2. LITERATURE REVIEW

2.1. Etiology of ovine pneumonic pasteurellosis

The major bacterial pathogen involved in pneumonic pasteurellosis of sheep is *Mannheimia haemolytica* (*Pasteurella haemolytica*) serotypes (Radostits *et al.*, 1994; Hodgson *et al.*, 2005). It is small, non-spore forming, gram-negative rods or coccobacilli and facultative anaerobic bacteria (Quinn *et al.*, 2002). *Mannheimia haemolytica* is weakly hemolytic and the principal casual agent associated with the respiratory-disease complex in ruminants (Miller, 2001).

Mannheimia haemolytica with the following complete taxonomy: Super kingdom *Bacteria*; phylum *Proteobacteria*; class *Gammaproteobacteria*; order *Pasteurellales*; family *Pasteurellaceae*; genus *Mannheimia* (The NCBI taxonomy browser, 2011).

The bacterium has been the subject of extensive reclassification in the past: first called *Bacterium bipolar multocidum* by Theodore Kitt in 1885 (Kitt, 1885). It was then renamed *Pasteurella haemolytica* in 1932 (Newson and Cross, 1932) and classified into two biotypes, A and T, based on its ability to ferment arabinose and trehalose, respectively (Smith, 1961).

There were 13 A serotypes and 4 T serotypes identified (Younan and Fodor, 1995), the latter being reclassified as *Pasteurella trehalosi* in 1990 (Bingham *et al.*, 1990; Sneath and Stevens, 1990). Nine years later, studies based on DNA-DNA hybridizations and 16S RNA sequencing led to renaming previous A serotypes (A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17) as *Mannheimia haemolytica*. Among A serotypes, serotype 3, 4, 10 and 15 are classified as *Pasteurella trehalosi*, and the remaining A serotype, A11, which is unrelated to *Mannheimia haemolytica* is renamed *M. glucosida* (Younan and Fodor, 1995; Angen *et al.*, 1999). Currently both genotypic and phenotypic testing supports the separate and distinct nature of *P. trehalosi* to a new genus, *Bibersteinia*, as *Bibersteinia trehalosi* (Blackall *et al.*, 2007). The name *Mannheimia* was given in tribute to Walter Mannheim, a German biologist whose research has improved the understanding of the taxonomy of the *Pasteurellaceae* family (Angen *et al.*, 1999).

2.2. The disease

Mannheimia haemolytica is a normal commensal of the upper respiratory tract and tonsillar crypts in healthy ruminants. However, in the case of animals with compromised pulmonary defense mechanisms and stress, it can migrate into the lungs and cause acute fibrinous pleuropneumonia or pasteurellosis, commonly known as “shipping fever” (Miller, 2001). Of the various microorganisms causing respiratory diseases, *Mannheimia haemolytica* is one of the most important and the lesions it produces are characterized by fibrinonecrotic pleurobronchopneumonia (Trigo and Romero, 1986; Martin, 1996; Brogden *et al.*, 1998).

From the twelve serotypes described, A1 and A2 are the most prevalent serotypes and are normal residents of the upper respiratory tracts of healthy cattle and sheep, worldwide. Both are able to colonize the upper respiratory tract of cattle and sheep, but they are generally, but not exclusively, species-specific in their ability to cause pneumonia (Highlander, 2001; Zecchinon *et al.*, 2005). Bovine pneumonic pasteurellosis is mostly caused by *M. haemolytica* serotype A1, while the serotype A2 frequently causes pneumonia in sheep (Lawrence *et al.*, 2010).

Healthy cattle frequently carry serotype A2 in their upper respiratory tract, but following a stress or a co-infection, A1 quickly takes the place of A2 as the main serotype (Frank and Smith, 1983), probably by horizontal transfer from ill animals (Highlander, 2001).

Table 1: The major pathogenic *Pasteurella* and *Mannheimia* species, their principal hosts and associated diseases

Pasteurella species	Host	Disease condition
<i>P. multocida</i> Type A	Cattle	Bovine pneumonic pasteurellosis (shipping fever complex); enzootic pneumonia complex of calves
	Sheep	Pneumonia and mastitis
	Pig	Pneumonia and atrophic rhinitis
	Poultry	Fowl cholera
	Rabbits	Snuffles
	Other animal species	Pneumonia following stress
<i>P. multocida</i> Type B	Cattle and buffaloes	Hemorrhagic septicemia (Asia)
<i>P. multocida</i> Type D	Pigs	Atrophic rhinitis and pneumonia
<i>P. multocida</i> Type E	Cattle and buffaloes	Hemorrhagic septicemia (Africa)
<i>M. haemolytica</i> [<i>M. haemolytica</i> biotype A]	Cattle	Bovine pneumonic pasteurellosis (shipping fever complex)
	Sheep	Septicemia (under 3 months of age); pneumonia; gangrenous mastitis
<i>B. trehalosi</i> [<i>M. haemolytica</i> biotype T]	Sheep	Septicemia (5-12 months of age)

Source: (Quinn *et al.*, 2002).

2.3. Epidemiology

2.3.1. Occurrence

Recent surveys have shown that serotype A6 is increasingly prevalent in the United Kingdom (Donachie, 1998) and in the USA and A5, A8, A13, and A14 were the least (Gilmour and Gilmour, 1989; Purdy *et al.*, 1997; Al-Ghamdi *et al.*, 2000) with about 30% of strains serotyped. In Sudan serotypes A2, A6, and A12 have been shown to be the most prevalent and A5, A7, and

All were the least (Hussein and Mohamed, 1984a). Nevertheless, based on lipopolysaccharide profiles and outer membrane proteins within each serotype, it has been concluded that, apart from the nature of their capsules, serotypes A1 and A6 are extremely similar (Davies and Donachie, 1996; Morton *et al.*, 1996).

The prevalence of pneumonic pasteurellosis and the frequency of Mannheimia and Pasteurella isolation in the farm animals vary among various years and seasons. Generally, a high incidence of isolates of Mannheimia and Pasteurella may be attributed to highly stressful conditions (Radostits *et al.*, 1994) and nasal carriage rates in ovine pasteurellosis were found to be higher during outbreaks of pneumonia and systemic pasteurellosis (Gilmour and Gilmour, 1989).

2.3.2. Mode of transmission

Most ruminants are asymptomatic carrier of *M. haemolytica* or *B. trehalosi* as a commensal on mucus membranes; this occurs very shortly following birth, probably from the ewe and carriage persists through adult life. There is an endogenous infection occurs when the defense mechanism of the host is compromised. Exogenous transmission can occur either by direct contact, through aerosols or consumption of contaminated feed and water (Quinn *et al.*, 1994; Biberstein and Thomson, 1996).

2.3.3. Epidemiological risk factors

Viral infections

Viruses are believed to predispose to bacterial infection in 2 distinct ways. First, viral agents can cause direct damage to respiratory clearance mechanisms and lung parenchyma, facilitating translocation of bacteria from the upper respiratory tract and establishment of infection in compromised lung. Secondly, viral infection can interfere with the immune system's ability to respond to bacterial infection (Martin and Bohac, 1986; Czuprynski *et al.*, 2004). Some viruses such as Parainfluenza virus type 3, adenovirus, sheep pulmonary adenomatosis and respiratory

syncytial virus have been shown to have a temporal relationship with *M. haemolytica* in ovine pneumonia (Gilmour and Gilmour, 1989; Blood and Radostits, 1994).

Shipping

Transportation is the most universally accepted non-infectious risk factor for pasteuriosis and commonly it is termed as “shipping fever”. Most researchers have attempted to identify what component of transportation has the greatest effect on incidence of respiratory disease. The distance and/or time in transit have been examined in various studies. A large survey found a positive association between distance transported and morbidity (Sanderson *et al.*, 2008). Dehydration is a frequent sequel to transportation and has been suggested as a mechanism by which transport impacts disease. Two studies found a positive correlation between drop-off in weight and stress associated with long transportation time (Schake *et al.*, 1980; Ribble *et al.*, 1995).

Poor Management

Management practices that are thought to increase the risk of pneumonia include tail docking, castration, crowding, handling, transport and weaning. Such practices cause stress, and stress can lead to increased secretion of stress related hormones which, in turn, increases susceptibility to infection. Many of these practices are unavoidable under different farming conditions. It is still poorly understood how management-related stress leads to the development of pneumonia in lamb flocks, due to a lack of field studies. Other management practices that should be avoided as much as possible to help control pneumonia include: sudden changes in diet; mixing animals from different sources; excessive heat; poor ventilation and exposure to low temperatures (Knowles *et al.*, 1995; Cole, 1996).

2.4. Pathogenesis and virulence factors

2.4.1. Pathogenesis

Regardless of the economic importance of this pathogen, relatively little is understood about the precise mechanisms of pathogenesis, specifically how *M. haemolytica* adheres to the respiratory tract epithelium. Previous studies have demonstrated the ability of *M. haemolytica* to adhere to epithelial cells *in vitro* (Kisiela and Czuprynski, 2009). Shipping fever pneumonia precipitated by stress inducing conditions such as shipping, viral infections, inclement weather and overcrowding. Moreover, stress also resulted in nasopharyngeal overgrowth of bacteria, including *M. haemolytica* which results in more bacteria reaching the lungs via inhalation of infected droplets. Cells proliferate under stressful conditions and are erotized in large numbers into lung alveoli, where they cause the disease (Frank and Briggs, 1992). In addition, respiratory viruses can damage the ciliated epithelium (Andrews and Kennedy, 1997) and compromising the normal defense mechanisms these allowing *M. haemolytica* to colonize the lung. Several mechanisms have been proposed to explain the phenomenon of viral-bacterial synergism in respiratory infection. These include selection of pathogenic form of bacteria, reduced efficiency of lungs in clearing bacteria, depressed phagocytosis or bactericidal potential of alveolar macrophages, depression of ciliary activities and suppression of the immune response (Sharma *et al.*, 1990).

The first stage of infection is the adherence of a pathogen to the surface of target cells, usually facilitated by specific interactions between a cell surface protein on the bacterium and the extracellular matrix in the target cell. The extracellular matrix is composed of a mixture of many glycoproteins including, fibronectin and collagen, with the former recently shown to form a specific interaction with *M. haemolytica*'s outer membrane protein A (OMP A) (Lo and Sorensen, 2007). OMP A has been shown to be extremely important in host-specific ligand binding and there is indirect evidence that *M. haemolytica* achieves its host specificity in the same manner (Ojha *et al.*, 2010). This hypothesis is strongly supported by the fact that bovine and ovine strains possess different OMP A types (OMP A1 and OMP A2, respectively) (Haig, 2011).

2.4.2. Virulence factors

Leukotoxin

Leukotoxin (LKT) appears to be the main virulence factor. Indeed, inactivation of *M. haemolytica* LKT by a gene knockout hardly causes any further pulmonary lesions, although the wild-type and mutant strains were equally capable of colonizing the upper respiratory tracts (Tatum *et al.*, 1998). Moreover, necrosis of neutrophils can be reproduced *in vitro* with purified LKT (Deshpande *et al.*, 2002; Davies and Baillie, 2003). LKT belongs to the repeats-in-toxin family of pore-forming exotoxins. Although these toxins have broad target cell specificity, *M. haemolytica* LKT is specific for ruminant macrophages, neutrophils and all leukocyte subsets (Shewen and Wilkie, 1985). Biologically, it is worth noting that even if LKT is able to bind leukocytes from various animal species, it is only cytotoxic for ruminant leukocytes, suggesting that the interaction specificity between LKT and ruminant leukocytes could be responsible for the ruminant-specificity of *M. haemolytica* (Sun *et al.*, 1999). Amongst leukocytes, macrophages are more resistant than neutrophils to the lytic effect of LKT (O'Brien and Duffus, 1987).

Iron-regulated outer-membrane proteins

Pathogenic bacteria often use iron as an environmental signal for the regulation of virulence genes (Mekalanos, 1992; McIver *et al.*, 1995). In mammalian host, pathogens from the families of *Neisseriaceae* and *Pasteurellaceae* frequently deal with the lack of free iron for uptake, as most of it is stored in intracellular or extracellular (transferrin, lactoferrin, haemopexin and haptoglobin) bound forms (Litwin and Calderwood, 1993; Braun *et al.*, 1996). To overcome this problem, Gram-negative pathogenic bacteria have evolved an elaborate iron regulatory system, to acquire this element from the host. These include the direct binding of iron containing proteins to outer-membrane receptors and the secretion of siderophores or haemophores (Stojiljkovi *et al.*, 1999). Although *M. haemolytica* has no known siderophores, these bacteria produce two highly conserved transferrin-binding proteins that specifically bind the transferrin of their particular host (Morton and Williams, 1989; Fuller *et al.*, 1998). Iron is required for the proper growth and LKT production in *M. haemolytica*, as pathogenic gonococcal mutants devoid of these proteins lose

their virulence (Gentry *et al.*, 1986; Cornelissen *et al.*, 1998). The presence of an elaborate set of iron-acquisition genes, therefore, reiterates the importance of iron in controlling the transcription and expression of LKT and other virulence factors in modulating *M. haemolytica* pathogenesis. Expression of these proteins allows *M. haemolytica* to acquire iron from host the hemoglobin, hemopexin and transferrin (Lawrence *et al.*, 2010).

Lipopolysaccharides

Lipopolysaccharide (LPS) is an integral part of the Gram negative bacterial cell wall estimated as 10-25% of the dries cell wall and is the chief endotoxin that contains pathogen-associated molecular patterns (PAMPs) (Raetz and Whitfield, 2002). The LPS constituted a major surface antigen and the somatic serotype was defined according to the antigenic components in their structure. *M. haemolytica* serotypes of biotype (A) possessed rough type LPS while biotype (T) contains smooth LPS. It was demonstrated that LPS could directly produce bovine endothelial cell injury *in vitro*. The mechanism of injury was not clarified, but it was observed that this toxic effect can be reduced by neutrophils. LPS was also found to stimulate tumor necrosis factor release from bovine alveolar macrophages as well as other cytokines. (Highlander, 2001 ; Lo, 2001). LPS activates the macrophages through toll like receptors and elicits inflammatory cytokine production resulting in septicemia (Imler and Hoffmann, 2001; Raetz and Whitfield, 2002). *M. haemolytica* LPS also induces an inflammatory cytokine response leading to increased expression of b2-integrins in the host (Yoo *et al.*, 1995).

Mannheimia haemolytica capsular polysaccharide

The capsular polysaccharide (CP) of *M. haemolytica* serotype A1 is the initial contact point between pathogen and host. An immune response to CP is important in protecting the lung from bacterial infection and colonization through activation of antibody-mediated killing and phagocytic clearance mechanisms. There is strong evidence to correlate high anti-CP antibody titers with resistance (Gilmour *et al.*, 1979; Conlon *et al.*, 1991; Fraser, 1991; Conlon and Shewen, 1993; Littledike, 1993; Robinson and Melling, 1993).

Adhesins

Adhesins help in tethering the bacteria to the host cell surface in a manner similar to a grappling hook. Filamentous hemagglutinin is a major cell surface - associated adhesin that attaches to the host ciliary epithelial cells and is a virulence determinant (Davies and Donachie, 1996; Coutte *et al.*, 2003). Two types of fimbriae were demonstrated in *M. haemolytica*, the large and rigid which measured 12nm width, while the smaller and flexible measured 5nm width. *In vivo* similar structures were seen especially when the organisms were recovered from lavage fluids from an experimentally or naturally infected calves and in organism's adherent to tracheal epithelium in a naturally infected calf. The large rigid fimbriae are composed of 35 kDa subunits these fimbrial proteins were proved to be highly immunogenic and provided high antibody titers in the serum of immunized animals as well as contributed in the blockage of colonization of invading pathogens. These characters facilitated the usage of fimbrial protein in vaccine production failure to demonstrate the fimbrial protein may reflect failure in the method of preparation rather than a variable capacity of the organisms to produce them (Highlander, 2001; Lo, 2001).

