

**ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF VETERINARY MEDICINE**

**A COMPARATIVE STUDY ON BACTERIAL DISTRIBUTION IN THE
RESPIRATORY TRACTS OF RUMINANTS**

By
Biruk Tesfaye

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**BY
BIRUK TESFAYE**

Board of Examiners

Signature

1. Dr. Nigussie Dugassa

School of Public health, College of Health Sciences

2. Dr. Azage Tegegne

International Livestock Research Institute

3. Prof. Getachew Tilahun

Aklilu Lemma Institute of Pathobiology, College of Health Sciences_____

4. Dr. Genene Tefera

Institute of Biodiversity Conservations

Academic Advisors:

Dr. Tesfaye Sisay (DVM, MSc, PhD, Asst. Professor)

Dr. Genene Tefera (DVM, PhD)

Dedication

This paper is dedicated to my late father, Tesfaye Birhanu, who lived his life for me and showed me a way through education but unable to see his fruit.

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God, Your eternal mercy, love and protection through Your Son Lord Jesus Christ throughout my life made all this possible and "I will praise You, O Lord, with my whole heart; I will shew forth all thy marvellous works." (Psalm 9:1).

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphism;
a.s.l.	Above sea level
<i>bvg</i>	Bordetella virulence gene
BUG	Biolog Universal Growth
Cbp	Collagen binding protein
CyaA	Adenylate cyclase/hemolysin
DNT	Dermonecrotic toxin
FIM	Fimbriae
FHA	Filamentous hemagglutinin
hr	Hour
Ig	Imunoglobulin
IgBPs	Immunoglobulin-binding proteins
Kbp	Kilo base pair
LKT	Leukotoxin
LOS	Lipooligosaccharide
LPS	Lipopolysacharides
Lpp	Lipoprotein
OF	Oxidative-fermentative test
OMP	Outer membrane protein
PRN	Pertactin
RDC	Respiratory disease complex
RTX	Ruminant-specific toxin
subsp.	Subspecies

ABSTRACT

For this study pneumonic lung tissue and tracheal swabs were collected from sheep, goat and cattle slaughtered at Addis Ababa abattoir. From the total of 170 bacterial isolates from sheep lung tissue 42.4% *Staphylococcus* species, 14.7% *Mannheimia haemolytica* and 10.6% *Bacillus* species were isolated aerobically. The tracheal cultures also indicate that 66.7% were *M. haemolytica*, 13% were *Bibersteinia trehalosi* and 5.8% were identified as *Enterobacter* species from the total of 69 cultures that cultured aerobically. Out of 192 bacteria isolated from aerobic inoculation of goat lung 45.3% were *Staphylococcus* species followed by *Bacillus* species 19.8%, *Micrococcus* species, 12.5% and *M. haemolytica* 6.3% of the total isolates. The tracheal culture showed *Enterobacter* species 30.1%, *M. haemolytica* 27.7% and *Bacillus* species 26.5%. Twenty-nine *Staphylococcus* species were among 93 bacterial isolates of aerobic cultures of cattle lung followed by *Bacillus* species, 17 and *M. haemolytica*, 8. While *Staphylococcus*, *B. trehalosi*, and *M. haemolytica* were account for 18, 14, and 8 out of the total 57 tracheal cultures respectively. A total of 522 bacterial isolates were detected via anaerobic culture, in which 40% of the 131 isolates from sheep lung were *Staphylococcus* species, 25.4% were *M. haemolytica*, and 6.2% were *Bacillus* species while *B. trehalosi* and *Micrococcus* species took 5.4% each from the total isolates. Whereas, 60.6% *M. haemolytica*, 19.7% *B. trehalosi* and 12.7% *Enterobacter* species were isolated from a total of 71 sheep trachea culture isolates. Among anaerobic cultures of goat lung tissue *Staphylococcus* species accounts for 36% followed by *Bacillus* species, 25.6% and *M. haemolytica* 11.2% out of the total 125 bacterial species. In the tracheal culture *M. haemolytica* counts for 33 followed by *Bacillus* species, 14 and *Enterobacter* species, 7 from 61 isolates. 42.9% *Staphylococcus*, 23.4% *Bacillus* species and 7.8% *B. trehalosi* were isolated species from the lung of the cattle, while from the trachea 25.0% *M. haemolytica* and *Staphylococcus* each were isolated anaerobically. A total of 1556 bacteria were isolated from 960 nasal swabs from three different areas. In Debre Berhan 140 *M. haemolytica*, 81 *Histophilus somni*, 57 *Staphylococcus* species and 52 *B. trehalosi* were isolated. While from Gimba *M. haemolytica*, *Staphylococcus*, *Streptococcus* and *H. somni* of which contribute for 25.2%, 15.9%, 11.4 % and 5.9% of the total 647 bacterial species were observed respectively. In Asella from 352 bacterial species isolated 93 were *M. haemolytica*, 48 were *Staphylococcus* species, 26 were *B. trehalosi* and 17 *H. somni* were recognized. Further identification and characterization was

made using BIOLOG identification system at the Institute of Biodiversity Conservation, Addis Ababa, and the result indicate that *Enterococcus avium* and *Sphingomonas sanguinis* were identified at 100% probability, while, *H. somni* and *Actinobacillus lignerisii* were suggested by the system.

Key words: Abattoir, nasal swab, *H. somni*, Aerobic bacteria, Anaerobic bacteria, Highland, Respiratory tract, Ruminant.

1. INTRODUCTION

Ethiopia is home for diverse indigenous livestock, parallel to its diverse ecology, production systems and ethnic communities. The estimates of total livestock population are 50 884 005, 25 979 919 and 21 960 706 for cattle, sheep and goats, respectively (CSA, 2010). According to FAO (2004), the total annual meat production comes from cattle (63%), sheep (25%) and goats (12%).

Livestock are also a major source of cash income. In large parts of the highlands of Ethiopia, livestock provide about 53% of the value of the total farm output (excluding the value of draught power) and the sale of livestock and livestock products provide up to 80% of farm cash income in a normal year. In summary, livestock produce a range of intermediate and final products in the traditional farming systems of Ethiopia and the presence of livestock on smallholders' farms enables them to be more productive and stable over time than would be the case in their absence (Rodriguez and Anderson, 1985).

The highland of Ethiopia comprises approximately 35% of the country using 1500 meters above sea level elevation as a crude threshold and is known for high production of livestock but majorly sheep production, which is an important part of the national economy (Alemayehu and Fletcher, 1991). Approximately 75% of the nation's sheep are found in the Ethiopian highland region. Although ruminants represent a great resource for Ethiopia, the rate of productivity per animal is low. The levels of foreign exchange earnings from livestock and livestock products are also much lower than would be expected hence; the current contributions of the livestock sector in Ethiopia, at either the macro or micro level is below potential given the size of the livestock population (Berhanu *et al.*, 2007). Disease and poor animal management is largely responsible for this reduced productivity (Ademosun, 1992).

Depending on the level of management, annual mortality rates may range from 7 to 44% in sheep (Njau *et al.*, 1988a; Bekele *et al.*, 1992a; Mukasa-Mugerwa *et al.*, 2000; Tibbo *et al.*, 2001). Moreover, respiratory diseases account for up to 54% of overall mortality rates in the Ethiopian highlands (Mukasa-Mugerwa *et al.*, 2000). The impact of respiratory disease is extensive and can

be measured as the sum of the direct economic losses occurring due to mortality, morbidity, treatment, prevention costs and loss of production (reduced animal performance and carcass quality) and the indirect costs like labor, infrastructure and intangibles (Jim, 2009). Pneumonia has been noted to be the most prominent infectious cause of mortality, both on-farm and on-station, in Ethiopian highland (Bekele *et al.*, 1992b; Mukasa-Mugerwa *et al.*, 2000). The causes of mortality due to respiratory diseases in the highlands of Ethiopia are multi-factorial. Hence, the term respiratory disease complex (RDC) is used for the condition conventionally known as bronchopneumonia. It is locally identified as 'Engib', 'Wozuwuz/Wotwut', and 'Gifaw' (Njau *et al.*, 1988a; Bekele *et al.*, 1992a, b; Ayelet *et al.*, 2004).

RDC is a polymicrobial infection that develops when the immune system of the animal is compromised by stress factors such as crowding, transportation, draught, and inclement weather combined with increased exposure to pathogens, may lead to respiratory infection. The importance of bacterial-viral synergism in RDC has been recognized for a long time (Fulton, 2009). Concurrent respiratory infestations by viruses (parainfluenza-3, reovirus, adenovirus, and respiratory syncytial virus), *Mycoplasma* species (*M. ovipneumoniae*, *M. arginini*, *M. agalactiae*, and others), lungworms (particularly *Dictyocaulus filaria*) and bacteria like *Mycobacterium*, *Fusobacterium*, *Arcanobacterium*, *Pasteurella multocida*, *Mannheimia haemolytica*, *Chlamydia psittacci*, *Bordetella parapertusis*, *Histophilus somni* can suppress the animal's immune system and inflict damage on the pulmonary tissues of most domestic animals allowing opportunistic microorganisms to colonize the lung and cause the disease (Andrews and Kennedy, 1997; Angen *et al.*, 2003).

In the highlands of Ethiopia, despite annual vaccination against different respiratory diseases mostly, pneumonic pasteurellosis with a monovalent vaccine (inactivated *P. multocida* biotype A), there are high rates of mortality and morbidity following respiratory distress. Since, outbreaks of respiratory diseases occur frequently, killing significant numbers of livestock, hence, respiratory diseases are a great economic concern for livestock producers in the highlands of Ethiopia (Ayelet *et al.*, 2004).

Nonetheless, it is becoming increasingly difficult to make an etiological diagnosis because, although a single agent may be a primary invader, when the local resistance of respiratory

mucosa is lowered, bacteria growing in the nose and throat extend downwards; usually producing multiple bacterial infections (McSween and Whaley, 1992). Besides, most of the infectious agents that cause respiratory disease are ubiquitous in nature and are normal inhabitants of the nasopharynx (Radostits *et al.*, 1994). Although losses due to pneumonia have been widely reported to be high in Ethiopian highlands (Njau *et al.*, 1988a; Bekele *et al.*, 1992a,b; Mukasa-Mugerwa *et al.*, 2000; Ayelet *et al.*, 2001; Tibbo *et al.*, 2001), specific causative agents of the disease have not been systematically studied.

Most studies conducted on the bacterial flora of the respiratory tract of domestic animals in Ethiopia mainly focus only on pneumonic lungs of sheep, cattle, goat and camel (Abayneh, 1999; Aschalew, 1998; Ayelet, 1996; Kinde, 1997; Mohammed, 1999; Sisay and Zerihun, 2003), and there are no published reports regarding the microflora of apparently healthy as well as pneumonic respiratory passageways. Hence, the importance and contribution of each respiratory bacterial species for the occurrence of RDC in ruminants, in general, and in sheep, in particular, of our country is not well documented yet. In addition, recent progress in epidemiologic surveillance and molecular biology has allowed the rapid recognition and identification of several newly emerging respiratory pathogens. The contribution of most of these bacteria in the occurrence of RDC is still undercover.

Therefore, the objectives of this paper were:

General objective:

- To isolate and characterize bacterial species that either can cause respiratory disease or live commensally in the respiratory tracts of ruminants.
- To isolate and characterize *Histophilus somni* from the respiratory tracts of ovine species.

Specific objectives:

- To isolate and characterize respiratory tract bacterial species from trachea and lungs of sheep, goat and cattle with pneumonic lungs.
- To isolate and characterize *H. somni* and other respiratory tract bacterial pathogens from the nasal cavity of apparently healthy and pneumonic ovine species.

2. LITERATURE REVIEW

Respiratory tract infections are of a common occurrence in various species of domestic and farm animals. As in cattle, causes of pneumonia in small ruminants reflect a mixture of inter-related variables and stress factors that culminate in bacterial species. Respiratory diseases in sheep and goats have not attained the economic significance as they have in the cattle industry but are still a major cause of morbidity, mortality, and economic losses. Pneumonias in ruminants are most prevalent when animals are confined in feedlot situations, in dry-lots, or kept indoors in crowded situations (Boudreaux, 2004).

Different species of bacteria are involved in causing RDC in ruminants among these bacteria which belongs to the family Pasteurellaceae are the major one.

2.1. Taxonomic Classification and Characteristics of Respiratory Tract Pathogens

2.1.1. *Pasteurellaceae*

Organisms belonging to the bacterial family *Pasteurellaceae* are ubiquitously present in the respiratory, alimentary and reproductive tracts of different avian, mammalian, reptilian, and likely amphibian hosts (Shewen and Conlon, 1993; Rycroft and Garside, 2000; Christensen *et al.*, 2003b; 2004b) mostly as constituents of commensal bacterial flora. However, at the same time, some species also have the ability to cause serious diseases. For example, *Mannheimia haemolytica* and *Histophilus somni* cause pneumonia in cattle (Czuprynski *et al.*, 2004); *Pasteurella multocida* is a common animal pathogen but occasionally causes meningitis in humans (Kimura *et al.*, 2004; Wade *et al.*, 1999). Other *Actinobacillus* species, such as *A. pleuropneumoniae*, *A. lignieresii*, *A. suis*, and *A. hominis*, also cause disease in animals.

A member of the family *Pasteurellaceae* as defined by Olsen *et al.* (2005) is Gram-negative, non-motile, coccobacilli or rod-shaped or pleomorphic with cells occurring singly and in pairs or short chains depending upon the growth stage. Growth on artificial media is enhanced by the addition

of serum or blood and colonies on blood agar are round, regular, grayish or yellowish, semi-transparent at the periphery and are about 2 mm in diameter after 24 hr at 37°C. Some isolates are haemolytic and are CAMP-positive. Endospores are not formed. Growth is mesophilic and facultatively anaerobic or microaerophilic. Nitrate is reduced without gas production. The reaction in Hugh–Leifson medium with (+)-D-glucose is fermentative without gas production. Porphyrin, phosphatase and alanine aminopeptidase tests are positive. Negative reactions occur for Simmons' citrate, malonate-base, growth in the presence of KCN, Voges–Proskauer, methyl red and urease tests. Negative tests are further observed with ONPG, arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, indole, gelatinase and hydrolysis of Tweens 20 and 80. Acid is formed from (–)-D-ribose, (–)-D-mannitol, (–)-D-sorbitol, (–)-D-fructose, (+)-D-glucose, (+)-D-mannose, maltose, sucrose, (+)-D-*trehalosi* and dextrin. Acid is not produced from adonitol, (+)-D-arabitol, (–)-D-arabinose, (+)-L-arabinose, *myo*-erythritol, dulcitol, (+)-D-fucose, (–)-L-fucose, (+)-D-galactose, D-glycogen, inulin, lactose, (+)-D-melibiose, (+)-D-melezitose, (+)-L-rhamnose, (–)-L-sorbose, (+)-D-turanose, xylitol, (+)-D-xylose or (–)-L-xylose. Reactions for α -fucosidase, α -galactosidase β -galactosidase, β -glucuronidase (PGUA), α -mannosidase and β -xylosidase are negative. Variable reactions occur for the catalase and oxidase tests and the production of acid from glycerol, *myo*-inositol, cellobiose, raffinose, aesculin, amygdalin, arbutin, gentiobiose and salicin. Variable reactions are also obtained in the α -glucosidase and β -glucosidase tests.

Although for several groups of *Pasteurellaceae*, host range and disease manifestations are not constant, the host animal species is indicative for routine bacterial identification (Christensen and Bisgaard, 2004). In accordance with other members of the *Pasteurellaceae* (Kuhnert *et al.*, 2004; Christensen *et al.*, 2004b), the predominant associated infections are localized in the respiratory tract or are to a lesser extent septicaemic in nature. A list of species within the family *Pasteurellaceae* that are associated with ruminants is given in Table 1.

The term pasteurellosis was broadly used to designate a number of infections in domestic animals and the causative agent of the disease was grouped under the genus *Pasteurella*. Hence, for several decades, the genus *Pasteurella* was believed to be only one single genus with numerous species responsible for or associated with a wide range of systemic, pulmonary and septicaemic

infections in various species of farm animals, particularly ruminants. Thus, ever since the isolation and recognition of the first pathogenic *Pasteurella* organism by Louis Pasteur at the end of the 19th century, a proper identification of members of the family *Pasteurellaceae* has been the source of much debate. Remarkably, and in contrast with other bacterial families of pathogenic importance, commercial identification kits were found not to be reliable for species identification of members of this bacterial family (Christensen *et al.*, 2003b).