Table 2: Virulence factors/ antigens of *M. haemolytica* A1

Virulence factors	Activity or contribution to pathogenesis	Reference
Leukotoxin	Pore forming cytotoxin destroy leukocytes	Lo <i>et al.</i> , 1985, 1987; Strathdee and Lo, 1989
O-sialoglycoprotease	Cleavage of glycoproteins from macrophages or other leukocytes	Abdullah <i>et al.</i> , 1991
Transferrin binding proteins	Iron acquisition	Ogunnariwo <i>et al.</i> , 1997
lipopolysacchride	Modulate leukocyt activities Endotoxic activity	Potter and Lo, 1995, 1996
Capsular polysaccharide	Masking of cell surface Prevention of phagocytosis Resistance to complement lysis	Lo <i>et al.</i> , 2001
Adhesin	Colonization	Lo, 2001
Outer-membrane lipoproteins	Activity not known, but are antigenic molecules	Cooney and Lo, 1993; Nardini <i>et al.</i> , 1998
Other outer membrane proteins	Activity not known, but are antigenic molecules.	Gonzalez- Rayos <i>et al.</i> , 1986; Lo and Mellors, 1996

Source: (Lo, 2001).

2.5. Clinical signs

Mannheimia haemolytica is associated with pneumonic disease in all ages of sheep although a more severe form of the disease involving septicaemia, pleurisy and, pericarditis also occurs in young lambs. The natural disease is sporadic, erupts suddenly and affects up to 10% of the flock (Gilmour and Gilmour, 1989). Flock outbreaks usually start suddenly with deaths, often in young lambs in which the disease is hyperacute and septicaemic rather than pneumonic. As lambs get older the disease becomes more confined to the thorax with prominent lesions of pleurisy and pericarditis. Beyond 3 months of age most cases are pneumonic although sudden deaths with

septicaemia, rather than pneumonia, may still occur. As the outbreak progresses over the next few days a number of sheep will be noticed with clinical signs of pneumonia. Observations of the flock show that some sheep have an occasional cough and slight oculo-nasal discharges (Donachie, 2003).

2.6. Pathology

Gross lesions

Presence of severe fibrinous pneumonia in antero- ventral of the lung can be seen. The pneumonic lobe exhibits advanced and fatal lesions involving most ventral tissue of the cranial (apical), intermediate (cardiac), and caudal (diaphragmatic) lobes. The lungs were characterized by well demarcated dark areas of consolidation covered by a layer of fibrin. Upon incision, the dark areas showed well circumscribed hemorrhagic areas with some creamy red tinge exudates coming out from the bronchi and bronchioles. Mucopurulent exudates oozed out from the bronchioles and bronchi. Pleuritis can be also evident grossly (Tehrani *et al.*, 2004).

Histopathology

Histopathological examination of the lungs with pneumonic pasteurellosis showed acute fibrinous pneumonia with pleuritis. The lesions were characterized by irregular shaped areas of neutrophil and lymphocyte infiltration with only a few macrophages. The bronchi and bronchioles were desquamated and were plugged by inflammatory exudates and cellular debris. They were also surrounded by inflammatory cells (Tehrani *et al.*, 2004).

2.7. Diagnosis and differential diagnosis

2.7.1. Laboratory diagnosis

Bacteriological diagnosis

Microscopically, the organism is typically gram negative rods, coccobacillary or bacillary and having darkly stained ends giving it a bipolar appearance, that is evident in Giemsa-stained or Leishman-stained smears (Shreeve *et al.*, 1970; Quinn *et al.*, 1999).

Biochemically, possible *Mannheimia haemolytica* isolates were selected on the basis of fermenting different sugars then observed for the typical reaction caused by the species. *Mannheimia haemolytica* reacted by fermenting glucose, lactose and sucrose with out gas formation. Generally the organism is indole and urease negative, but positive fermentation test for sugars like arabinose, raffinose, maltose and xylose (Timoney and Gillespie, 1981; Quinn *et al.*, 1999).

Cultural observation of the texture of the colonial surface one of the most diagnostic features revealed a smooth, less mucoid and grayish pinpoint colony with no odor. These colonial properties are reflection of certain bacterial characteristic and are attributed mainly to the presence of capsule. Capsular materials promote cellular orientation, hence, the smoothness and mucoid appearance of the growth on blood agar plate. *M. haemolytica* on blood agar show a narrow zone of β -haemolysis. This organism also grow on MacConkey agar because it able to ferment lactose (Stanier *et al.*, 1965; Quinn *et al.*, 1999).

Serology

The ability to identify *M. haemolytica* by serotype is crucial in the understanding of the epidemiology of the disease and in the development of an effective bacterin or vaccine. Most veterinary investigation frequently isolate *M. haemolytica* from pneumonic lungs of sheep, but the laboratories do not routinely serotype the isolates owing to time and expense involved.

However to be able to formulate an effective bacterin for use in the remission of the disease in sheep in a target area it is necessary to determine which serotypes are commonly involved (Odugbo *et al.*, 2004). Several methods for the serological typing of *Pasturella/Mannheimia* like rapid slide agglutination test (De Alwis, 1993), indirect haemagglutination (IHA) to detect serum antibody responses to individual serotype for vaccine evaluation and in sero-epidemiological investigation of pasteurellosis (Hussein and Mohammed, 1984b) and indirect enzyme linked immunosorbent assay (I- ELISA) (OIE, 1992; Rimler, 1993).

Molecular techniques

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 (McPherson and Moller, 2000). PCR assay has been used for detection of both species with the development of a rapid and multiple detection method for *P. multocida* and *M. haemolytica* in sheep, especially during acute cases of respiratory diseases (Deressa *et al.*, 2010).

Direct specimen analysis using PCR is more rapid and specific than standard bacteriological and serological methods of identification, hence, facilitating clinical diagnosis. The strains of *P. multocida* and *M. haemolytica* were successfully detected directly from clinical and tissue section materials (Deressa *et al.*, 2010; Lawrence *et al.*, 2010).

2.7.2. 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 reexamination should be carried out for confirmation (Gilmour, 1993). *Mycoplasma ovipneumoniae* has been reported in sheep but is

often overlooked because of apparent greater involvement of pasteurellosis. *M. ovipneumoniae* is the most commonly isolated *Mycoplasma* from the respiratory tract of sheep often in association with *M. arginini* that may exacerbate disease signs (Ayling and Nicholas, 2007). Respiratory disease associated with *M. ovipneumoniae* has been referred to as atypical pneumonia of sheep, *Mycoplasma* pneumonia, nonprogressive (atypical) pneumonia of sheep, proliferating exudative pneumonia, though the preferred scientific name is atypical non-progressive proliferative pneumonia (Gonçalves *et al.*, 2010).

2.8. The current situation in Ethiopia

Respiratory diseases, including pasteurellosis, are a great economic concern for sheep producers of Ethiopia. Despite annual vaccination against pneumonic pasteurellosis with a monovalent vaccine (inactivated *P. multocida* biotype A), there are high rates of mortality and morbidity following respiratory distress. Pasteurellosis is, therefore, a high priority issue at the national level due to the significant economic losses through mortality, morbidity, and the high cost of treatment (Ayelet *et al.*, 2004).

In sheep, respiratory infections and pneumonia caused by *Mannheimia* pathogens is prevalent in Ethiopia (Sisay and Zerihun, 2003). All prevalence studies were carried using standard bacteriological and serological techniques; to date no PCR assay has been used for detection of *Pasteurella* pathogens in Ethiopia except the one that was carried out to detect *Pasteurella* pathogens involved in sheep respiratory infections using PCR assay (Deressa *et al.*, 2010).

M. haemolytica biotype A serotypes A2, A7 and A1 are the most prevalent in Ethiopia, while A14, A11, and A9 were the least prevalent (Pegram *et al.*, 1980). In other study in central highland of Ethiopia (Ayelet *et al.*, 2004) shows the prevalence rates of *M. haemolytica* A2 and A8 serotypes were the highest (36% and 35%, respectively), while serotype A9 was the least prevalent (2%). Currently, the available vaccine using *Pasteurella multocida* type A, which do not coincide with the exact causal agent that does not confer effective protective immunity against the field case (Ayelet *et al.*, 2004).

Table 3: Micro-organisms identified and their prevalence in ovine pneumonic pasteurellosis from different parts of Ethiopia

Isolated spp.	Serotypes	Place	Prevalence	Author(s)
<i>M. haemolytica</i>	A1, A2, A5-8, A11, A13 and A14	North Shoa	Between 2 and 36	Ayelet (1996)
<i>M. haemolytica</i>	A1, A2, A5-8 and A12	North Shoa	50.8	Zelege (1998)
<i>B. trehalosi</i>	T3, T4 and T10	North Shoa	38.6	Zelege (1998)
<i>M. haemolytica</i>	A1, A2, A5, A7-9, A11-13	Arsi	85	Teferi (2000)
<i>B. trehalosi</i>	T15		12	Teferi (2000)
<i>M. haemolytica</i>	A1, A2, A5-8 and A12	Wollo	59	Sisay and
<i>B. trehalosi</i>	T3, T4, T10 and T15		32	Zerihun (2003)
<i>M. glucosidal</i>	A11		2	
<i>M. haemolytica</i>	A1, A2, A5-9, A11, A13 and A14	North Shoa	36 (A2) 2 (A9)	Ayelet <i>et al.</i> , (2004)
<i>M. haemolytica</i>	A1, A2, A5-8 and A12	Debre Zeit	5.6	Abera (2005)
<i>B. trehalosi</i>	T3, T4, T10 and T15		1.1	
<i>M. haemolytica</i>	A1, A2, A5-9, A12-14	Wollo	20	Mulate (2007)

2.9. Treatment, control and prevention

2.9.1. Prophylaxis/Metaphylaxis

Isolates of *M. haemolytica* from sheep are generally more susceptible to antibiotics than those recovered from cattle (Gilmour and Gilmour, 1989). Treatment of ovine pasteurellosis can be very effective and the use of oxytetracycline in prophylaxis, metaphylaxis and therapy is well documented (Gilmour *et al.*, 1982; Gilmour *et al.*, 1988). Although antibiotic treatment is of great value during an outbreak, it is not a realistic way of controlling a disease which is sporadic and unpredictable in nature and which has such a short clinical course. It is also an inappropriate use of anti-microbials which encourages the development of antibiotic resistance (Donachie, 2003).

2.9.2. Control and prevention

Although safe management practices are still likely to be the most economic way of controlling pasteurellosis it is generally believed that vaccination offers the best practical means of control. In view of the economic importance of the disease and its severe effects on health and welfare the development and use of effective vaccines is essential (Donachie, 2003).

Management factors such as avoiding crowding, mixing of different flocks, deprivation of feed and water, exposure to aerosol infection from other sheep and providing shelter especially during extremes of weather condition reduce the outbreak of the disease and used as a preventive measures (Gilmour and Gilmour, 1989; Kehrenberg *et al.*, 2000).

The development of a vaccine that is effective worldwide has been hindered by the multiple serotypes of *M. haemolytica* and *B. trehalosi* capable of causing pasteurellosis (Mosier, 1993). These bacteria also produce several virulence factors, such as an exotic LKT, LPS with endotoxic activity, capsular antigen and iron- regulated OMP, which have to be considered during vaccine development (Sreevatsan *et al.*, 1996). Most vaccines incorporate several of the important serotypes of these organisms, so as to provide cover against infection by the various common serotypes (Mosier, 1993). Vaccines used to prevent pasteurellosis consist of bacterins, attenuated live bacteria, cell extracts or culture supernatant (Confer, 1993). Most preparations give variable and unreliable results, especially against serotype A2, if they lack prominent antigens that trigger a sufficient immune response (Adlam, 1989). Therefore, the search for improved vaccine development continues. Gilmour and Gilmour (1989) reported that serotype- specific multivalent vaccines prepared from the most common serotypes using sodium salicylate extract (SSE) and a heat- killed vaccine with oil adjuvant gave better protection.

Immunity against *M. haemolytica* is thought to be primarily through production of serum antibodies that neutralize the secreted LKT and antibodies against surface antigens (Shewen and Wilkie, 1988). The specific surface antigens that are important in stimulating host immunity to *M. haemolytica* are not known; however, several studies point towards the importance of outer membrane proteins (Pandher *et al.*, 1999). However, LKT neutralizing antibodies alone are

insufficient for optimum protection against pneumonic pasteurellosis (Mosier *et al.*, 1998). To control pasteurellosis it is very important that one not rely on vaccine induced immunity, as excessive challenge or stressors can overwhelm this immunity (Seifert, 1996).

2.10. Development of *Mannheimia* vaccines

2.10.1. *Mannheimia* vaccine world wide

Trials investigating vaccination as a potential tool to control ovine pasteurellosis have been variable. Most available vaccines against *M. haemolytica* A2 have failed to meet efficacy requirements in the field. In many studies that have been conducted a poor immune response to the Sero-type specific capsular polysaccharide of *M. haemolytica* A2 has been evident (Gilmour *et al.*, 1979; Adlam, 1989; Zamri-Saad *et al.*, 1994). If antigens common to all serotypes were identified a vaccine could be developed to provide better protection.

A controlled environment is required for challenge experiments with infectious pathogens if vaccine efficacy is to be demonstrated in small groups of fully susceptible experimental animals (Roth and Flaming, 1990), in addition vaccines that have been evaluated in a controlled environment have generally been successful but efficacy has been questionable in the field due to the varying extent of susceptibility and pathogen challenge. Generally vaccination may regard as the only solution in controlling this complex disease. However, the prevention of causes other than specific pathogens may have greater benefit in controlling ovine pasteurellosis in the field.

Mannheimia vaccines using antigens derived from killed whole bacterial cells, or cell wall extracts, administered with and without adjuvants have been studied (Donachie *et al.*, 1983; Gilmour *et al.*, 1983; Wells and Robinson, 1984; Jones *et al.*, 1986). Some have been shown to protect sheep against challenge with virulent *M. haemolytica* organisms (Donachie *et al.*, 1983; Gilmour *et al.*, 1983; Wells and Robinson, 1984). *Mannheimia* vaccines are commercially available worldwide, although not available in Ethiopia at present (Table4).

Table 4: *Mannheimia* vaccines commercially available throughout the world

Vaccine	Country	Antigens	Reference
Ovipast Plus	New Zealand/ UK/Spain	Serotypes of <i>M. haemolytica</i> and <i>B. trehalosi</i>	Intervet Ltd., 2011; Intervet UK Ltd., 2011b; Laboratories Intervet S.A., 2011
Multivax-P	South Africa	T serotypes of <i>Mannheimia (Pasteurella) haemolytica</i> <i>Clostridium perfringens</i> type D, <i>Cl. septicum</i> , <i>Cl. tetani</i> , <i>Cl. chauvoei</i>	Intervet South Africa (Pty.) Ltd., 2011
Ovivac-P Plus	UK	Serotypes of <i>M. haemolytica</i> and <i>B. trehalosi</i>	Intervet UK Ltd., 2011c
Heptavac-P Plus	UK	Serotypes of <i>M. haemolytica</i> and <i>B. trehalosi</i> , <i>Cl. perfringens</i> types B, C and D, <i>Cl. septicum</i> , <i>Cl. tetani</i> , <i>Cl. novyi</i> type B and <i>Cl. chauvoei</i>	Intervet UK Ltd., 2011a
Poly serum	United States	<i>M. haemolytica</i> , <i>P. multocida</i> , <i>Actinomyces pyogenes</i> , <i>E. coli</i> and <i>Salmonella typhimurium</i>	Novartis Animal Vaccines Inc., 2011
Ovine <i>M. haemolytica</i> pneumonia vaccine	United States	<i>M. haemolytica</i>	Pipestone Veterinary Supply, 2011

Source: (Goodwin-Ray, 2006)

2.10.2. Considerations in the development of vaccines for ovine pasteurellosis

There are a number of considerations for determining how effective any new *Mannheimia* vaccine should be. Firstly, efficacy has to be determined in an acceptable and relevant experimental system. This should be as close to a natural experience as can be obtained. An aerosol inoculum, which gives rise to minimal invasion and trauma, is recognized as being similar to the proposed natural route for this challenge. Another consideration is protective efficacy should be determined against the most prevalent serotypes. In the case of ovine pasteurellosis serotype A2 is by far the most prevalent with A1, A6, A7 and A9 of lesser importance. However, most serotypes can occur in sheep disease. Any new vaccine should be able to demonstrate good protective efficacy against them all. The specific antigenic basis for protection should be demonstrable and any new vaccine should be able to show that immune responses to the selected antigen/serotypes are present in protected animals. The demonstration by Gilmour *et al.* (1983) showed cell-free extracts of *M. haemolytica* elicited serotype-specific protection in specific pathogen free (SPF) lambs indicated that representative strains of all the serotypes should be included in sheep vaccines if protection against all strains was to be expected. The traditional approach to vaccine formulation has been to incorporate all of the most commonly isolated serotypes but this leads to complex formulations which imposes a very heavy antigenic load on vaccinated animals. The identification of shared protective antigens is therefore important in the development and improvement of this type of vaccine as their inclusion should reduce the overall quantity of antigen required.