However, with more recent advancements in molecular biology involving DNA hybridization studies and 16S rRNA sequencing, most of the formerly recognized species were found to share a number of common features and became the subject of intensive revision and reclassification (Dewhirst *et al.*, 1992; Christensen and Bisgaard, 2004), and has led to several new genera and over 58 formally recognized species and 25 unnamed taxa within the family *Pasteurellaceae* (Christensen and Bisgaard, 2004). The so far designated genera within this family are *Pasteurella* (Mutters *et al.*, 1985a), *Mannheimia* (Angen *et al.*, 1999), *Actinobacillus* (Christensen and Bisgaard, 2004), *Haemophilus* (Broom and Sneath, 1981), *Histophilus* (Angen *et al.*, 2003), *Lonepinella* (Osawa *et al.*, 1995), *Phocoenobacter* (Foster *et al.*, 2000), *Gallibacterium* (Christensen *et al.*, 2003a), *Volucribacter* (Christensen *et al.*, 2004b), *Nicoletella* (Kuhnert *et al.*, 2004), *Avibacterium* (Blackall *et al.*, 2005) and *Biebersteinia* (Blackall *et al.*, 2007) (Table 1).

It is worth mentioning that *M. haemolytica*, *P. multocida* and *B.trehalosi* constitute the most important members of the family *Pasteurellaceae* that pose serious hazards in livestock industry. These species are commensally resident in the animal body as normal constituents of the nasopharyngeal microflora and are all capable of causing infection when the body defense mechanisms are impaired. Their presence is mainly confined to ruminants with most adequately characterized strains originating from cattle, sheep and goats (Biberstein and Hirsh, 1999).

Table 1: Documented relationships between taxa of Pasteurellaceae and ruminants

Taxa	Origin/Pathology	Former classification	Ref. ^a
<i>Actinobacillus lignieresii</i>	Bovine pyogranulomatous lesions (Actinobacillosis)		[5]
[<i>Actinobacillus</i>] ^b <i>seminis</i>	Ovine epididymitis, Orchitis, mastitis		[5,12]
[<i>Actinobacillus</i>] <i>succinogenes</i>	Bovine rumen (lesions not reported)		[10]
<i>Histophilus somni</i>	Bovine meningo-encephalitis, bovine (BRD ^c), ovine and caprine pneumonia, septicaemia, mastitis, arthritis, otitis	[<i>Haemophilus</i>] <i>somnus</i> , <i>Haemophilus agni</i> , <i>Histophilus ovis</i>	[3,10]
[<i>Pasteurella</i>] <i>aerogenes</i>	Bovine bone marrow		[6]
<i>Pasteurella lymphangitidis</i>	Bovine lymphangitis		[19]
<i>Pasteurella multocida</i> capsular type A, D capsular type B, E	Bovine (BRD), ovine, and caprine pneumonia, mastitis Bovine haemorrhagic septicaemia, otitis	Indole negative variants of <i>P. multocida</i> : [<i>Pasteurella</i>] <i>avium</i> biovar 2, <i>Pasteurella canis</i> biovar 2 ^d	[8,9,11]
[<i>Bibersteinia</i>] <i>trehalosi</i>	Ovine pneumonia, septicaemia in lambs	[<i>Pasteurella</i>] <i>haemolytica</i> serogroups 3,4,10,15 (biotype T); (<i>Pasteurella trehalosi</i>)	[1,2,12, 13]
<i>Mannheimia glucosida</i>	Ovine pneumonia and BRD	[<i>Pasteurella</i>] <i>haemolytica</i> serogroups 11 (biotype 3A-3H,9)	[1,2]
<i>Mannheimia granulomatis</i>	Bovine panniculitis (fibrogranulomas) pneumonia in deer	[<i>Psteruella</i>] <i>haemolytica</i> (biogroup 3J)	[1]
<i>Mannheimia haemolytica</i>	BRD, ovine, and caprine pneumonia and septicaemia Ovine arthritis and gangrenous mastitis, bovine otitis	[<i>Pasteurella</i>] <i>haemolytica</i> serogroups 1,2,5-9,12-12-14,16 (biotype 1)	[1,2,7]
<i>Mannheimia ruminalis</i>	Bovine and Ovine rumen (lesions not reported)	[[<i>Psteruella</i>] <i>haemolytica</i> (biogroup 8D]	[1,2]
<i>Mannheimia varigena</i>	BRD, bovine mastitis, septicaemia, meningitis, abortion	[[<i>Psteruella</i>] <i>haemolytica</i> (biogroup 6]	[1,4]

^aReferences: [1] Angen *et al.*, 1999; [2] Angen *et al.*, 2002; [3] Angen *et al.*, 2003; [4] Catry *et al.*, 2008; [5] Christensen and Bisgaard, 2004; [6] Culture Collection University of Göteborg, Sweden; [7] Highlander, 2001; [8] Mitters *et al.*, 1985a; [9] Quinn *et al.*, 1994; [10] Rycroft and Garside, 2000; [11] Shewen and Conlon, 1993; [12] Sneath and Stevens, 1990; [13] Blackall *et al.*, 2007. ^b Brackets indicate the species was excluded from the genus *sensu stricto*. ^c BRD: bovine respiratory disease. ^d Biovars 2 of *P. avium* and *P. canis* have been proposed to be included in the species *P. multocida* (Christensen *et al.*, 2004b).

Other *Pasteurella* species of pathogenic significance in domestic animals include *P. caballi* which causes pneumonia and peritonitis in horses, *P. canis*, *P. stomatis* and *P. dagmatis* and these are associated with pneumonia and oral infections in dogs and cats. In addition, there are many other *Pasteurella* and *Mannheimia* species which can cause occasional infections in domestic and laboratory animals such as *M. granulomatis*, the causative agent of fibrogranulomatous panniculitis (lechiguana) in cattle (Riet-Correa *et al.*, 1992) and *P. pneumotropica* that causes secondary pneumonia in rats, mice and rabbits (Biberstein and Hirsh, 1999; Quinn *et al.*, 2002).

Pasteurella multocida

Taxonomy: A type genus *Pasteurella* identified in 1887 was classified into Gram negative facultative anaerobic and rods that formed the *Actinobacillus*, *Haemophilus* and *Pasteurella* group. The group has been examined on the basis of numerical phenotypic analysis, which has indicated that the three genera could form a family. This has been conformed by recent phenotypic comparison and by DNA-DNA hybridization using optical method for determining quantitative renaturation rates. The oldest recognized name within the group, *Pasteurella*, was used as a basis for the name of the family *Pasteurellaceae* (Mannheim, 1981).

Characteristics: *P. multocida* typically grows as mucoid confluent colonies, and in routine diagnostic bacteriology it is distinguished from *M. haemolytica* by production of indole from tryptophan, and by lack of haemolysis. A typical sweetish odour can also be present (Shewen and Conlon, 1993; Quinn *et al.*, 1994).

Habitat: Mutters *et al.* (1985a) demonstrated that *P. multocida* can be categorized upon sugar fermentation patterns (dulcitol and sorbitol) into three subspecies (subsp.) *multocida*, *septica*, and *gallicida*. However, host specificity can only be attributed to subsp. *gallicida*, which is a typical fowl-associated organism. In contrast, subsp. *septica* isolates are mainly recovered from dogs, cats, birds and man, while subs *P. multocida* causes significant diseases in many domestic animal species (Mutters *et al.*, 1985a; Quinn *et al.*, 1994). Although in 2002 a fourth novel subspecies *tigris* was proposed by Capitini *et al.* (2002) on the basis of 16S rRNA sequencing, this

subspecies associated with an infected tiger bite has so far not been referred to in later investigations. In general, capsular type A is mainly associated with fowl cholera, recurrent purulent rhinitis in rabbits ('snuffles'), and ruminant, porcine, canine, and feline respiratory disorders. Although type D is the predominant capsular type associated with atrophic rhinitis of pigs, this strain is occasionally isolated from pneumonic lungs in other animal species, including ruminants. Capsular types B and E are restricted to haemorrhagic septicaemia in bovine species from tropical regions, while *P. multocida* capsular type F is isolated mainly from diseased turkeys (Shewen and Conlon, 1993; Quinn *et al.*, 1994).