3. MATERIALS AND METHODS

3.1. Experiment station

This experimental study was conducted at National Veterinary Institute (NVI), Debre Zeit, Ethiopia. It is located in Oromia National Regional State, East Showa Zone, 46 km South-East of Addis Ababa, 8° 44' N and 39° 02' E, at an altitude of 1,880 m above sea level. The area receives mean annual rain fall of 865 mm in a bimodal distribution with mean minimum and maximum temperatures of 15°C and 28°C, respectively (CSA, 2009).

3.2. Experimental animals

A total of 47 clinically healthy young, male lambs of approximately 6 - 9 months old were selected for the trial. All of them were purchased from local market around Debre Zeit. Immediately upon arrival at the experimental station, they were allowed to adapt the environment and acclimatized for the trial. They were dewormed with albendazole (7.5 mg/kg body weight) upon arrival at the institute. The trial was started only when all lambs were kept for two weeks of adaptation period. They were individually identified with ear tags.

3.3. Experimental animal management

The experimental animals were divided into four groups, but were kept in the same paddock. The lambs were left to graze during daytime and kept in confined areas at night. While in confinement, they were fed concentrate feed twice daily first at mid day then before confinement at night, while drinking water was supplied ad libitum. The animals were kept in a clean house and the house provides sufficient ventilation, feeding area, and resting space.

3.4. Experimental design

At the start of the experiment, animals in groups 1-3 were injected subcutaneously with 1 ml of the capsular antigen vaccine prepared for the same purpose. Those in group 1 received the vaccine containing the capsular antigen of *M. haemolytica* serotype A2; group 2 received the vaccine containing the capsular antigen of *M. haemolytica* serotype A7 while group 3 received the vaccine containing the combined capsular antigens of *M. haemolytica* serotypes A2 and A7. Group 4 was inoculated the same manner with sterile saline water and left as unvaccinated controls. Serum samples were collected from all lambs before vaccination for screening purpose and then at weekly interval post-vaccination throughout the 5 weeks study periods. Five weeks after the vaccination, 5 animals from group 1 were challenged intra-tracheal with 4 ml inoculums containing 5.2×10^9 colony forming unit (CFU) of live *M. haemolytica* A2. Similarly, 5 animals from group 2 were challenged intra tracheal with 4 ml inoculums containing live *M. haemolytica* serotype A7. Group three was split into two groups each containing 5 animals and one of the group was challenged by A2 and the other group by A7. Finally, half of the control (4 animals each) was challenged by A2 and the other half by A7 (Table 5). Following the intra tracheal challenge, all lambs were observed daily for general signs of illness; however, signs related to respiratory problems had been given particular attention. Those that had survived were slaughtered on day 12 post-challenge. The entire respiratory tracts were examined and the extent of the lung lesions was determined according to the method described by Akan *et al.* (2006).

Table 5: Summary of the number of sheep used in each treatment group and volume of inoculums used

Treatment groups	Type of serotype	No. of sheep	Vaccine dose	Vaccination route	Type of challenge	Inoculums Volume(ml)
1	A2	14	1ml	Sc	Live <i>M. haemolytica</i> A2 (5 animals)	4
2	A7	13	1ml	Sc	Live <i>M. haemolytica</i> A7 (5 animals)	4
3	Bivalent (A2 and A7)	11	1ml	Sc	Live <i>M. haemolytica</i> A2 (5 animals) and A7 (5 animals)	4
4 (control)	Sterile saline water	9	-	-	Live <i>M. haemolytica</i> A2 (4 animals) and A7(4 animals)	4

3.5. Preparation of experimental vaccines

The experimental vaccine used in this study was prepared at NVI bacteriology laboratory.

3.5.1. Selection of vaccine strain

Mannhemia haemolytica serotypes A2 and A7 were selected and used as candidate vaccine based on their higher rate of isolation from different parts of the country. These strains were isolated and identified from different outbreaks of pneumonic pasteurellosis in the country and kept at NVI germ bank in a lyophilized form at -20°C.

3.5.2. Mass cultivation

The experimental vaccines were prepared in tryptose broth enriched with 10% horse serum. The media was pre-incubated for 48 hours to check its sterility. The media that had passed this sterility test was used for our purpose. The selected serotypes were inoculated into the above media separately and incubated at 37°C for 18 hours. At the end of this incubation time, bacterial growth was checked by looking at the turbidity of the media, pH of the media and examination of smears from the culture. The bacterial growth suspensions were harvested at logarithmic growth phase as determined by pH of the culture media (Annex II).

3.5.3. Bacterial mass determination

This was performed using titration and standard plate count method as described by Quinn *et al.* (2002). Counts were made on titrations that had yield 30 and 300 colonies per plate. The required bacterial growth mass was 10^7 or more which is considered as a minimum standard to prepare a vaccine suspension. In this study the viable count for each serotype used in the vaccine was 5×10^8 CFU/ml (Annex I).

3.5.4. Inactivation of the live bacterial suspension and tests on inactivated material

For the purpose of inactivation 0.5% formaldehyde was used. The total volume of formaldehyde needed to inactivate the culture was calculated and the whole bacterial suspension was exposed for 72 hours at 37 °C. To check the complete inactivation of the culture suspension, samples were taken from the suspensions and cultured onto the above media and incubated overnight at 37 °C.

Purity test

As a standard procedure, before inactivation of the culture the purity of the culture was tested on tryptose agar media enriched with 10% horse serum. A loop full of sample was taken from the broth culture and transferred to the agar media just to see whether there was any bacterial

contaminant. To double check the purity, smear was also made from a drop of inactivated material and stained by Gram's stain.

3.5.5. Adjuvant preparation

10% Alum (aluminum potassium sulfate) suspension was prepared as stock solution. Then, the alum was mixed with saline water to get 1% final concentration of the adjuvant in the vaccine.

3.6. Vaccine efficacy test by monitoring the antibody titer response

Since there are problems of producing the disease in target animals and no other appropriate model animals available, the evaluation of the vaccine was mainly depend on post vaccination serological analysis. For this purpose indirect haemagglutination (IHA) test was used to determine the antibody titer against capsular antigen of the organism.

3.6.1. Blood sample collection for serology

About 5-8 ml of blood sample for serum extraction was collected directly from jugular vein using sterile needles and plain vacutainer tubes and the tubes were left to stand in inclined position over night at +4°C. Then, the sera were harvested using sterile pasture pipette tips and transferred to sterile cryo tubes bearing the animal identification. Sera were stored at -20°C until assayed. This was done one week before vaccination and then weekly after vaccination for 5 weeks.

3.6.2. Indirect Haemagglutination test

Modified IHA (Indirect Hemagglutination) test that uses glutaraldehyde fixed sheep red blood cells (GA-SRBC) as described by Sawada *et al.* (1982) was used to measure the antibody titer following vaccination. The test was conducted at NVI serology laboratory. The IHA antibody titer was displayed as the reciprocal of its end point dilution. The IHA titers of all the serum

samples obtained were recorded and geometrical means of titers were calculated and analyzed statistically.

Materials and reagent

- Test sera from the experiment sheep's
- V- shaped microplates, multichannel micropipets (Titertek^R) and tips
- Phosphate buffered saline at PH 7.2 and alsever's solution
- Tryptose serum agar and broth
- Water bath and incubator with agitator
- Centrifuge (CRYOFUGE 2000-5000)
- Sheep erythrocyte (NVI sheep flock)
- 50% gluteraldehyde
- Syringe with needle
- Refrigerator
- Distilled water
- *Mannheimia haemolytica* serotypes (CIRAD-EMVT, France)
- Positive sera (hyper immune sera of each serotypes) (CIRAD-EMVT, France)

Antigen preparation

The culturing was performed in microbiology laboratory at NVI in biological safety II cabinet. Then, the reference strains were seeded on tryptose- serum agar and incubated at 37°C for 18-20 hours (we deal with two serotypes A2 and A7). The growth was then harvested in phosphate buffered saline (PBS) in proportion of 20-30 colonies in 10ml of PBS. This suspension was then heated in a water bath at 60°C for one hour to kill viable organisms and then, centrifuged at 2,500 rpm and 5,000 rpm for 20 minutes and 15 minutes, respectively. Finally, the clear supernatant fluid was discovered and used as capsular antigen extract

Sensitization of sheep RBC

Blood was taken from the jugular vein of male sheep freely flowing into a syringe containing Alsever's solution (take 3ml blood in 5ml Alsever's solution in proportion) and stored at +4°C at least one day overnight. This was washed three times in 5ml PBS by centrifugation at 2,000 rpm for 10 minutes. 100µl of the prepared packed RBC were added to 10ml of each antigen. Then, 50µl of 50% glutaraldehyde was added and homogenized by gentle shaking and incubated for one hour at 37°C with periodic shaking. This was centrifuged at 2,000 rpm for 10 minutes and washed two times in PBS by centrifugation similarly. Finally, 10ml of PBS was added to the final sediment and made up to 1% suspension.

Test procedure

Titerteck microtiter plates (Flow Laboratories, UK) with 8 rows and 12 columns of wells were used in the test capable of titrating 10 serum samples column- wise simultaneously. Both positive and negative control sera were included in each plate and the first two columns of the wells were left for positive and negative serum controls. 90 µl of PBS in the first wells and 50 µl to the remained wells were transferred. About 10 µl of test sera in the first row starting from the third column was mixed. Control tests in which sensitized and unsensitized SRBC's were added to respective positive and negative sera parallel in every test. Serial dilution of the sera was done by pepetting 50 µl from the first row to second, third wells until the last well (1:10, 1:20, 1:40, etc...) and discard the last 50 µl and then 50 µl of sensitized sheep RBC to all wells were added. The plates were covered with micro plate sealer to prevent evaporation and incubated at 37°C for 1 hour in moist chamber with constant agitation. Then, the result was read and about 50% or more agglutination of red cells were indicated as a positive reaction and small button of deposited cells were a negative reaction. The highest dilution of each serum sample causing a clear agglutination pattern was taken as end point.

3.7. Clinical investigation and necropsy after artificial challenging

All groups were challenge by intra tracheal route with live bacterial culture containing ID_{50} 5.2×10^9 . Rectal temperatures were recorded every morning for three consecutive days and on 5th, 7th and 10th day post challenge and lambs were observed closely for clinical symptoms including death if present. Necropsies were performed 10 days post challenge and lung lesions were recorded and evaluated accordingly. Based on the percentage of consolidated lung masses lung lesions were scored as follows: 0 (lungs without lesions), +1 (1–4% consolidation), +2 (5–14% consolidation) and +3 ($\geq 15\%$ consolidation) (Akan *et al.*, 2006).

3.8. Statistical analysis

Data generated from all experiments were recorded and stored in MS Excel 2007. Results were statistically analyzed using SPSS software versions 16.0 for MS windows. Serological data obtained from this experiment is transformed to normal distribution so as to use parametric statistical test. The best method of transformation for this type of data is logarithmic transformation and hence, normalized by $\log_{10}$ transformation. The mean serum antibody IHA titers were compared by General Linear model at 95% confidence interval. Descriptive statistics were used to analyze the clinical parameter post challenge of the control and the vaccinated groups and Post Hoc test was used to determine statistical difference at 95% confidence interval. Differences between groups of means were determined by one- way ANOVA test and paired wise comparison of means. Wilcoxon rank test was used for pair wise comparison of lung lesion score among the different treatment groups. Correlation between the antibody response and extent of lung lesions was also done.

4. RESULTS

4.1. Serological evaluation of experimental *M. haemolytica* serotype A2 and A7 vaccines

4.1.1. Pre vaccination antibody titer

Prior to vaccination, all animals were showed moderate antibody levels against the capsular antigen of *M. haemolytica* A2 and A7. However, following vaccination, the antibody levels against A2 and A7 serotypes were significantly ($p < 0.05$) increased after the third week of vaccination above the levels before vaccination. Hence, the geometric mean antibody titer before vaccination was higher in the unvaccinated control group followed by the combined serotype vaccinated groups 1.46 ± 0.3 and 1.33 ± 0.5 , respectively, as shown in Figure 1.

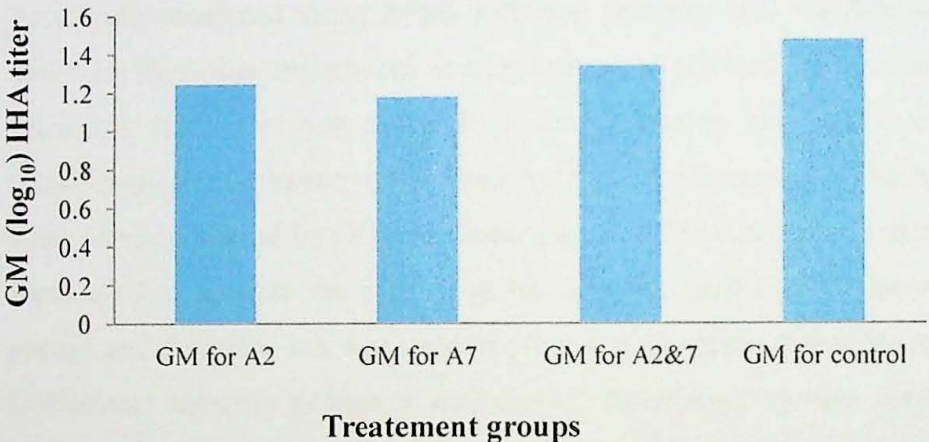


Figure 1: GM (log₁₀) antibody titer before vaccination against *M. haemolytica*

4.1.2. Serological response following vaccination

Post vaccination IHA titer depicted that all vaccinated animals demonstrated an increased level of antibody against the capsular antigens of *M. haemolytica* serotype A2 and A7 respective to their group. This rise in geometric mean titer was significant ($p < 0.05$) at the third and fourth week of vaccination. In contrast, there was no significant difference between the vaccinated groups ($p > 0.05$). The combined A2 and A7 vaccinated groups elicit response against both serotypes which

was evaluated by IHA using the antigen of both serotypes. Generally, the difference in the GM antibody titer level against serotype A2 and A7 vaccinated groups across the study periods was insignificant ($p > 0.05$), except at the 1st week post vaccination. A significantly higher GM titer was detected in A2 vaccinated group ($\log_{10}^{1.37}$) than A7 ($\log_{10}^{1.07}$) at first week of vaccination as shown in table 6 and 7.

Table 6: Post vaccination GM antibody titer for the treatments across the study periods

Treatment groups	N	Geometric mean	Std. deviation	Std. Error	95% CI for Mean	
					Lower bound	Upper bound
GM antibody titer for week 1						
A2	14	1.366	0.241	0.065	1.226	1.505
A7	13	1.069	0.132	0.037	0.989	1.149
A2 and A7	11	1.164	0.157	0.047	1.059	1.269
Control	9	1.268	0.235	0.078	1.087	1.448
Total	47	1.218	0.224	0.033	1.152	1.283
GM antibody titer for week 2						
A2	14	1.495	0.224	0.059	1.365	1.624
A7	13	1.393	0.226	0.062	1.257	1.530
A2 and A7	11	1.493	0.203	0.061	1.356	1.629
Control	9	1.268	0.279	0.093	1.053	1.482
Total	47	1.423	0.240	0.035	1.352	1.493
GM antibody titer for week 3						
A2	14	1.602	0.312	0.083	1.421	1.782
A7	13	1.648	0.167	0.046	1.547	1.749
A2 and A7	11	1.657	0.182	0.055	1.535	1.779
Control	9	1.334	0.181	0.060	1.195	1.474
Total	47	1.576	0.249	0.036	1.503	1.649
GM antibody titer for week 4						
A2	14	1.452	0.349	0.093	1.249	1.653
A7	13	1.579	0.193	0.053	1.462	1.695
A2 and A7	11	1.575	0.284	0.086	1.384	1.766
control	9	1.134	0.159	0.053	1.012	1.256
Total	47	1.455	0.307	0.045	1.365	1.545
GM antibody titer for week 5						
A2	14	1.301	0.289	0.077	1.134	1.468
A7	13	1.093	0.145	0.040	1.005	1.180
A2 and A7	11	1.164	0.157	0.047	1.059	1.269
Control	9	1.134	0.219	0.073	0.966	1.302
Total	47	1.179	0.223	0.033	1.114	1.245

Lower GM antibody titer was observed in serotype A2 vaccinated group at the 3rd and 4th week of post vaccination ($\log_{10}^{1.60}$ and $\log_{10}^{1.45}$ for the respective weeks) than serotype A7 ($\log_{10}^{1.65}$ and $\log_{10}^{1.58}$ for the respective weeks) and the combined serotype (A2 and A7) vaccinated groups ($\log_{10}^{1.66}$ and $\log_{10}^{1.57}$ for the respective weeks) as shown in figure 4. This indicated that at third and fourth weeks of post vaccination all the vaccinated groups were statistically different ($p < 0.05$) in GM antibody titer from the control group. Moreover, A7 and combined serotype vaccinated groups had better GM antibody titer than A2 vaccinated group (Table 7).

Table 7: Summary result for pair wise comparison of log titer among treatment groups and controls

Log titer	Variable constant	Variable compared	Mean difference	Std. Error	P-value	95% CI	
						Lower bound	Upper bound
Week 1	A2	A7	0.296*	0.076	0.002	0.094	0.498
		A2 and 7	0.201	0.079	0.067	-0.010	0.412
		control	0.098	0.084	0.650	-0.126	0.322
	A7	A2	-0.296*	0.076	0.002	-0.498	-0.094
		A2 and 7	-0.95	0.080	0.644	-0.309	0.120
		control	-0.198	0.085	0.108	-0.426	0.029
	A2 and7	A2	-0.201	0.079	0.067	-0.413	0.010
		A7	0.095	0.080	0.644	-0.120	0.309
		Control	-0.103	0.088	0.648	-0.339	0.132
	Control	A2	-0.098	0.084	0.650	-0.322	0.126
		A7	0.198	0.085	0.108	-0.029	0.426
		A2 and 7	0.103	0.088	0.648	-0.132	0.339
Week 2	A2	A7	0.101	0.089	0.672	-0.137	0.339
		A2 and 7	0.002	0.093	1.000	-0.247	0.251
		control	0.227	0.099	0.115	-0.037	0.491
	A7	A2	-0.101	0.089	0.672	-0.339	0.137
		A2 and 7	-0.099	0.095	0.725	-0.352	0.154
		control	0.126	0.100	0.595	-0.142	0.394
	A2 and7	A2	-0.002	0.093	1.000	-0.251	0.247
		A7	0.099	0.095	0.725	-0.154	0.352
		Control	0.225	0.104	0.150	-0.053	0.503
	Control	A2	-0.227	0.099	0.115	-0.491	0.037
		A7	-0.126	0.100	0.595	-0.394	0.142
		A2 and 7	-0.225	0.104	0.150	-0.503	0.053

Week 3	A2	A7	-0.046	0.087	0.951	-0.279	0.186
		A2 and 7	-0.055	0.091	0.931	-0.298	0.189
		control	0.268*	0.097	0.039	0.009	0.526
	A7	A2	0.046	0.087	0.951	-0.186	0.279
		A2 and 7	-0.008	0.093	1.000	-0.256	0.239
		control	0.314*	0.098	0.013	0.052	0.576
	A2 and7	A2	0.055	0.091	0.931	-0.189	0.298
		A7	0.008	0.093	1.000	-0.239	0.256
		Control	0.322*	0.102	0.014	0.051	0.594
	Control	A2	-0.268*	0.097	0.039	-0.526	-0.009
		A7	-0.314*	0.098	0.013	-0.576	-0.052
		A2 and 7	-0.322*	0.102	0.014	-0.594	-0.051
Week 4	A2	A7	-0.127	0.102	0.603	-0.401	0.146
		A2 and 7	-0.123	0.107	0.661	-0.409	0.163
		control	0.318*	0.114	0.037	0.014	0.621
	A7	A2	0.127	0.102	0.603	-0.146	0.401
		A2 and 7	0.004	0.109	1.000	-0.287	0.295
		control	0.445*	0.115	0.002	0.137	0.753
	A2 and7	A2	0.123	0.107	0.661	-0.163	0.409
		A7	-0.004	0.109	1.000	-0.295	0.287
		Control	0.441*	0.119	0.003	0.121	0.760
	Control	A2	-0.318*	0.114	0.037	-0.621	-0.014
		A7	-0.445*	0.115	0.002	-0.753	-0.137
		A2 and 7	-0.441*	0.119	0.003	-0.760	-0.121
Week 5	A2	A7	0.208	0.082	0.069	-0.012	0.429
		A2 and 7	0.137	0.086	0.396	-0.094	0.367
		control	0.167	0.091	0.274	-0.077	0.412
	A7	A2	-0.208	0.082	0.069	-0.429	0.012
		A2 and 7	-0.072	0.088	0.846	-0.306	0.163
		control	-0.041	0.093	0.970	-0.289	0.207
	A2 and7	A2	-0.137	0.086	0.396	-0.367	0.094
		A7	0.072	0.088	0.846	-0.163	0.306
		Control	0.030	0.096	0.989	-0.227	0.287
	Control	A2	-0.167	0.091	0.274	-0.412	0.077
		A7	0.041	0.093	0.970	-0.207	0.289
		A2 and 7	-0.030	0.096	0.989	-0.287	0.227

*. The mean difference is significant ($p < 0.05$)

Statistically significant difference ($p < 0.05$) for IHA log titer among the four treatment groups across the study weeks was observed at the 3rd and 4th weeks of post vaccination as shown in Table 8. Furthermore, GM log antibody titer on the 1st week had also showed significant ($p < 0.05$) difference among the treatment groups. Multiple comparison of the general linear model showed that these groups variation on the 1st week comes mainly by serotype A2 and A7 vaccinated groups having a GM titer of $\log 10^{1.37}$ and $\log 10^{1.07}$, respectively, with a mean difference of 0.3 (Table 6 and 7).

Table 8: ANOVA result of IHA log titer among the four treatment groups across the study weeks

Source of Differences variation	Sum of squares	df	Mean square	F	p- value
Log titer for week 1					
Between groups	0.646	3	0.215	5.585	0.003
Within groups	1.657	43	0.039		
Total	2.302	46			
Log titer for week 2					
Between groups	0.354	3	0.118	2.200	0.102
Within groups	2.303	43	0.054		
Total	2.657	46			
Log titer for week 3					
Between groups	0.674	3	0.225	4.405	0.009
Within groups	2.195	43	0.051		
Total	2.869	46			
Log titer for week 4					
Between groups	1.286	3	0.429	6.062	0.002
Within groups	3.041	43	0.071		
Total	4.327	46			
Log titer for week 5					
Between groups	0.326	3	0.109	2.376	0.083
Within groups	1.968	43	0.046		
Total	2.294	46			

df: degree of freedom

The antibody titers in all groups of vaccinated lambs were dropped at 5th week of the study period. But, the lambs vaccinated with serotype A2 was showed relatively higher GM antibody titer ($\log 10^{1.3}$) at the 5th week than the other groups; however, it was not significant difference from the other groups ($p > 0.05$) as demonstrated in Figure 2 and Table 8.

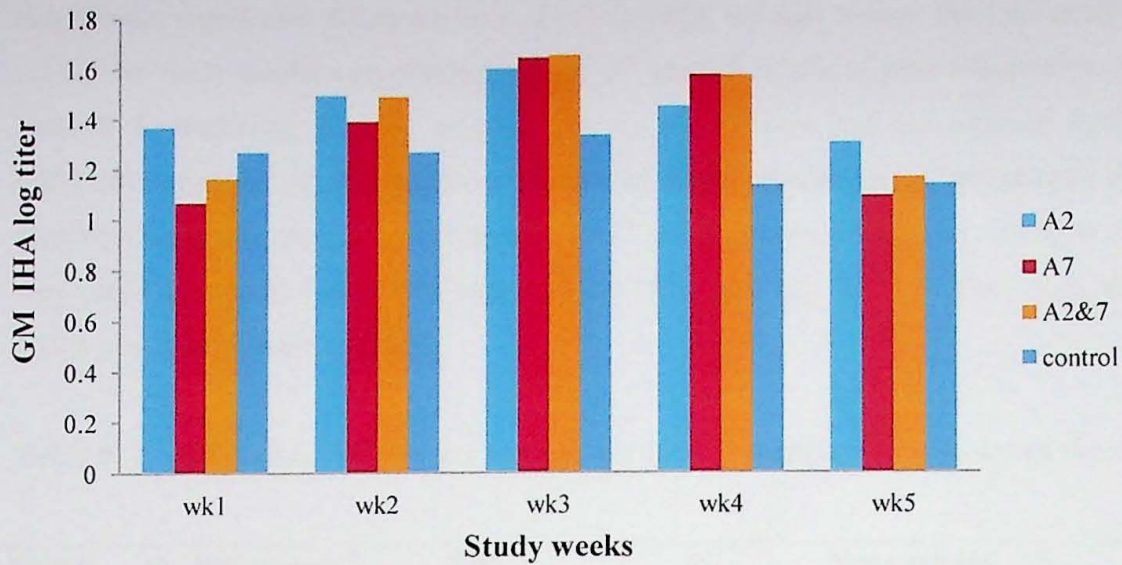


Figure 2: GM antibody titer of the treatment groups across the study weeks.

4.2. Clinical investigation and necropsy following challenge

All animals were apparently healthy at the time of challenge as evidenced by detailed clinical examination of individual animals. On day 1 post challenge, the mean rectal temperature for the control and all vaccinated groups were 39.5 and 39.6^oC, respectively. But, on day 10 after challenge exposure a 1.5^oC rise were observed in the controls than all the vaccinated groups (Table 9).

Table 9: Post challenge mean values of rectal temperature (in ^oC)

Days post challenge			A2 and A7 combined		Control		Day mean $\pm$
	A2	A7	A2	A7	A2	A7	SD
1	39.6	39.2	40.4	39.0	39.8	39.1	39.5 $\pm$ 0.7
2	38.7	39.2	39.4	39.1	39.0	39.1	39.1 $\pm$ 0.6
3	38.8	39.4	39.7	39.0	39.1	39.0	39.2 $\pm$ 0.6
5	38.9	39.0	39.2	38.9	38.9	39.1	39 $\pm$ 0.4
7	38.4	38.5	38.7	38.8	39.9	39.7	39 $\pm$ 0.7
10	38.4	38.7	38.5	38.8	40.0	40.1	39 $\pm$ 0.7
Group mean $\pm$ SD	38.8 $\pm$ 0.4	39 $\pm$ 0.3	39.3 $\pm$ 0.7	38.9 $\pm$ 0.1	39.5 $\pm$ 0.5	39.4 $\pm$ 0.4	

Following challenge no mortalities were observed in all treatment groups until necropsy performed, but there was a significantly higher morbidity rate in the control group as manifested by a rise in rectal body temperature and respiratory signs (Table 10). There was a significant difference ($p < 0.05$) in rectal temperature at day 1 post challenge in group 3 with those challenged by serotype A2 and A7. At day 7 and 10 post challenge there were a rise in rectal temperature in the control groups challenged by serotype A2 and A7 and found statistical significance difference ($p < 0.05$) in rectal temperature between the vaccinated and control groups (Figure 3 and Annex 3).

Table 10: Challenge protection test of experimental vaccines 10 days post challenge

Vaccine used	Challenge serotype	Outcome from challenge	No. of animals
A2	A2	Died	0
		Sick	1
A7	A7	Died	0
		Sick	0
A2 and A7 combined	A2	Died	0
		Sick	1
	A7	Died	0
		Sick	0
Control	A2	Died	0
		Sick	3
	A7	Died	0
		Sick	3

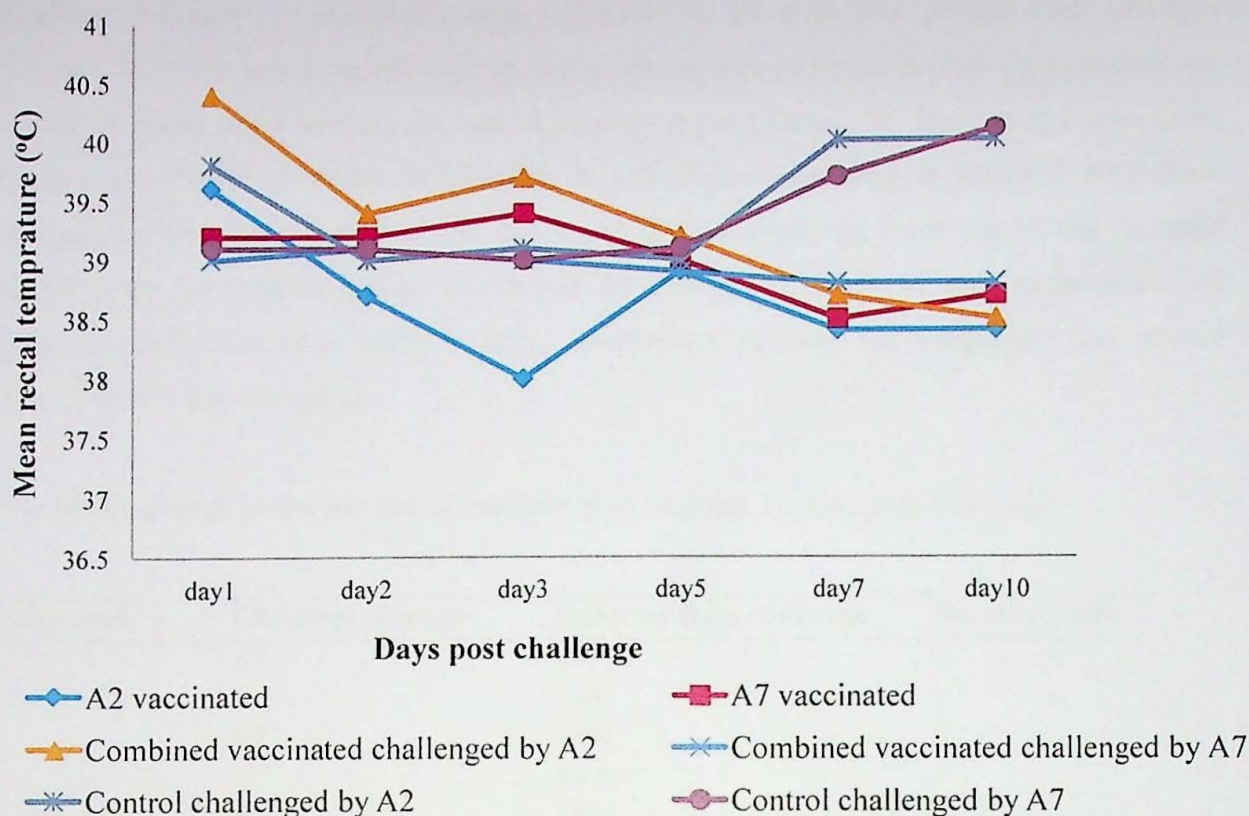


Figure 3: Post challenge mean rectal temperature

Animals vaccinated with *M. haemolytica* A2 showed no lung lesion except one animal, although less severe (+1) pneumonic lung lesions following intra-tracheal challenge with live *M. haemolytica* A2 compared to the control group. None of the animals that were vaccinated with the combined *M. haemolytica* A2 and A7 serotypes developed pneumonic lesions following intra-tracheal challenge with live *M. haemolytica* A7. However, Following intra-tracheal challenge with *M. haemolytica* A2, only one animal showed mild lung lesions (+1) which were significantly ($p < 0.05$) less severe than the unvaccinated control group. Similarly vaccination with the *M. haemolytica* A7 significantly ($p < 0.05$) reduced the severity of lung lesions following challenge with live *M. haemolytica* A7.

Only 3 animals (15%) from vaccinated groups with +1 scored lung lesions. All the lambs in unvaccinated control group were found to have lung lesions of scores between +2 and +3 (Figure 4). 50% of animals from unvaccinated control group have showed sever lung lesion (+3). When

the necropsy findings were evaluated comparatively, there was a significant statistical difference ($p < 0.05$) between the control and the trial groups concerning lung lesion scores, while there was no statistically significant difference ($p > 0.05$) between the 3 vaccinated groups. An important decrease was observed in lung lesions following vaccination. Lung lesions were detected in all of the 8 (100%) lambs in unvaccinated control group, while they were detected in 1 (20%) lamb in serotype A2 and A7 vaccinated, while only 10% from combined A2 and A7 serotypes vaccinated group (Table 11).