Virulence factors: *P. multocida* virulence factors are less numerous than those identified in *M. haemolytica*. Several adhesins, a thick polysaccharide capsule and lipopolysaccharides (LPS) are the major factors. Adhesins responsible for adherence of the bacterium to cell surfaces include type IV fimbriae, outer membrane protein A (OmpA), neuraminidase and filamentous hemagglutinin (FHA); the latter is similar to FHA found in *Bordetella* species. In addition, OmpA, various iron-binding proteins such as hemoglobin-binding protein A and transferrin-binding protein A bind fibronectin and other extracellular matrix proteins suggesting that they aid in invasion. *P. multocida* LPS induces classic signs of endotoxic shock and has biological and chemical properties and R-type LPS structure (with no polymeric O-antigen) similar to those found in many Gram-negative bacteria (Harper *et al.*, 2006). *P. multocida* LPS is a potent stimulator of inflammatory cytokines and a predominant inciter of pulmonary inflammation. The importance of capsule as a virulence determinant in the pathogenesis of *P. multocida* infection is due to its antiphagocytic properties (Dabo *et al.*, 2007).

Pathology: *P. multocida* has been isolated from up to 40% of the cases of enzootic and shipping fever pneumonia (Welsh *et al.*, 2004). This bacterium is acquired at an early age from the dam and is a common nasopharyngeal commensal. Serotype A-3 is the most common *P. multocida* isolate from bovine pneumonia, and pneumonia associated with *P. multocida* infections (whether in young dairy calves, in weaned and shipped beef cattle, or in calves experimentally challenged) is often difficult to discern from pneumonia associated with other respiratory bacterial pathogens, i.e. *M. haemolytica* and *H. somni*. In fact, isolation of more than one bacterial pathogen from pneumonic lungs in conjunction with respiratory viruses and/or *Mycoplasma* species occurs

frequently (Gagea *et al.*, 2006b). Various authors have described the *P. multocida* associated gross pulmonary pathological changes differently, and those designations probably reflect; the age of the lesion, whether or not other infectious agents were involved but not identified, and descriptive preferences of the various authors. The lesion is a typical cranioventral bronchopneumonia and has been characterized simply as bronchopneumonia or as a bronchopneumonia with various descriptive modifiers including acute fibrinosuppurative, subacute to chronic fibrinopurulent, fibrinous to fibrinopurulent, suppurative and fibrino-necrotizing (Dabo *et al.*, 2007). The presence of fibrinous to fibrinopurulent pleuritis, distended interlobular septa with edema or fibrin, and/or abscesses is variable with *P. multocida* infection (Caswell and Williams, 2007). *P. multocida* is associated with haemorrhagic septicaemia in cattle and buffaloes and enzootic pneumonia complex in young ruminants (Jones *et al.*, 1997). Other diseases such as fowl cholera and snuffles (an upper respiratory tract infection occasionally accompanied by pleurisy, pneumonia or fatal septicaemia in rabbits) are also caused by *P. multocida*.

Mannheimia haemolytica

Taxonomy: The most substantial reclassification in relation to respiratory diseases was the redesignation of organisms formerly known as [*Pasteurella*] *haemolytica* biotype A into the new genus *Mannheimia*, with *Mannheimia haemolytica* as the type species besides four other new species (Table 1). This new genus now contains several species including *M. haemolytica*, *M. granulomatis*, *M. glucosida*, *M. ruminalis* and *M. varigena* (Angen *et al.*, 1999). The name *Mannheimia* was given in tribute to the German scientist Walter Mannheim for his significant contributions in the recent taxonomy of the family *Pasteurellaceae*.

Organisms previously identified as *M. haemolytica*, have earlier been categorized into 2 biotypes: A and T, based on sugar fermentation of arabinose (A) and trehalosi (T), 12 biogroups based on extended phenotyping including fermentation patterns of sugars and glucosides, and 17 serotypes were further identified on basis of soluble or extractable surface antigens by passive haemoagglutination procedure or rapid plate agglutination test (Carter and Chengappa, 1991; Shewen and Conlon, 1993; Highlander, 2001; Angen *et al.*, 2002). Serotypes 1, 2, 5, 6, 7, 8, 9,

11, 12, 13, 14, 16 and 17 belong to biotype A which was reclassified as *M. haemolytica*. However, serotype A11 was later reclassified as *M. glucosida* (Younan and Fodor, 1995). In Ethiopia the most prevalent serotypes of recorded are *M. haemolytica* biotype A1, A2 and A8, while A14, A11 and A9 are the least (Ayelet, *et al.*, 2004; Sisay and Zerihun, 2003).

Characteristics: Mostly, the primary identification of *M. haemolytica* is based on the isolation of round grayish colonies of moderate size with a small pronounced surrounding zone of haemolysis after 24hr of aerobic incubation on sheep blood agar. *M. haemolytica* does not produce indole and tolerates bile salts. This Gram-negative bacterium has 12 representative capsular serotypes, but serotype 1 and 6 are the most prevalent in bovine pneumonia (Blackall *et al.*, 2007).

Habitat: The habitat and clinical relevance of *M. haemolytica* is predominantly restricted to ruminants, and more particularly to cattle. In early life, calves acquire *M. haemolytica* through contact with their dams and other cattle. The bacterium resides in the nasopharynx and tonsils with the latter considered the major reservoir. Of the pathogenic bacteria discussed herein, *M. haemolytica* is arguably the most important of the group and has traditionally been associated with the acute fulminating pleuropneumonia seen within the first weeks of cattle feeding (Hunt *et al.*, 2000).

Virulence factors: Virulence factors are many and include protein adhesins, capsular polysaccharide, LPS, iron-binding proteins, secreted enzymes and a ruminant-specific toxin (RTX) leukotoxin (LKT). Specific adhesins include a glycoprotein, N-acetyl-D-glucosamine, that mediates adherence to tracheal epithelial cells and activates the oxidative burst of bovine neutrophils. Heat-modifiable OmpA and the surface lipoprotein 1 (Lpp1) mediate *M. haemolytica* binding to bronchial epithelial cells. In addition, the *M. haemolytica* capsule may function as an adhesin in addition to its antiphagocytic properties. Neuraminidase and sialoglycoprotease modify cell surfaces and may enhance bacterial adhesion. *M. haemolytica* LPS has typical endotoxic and proinflammatory properties, causes vasculitis, and complexes with LKT enhancing LKTreceptor production and augmenting LKT activity. LKT induces dose-related changes in bovine leukocytes. At high LKT concentrations, cells undergo rapid osmotic swelling, membrane pore formation and necrosis. At reduced doses, LKT can induce leukocyte apoptosis or release of

proinflammatory cytokines, oxygen free radicals and cellular proteases. The sum result of these virulence factors is alveolar and vascular damage with pulmonary inflammation (Confer *et al.*, 1995; Czuprynski and Welch, 1995; Czuprynski *et al.*, 2004; Blackall *et al.*, 2007).

Pathology: *M. haemolytica*-induced pneumonia is characterized by acute cranioventral fibrinous to fibrinopurulent pleuropneumonia. On the cut surface, there is characteristic 'marbling' of the lung with lobules varying from normal, gray, or red. Whole lobules undergo hemorrhage or coagulation necrosis. Interlobular septa are usually distended with fibrin-rich edema fluid, and interlobular lymphatics may contain fibrin thrombi. Histologically, alveoli are flooded with fibrin-rich exudate, a variable neutrophil and macrophage infiltrate, and the traditional 'oat cells' that are flattened and streaming necrotic macrophages within the affected alveoli. Lobules undergoing coagulation necrosis appear as infarcts with thick neutrophilic infiltrates around the periphery of the necrotic lobule. Vasculitis with fibrin thrombi is frequently seen. Bronchioles often are spared or contain fibrin-rich exudate that may extend from alveoli (Caswell and Williams, 2007). Examples of the most commonly recognized diseases associated with *M. haemolytica* include shipping fever in cattle, primary and secondary pneumonia in cattle, sheep and goats, septicaemia and mastitis in sheep and a number of non-specific inflammatory lesions in various species of domestic animals (Quinn *et al.*, 2002).

Bibersteinia trehalosi

Taxonomy: Blackall and his coworker (2007) on the basis of their results, plus other existing evidence, they propose that [*P.*] *trehalosi* should be housed within a separate genus, for which they propose the name *Bibersteinia* gen. nov. using 16S rRNA gene sequence analysis, amplified fragment length polymorphism (AFLP) typing and phenotypic characterization is in full accord with all existing evidence as [*P.*] *trehalosi* represents a distinct and separate genus within the family *Pasteurellaceae*. The rest of serotypes (3, 4, 10 and 15) belong to biotype T, which was initially reclassified as *P. trehalosi* before being recently revised and moved to a separate new genus by the name of *Bibersteinia trehalosi* (Blackall *et al.*, 2007). On the other hand, *Pasteurella haemolytica* biotype T was first reclassified as *P. trehalosi*. In addition, avian

Pasteurella species including *P. gallinarum*, *P. paragallinarum* and *P. volantium* were similarly removed to a new separate genus named as *Avibacterium* (Blackall *et al.*, 2005).