Figure 4: Lung lesions observed and their scores following challenge (a, lesion +2; b, lesion +3)

Table 11: Lung lesion scores following intra tracheal challenge of vaccinated and unvaccinated animals with live *M. haemolytica* A2 and A7

Group	Vaccination used	type	No. of animals challenged	Challenge serotype	No. of animals with lung lesion
1	Capsular antigen of <i>M. haemolytica</i> A2	serotype A2	5	<i>M. haemolytica</i> A2	1
2	Capsular antigen of <i>M. haemolytica</i> A7	serotype A7	5	<i>M. haemolytica</i> A7	1
3	Capsular antigen of <i>M. haemolytica</i>		5	<i>M. haemolytica</i> A2	1
	Serotype A2 and A7		5	<i>M. haemolytica</i> A7	0
4	Sterile saline water		4	<i>M. haemolytica</i> A2	4
			4	<i>M. haemolytica</i> A7	4

Pair wise comparison of lung lesion scores among the different treatment groups were compared by the Wilcoxon rank test. Statistical significance ($p < 0.05$) difference in lung lesion scores were observed between the vaccinated and unvaccinated control group as shown in table 12. A very strong difference of ranking revealed between the combined A2 and A7 vaccinated and the control ($p = 0.010$). Moreover, animals vaccinated with combined A2 and A7 were showed the least lung lesion score when compared to separate serotype A2 and A7 vaccinated groups. However, the difference was not statistically significant ($p > 0.05$) between vaccinated groups.

Table 12: Lung lesion score pair wise comparison between treatment groups

Groups	Compared groups	Standard deviation		Mean ranks		Sum of ranks		P value
		Group	Compared group	Positive rank	Negative rank	Positive rank	Negative rank	
A2	Control	0.447	0.535	0	3	15	0	0.039
	A7	0.447	0.447	1.5	1.5	15	1.5	1.000
	A2and A7	0.447	0.316	0	0	0	0	1.000
A7	Control	0.447	0.535	0	3	15	0	0.039
	A2 and A7	0.447	0.316	1.5	1.5	1.5	1.5	1.000
	A2	0.447	0.447	1.5	1.5	1.5	1.5	1.000
A2 and A7 combined	Control	0.316	0.535	0	4.5	36	0	0.010
	A2	0.316	0.447	0	0	0	0	1.000
	A7	0.316	0.447	1.5	1.5	1.5	1.5	1.000
Control	A2	0.535	0.447	3	0	0	15	0.039
	A7	0.535	0.447	3	0	0	15	0.039
	A2 and A7	0.535	0.316	4.5	0	0	36	0.010

4.3. Correlation between the antibody response and extent of lung lesions

Generally there was a moderately weak correlation between antibody titer and lung lesion score following challenge ($r^2 = 0.314$). However, negative correlation was observed between the two variables (Figure 5).

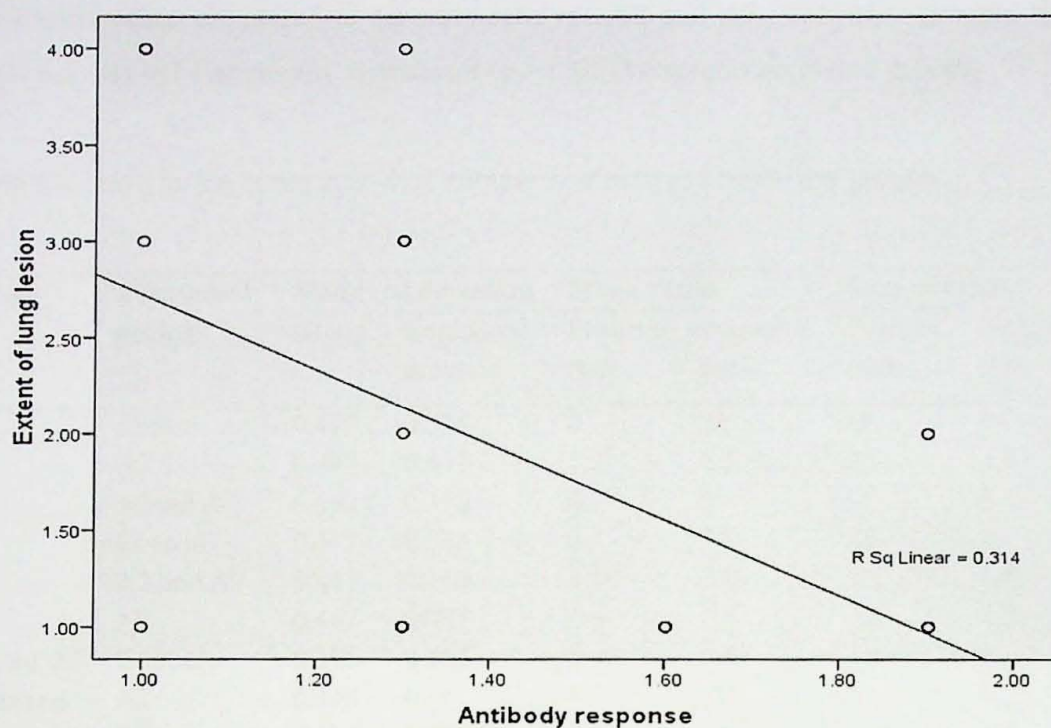


Figure 5: Correlation between antibody response and extent of lung lesion

5. DISCUSSION

Genus *Mannheimia haemolytica* (formerly called *Pasteurella haemolytica*) is the most important respiratory pathogens affecting domestic ruminants, especially in sheep and cattle, causing fibrinous, necrotic pneumonia. *M. haemolytica* constitutes the most significant etiology of respiratory system infection, which is a major problem for sheep breeders in all over the world. This disease, commonly called “shipping fever”, is a leading cause of economic loss. Factors such as transportation, environmental factors, viral infection, overcrowded housing and other stress factors also may predispose the opportunistic infection by the pathogen to cause sheep pneumonia. At present, several commercial vaccines have been developed to control pasteurellosis in sheep, such as; bacterins, live attenuated, leukotoxin, capsule, lipopolysaccharide, subunit vaccines, sodium salicylate extract and potassium thiocyanate.

In this study, efficacy of an experimental vaccine against experimental *M. haemolytica* infection in sheep and serological determination of antibody titers following vaccinations were investigated. This study was performed with four different trial groups of lambs kept in appropriate environmental and management facilities. The capsular antigens of *M. haemolytica* serotype A2, A7 and the combined serotype (A2 and A7) were vaccinated from lambs in groups 1, 2 and 3, respectively, and unvaccinated control group (group 4), followed by challenge with homologues serotype of *M. haemolytica* in all groups and at last necropsies were performed.

The experimental vaccine was prepared in *Pasteurella* vaccine production medium employed for the mass cultivation of *M. haemolytica* serotypes at 37°C for 24 hours of incubation. The growth was insured through morphological examination of slide smear under the microscope and confirmed for the homogeneous and pure growth of the organism. The bacterial count was adjusted so that 1ml of each suspension contained 5×10^8 CFU/ml. This is virtually the same with the work of Jaramillo-Meza *et al.* (2007) with the exception of using different inoculums prepared at different growth phase of the bacteria. Antibody response was evaluated using IHA test which is a widely used technique and reliable for measuring immunity against *M. haemolytica* (Sawada *et al.*, 1982; Al- Tarazi, 2002; Jaramillo-Meza *et al.*, 2007).

Potentially, the vaccines tested in this experimental study might be useful because many of the serotypes of *M. haemolytica* present in the vaccine are known to cause pneumonic pasteurellosis in sheep in Ethiopia (Pegram *et al.*, 1980; Sisay and Zerihun, 2003; Ayelet *et al.*, 2004). *M. haemolytica* is a commensal organism of the nasopharynx of ruminants (Carter *et al.*, 1995; Quinn *et al.*, 2002). At the start of this study the pre vaccination IHA test suggested that there was a circulating antibody against both serotypes A2 and A7 in all lambs. This raises the possibility of the presence of antibodies against these organisms in conventional animals used in vaccine trial that might complicate the interpretation of the result. Similarly the work of Sabri *et al.* (2000) indicated that prior to vaccination all animals showed low antibody level against the OMP of *M. haemolytica* A2, A7 and A9, but their result was consistent with this study in a way that following vaccination the antibody levels significantly increased in all vaccinated animals above the levels shown by the control unvaccinated lambs. This indicated that the development of higher post vaccination titer shows the vaccine induce sero-conversion against the capsular antigens of *M. haemolytica*.

On this experimental study a significant higher GM titer was observed in A2 vaccinated group than A7 vaccinated group at the first week and this might be due to a relatively higher titer at the start of the experiment in A2 group. A considerable difference between vaccinated and control groups were observed after the 2nd week. This might be due to poor immunogenic nature of the capsular antigen forming polysaccharide as described by Townsend *et al.* (1987).

The current study describes the comparison of one time vaccination because of difficulty to prepare the antigen, but Sabri *et al.* (2000), Al- Tarazi (2002) and Akan *et al.* (2006) were used booster dose in their work and found an increased titer following the booster compared to one time vaccination. In this study, the significance higher titer between the vaccinated and controls persists for only 2 weeks and this may indicated that one inoculation is not enough to induce a high antibody titer as reported by Jones *et al.* (1989).

Though significant variation was noticed in GM antibody titer, but not as such strong and this might be attributed to different factors like age and sensitivity of the test. This hypothesis substantiated by Donachie (1994) as reported that a vaccine prepared from the capsule and OMPs

from serotype A2 inoculated subcutaneously into ewe's stimulated high titers of anti-capsular antibodies while serum collected from similarly vaccinated 10-week-old lambs did not show high anti-capsular antibodies. Another reason for the low IHA titer detected might be due to the lack of sensitivity of the test, as Burrells *et al.* (1979) described that the ELISA test was 160 times more sensitive than the IHA test in measuring antibody titer against *M. haemolytica* A1 in sera of vaccinated SPF lambs.

There was no any other work done so far on immunization of lambs using the combined serotypes A2 and A7, but this research revealed that a promising result (GM titer $\log_{10}^{1.66}$ and $\log_{10}^{1.57}$ at the 3rd and 4th weeks, respectively) which was significantly higher than the control group. But, to say more a thorough investigation recommended on whether the combined efficacy is better in protection by both challenge simultaneously.

Research on pneumonia caused by *M. haemolytica* was impeded for many years by the absence of reproducible and standardizable methods for experimental infection to understand the pathogenesis and immunity. Production of pneumonia proved difficult in conventional sheep, unless very high number of organisms were given intra tracheally. The use of SPF lambs pre-infected by other biological pre-disposing factors such as viruses (*Parainfluenza virus type 3*), *Mycoplasmas* or *Chlamydia* and 7 days later exposed to a high challenge dose of *M. haemolytica* by aerosol for not less than 15 min within a chamber have proved to be a generally reliable model of the natural disease (Gilmour *et al.*, 1983; Donachie *et al.*, 1986; Jones *et al.*, 1989). In this study because of limited resource and lack of facility intra tracheal challenge with a higher dose of *M. haemolytica* was used, this is exactly similar to the work done by Sabri *et al.* (2000) and Akan *et al.* (2006) though they use relatively lower challenge dose (3ml of 1.3×10^9).

Even though no mortality was observed after challenge in all treatment groups, 75% (N = 6) morbidity was observed in the control group in contrast to the vaccinated 10% (N = 2). There was a mean rise rectal temperature of the controls challenged by A2 (40°C) and A7 (40.1°C), indicated that the challenged unvaccinated group might develop clinical signs. Generally vaccinated lambs developed less severe clinical signs of respiratory disease and subsequently had lower lung lesion scores than the unvaccinated control group. This clearly indicated that the

vaccine could provide protection against a severe *M. haemolytica* challenge. Crouch *et al.* (2012) found that both vaccinated and unvaccinated groups showed that there was no rise in mean rectal temperature at the first 2 days post challenge, but gradually increases in control group post challenge.

The result of the lung lesion score post challenge showed that only 20% (N = 1) in A2 and A7 serotype vaccinated groups and 10% (N = 1) in the combined serotype vaccinated group were recorded. Moreover in all the above cases +1 degree of lesion was observed which is represented as a very small portion of the lung mass is involved (only 3% of the cranial portion of the lung lobe). In contrast, 100% (N = 8) of the control group showed an intense degree of lesion +2 and +3 which constituted large mass of the lung portion. In case of the combined vaccinated group after challenge with *M. haemolytica* serotype A7, there was no lamb that showed a lesion (N = 0). This result is consistent with the finding of Akan *et al.* (2006), following the *M. haemolytica* challenge of two vaccinated groups with a commercial vaccine (One shot Ultra 8) (Pfizer Animal Health Inc. USA, 2011) comprised of a *M. haemolytica* A1 bacterins + toxoid and the control group, necropsies were performed and they were observed lung lesion in all the lambs in control group. Similar studies were performed with other experimental models of other animals. Sabri *et al.* (2000) also performed challenge studies on vaccinated sheep with homologous strains by using OMP extracted from *M. haemolytica* A2, A7 and A9 serotypes as a vaccine and they found that OMP vaccine prepared from A7 serotype confers good protection (none of the animals were develop pneumonic lesion). They also found similar result in OMP A2 serotype vaccinated group only 1% pneumonic lesion. In contrary Goodwin- Ray *et al.* (2008) came upon a result of no significance difference by vaccinating lambs with Ovipast Plus for preventing development of pneumonic lesion. The reason they propose was due to censoring of those lambs that had good body condition. Besides the manufacturer claims regarding the vaccine were it reduces mortality but without published evidence of its efficacy on preventing morbidity.

In the current study correlation between the lung lesion and the GM titer was not as such strong ($r^2=0.314$), but was found negative correlation indicates the higher the antibody titer the lower the extent of lung lesion. This could be due to measuring of antibody titer against capsular antigen of the organism (but antigenic components such as LKT and OMP can show a higher antibody titer)

and due to using of lower number of lambs in the control group. Similar low-to-moderate correlations were observed in earlier vaccination trials involving pasteurella vaccines (Effendy *et al.*, 1998; Zamirah, 1998). In contrary, the work of Sabri *et al.* (2000) revealed that vaccination with the OMPs of *M. haemolytica* A2 ($r^2 = 0.78$), A7 ($r^2 = 0.92$), and A9 ($r^2 = 0.85$) was generally provided good correlation between the antibody responses and the extent of lung lesions following homologous challenge. The other reason for weak correlation between the lung lesion and the GM titer might be due to the presence of other organisms that could result lung lesions other than serotypes of *M. haemolytica*.

This current study showed that the vaccine induced sero-conversion as a measure of efficacy against the surface antigen, but still the use of good antigenic component in the vaccine is a requisite which lead to a strong antibody response.

6. CONCLUSION AND RECOMMENDATIONS

Respiratory diseases are of great economic concern for sheep producers in different parts of Ethiopia. One of the potential respiratory diseases of sheep is pneumonic pasteurellosis. Serotypes of *Mannheimia haemolytica* are the primary etiological agent of pneumonic pasteurellosis in sheep. The control of the disease mainly relies on vaccination. Vaccine research on pneumonic pasteurellosis in sheep has primarily focused on *M. haemolytica* serotype A2 followed by A7; furthermore, vaccine containing different serotypes should be incorporated in the effective control of the disease. The present investigation showed that the tested experimental vaccine induced a moderate sero-conversion against the capsular antigen in vaccinated lambs. Besides, the titer will not stay longer ensuing one injection of the vaccine is not enough to induce a high titer. Lambs vaccinated by A7 and the combined serotype showed better titer than A2 serotype vaccinated group. However, serotype A2 had induced and reached its peak antibody titer earlier and stayed longer compared to other vaccinated groups. Though tried on the first time the combined serotype vaccine showed a promising result, but still necessitates further detail study on the immunological interaction of the body to the combined antigen. The efficacy of the vaccine is also measured by challenge protection test and all tested vaccines sufficiently protect the homologues challenge, but superior protection obtained by the combined serotype vaccinated group. Although lung lesion and antibody titer were negatively correlated, the correlation was moderately weak. Hence, the significantly lower occurrence of lung lesions in all vaccinated groups compared to control groups but a weak correlation implies that, other component of the vaccine might have greater role in protecting lambs from challenge trial.