Bibersteinia bacterial genus named after Ernst L. Biberstein, who did much of the early characterization work on this organism, including the creation of the serotyping scheme and some of the earliest DNA–DNA relatedness studies that indicated the unique nature of this taxon) (Blackall *et al.*, 2007). In Ethiopia *B. trehalosi* serotypes T4 and T3 are the predominant serotypes while, serotype T15 is less commonly encountered (Sisay and Zerihun, 2003).

Characteristics: At the genus level, the genus *Bibersteinia* is phenotypically distinguishable clearly from all other genera in the family *Pasteurellaceae*. Thus, separated from the existing genera of *Pasteurellaceae* by catalase, porphyrin test, urease, indole production, phosphatase, α -glucosidase and production of acid without gas from dulcitol, (+)-D-galactose, (+)-D-mannose, (+)-D-trehalose and glycosides. For characteristics showing variation in Table 2, the type strain shows the following reactions: catalase and oxidase-negative, weak haemolysis on bovine blood agar, CAMP-positive, weak growth on MacConkey agar, yellowish pigment, production of acid from cellobiose, raffinose, aesculin, amygdalin, arbutin, gentiobiose and salicin, no acid production from glycerol and *myo*-inositol (Blackall *et al.*, 2007). Key tests that allow the separation of *B. trehalosi* from the other genera of the *Pasteurellaceae* are shown in Table 2.

Habitat: Most of the bovine isolates of *B. trehalosi* were isolated from the respiratory tract and it is difficult to assess their role as primary pathogens. While *B. trehalosi* does not appear to have been commonly reported from cattle, the organism was found to be the most common member of the family *Pasteurellaceae* to be cultured from the tonsils of commercially reared American bison (Ward *et al.*, 1999). The past tendency to lump field isolates within the *M. haemolytica* complex may have obscured the role of *B. trehalosi* in bovine disease (Blackall *et al.*, 2007).

Virulence factors: The virulence factors of *B. trehalosi* is mostly similar to the factors associated with *M. haemolytica* except in that the regulatory gene in *B. trehalosi* has an inhibiting factor on the growth of *M. haemolytica* (Christensen and Bisgaard, 2004).

Table 2: Key characteristics for the differentiation of genera within the family Pasteurellaceae

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Catalase	D	D	D	D	–	–	+	D	–	D	+	D
NAD requirement	–*	–	+ [D]	–	–	–	–	–	–	+	–	–
Porphyrin	+	+	– [+]	+	+	+	+	+	–	+	ND	+
Methyl red	–	–	ND	–	–	ND	W	+	ND	–	ND	–
Voges–Proskauer	–	–	ND	–	D	+	–	–	–	–	ND	–
Urease	+	–	+ [D]	–	–	–	–	–	–	–	+	–
Indole	–	+	D	–	–	–	–	–	+	–	–	–
Phosphatase	+	+	+	+	–	+	+	+ / W	+	+	D	+
Pigment	–	–	ND	–	–	ND	–	–	+	D	ND	D
Acid from:												
(–)-D-Arabinose	–	D	ND	D	(+)	ND	(+)	–	ND	D	ND	–
Dulcitol	–	D	–	–	+	–	–	–	ND	–	–	–
(–)-D-Mannitol	+	D	–	+	–	–	+	–	ND	D	–	+
(–)-D-Fructose	+	+	– [D]	+	+	ND	+	+	–	+	–	+
(+)-D-Galactose	D	+	+	+	ND	ND	+	+	ND	D	–	–
(+)-D-Mannose	D	+	– [+]	–	+	–	+	+	ND	+	–	+
Maltose	+	–	+	D	+	–	D	D	–	D	–	+
Sucrose	+	+	– [D]	+	D	–	+	+	–	+	–	+
(+)-D-Trehalosi	D	D	–	–	–	–	D	–	–	D	–	+
Glycosides	D	–	–	D	+	D	–	–	ND	–	ND	D
ONPG	D	D	– [D]	D	+	+	+	D	ND	D	D	–
-Glucosidase	D	+	–	–	–	ND	+	–	ND	+	ND	D
-Glucuronidase –	–	–	–	+	ND	–	–	ND	–	ND	–	–

Genera: 1, *Actinobacillus* sensu stricto; 2, *Pasteurella* sensu stricto; 3, *Haemophilus* sensu stricto (includes *H. influenzae*, *H. haemolyticus* and *H. aegypticus*; results for *H. parainfluenzae* and *H. pittmania* are given in []); 4, *Mannheimia*; 5, *Lonepinella*; 6, *Phocoenobacter*; 7, *Gallibacterium*; 8, *Volucrobacter*; 9, *Histophilus*; 10, *Avibacterium*; 11, *Nicoletella*; 12, *Bibersteinia* gen. nov. Data are based on Angen *et al.* (1999, 2003), Blackall *et al.* (2005), Christensen and Bisgaard (2004), Christensen *et al.* (2003a, b, 2004b), Kuhnert *et al.* (2004), Mutters *et al.* (1985a) and this study. +, 90 % or more of the strains positive within 1–2 days; (+), 90 % or more of the strains positive within 3–14 days; –, less than 10 % of the strains are positive within 14 days; D, 11–89 % of the strains are positive; W, weakly positive.

Pathology: *B. trehalosi* is primarily a sheep pathogen, especially associated with septicemia but the organism is also isolated from cattle. *B. trehalosi* is frequently associated with acute systemic disease or septicaemia in young sheep (Dyson *et al.*, 1981; Jones *et al.*, 1997) it also associated with severe pneumonia in sheep and recently, *B. trehalosi*-associated pneumonia in young dairy calves has been also described (Goodwin-Ray *et al.*, 2008).

Histophilus somni

Taxonomy: Isolation of an organism identified as *Histophilus ovis* was first reported in Australia in 1956 (Roberts, 1956). Two years later, it was reported that a similar organism, identified as *Haemophilus agni*, was isolated from sheep with septicemia in the United States (Kennedy *et al.*, 1958). In 1960, an organism with similar characteristics was isolated from bovine cases of thrombotic meningoencephalitis (Kennedy *et al.*, 1960). This organism was later given the name *Haemophilus somnus* (Bailie *et al.*, 1969). It was subsequently concluded that all the three organisms were closely related and possibly representative of a single taxon (Stephens *et al.*, 1983).

Histophilus is a novel genus within the family *Pasteurellaceae*. The genus is not closely phylogenetically related to any other taxon within the *Pasteurellaceae*. The type species of the genus is *Histophilus somni* (Angen *et al.*, 2003).

Characteristics: The genus consists of Gram-negative, non-motile rods or coccobacilli. Endospores are not formed. Characteristics at the genus level, most isolates produce indole and yellow pigment ('H. agni' has been described as indole-negative). Considerable phenotypic variability has been reported between strains and laboratories (Kilian, 2003). Growth of most strains requires addition of 5–10% CO₂. Haemolysis may vary on calf blood agar. Colonies of most strains are pinpoint after 24 hr and up to 1–1.5 mm in diameter after 48 hr. Glucose is fermented without gas production. Oxidase reaction is normally positive. Alkaline phosphatase test is positive and nitrate is reduced. Simmons' citrate, Voges–Proskauer, arginine dihydrolase and ornithine decarboxylase tests are negative and there is no fermentation of adonitol or L-sorbose. Urease reaction is negative (Angen *et al.*, 2003). Phenotypic characters that separate the genus *Histophilus* from other genera of the family *Pasteurellaceae* are given in Table 2.

Habitat: *Histophilus* species are members of the normal flora in the upper respiratory tract and occasionally the genital area and gastrointestinal tract. *H. somni* occupies the respiratory tract and the genital tract of cattle, while the reservoir for *H. agni* was presumed to be a mucosal surface in sheep (Angen *et al.*, 2003).

Virulence factors: *H. somni* are non-encapsulated, and the virulence factors include lipooligosaccharide (LOS) and various Omps especially transferrin-binding proteins and immunoglobulin-binding proteins (IgBPs). Studies of adhesion proteins are generally lacking. Of particular interest with respect to virulence are LOS and IgBPs. LOS can mediate endothelial cell apoptosis, and through antigenic phase variation can assist the bacterium to escape the host immune response. IgBPs are surface exposed fibrillar protein networks that bind the Fc domain of bovine IgG2 and are responsible for resistance to complement-mediated serum killing, a characteristic of pathogenic strains of *H. somni* (Corbeil, 2007). Recently, studies demonstrated *H. somni* wild-type strain was cytotoxic for macrophages and inhibited phagocytosis of microspheres through disruption of the actin-filament structure. Conversely, cytotoxicity and phagocytosis inhibition were absent in an IgBPA – deficient mutant (Hoshinoo *et al.*, 2009). *H. somni* produces histamine, which in conjunction with anti-major Omp IgE, may account for early respiratory lesions (Corbeil, 2007). Recently, biofilm production by *H. somni* within the host has been documented, and FHA proteins may be involved in that process (Sandal *et al.*, 2009).

Pathology: *H. somni* induces respiratory disease in sheep and cattle, as well as infertility, septicemia, abortion, myocarditis, arthritis, mastitis, conjunctivitis, otitis, and meningoencephalitis in cattle (Ward *et al.*, 1995, 1999, 2006; Dyer, 2001). The bacterium is associated with enzootic or shipping fever pneumonia with epidemiological studies demonstrating marked variation in its prevalence compared to *M. haemolytica* or *P. multocida* in shipping fever pneumonia (Corbeil, 2007). Not only is *H. somni* the etiologic agent in all of these syndromes but also it commonly exists in an asymptomatic carrier state on the reproductive and respiratory mucosa (Humphrey and Stephens, 1983; Humphrey *et al.*, 1985). Several reviews of the naturally occurring disease are available (Humphrey and Stephens, 1983; Miller *et al.*, 1983; Harris and Janzen, 1989; Kwiecien and Little, 1991). Respiratory disease due to *H. somni* is sometimes seen in young calves (enzootic pneumonia) but most often in feedlot cattle (Humphrey and Stephens, 1983; Harris and Janzen, 1989). A study of haemophilosis in feedlot calves showed that *H. somni* was a significant cause of death even

though calves were immunized with a commercial *H. somni* vaccine on arrival (Van Donkersgoed *et al.*, 1990).

Gross lesions of acute fulminating pneumonia due to *H. somni* are similar to those seen with *M. haemolytica* infection, i.e. a cranioventral fibrinous pleuropneumonia with hemorrhage and coagulation necrosis that can involve entire lobules. Pulmonary vasculitis is often seen, and those vessels may contain fibrin thrombi. In several studies, *H. somni* was isolated later in the feeding period than was *M. haemolytica*, and in *H. somni* cases, the pneumonic lesions included fibrosis, fibrous pleural adhesions and bronchiolitis obliterans (Caswell and Williams, 2007).

2.1.2. Other bacterial species

Arcanobacterium pyogenes

Taxonomy: The family Actinomycetaceae is restricted to bacterial species that appeared to be linked taxonomically by the following phenotypic characteristics: ability to produce Gram positive, branching and, later on, fragmenting filaments without aerial hyphae and spores; comparatively exacting nutritional requirements; facultatively anaerobic (capnophilic) to anaerobic growth; and fermentative carbohydrate metabolism. The early description of the genus was mainly based upon morphological characteristics together with some chemotaxonomic information such as peptidoglycan composition, longchain fatty acid content and respiratory quinone (Slack, 1974; Slack and Gerencser, 1975).

The genus *Arcanobacterium* currently comprises six validly described species, namely *A. bernardiae*, *A. haemolyticum*, *A. hippocoleae*, *A. phocae*, *A. pluranimalium* and *A. pyogenes*. This current taxonomic structure has involved the inclusion of species that were transferred to *Arcanobacterium* from other genera. Thus, *A. pyogenes* and *A. bernardiae* were transferred to this genus by Pascual Ramos *et al.* (1997b) after they had meanwhile been included in the genus *Actinomyces* as “*Actinomyces pyogenes*” and “*Actinomyces bernardiae*,” respectively.

Characteristics: Morphological Characteristics introduced for the genus *Arcanobacterium* for bacteria formerly named “*Corynebacterium haemolyticum*,” the cellular morphology of the genus was described as follows: On blood agar plates, slender, irregular, bacillary forms

predominate during the first 18 hr; many cells are arranged at an angle to give V-formations. As growth proceeds, organisms become granular and segmented so that they resemble small and irregular cocci. Both rods and coccoid cells are Gram-positive, non-acid fast and nonmotile; endospores are not formed (Reddy *et al.*, 1982;).

Habitat: Information on the natural habitat of *Arcanobacteria* is scarce, although it has been assumed that these organisms occur as commensals of the mucous membranes of warm-blooded animals, in particular domestic animals such as cattle, sheep, swine and goats (Reddy *et al.*, 1982; Billington *et al.*, 2002a). Thus, Baele *et al.* (2001), in a study on the Gram-positive tonsillar and nasal flora of piglets before and after weaning, found that *A. pyogenes* was one of the common colonizers of the tonsils of pigs before and after weaning but not of the nasal conchae. Furthermore, this organism was also isolated from semen of beef breed bulls (Sprecher *et al.*, 1999) without a significant relationship between the cultural results and possible sperm abnormalities.

A. pyogenes is an inhabitant of many mucosal surfaces in cattle including the nasopharynx and appears to be a secondary invader into a lung already pneumonic due to other infectious agents. Factors precipitating its proliferation and inhalation are not known; however, Catry *et al.* (2008) recently demonstrated that fluoroquinolone administration to healthy cattle diminished upper respiratory *P. multocida*, whereas *A. pyogenes* became the predominant nasopharyngeal isolate. Therefore, it may survive and proliferate in cattle treated for RDC.

Virulence factors: *A. pyogenes* has several important virulence factors. These include a collagen-binding protein (CbpA) that allows it to bind collagen and promote adhesion to host cells (Pietrocola *et al.*, 2007). A cholesterol-dependent cytolysin (pyolysin) that is a pore-forming cytolysin for immune cells and is also a hemolysin has been characterized (Rudnick *et al.*, 2008). Several adhesins have been identified including two neuraminidases, which cleave sialic acids and expose cell receptors, and type 2 fimbriae. Several extracellular matrix-binding proteins that bind to collagen or fibronectin and exoenzymes (DNase and proteases) assist in invasion of tissue and degradation of proteins and nucleic acids. In addition, *A. pyogenes* can evade host defenses by invasion of epithelium, intracellular survival in macrophages and formation of biofilm (Jost and Billington, 2005).

Pathology: *A. pyogenes* is a common pathogen in bovine abscesses. In the lungs, *A. pyogenes* is associated with chronic abscessing pneumonia. Transmission of *A. pyogenes* occurs at a young age with calves acquiring the bacterium from their dams. Pulmonary lesions ascribed to *A. pyogenes* are primarily severe abscesses within areas of chronic bronchopneumonia or chronic pleuropneumonia (Lopez, 2007). These abscesses are typically characterized by liquefactive necrosis surrounded by a thick fibrous connective tissue band, whereas they are often larger and less caseous than *Mycobacterium bovis*-induced lesions (Confer, 2009).

***Bordetella bronchiseptica* cluster**

Taxonomy: The systematic analysis of 16S rDNA sequences has established *Achromobacter*, not *Alcaligenes* as previously reported, as the bacterial species most closely related to the *Bordetella*. *Achromobacter* are free-living organisms that are widespread in soil and water but are also commonly isolated from the hospital environment. They only rarely cause disease, and then in compromised individuals, and appear to lack the constellation of virulence factors associated with *Bordetella* (Gerlach *et al.*, 2001).

The three main species of *Bordetella*; *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, are respiratory pathogens of mammals and have an important economic impact on both human and animal health. Historically, the classification into species was based on host range and severity of clinical disease. However, it is now apparent that by true genetic criteria, they actually comprise a single group of highly related subspecies. *Bordetella pertussis* and *B. parapertussis* appear to be more host-adapted and differentiated and are less representative of the group as a whole than are *B. bronchiseptica* strains. As a result, the designation “*B. bronchiseptica* cluster” has been adopted to more accurately refer to this group of highly related organisms (Mattoo and Cherry, 2005).