Hence, based on the above conclusion, the following recommendations are forwarded:

- ❖ Extensive field study should be supplemented in order to observe the real challenge protection at natural infection.
- ❖ The geographical distribution of different serotypes of this organism should be investigated so that local ecotype based vaccine is prepared.

- ❖ These vaccines, preferably the combination serotype, should be produced in bulk and made available for customers so as to reduce the current problem of the disease.
- ❖ Further investigation on other antigenic components of the organism especially leukotoxin and iron regulated outer membrane proteins should be done to utilize as a vaccine.

7. REFERENCES

- Abdullah, K. M., Lo, R. Y. C. and Mellors, A. (1991): Cloning, nucleotide sequence and expansion of the *Pasteurella haemolytica* A1 glycoprotease gene. *J. Bacteriol.*, **173**: 5597-5603.
- Abebe, M., Alemu, Y. and Hegde, P. B. (2002): Growth performance and mortality of Menz sheep in the traditional sector of Lallo, Mama Mider Woreda, North Shoa. In: Proceedings of the 8th Annual Conference of Ethiopian Society for Animal Production (ESAP), May 18-20, 2001, Addis Ababa, Ethiopia, pp 85-98.
- Abera, M. (2005): Bacteriological study of the respiratory tract of sheep slaughtered at Debre zeit ELFORA export abattoir. MSc thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre zeit, Ethiopia.
- Adlam, C. (1989): The structure, function and properties and extracellular components of *Pasteurella haemolytica*. In: Adlam, C. and Rutter, J. M. (Eds.), *Pasteurella* and pasteurellosis, (Academic Press, London, UK), pp 75-92.
- Akan, M., Öncel, T., Sareyyüpoğlu, B., Hazıroğlu, R., Yaşar Tel, O. and Cantekin, Z. (2006): Vaccination studies of lambs against experimental *Mannheimia* (*Pasteurella*) *haemolytica* infection. *Small Rum. Res.*, **65**: 44–50.
- Al-Ghamdi, G. M., Ames, T. R., Baker, J. C., Walker, R., Chase, C. C., Frank, G. H. and Maheswaran, S. K. (2000): Serotyping of *Mannheimia* (*Pasteurella*) *haemolytica* isolates from the upper Midwest United States, *J. Vet. Diagn. Invest.*, **12**:576–578.
- Al-Tarazi, Y.H.M. (2002): The efficacy of an experimental *Pasteurella hemolytica* vaccine as measured by sero-conversion in Awassi lambs in Jordan. *Small Rum. Res.*, **45**: 201-208.
- Andrews, G. A. and Kennedy, G. A. (1997): Respiratory diagnostic pathology. *Vet. Clin. North Am. Food Anim. Pract.*, **13**: 515-547.
- Angen, O., Mutters, R., Caugant, D. A., Olsen, J. E., Bisgaard, M. (1999): Taxonomic relationships of the *Pasteurella haemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia haemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp.

- nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. Nov. *Int. J. Syst. Bacteriol.*, **49**: 67–86.
- Ayelet, G. (1996): Epidemiological and serological investigation on multi factorial ovine respiratory diseases and vaccine trials on the highlands of North Shoa, Ethiopia. DVM thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre zeit, Ethiopia.
- Ayelet, G., Yigezu, L., Gelaye, E., Tariku, S. and Asmare, K. (2004): Epidemiologic and Serologic Investigation of Multifactorial Respiratory Disease of Sheep in the Central Highland of Ethiopia. *Intern. J. Appl. Res. Vet. Med.*, **2**: 274 - 278.
- Ayling, R. D. and Nicholas, R. A. J. (2007): Mycoplasma respiratory infections. In: Aitken, I.D. (Ed.), *Diseases of Sheep*, 4th ed. Blackwell Publishing, Moredun, pp 231– 235 (Chapter 33).
- Bahaman, A. R., Nurida, A. B., Sheikh-Omar, A. R. and Zamri-Saad, M. (1991): Biotypes and serotypes of *Pasteurella haemolytica* and their importance in the production of vaccines for pneumonic pasteurellosis in sheep. *J. Vet. Malaysia*, **3**: 33-35.
- Biberstein, E. L. and Thomson, D. A. (1996): Epidemiological studies on *Pasteurella haemolytica* in sheep. *J. Comp. Pathol.*, **71**: 83–94.
- Bingham, D. P., Moore, R. and Richards, A. B. (1990): Comparison of DNA: DNA homology and enzymatic activity between *Pasteurella haemolytica* and related species, *Am. J. Vet. Res.*, **51**:1161–1166.
- Blackall, P. J., Bojesen, A. M., Christensen, H. and Bisgaard, M. (2007): Reclassification of [*Pasteurella*] *trehalosi* as *Bibersteinia trehalosi* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.*, **57**: 666 – 674.
- Blood, D. C. and Radostits, O. M. (1994): Disease caused by *Pasteurella* species. In: *A Textbook of the diseases of Cattle, Sheep, Pigs, Goats and Horses*. (8th Ed.). Brailliere Tindal, London, UK, pp. 748–785.
- Braun, V., Gaisser, S., Herrmann, C., Kampfenkel, K., Killmann, H. and Traub, I. (1996): Energy coupled transport across the outer membrane of *Escherichia coli*: ExbB binds ExbD and TonB *in vitro*, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. *J. Bacteriol.*, **178**:2836-2845.
- Brogden, K. A., Lehmkuhl, H. D. and Cutlip, R. C. (1998): *Pasteurella haemolytica* complicated respiratory infections in sheep and goats. *Vet. Res.*, **29**: 233–254.

- Burrells, C., Wells, D. W. and Dawson, A. M. (1979): The quantitative estimation of antibody to *Pasteurella haemolytica* in sheep sera using a micro enzyme linked immunosorbent assay (ELISA). *Vet. Microbiol.*, **3**: 291–301.
- Carter, G. R., Chengappa, M. M., Roberts, A. W., William Claus, G. and Rikihisa, Y. (1995): In: *Pasteurella and Francisella, Essential of Veterinary Microbiology*. (5th Ed.). Williams and Wilkins, Baltimore, USA, pp 171–179.
- Chasdrasekaran, S., Hizat, K., Saad, Z., Johara, M. Y. and Yeap, P. C. (1991): Evaluation of combined *Pasteurella* vaccines in control of sheep pneumonia. *Br. Vet. J.*, **147**: 437–443.
- Cole, N. A. (1996): Metabolic changes and nutrient repletion in lambs provided with electrolyte solutions before and after feed and water deprivation. *J. of Animal Sci.*, **74** (2): 287–294.
- Confer, A. W. (1993): Immunogens of *Pasteurella*. *Vet. Microbiol.* **37**: 353- 368.
- Conlon, J. A. R. and Shewen, P. E. (1993): Clinical and serological evaluation of a *Pasteurella haemolytica* A1 capsular polysaccharide vaccine. *Vaccine*, **11**: 767-772.
- Conlon, J. A., Shewen, P. E. and Lo, R. Y. C. (1991): Efficacy of recombinant leukotoxin in protection against pneumonic challenge with live *Pasteurella haemolytica* A1. *Infect. Immun.*, **59**(2): 587-591.
- Cooney, B. J. and Lo, R. Y. C. (1993): Three contiguous lipoprotein genes in *Pasteurella haemolytica* A1 which are homologous to a lipoprotein gene in *Haemophilus influenzae* type b. *Infect. Immun.*, **61**: 4682- 4688.
- Cornelissen, C. N., Kelley, M., Hobbs, M. M., Anderson, J. E. and Cannon, J. G. (1998): The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. *Mol. Microbiol.*, **27**:611-616.
- Coutte, L., Alonso, S., Reveneau. N., Willery, E., Quatannens, B., Loch, C. and Jacob-Dubuisson, F. (2003): Role of adhesin release for mucosal colonization by a bacterial pathogen. *J. Exp. Med.*, **197**:735-742.
- Crouch, C. F., LaFleur, R., Ramage, C., Reddick, D., Murray, J., Donachie, W. and Francis, M. J. (2012): Cross protection of a *Mannheimia haemolytica* A1 Lkt-/ *Pasteurella multocida* _hyaE bovine respiratory disease vaccine against experimental challenge with *Mannheimia haemolytica* A6 in calves. *Vaccine*, **30**: 2320-2328.
- CSA (2009): Central Statistical Authority of Ethiopia, Addis Ababa, Ethiopia.

- Czuprynski, C. J., Leite, F. and Sylte, M. (2004): Complexities of the pathogenesis of *Mannheimia haemolytica* and *Haemophilus somnus* infections: Challenges and potential opportunities for prevention. *Anim. Health Res. Rev.*, **5**: 277–282.
- Davies, R. L. and Baillie, S. (2003): Cytotoxic activity of *Mannheimia haemolytica* strains in relation to diversity of the leukotoxin structural gene LKT A. *Vet. Microbiol.*, **92**:263–279.
- Davies, R. L. and Donachie, W. (1996): Intra-specific diversity and host specificity within *Pasteurella haemolytica* based on variation of capsular polysaccharide, lipopolysaccharide and outer membrane proteins. *J. Microbiol.*, **142**:1895–1907.
- De Alwis, M. C. (1993): Pasteurellosis in production animals. A review in: Patent, B. E., Spencer, T. L. and Jonson, R. B. (Eds.). ACIAR Monograph No. 43, Australia, Canberra. Pp 11–12.
- Deressa, A., Asfaw, Y., Lubke, B., Kyule, M. W., Tefera, G. and Zessin, K. H. (2010): Molecular Detection of *Pasteurella multocida* and *Mannheimia haemolytica* in Sheep Respiratory Infections in Ethiopia. *Intern. J. Appl. Res. Vet. Med.*, **8**: 101–108.
- Deshpande, M. S., Ambagala, T. C., Ambagala, A. P., Kehrli, M. E. and Srikumaran, S. (2002): Bovine CD18 is necessary and sufficient to mediate *Mannheimia* (*Pasteurella*) *haemolytica* leukotoxin- induced cytolysis. *Infect. Immun.*, **70**:5058–5064.
- Diker, K. S., Akan, M. and Kaya, O. (2000): Evaluation of immunogenicity of *Pasteurella haemolytica* serotypes in experimental models. *Turkey J. Vet. Anim. Sci.*, **24**: 139–143.
- Donachie, W. (1994): Vaccine Development against *Pasteurella haemolytica* infections in Sheep, *Haemophilus*, *Actinobacillus* and *Pasteurella* (H.A.P.). In: Proceedings of the International Conference on Abstract Book and Programme, 31 July–4 August, 1994, Edinburgh, Scotland, pp 121–167.
- Donachie, W. (1998): Bacteriology of bovine respiratory disease. *UK Vet.*, **3**:44–46.
- Donachie, W. (2003): The development of iron regulated protein vaccines against pasteurellosis. *J. of the Royal Agri. Soc. of Eng.*, **164**: 1–8.
- Donachie, W., Burrells, C. and Dawson, A. M. (1983): Specificity of the enzyme-linked immune sorbent assay (ELISA) for anti bodies in the sera of specific pathogen-free lambs vaccinated with *Pasteurella haemolytica* antigens. *Vet. Microbiol.*, **8**: 199 – 205.

- Donachie, W., Burrells, C., Sutherland, A. D., Gilmour, J. S. and Gilmour, N.J.L. (1986): Immunity of specific pathogen-free lambs to challenge with an aerosol of *Pasteurella haemolytica* biotype A serotype. Part 2. Pulmonary antibody and cell responses to primary and secondary infections. *Vet. Immunol. Pathol.*, **11**: 265–279.
- Effendy, A. W., Zamri-Saad, M. and Rosiah, S. (1998): Efficacy of intranasal administration of formalin-killed *Pasteurella haemolytica* A2 against intra tracheal challenge in goats. *Vet. Rec.*, **142**: 428-431.
- FAO (2004): FAO Production Yearbook, **41**: Rome, Italy.
- Frank, G. H. (1989): Pasteurellosis of cattle. In: Adlam, C. F. and Rutter, J. M. (1st Ed): *Pasteurella* and Pasteurellosis. London: Academic Press. Pp. 197-222.
- Frank, G. H. and Briggs, R. E. (1992): Colonization of the tonsils of calves with *Pasteurella haemolytica*. *Am. J. Vet. Res.*, **53**: 481-484.
- Frank, G. H. and Smith, P. C. (1983): Prevalence of *Pasteurella haemolytica* in transported calves. *Am. J. Vet. Res.*, **44**:981–985.
- Fraser, C. (1991): In: The Merck Veterinary Manual. Merck and Co., Inc., Rahway, New Jersey, pp. 723-724.
- Fuller, C. A., Yu, R., Irwin, A. W. and Schryvers, A. B. (1998): Biochemical evidence for a conserved interaction between bacterial transferrin binding protein A and transferrin binding protein B. *Microbiol. Pathol.*, **24**:75-78.
- Gatewood, D. M., Fenwick, B.W. and Chengappa, M. M. (1994): Growth condition dependent expression of *Pasteurella haemolytica* A1 outer membrane protein, capsule, and leukotoxin. *Vet. Microbiol.*, **41**: 221-233.
- Gentry, M. J., Confer, A. W., Weinberg, E. D. and Homer, J. T. (1986): Cytotoxin (leukotoxin) production by *Pasteurella haemolytica*: requirement for an iron containing compound. *Am. J. Vet. Res.*, **47**:1919-1923.
- Gilmour, N. J. (1993): *Pasteurella*. In: Pasteurellosis in production animals. A review in: Patten, B. E., Spencer, T. L. (Ed). *ACIR proceedings*. Australia, **43**: Pp 84- 89.
- Gilmour, N. J. L. and Gilmour, J. S. (1989): Pasteurellosis of sheep. In: Adlam, C. and Rutter, J. M. (1st Ed.): *Pasteurella* and Pasteurellosis. Academic Press, London, UK. Pp. 223–254.

- Gilmour, N. J. L., Donachie, W., Sutherland, A. D., Gilmour, J. S., Jones, G. E. and Quirie, M. (1991): Vaccine containing iron-regulated proteins of *Pasteurella haemolytica* A2 enhances protection against experimental pasteurellosis in lambs. *Vaccine*, **9**: 137–140.
- Gilmour, N. J. L., Martin, W. B., Sharp, J. M., Thompson, D. A., Wells, P. W. and Donachie, W. (1983): Experimental immunization of lambs against pneumonic pasteurellosis. *Res. Vet. Sci.*, **35**(1): 80–86.
- Gilmour, N. J. L., Martin, W. B., Sharp, J. M., Wells, P. W. and Thompson, D. A. (1979): The development of vaccines against pneumonic pasteurellosis in sheep. *Vet. Rec.*, **104**: 15.
- Gilmour, N. J. L., Quirie, M., Jones, G. E. and Gilmour, J. S. (1988): Metaphylactic use of long acting oxytetracycline in pasteurellosis in lambs. *Vet. Rec.*, **123**:443-444.
- Gilmour, N. J. L., Sharp, J. M. and Gilmour, J. S. (1982): Effect of oxytetracycline therapy in experimentally induced pneumonic pasteurellosis. *Vet. Rec.*, **111**: 97-99.
- Gonçalves, R., Mariano, I., Núñez, A., Branco, S., Fairfoul, G. and Nicholas, R. (2010): Atypical non-progressive pneumonia in goats. *The Vet. J.*, **183**: 219-221.
- Gonzalez- Rayos, C., Lo, R. Y. C., Shewen, P. E. and Beveridge, T. J. (1986): Cloning of a Serotype- specific antigen from *Pasteurella haemolytica* A1. *Infect. Immun.*, **53**: 505-510.
- Goodwin-Ray, K. A. (2006): Pneumonia and pleurisy in sheep: Studies of prevalence, risk factors, vaccine efficacy and economic impact. PhD Thesis, Massey University, Palmerston North New Zealand.
- Goodwin-Ray, K. A., Stevenson, M. A. and Heuer, C. (2008): Effect of vaccinating lambs against pneumonic pasteurellosis under New Zealand field conditions on their weight gain and pneumonic lung lesions at slaughter. *Vet. Rec.*, **162**: 9-11.
- Haig, S. J. (2011): Adherence of *Mannheimia haemolytica* to ovine bronchial epithelial cells. *Biosci. Horizons*, **4**: 50- 60.
- Highlander, S. K. (2001): Molecular genetic analysis of virulence in *Mannheimia (Pasteurella) haemolytica*. *Front Biosci.*, **6**:1128-1150.
- Hodgson, D. P., Aich, P., Manuja, A., Hokamp, K., Roche, M. F., Brinkman, L. S. F., Potter, A., Babiuk, A. L. and Griebel, J. P. (2005): Effect of stress on viral- bacterial synergy in bovine respiratory disease: Novel mechanisms to regulate inflammation. *J. Comp. Fun. Gen.*, **6**: 244- 250.

- Hussein, A. M. and Mohamed, E. O. (1984a): Serological survey of sheep sera for antibodies to *Pasteurella haemolytica* serotypes in Sudan. *Rev. Elev. Med. Vet. Trop.*, **37**:418–421.
- Hussein, A. M. and Mohamed, E. O. (1984b): Serology and Mycoplasma. *Vet. Sci.*, **47**: 84- 89.
- Imler, J. L. and Hoffmann, J. A. (2001): Toll receptors in innate immunity. *Trends in Cell Biol.*, **11**:304-311.
- Intervet Ltd. (2011): Ovipast Plus Intervet Limited, New Zealand. http://www.intervet.co.nz/products/ovipast_plus/010/overview.asp. Accessed on 18 December, 2011.
- Intervet South Africa (Pty.) Ltd. (2011): Multivax P Intervet South Africa Limited. http://www.intervet.co.za/products/multivax_p/020/product_details.asp. Accessed on 18 December, 2011.
- Intervet UK Ltd. (2011b): Ovipast Plus Intervet United Kingdom Limited. http://www.intervet.co.uk/products_public/ovipast_plus/010/overview.asp. Accessed on 18 December, 2011.
- Intervet UK Ltd. (2011c): Ovivac-P Plus Intervet United Kingdom Limited. http://www.intervet.co.uk/Products_Public/Ovivac_P_Plus/O10/Overview.asp. Accessed on 18 December, 2011.
- Intervet UK Ltd. (2011a): Heptavac P Plus Intervet United Kingdom Limited. http://www.intervet.co.uk/Products_Public/Heptavac_P_Plus/O10/Overview.asp. Accessed on 18 December, 2011.
- Jaramillo-Meza, L., Aguilar-Romero, F., Su'arez-G'üemes, F. and Trigo-Tavera, F. (2007): Challenge exposure of sheep immunized with live vaccine and culture supernatant of *Mannheimia haemolytica* A1: Effects of revaccination. *Small Rum. Res.*, **70**: 209-217.
- Jones, G. E., Donachie, W., Gilmour, J. S. and Rae, A. G. (1986): Attempt to prevent the effects of experimental chronic pneumonia in sheep by vaccination against *Pasteurella haemolytica*. *British Vet. J.*, **142**: 189 - 194.
- Jones, G. E., Donachie, W., Sutherland, A. D., Knox and Gilmour (1989): Protection of lambs against experimental pneumonic pasteurellosis by transfer of immune serum. *Vet. Microbiol.*, **20**: 59–71.
- Kassahun, A., Getachew, G., Zelalem, A., Negussie, A. and Fletcher, I. (1989): Small ruminant production in Ethiopia: Constraints and future prospects. In: Proceedings of the 3rd

- National Livestock Improvement Conference. Institute of Agricultural Research, Addis Ababa, Ethiopia.
- Kehrenberg, C. G., Gundula, S. T., Jean- Louis, M., Elisabeth, C. D. and Schwarza, S. (2000): Antimicrobial resistance of *Pasteurella* and *Mannheimia*: Epidemiology and genetic basis. *Vet. Res.*, **32**: 323- 329.
- Kisiela, D. and Czuprynski C. J. (2009): Identification of *Mannheimia haemolytica* adhesins involved in binding to bovine bronchial epithelial cells. *Infect. Immun.*, **77**: 446–455.
- Kitt, T. (1885): Uber eine experimentelle, der Rinderseuche ahnliche Infektionskrankheit, Sitzunbsber. Ges. Morphol. Physiol. *Muenchen*, **23**: 140–168.
- Knowles, T. G., Brown, S. N., Warriss, P. D., Phillips, A. J., Dotan, S. K., Hunt, P., Ford, J. E., Edwards, J. E. and Watkins, P. E. (1995): Effects on sheep of transport by road for up to 24 hours. *Vet. Rec.*, **136**(17): 431 - 438.
- Laboratories Intervet S.A. (2011): Ovipast Plus Intervet Limited Spain. http://www.intervet.es/products/ovipast_plus/020_ficha_tcnica.asp. Accessed on 18 December, 2011.
- Lawrence, P. K., Kittichotirat, W., McDermott, J. E. and Bumgarner, R. E. (2010): A three-way comparative genomic analysis of *Mannheimia haemolytica* isolates. *Bio. Med. Cent. Ltd. Geno.*, **11**:535.
- Littledike, E. T. (1993): Variation of abscess formation in cattle after vaccination with a modified-live *Pasteurella haemolytica* vaccine. *Am. J. of Vet. Res.*, **54**: 1244 - 1248.
- Litwin, C. M. and Calderwood, S. B. (1993): Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.*, **6**:137-149.
- Lo, R. Y. C. (2001): Genetic analysis of virulence factors of *Mannheimia (Pasteurella) haemolytica* A1. *Vet. Microbiol.*, **83**: 23-35.
- Lo, R. Y. C. and Mellors, A. (1996): The isolation of recombinant plasmids expressing secreted antigens of *Pasteurella haemolytica* A1 and the characterization of an immunogenic 60 KDa antigen. *Vet. Microbiol.*, **51**: 381-391.
- Lo, R. Y. C., Mckerral, L. J., Hills, T. L. and Kostrzynstra, M. (2001): Analysis of the capsule biosynthetic locus of *Mannheimia (Pasteurella) haemolytica* A1 and proposal of a nomenclature system. *Infect. Immun.*, **69**(7): 4458- 4464.

- Lo, R. Y. C., Shewen, P. E., Strathdee, C. A. and Greer, C. N. (1985): Cloning and expression of the leukotoxin gene of *Pasteurella haemolytica* A1 in *Escherichia coli* K- 12. *Infect. Immun.*, **50**: 667-671.
- Lo, R. Y. C., Strathdee, C. A. and Shewen, P. E. (1987): Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. *Infect. Immun.*, **55**: 1987- 1996.
- Lo, R.Y.C. and Sorensen, L. S. (2007): The outer membrane protein OmpA of *Mannheimia haemolytica* A1 is involved in the binding of fibronectin. *FEMS Microbiol. Lett.*, **274**: 226-231.
- Martin, S. W. and Bohac, J. G. (1986): The association between serological titers in infectious bovine rhinotracheitis virus, bovine virus diarrhea virus, *Parainfluenza- 3 virus*, respiratory syncytial virus and treatment for respiratory disease in Ontario feedlot calves. *Can. J. Vet. Res.*, **50**:351-358.
- Martin, W. B. (1996): Respiratory infections of sheep. *Comp. Immunol. Microbiol. Infect. Dis.*, **19**:171-179.
- McIver, K. S., Heath, A. S. and Scott, J. R. (1995): Regulation of virulence by environmental signals in group A streptococci: influence of osmolarity, temperature, gas exchange, and iron limitation on transcription. *Infect Immun.*, **63**:4540-4542.
- McPherson, M. J. and Moller, S. G. (2000): Polymerase Chain Reaction. BIOS Scientific Publishers Ltd., Oxford, pp 1-18.
- Mekalanos, J. J. (1992): Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.*, **174**:1-7.
- Miller, M. W. (2001): Pasteurellosis. In: Williams, E. S. and Barker, I. K. (3rd Ed.): Infectious Diseases of Wild Mammals. Iowa: Iowa State University Press. Pp 330-339.
- Morton, D. J. and Williams, P. (1989): Utilization of transferrin-bound iron by *Haemophilus* species of human and porcine origins. *FEMS Microbiol. Lett.*, **53**:123-127.
- Morton, R. J., Simons, K. R. and Confer, A. W. (1996): Major outer membrane proteins of *Pasteurella haemolytica* serovars 1-15: comparison of separation techniques and surfaceexposed proteins on selected serovars. *Vet. Microbiol.*, **51**:319-330.
- Mosier, A. D., Panciera, J. R., Rogers, P. D., Uhlich, A. G., Butine, D. M., Confer, W. A. and Basaraba, J. R. (1998): Comparison of serology and protective response induced by two *Pasteurella* vaccines. *Can. J. Vet. Res.*, **62**: 178 - 182.

- Mosier, D. A. (1993): Prevention and control of Pasteurellosis. In: Pasteurellosis in Production Animals. ACIAR Proceedings, (Watson and Fergusson, Brisbane), Pp 121-135.
- Mulate, B. (2007): Study of ovine Pasteurellosis in highlands of South Wollo Zone of the Amhara Region, Ethiopia. MSc thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre zeit, Ethiopia.
- Nardin, P. M., Mellors, A. and Lo, R. Y. C. (1998): Characterization of a fourth lipoprotein from the *Pasteurella haemolytica* A1 and its homology to the OMP A family of outer membrane proteins. *FEMS Microbiol. Lett.*, **165**: 71- 77.
- NBE (2008): National Bank of Ethiopia annual report.
- Newson, I. E. and Cross, F. (1932): Some bipolar organisms found in pneumonia in sheep. *J. Am. Vet. Med. Assoc.*, **80**:711–719.
- Novartis Animal Vaccines Inc. (2011): Poly Serum, Novartis Ltd., United kingdom <http://www.novartis.com/products/livestock.shtml>. Accessed on 18 December, 2011.
- O'Brien, J. K. and Duffus, W. P. (1987): *Pasteurella haemolytica* cytotoxin: relative susceptibility of bovine leucocytes. *Vet. Microbiol.*, **13**: 321–334.
- Odugbo, M. O., Okpara, J. O., Abechi, S. A. and Kumbish, P. R. (2004): An outbreak of pneumonic pasteurellosis in sheep due to *Mannheimia (Pasteurella) haemolytica* serotype 7. *The Vet. J.*, **167**: 214–215.
- Ogunnariwo, J. A., Woo, T. K. W., Lo, R. Y. C., Gonzalez, G. C. and Schryvers, A. B. (1997): Characterization of the *Pasteurella haemolytica* transferrin receptor genes and the recombinant receptor proteins. *Microbiol. Pathol.*, **23**: 273- 284.
- OIE (1992): Diagnosis of Pasteurellosis. In: Manual of standards for diagnostic test and vaccine in terrestrial animals, **2**: Pp 1-27.
- OIE *Terrestrial Manual* (2008): Principles of Veterinary Vaccine Production. Pp. 90-104.
- Ojha, S., Lacouture, S., Gottschalk, M. and MacInnes, J. I. (2010): Characterization of colonization deficient mutants of *Actinobacillus suis*. *Vet. Microbiol.*, **140**: 122–130.
- Pandher, K., Murphy, G.L. and Confer, A.W. (1999): Identification of immunogenic surface-exposed outer membrane proteins of *Pasteurella haemolytica* serotype 1. *Vet. Microbiol.*, **65**: 215–226.

- Pati, U. S., Srivastava, S. K., Roy, S. C. and More, T. (1996): Immunogenicity of outer membrane protein of *Pasteurella multocida* in buffalo calves. *Vet. Microbiol.*, **52**: 301-311.
- Pegram, R. G., Roeder, P. L. and Scott, J. M. (1980): The prevalence of serotypes of *Pasteurella haemolytica* in Ethiopia. *Ethiop. Vet. Bull.*, **4**:18-25.
- Pfizer Animal Health Inc. USA (2011): One Shot Ultra 8, Pfizer Animal Health Inc. USA. <https://animalhealth.pfizer.com/sites/pahweb/US/EN/Products/Pages/OneShotUltra%20AE8.aspx>. Accessed on 31 May, 2012.
- Pipestone Veterinary Supply (2011): Ovine *Pasteurella haemolytica* pneumonia vaccine Pipestone Veterinary Supply. <http://www.pipevet.com/content/products.asp>. Accessed on 18 December, 2011.
- Potter, M. D. and Lo, R. Y. C. (1995): Cloning and characterization of a gene from *Pasteurella haemolytica* A1 involved in lipopolysaccharide biosynthesis. *FEMS Microbiol. Lett.*, **129**: 75-82.
- Potter, M. D. and Lo, R. Y. C. (1996): Cloning and characterization of the galE locus of *Pasteurella haemolytica* A1. *Infect. Immun.*, **64**: 855-860.
- Purdy, C. W., Cooley, J. D. and Straus, D. C. (1998): Cross-protection studies with three serotypes of *Pasteurella haemolytica* in a goat model. *Curr. Microbiol.*, **36**: 207-211.
- Purdy, C. W., Raleigh, R. H., Collins, J. K., Watts, J. L. and Straus, D. C. (1997): Serotyping and enzyme characterization of *Pasteurella haemolytica* and *Pasteurella multocida* isolates recovered from pneumonic lungs of stressed feeder calves. *Curr. Microbiol.*, **34**:244-249.
- Quinn, P. J., Carter, M. E., Markey, B. k. and Carter, G. R. (1994): *Pasteurella* species. In: Clinical Veterinary Microbiology. Wolfe publishing, Mosby, London, UK. Pp 254- 258.
- Quinn, P. J., Carter, M. E., Markey, B. K. and Carter, G. R. (1999): Clinical Veterinary Microbiology. Harcourt Publishers Limited, Edinburgh, London, UK, Pp 13- 28.
- Quinn, P. J., Markey, B. K., Carter, M. E., Donnelly, W. J. C. and Leonared, F. C. (2002): *Pasteurella* species and *Mannheimia haemolytica*. In: Veterinary Microbiology and Microbial Disease. Blackwell science, London, UK, Pp 137 - 143.

- Radostits, O. M., Blood, D. C. and Gay, C. C. (1994): Diseases caused by *Pasteurella* species. In: veterinary Medicine, Text book of the disease of cattle, sheep, pigs, goats and horses. (9th Ed.). Brailliere Tindal, London, UK, pp 748–785.
- Raetz, C. R. H. and Whitfield, C. (2002): Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.*, **71**:635-700.
- Ribble, C. S., Meek, A. H, Shewen, P. E., Jim, G. K. and Guichon, P. T. (1995): Effect of transportation on fatal fibrinous pneumonia and shrinkage in calves arriving at a large feedlot. *J. Am. Vet. Med. Assoc.*, **207**:612–615.
- Rimler, R. B. (1993): Laboratory techniques for *Pasteurella* typing and diagnosis of infections. In: Pasteurellosis in production animals. A review in: Patent, B. E., Spencer, T. L. and Jonson, R. B. (2nd Ed.). ACIAR, Australia, Camberra. Pp 136- 200.
- Robinson, A. and Melling, J. (1993): Envelope structure and the development of new vaccines. *J. of App. Bacteriol.*, **74**: 435-515.
- Roth, J. A. and Flaming, K. P. (1990): Model systems to study immune modulation in domestic food animals. *Adv. in Vet. Sci. and Comp. Med.*, **35**: 21 - 41.
- Sabri, M. Y., Zamri-Saad, M., Mutalib, A. R., Israf, D. A. and Muniandyb, N. (2000): Efficacy of an outer membrane protein of *Pasteurella haemolytica* A2, A7 or A9-enriched vaccine against intra tracheal challenge exposure in sheep. *Vet. Microbiol.*, **73**: 13-23.
- Sanderson, M. W., Dargatz, D. A. and Wagner, B. A. (2008): Risk factors for initial respiratory disease in United States' feedlots based on producer-collected daily morbidity counts. *Can. Vet. J.*, **49**:373–378.
- Sawada, T., Rimler, R. B. and Rhoades, K. R. (1982): Indirect Haemagglutination test that uses Glutaraldehyde- fixed sheep erythrocytes sensitized with extract antigens for detection of *Pasteurella* antibody. *J. Cl. Microbiol.*, **15**: 752-756.
- Schake, L. M., Webster, W. W. and Conway, R. C. (1980): Effect of origin and shipping distance on feedlot performance of cattle, pp 90–92.
- Seifert, S. H. H. (1996): Pasteurellosis. In: Tropical animal health. (2nd Ed). Kluwer academic publishers. Dordrecht, the Netherlands, pp 373- 378.
- Sharma, R., Woldehiwet, Z. Spiller, D. G. and Warenius, H. M. (1990): Lymphocyte subpopulations in peripheral blood of lambs experimentally infected with bovine respiratory syncytial virus. *Vet. Imm. and immunopatho.*, **24** : 383 – 391.