Characteristics: The genus *Bordetella* encompasses a group of Gram-negative, small coccobacilli bacteria. They are catalase-positive, oxidase-positive and are motile peritrichous bacteria. They are obligate aerobes and fail to ferment carbohydrates such as glucose. Because they cannot utilize carbohydrates, they derive their energy mainly from the oxidation of amino acids and have no special growth requirements. They grow on MacConkey agar (Quinn *et al.*, 2002).

Habitat: Most members have adapted to live in close association with higher organisms, either as overt primary pathogens or in commensal associations that occasionally result in opportunistic diseases. All members of the *B. bronchiseptica* cluster have been implicated in respiratory disease in mammals (Mattoo and Cherry, 2005).

Virulence factors: *B. pertussis*, *B. paraptussis* (human and ovine), and *B. bronchiseptica* share a nearly identical virulence control system encoded by the *Bordetella* virulence gene AS (*bvgAS*) locus. Based on in vitro attachment assays and in vivo colonization experiments, several surface-exposed and secreted factors have been proposed to play a role in *Bordetella* pathogenesis. Putative adhesins commonly expressed in the Bvg₋ phase of all four subspecies of the *B. bronchiseptica* cluster include FHA, fimbriae (FIM), and pertactin (PRN), 1 of the 13 autotransporter proteins encoded in the *Bordetella* genomes: Additional autotransporters expressed by members of the *B. bronchiseptica* cluster include BrkA, SphB1, and Vag8. Commonly expressed Bvg₋ phase toxins include a bifunctional adenylate cyclase/hemolysin (CyaA) and dermonecrotic toxin (DNT) (Mattoo and Cherry, 2005).

Pathology: The genus *Bordetella* is responsible for notorious human and animal pathologies dominated by respiratory symptoms. Members of this group can infect many mammalian species, and the result can vary from persistent colonization without symptoms to serious, debilitating disease. Even though, *B. pertussis* and *B. paraptussis* are mainly the etiological agents for whooping cough in humans (Mastrantonio, 1998), *B. paraptussis* has also been isolated in sheep (Porter *et al.*, 1995). Considered an obligate respiratory tract pathogen for man (Linneman and Perry, 1977; Pittman, 1984) it has been also isolated from the respiratory tract of healthy and pneumonic lambs in New Zealand (Cullinane *et al.*, 1987).

On the other hand, *B. bronchiseptica* is responsible for a documented anthroponosis. It has been isolated from various domestic animal species (Le Blay, 1997) and is known to play a determinant role in the development of canine kennel cough (Thompson, 1976), of swine atrophic rhinitis (Magyar, 1988) and of sporadic human infectious respiratory syndromes (Lo Re, 2001). In addition with recent report from Senegal indicate that this species of *Bordetella* are isolated from the small ruminants (Ngom, 2006).

2.2. Respiratory disease complex in Ethiopian condition

In Ethiopia RDC in ruminants is not well documented. Yimer and Asseged (2007) reported the presence of aerobic bacteria from the respiratory tracts (from nasal cavity, tonsil, trachea and lung) of healthy sheep on their abattoir survey in Dessie. Based on their study they presented the presence of *E. coli* which is the predominant species, followed by Coagulase negative *Staphylococcus* and *Corynebacterium pseudotuberculosis* and *Bacillus* species; *Citrobacter* and *Klebsiella* were among the least encountered bacterial genera. In addition, *M. haemolytica*, *P. multocida*, *A. pyogenes*, *Enterobacter* species, *E. faecalis*, *Micrococcus*, *Pseudomonas* and *Streptococcus* species has been isolated from the respiratory passage ways, even though their proportion is lower than the other bacterial species.

On the other hand Ayelet and coworkers (2004) has showed that the predominant *M. haemolytica* serotypes are *M. haemolytica* A2 and A8 serotypes are among the most prevalent serotypes, while serotype A9 was the least prevalent. Ten percent of the sera were positive for *P. multocida* A from their epidemiologic and serologic investigation RDC in Debre Berhan town.

In another study conducted on goats from Dire Dawa, bacterial species identified were coagulase negative *Staphylococcus*, *M. haemolytica*, *S. aureus*, *P. multocida*, *C. pseudotuberculosis*, *Bacillus* species, *A. pyogenes*, *E. coli* and *Micrococcus* species are considered as a contributing factor for the occurrence of RDC (Megra *et al.*, 2006). In this study *S. aureus*, Coagulase Negative *Streptococcus*, *M. haemolytica*, *P. multocida*, *A. pyogenes*, and *Bacillus* species were isolated from nasal cavity, tonsil, trachea, and the lung. On the other hand, *C. pseudotuberculosis*, *E. coli*, and *Micrococcus* species were isolated only from the nasal cavity and tonsils.

3. MATERIALS AND METHODS

3.1. Study Area

Two types of studies were conducted in this work – a field as well as abattoir based. The field sampling areas include Debre Berhan, South Wollo (Gimba) and Asella towns while the abattoir work was conducted at Addis Ababa abattoir enterprise.

Debre Berhan is found in North Shoa, 120 km northeast of Addis Ababa, the capital of Ethiopia (fig. 1). It is located at 9°36' N latitude and 39°38' E longitude; and at an altitude of 2780 m above sea level (a.s.l.) with a bimodal rainfall pattern consisting of a long rainy season from June to September accounting for 75% of the annual rainfall, and a short rainy season in February/March to April/May and a dry season (October to January). The average monthly minimum temperature ranged from 2°C in November to 8°C in August, while the average monthly maximum temperature ranged from 18°C in September to 23°C in June leaving the area with mean annual temperature of 12.6°C, an average annual rainfall of 956 mm, and a mean relative humidity of 60% (Ayelet, 2004).

Gimba is located in South Wollo zone, Legambo Woreda at an altitude of 3170 m a.s.l., 525 km northeast of Addis Ababa (fig. 1). Gimba has a latitude and longitude of 11°4'N and 39°44'E and experienced a bimodal rain fall, the short and long rains with 39.63 mm and 1800mm precipitation respectively. Annual temperature ranges from 11.8°C-26°C and relative humidity of the region varies from 23.9%-79% (Teskaye, 1997).

Asella town is located in East Showa zone, at about 175 km south east of Addis Ababa; located at 7°57' N latitude and 39°7'E longitude. The city is positioned at the foot slopes of Mount Chilalo in the eastern side. The town is located in Dega agro-ecological condition with altitude of 2300-3200m a.s.l. (fig. 1). Asella has diverse climate condition ranging from Weyna Dega, Dega, Wurch, and Kur agro-ecologies. The town is characterized by 1300mm to 1350mm annual rainfall and average temperature of 18°C to 25°C during dry season and 5°C to 10°C during wet season (Tessema and Gashaw, 2010).