- Shewen, P. E. and Wilkie, B. N. (1985): Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. *Am. J. Vet. Res.*, **46**:1212-1214.
- Shewen, P. E. and Wilkie, B. N. (1988): Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. *Can. J. Vet. Res.*, **52**:30-36.
- Shreeve, B. J., Ivanov, I. N. and Thompson, D. A. (1970): Biochemical reactions of different biotypes of *Pasteurella haemolytica*. *J. Med. Microbiol.*, **30**: 356.
- Sisay, T. and Zerihun, A. (2003): Diversity of *Mannheimia haemolytica* and *pasteurella trehalosi* serotypes from apparently healthy sheep and abattoir specimens in the highlands of wollo, North East Ethiopia. *Vet. Res. Commun.*, **27**: 3-14.
- Smith, G. R. (1961): The characteristics of two types of *Pasteurella haemolytica* associated with different pathological conditions of sheep. *J. Pathol. Bacteriol.*, **81**:431-440.
- Sneath, P. H. and Stevens, M. (1990): *Actinobacillus rossii* sp. nov., *Actinobacillus seminis* sp. nov., nom. rev., *Pasteurella bettii* sp. nov., *Pasteurella lymphangitidis* sp. nov., *Pasteurella mairi* sp. nov., and *Pasteurella trehalosi* sp. Nov. *Int. J. Syst. Bacteriol.*, **40**:148- 153.
- Sreevatsan, S., Ames, T. R., Werdin, R. E., Yoo, H. S. and Maheswaran, S. K. (1996): Evaluation of three experimental subunit vaccines against pneumonic pasteurellosis in cattle. *Vaccine*, **14**: 147- 154.
- Stanier, R. Y., Doudoroff, M. and Adelberg, E. A. (1965): The microbial world. (2nd Ed.). Prentice Hall Inc., USA, pp 321- 341.
- Stojiljkovic, I., Kumar, V. and Srinivasan, N. (1999): Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. *Mol. Microbiol.*, **3**:429-442.
- Strathdee, C. A. and Lo, R. Y. C. (1989): Cloning, nucleotide sequence and characterization of the genes which encode the secretion function of the *Pasteurella haemolytica* leukotoxin determinant. *J. Bacteriol.*, **171**: 916-928.
- Sun, Y., Clinkenbeard, K. D., Cudd, L. A., Clarke, C. R. and Clinkenbeard, P. A. (1999): Correlation of *Pasteurella haemolytica* leukotoxin binding with susceptibility to intoxication of lymphoid cells from various species. *Infect. Immun.*, **67**:6264-6269.
- Tatum, F. M., Briggs, R. E., Sreevatsan, S. S., Zehr, E. S., Ling Hsuan, S., Whiteley, L. O., Ames, T. R. and Maheswaran, S. K. (1998): Construction of an isogenic leukotoxin

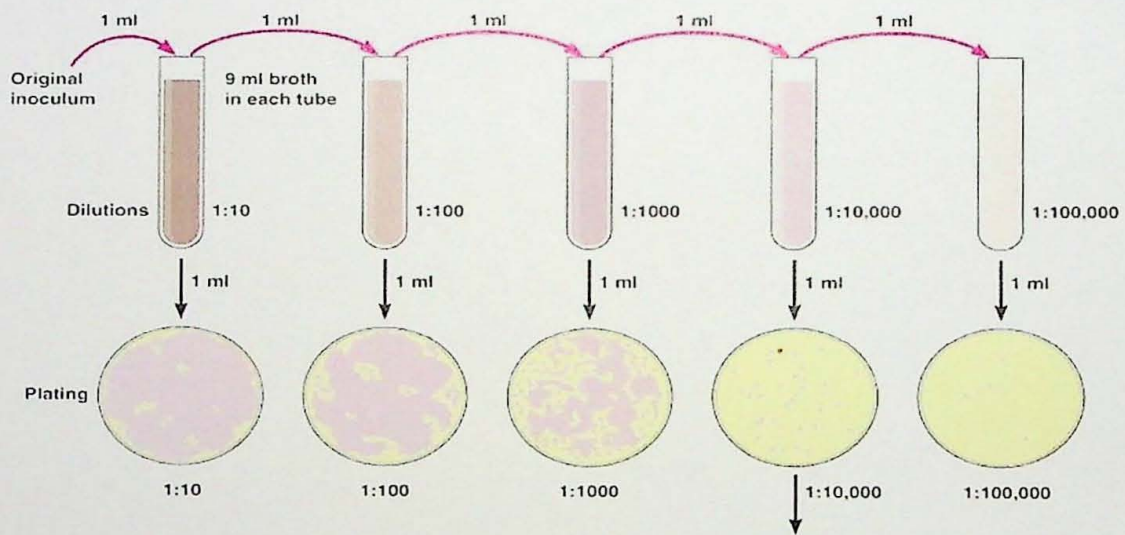
- deletion mutant of *Pasteurella haemolytica* serotype 1: characterization and virulence. *Microbiol. Pathol.*, **24**:37–46.
- Teferi, M. (2000): An Epidemiological study on ovine Pasteurellosis in Arsi, South- East Ethiopia. DVM thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre zeit, Ethiopia.
- Tehrani, A. A., Ras, M. B. and Niazy, H. (2004): Isolation and identification of *Pasteurella haemolytica* biotype A from sheep in Urmia, Iran. *Iranian J. of Vet. Res.*, **5**: 105- 109.
- The NCBI taxonomy browser (online), <http://www.ncbi.nlm.nih.gov/Taxonomy/>, Browser/ wwwtax.cgi. Accessed on 10 September, 2011.
- Timoney, G. F. and Gillespi, J. H. (1981): The genus *Pasteurella*. Hagan's infectious diseases of domestic animals. (7th Ed.) pp 105- 111.
- Townsend, E. L., Maheswaran, S. K., Leininger, J. R. and Ames, T. R. (1987): Detection of Immunoglobulin G to *Pasteurella haemolytica* Capsular Polysaccharide by Enzyme- Linked Immunosorbent Assay. *J. Cl. Microbiol.*, **25**(2): 242-247.
- Trigo, T. F. J. and Romero, J. (1986): La relevancia de las neumonías como causa de mortalidad en corderos. *Vet. Mex.*, **17**:116–119.
- USDS (2010): United States Department of State, Bureau of African Affairs. Background Note on Ethiopia, <http://www.state.gov/r/pa/ei/bgn/2859.htm#econ>. Accessed on 28 December, 2011.
- Wells, P. W. and Robinson, J. T. (1984): Development of a combined clostridial and *Pasteurella haemolytica* vaccine for sheep. *Vet. Rec.*, **114**: 266-269.
- Yoo, H. S., Maheswaran, S. K., Lin, G., Townsend, E. L. and Ames, T. R. (1995): Induction of inflammatory cytokines in bovine alveolar macrophages following stimulation with *Pasteurella haemolytica* lipopolysaccharide. *Infect. Immun.*, **63**:381-388.
- Younan, M. and Fodor, L. (1995): Characterization of a new *Pasteurella haemolytica* serotype A17. *Res. Vet. Sci.*, **58**: 98.
- Zamirah, Z. A. (1998): Cross-protection of goats against challenge with *Pasteurella haemolytica* A7 and A9 following vaccination with spray vaccine containing *Pasteurella haemolytica* A2. DVM final year thesis, FKVSP, University Putra Malaysia, Faculty of Veterinary Medicine, Putra, Malaysia.

- Zamri-Saad, M., Norizah, A. and Sheikh-Omar, A. R. (1994): Detection of immunogenic components of several serotypes of *Pasteurella haemolytica*. In: Proceedings of the 17th Malaysian Microbiology Symposium, June 18-20, 1994, Malaysia, pp 25-27.
- Zecchinon, L., Fett, T. and Desmecht, D. (2005): How *Mannheimia haemolytica* defeats host defence through a kiss of death mechanism. *Vet. Res.*, **2**: 133-156.
- Zelege, A. (1998): Study on ovine Pneumonic Pasteurellosis in North Shoa, Ethiopia. DVM thesis, Faculty of Veterinary Medicine, Addis Ababa University, Debre-zeit, Ethiopia.

8. ANNEXES

Annex 1: Standard plate count method for bacterial mass determination

- Label the bottom of six petri plates 1-6. Label four tubes of saline 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , 10^{-11} and 10^{-12} .
- Using aseptic technique, the initial dilution is made by transferring 1 ml of *M. haemolytica* isolate to a 9 ml tryptose broth just to make a $1/10$ or 10^{-1} dilution (Figure below).
- The 10^{-2} dilution is then shaken by grasping the tube between the palms of both hands and rotating quickly to create a vortex. This serves to distribute the bacteria and break up any clumps.
- Immediately after the 10^{-2} dilutions have been shaken, uncapped it and aseptically transfer 1 ml to a second 9 ml tryptose broth. Since this is a 10^{-2} dilution, this second blank represents a 10^{-3} dilution of the original sample.



Calculation: Number of colonies on plate $\times$ reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $1/10,000$ dilution, then the count is $32 \times 10,000 = 320,000$ bacteria/ml in sample.)

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Figure 6.15

Source: (Quinn *et al.*, 2002)

- Shake the 10^{-3} dilution vigorously and transfer 1ml to the 3rd 9ml tryptose. This 3rd dilution represents a 10^{-4} dilution of the original sample. Repeat the process once more to produce a 10^{-5} dilution, continue until 10^{-12} dilution.
- Shake the 10^{-4} dilution again and aseptically transfer 1ml to one petri plate and 0.1ml to another petri plate. Do the same for the 10^{-6} , 10^{-8} , 10^{-10} and 10^{-12} dilutions.
- Remove one agar pour tube from the 48 to 50°C water bath. Carefully remove the cover from the 10^{-4} petri plate and aseptically pour the agar into it. The agar and sample are immediately mixed gently moving the plate in a figure eight motion or a circular motion while it rests on the table top. Repeat this process for the remaining plates.
- After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 37°C for 24 hours.
- At the end of the incubation period, select all of the petri plates containing between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated too many to count. Plates with fewer than 30 colonies are designated too few to count. Count the colonies on each plate by using a Quebec colony counter.
- Calculate the number of bacteria (CFU) per milliliter or gram of a sample by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquefied agar.

$$\text{Number of bacteria/ml} = \frac{\text{number of colonies (CFUs)}}{\text{Dilution} \times \text{amount plated}}$$

- Record your result

Annex 2: *Mannheimia haemolytica* vaccine production media

Chemical type	Unit	Quantity in litter	Quantity in vaccine
Peptone	Gram	10	7
Casitone hydrolysate	Gram	5	3.5
Sodium chloride	Gram	5	3.5
Di sodium hydrogen sulphate (Na ₂ HPO ₄)	Gram	7	4.9
Potassium Di hydrogen sulphate (KH ₂ PO ₄)	Gram	2.5	1.75
Magnesium sulphate (MgSO ₄)	Gram	1	0.7
Yeast extract	Gram	2.5	1.75
D (+) glucose	Gram	5	3.5
Horse serum (10%)	ml	10	7
Distilled water	ml	990	693

Source: (OIE, 1992)

Annex 3: Pair wise comparison of mean temperature value post challenge

Days post challenge	Constant group	Compared group	Mean difference	SE	Sig .	95%CI	
						Lower bound	Upper bound
Day 1	A2 & 7 challenge d by A2	A2 & 7 challenged by A7	1.32*	0.39	0.03	0.1	2.54
		Control challenged by A2	0.59	0.42	0.73	-0.71	1.88
		Control challenged by A7	1.26	0.42	0.06	-0.04	2.56
	A2 & 7 challenge d by A7	A2 & 7 challenged by A2	-1.32*	0.39	0.03	-2.54	-0.1
		Control challenged by A2	-0.74	0.42	0.51	-2.03	0.56
		Control challenged by A7	-0.06	0.42	1.0	-1.36	1.24
	A2	Control challenged by A2	-1.56*	0.29	0.04	-2.45	-0.66
		Control challenged by A7	-1.23*	0.29	0.04	-2.13	-0.33
		A7	-1.44*	0.29	0.01	-2.33	-0.54
Day 7	A7	Control challenged by A2	-1.11*	0.29	0.010	-2.01	-0.21
		Control challenged by A7	-1.26*	0.29	0.03	-2.15	-0.36
		A2 & 7 challenge d by A2	-0.93*	0.29	0.04	-1.83	-0.03
	A2 & 7 challenge d by A7	Control challenged by A2	-1.18*	0.29	0.06	-2.07	-0.28
		Control	1.56*	0.29	0.0	0.66	2.45
		Control challenge d by A2	1.44*	0.29	0.01	0.54	2.33
	Control challenge d by A2	A2 & 7 challenged by A2	1.26*	0.29	0.03	0.36	2.15
		A2 & 7 challenged by A7	1.18*	0.29	0.06	0.28	2.07
		A2	1.23*	0.29	0.04	0.33	2.13
	Control challenge d by A7	A7	1.11*	0.29	0.010	0.21	2.01
		A2 & 7 challenged by A2	0.93*	0.29	0.039	0.03	1.83

Day 10	A2	Control	-1.59*	0.23	0.0	-2.29	-0.88
		challenged by A2					
	A7	Control	-1.61*	0.23	0.0	-2.32	-0.90
		challenged by A7					
	A2 & 7	Control	-1.37*	0.23	0.0	-2.07	-0.66
		challenged by A2					
	challenge	Control	-1.39*	0.23	0.0	-2.1	-0.68
		challenged by A7					
	d by A2	Control	-1.51*	0.23	0.0	-2.21	-0.8
		challenged by A2					
	d by A7	Control	-1.53*	0.23	0.0	-2.24	-0.82
		challenged by A7					
	Control	Control	-1.25*	0.23	0.0	-1.95	-0.54
		challenged by A2					
	challenge	Control	-1.27*	0.23	0.0	-1.98	-0.56
		challenged by A7					
	d by A2	A2	1.59*	0.23	0.0	0.88	2.29
		A7	1.37*	0.23	0.0	0.66	2.07
	d by A7	A2 & 7 challenged by A2	1.51*	0.23	0.0	0.8	2.21
		A2 & 7 challenged by A7	1.25*	0.23	0.0	0.54	1.95
	Control	A2	1.61*	0.23	0.0	0.9	2.32
		A7	1.39*	0.23	0.0	0.68	2.1
	challenge	A2 & 7 challenged by A2	1.53*	0.23	0.0	0.82	2.24
		A2 & 7 challenged by A7	1.27*	0.23	0.0	0.56	1.98

* The mean difference is significant at $p < 0.05$.

9. CURRICULUM VITAE

Personal data

Name	Kuastros Mekonnen Belaynehe
Date of birth	28 Dec, 1984
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Education/qualification

1990-1997	Primary education at Ewket Minch Elementary and junior secondary school.
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Academic awards certificate

Ethiopian school leaving certificate examination
A degree Doctor in Veterinary Medicine from AAU
Certificate in basic computer application
Certificate of training on teaching meted logy from Hawasa teachers Education College, Ethiopia

Communication

2006	Vaccination of chickens against infectious bursal diseases (IBD) using Immune complex vaccine (Seminar paper)
2007	Major Animal Health Problems in Market Oriented Livestock Development in Alaba Special woreda of the Southern Nations Nationalities and Peoples Region, Ethiopia. (Major research/ Thesis topic conducted to attain the degree of Doctor of veterinary medicine)
2011	Review on Ovine Pasturellosis: Epidemiology and Current Situation in Ethiopia (Postgraduate seminar paper)
2012	Experimental Vaccine Development and efficacy trial against Capsular antigen of <i>Mannheimia haemolytica</i> serotypes A2, A7 and their combination (MSc Thesis)

Work experience

November 22, 2006-July 13, 2007	Attachment associate, at ILRI/IPMS project in the Alaba special woreda
December 10-2007 up to now	Lecturer at Alage ATVET College

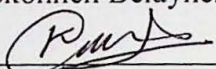
Membership

Ethiopian veterinary association	Full
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10. SIGNED DECLARATION SHEET

I under signed, declare that this thesis is my original work, has not been presented for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

Name: Kuastros Mekonnen Belaynehe.

Signature: 

Date of submission: JULY 102 / 2012

This thesis has been submitted for examination with our approval as university advisors.

Academic Advisors

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