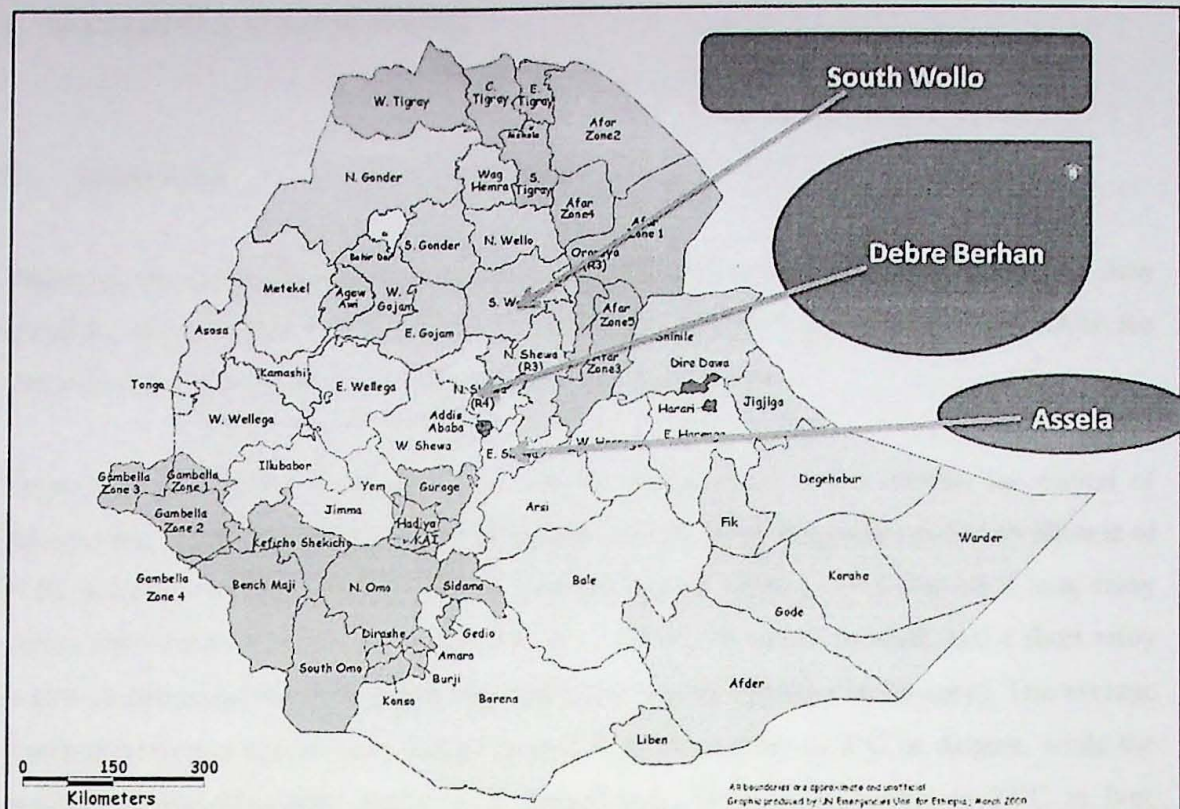


Figure 1: Map of the study areas

3.2. Study animals

Sheep with clinical signs of respiratory diseases and apparently healthy animals found in the study areas were used as sources of nasal swabs. In addition lungs with pneumonic lesions with their respective trachea from cattle, sheep and goats slaughtered at Addis Ababa abattoir were sampled for isolating the bacteria.

3.3. Sample size

The numbers of study animals were determined based on the expected prevalence of respiratory disease causing bacterial species and the desired absolute precision following the formula published in Thrusfield (2007).

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

n = required sample size

P_{exp} = expected prevalence

d = desired absolute precision

Therefore, based on this the total number samples to be collected with 50% prevalence rate, absolute precision value of 5% and 95% confidence interval was 384 from each sites except from Assella in which 192 nasal swabs were collected. In addition a total of 160 tracheal swabs and 160 lung tissues from cattle, sheep and goats were taken for identifying the bacteria.

3.4. Sample collection

Nasal swabs: Samples were taken with sterile cotton swabs moistened with Tryptose Soya broth from the nasal cavities of sheep. The external nares of the animal were cleansed with 70% denatured alcohol carefully. Then, the swab was introduced directly into nasal cavity and rubbed smoothly against the mucosa in a circular motion. The swab was allowed to remain in contact with the secretions for up to one minute, and the collected swab was kept into Tryptose Soya broth transport medium and transported to the laboratory in a cool box on ice without delay (Lees, 1990; OIE, 2008).

Post-mortem samples: samples of pneumonic lung tissue and tracheal swabs were collected at post-mortem for microbiological culture from Addis Ababa abattoir enterprise. Each piece of tissue was placed in a separate sterile screw-capped universal bottle. Containers were fully labeled with the date, tissue and animal identification. Sterile instruments (knife, scalpel, forceps and scissors) were used for collecting specimens for microbiological culture, while swabs were submitted in appropriate transport media. After collection, the tracheal swabs were incubated immediately at 37⁰C for 24 hr both aerobically and anaerobically, while the lung tissues were processed immediately.

3.5. Isolation and Identification of bacterial species

3.5.1. Isolation of bacteria

The outer surfaces of the lungs were first seared with a heated spatula before the cut inner surface of the lungs were minced by sterile scissors and forceps. The minced tissue was inoculated into tryptic soya broth and incubated at 37 ⁰C for 24-48 hr both aerobically and anaerobically. After the first incubation the sample was streaked on 7% sheep blood agar and incubated at 37 ⁰C for another 24-48 hr both, aerobically and anaerobically.

In case of *H. somni*, a cotton-tipped culture swab of the respiratory tract was streaked after collection onto chocolate agar plates and incubated in 5-10% CO₂ at 37 °C for 24-48 hr.

3.5.2. Morphological and cellular Identification

The plates were then examined for presence of growth and colony morphology, size, shape and presence or absence of haemolysis. After taking note of cultural growth characteristics, subcultures were subjected to Gram's staining to study staining properties and cellular morphology under 100X objective of light microscope. Mixed colonies and Gram-negative bacteria were sub-cultured on both blood and MacConkey agars and incubated aerobically as well as anaerobically for further 24 hr. Pure cultures of single colony type, from both blood and MacConkey agars, were transferred onto nutrient agar-slants for a series of biochemical tests including catalase, oxidase, and oxidative-fermentative (OF) tests for final identification, following standard procedures (Shewen and Conlon, 1993).

In case of *H. somni*, a presumptive identification of *H. somni* was made on the basis of morphology and biochemical reactions, i.e. small, circular, slow growing, butter-colored colonies of gram-negative coccobacilli/rods which produced positive indole and negative catalase reactions (Lees, 1990).

3.5.3. Fatty acid analysis using BIOLOG-Microstation Identification System

Pure isolates of bacterial species were prepared for BIOLOG- Microstation Identification System (Biolog, California, USA) which is the latest molecular identification techniques based on each microbial species surface fatty acid substrates, at the Institute of Biodiversity Conservation, Addis Ababa. Briefly, bacterial suspension was prepared with an appropriate level of bacterial density, as recommended in the manufacturer's protocol. After growth on Biolog Universal Growth (BUG) agar suspension was inoculated into MicroPlates aseptically. The MicroPlates were incubated at 30 °C. Then the MicroPlates were read by Microstation Reader and bacterial species were identified using the inbuilt BIOLOG database.

3.6. Data Analysis

The results of the collected data were entered in to Microsoft Excel spread sheet and finally were analyzed using SPSS version 15 software. Descriptive statistics was used to summarize the data generated from the study. The relative abundance of each species/genera was expressed as a percentage in comparison to the total number of isolates. In addition, chi-square test is computed in order to observe a relationship between the variants.

4. RESULTS

4.1. Isolation and identification of bacterial species from the respiratory tract of cattle, sheep and goats (abattoir survey)

4.1.1. Morphology, staining and cultural characteristics of the bacterial isolates

In this study a total of 1186 bacteria were isolated from both aerobic and anaerobic cultures of pneumonic lung and trachea of ruminants on abattoir based study. In general, fourteen different bacterial genera including *Histophilus*, *Bordetella*, *Pasteurella*, *Mannheimia*, *Bibersteinia*, *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Enterococcus*, *Arcanobacterium*, *Actinobacillus*, *Bacillus*, *Enterobacter*, *Klebsiella* and *Pseudomonas* were isolated.

The colonies of bacterial species which belongs to the family *Pasteurellaceae* were recognized as grayish with a little variation in size from very small to moderate size on blood and chocolate agar. They were all gram negative with variation in shape from coccobacillary to rods. But on MacConkey agar they have different growth characteristics with no growth for *P. multocida* and *H. somni* while *M. haemolytica* looked as pink and *B. trehalosi* took pale coloration after 24 hr of incubation.

Staphylococcal colonies were round and smooth usually appear as white and opaque but also sometimes they resemble golden yellow. Few species form haemolysis on blood agar after 24 hr of incubation both aerobically and anaerobically. All of the species were gram-positive cocci present in clusters resembling bunches of grapes.

Streptococcus species were small white translucent colonies usually haemolytic on blood agar and able to grow on MacConkey agar as red pin point colony unlike that of *Staphylococcus* species which were unable to resist the bile salt in the MacConkey agar. They were Gram-positive cocci and found in chains.

4.1.2. Biochemical characteristics

In the present study, once the bacterial colonies were identified at the genus level using gram stain, catalase, oxidase, and oxidation fermentation test the bacteria which belong to the family *Pasteurellaceae* were further subjected to secondary biochemical test in order to identify at the species level.

H. somni were recognized as Gram-negative, non-motile rods or coccobacilli, which were positive for oxidase test but negative for catalase. Haemolysis may vary and colonies of most strains were like pinpoint after 24 hr of incubation. It reduces nitrate into nitrite, produce indole and were negative for Simmons' Citrate, Voges–Proskauer and Urease reaction. In the present investigation, strains of *H. somni* did not ferment sucrose, D-galactose, D-fructose, maltose or trehalose but glucose was fermented without gas production.

P. multocida isolates were non motile, catalase and oxidase positive. They fermented glucose, mannose, sucrose, mannitol and sorbitol without any gas production. In addition they fermented maltose and trehalose and were positive to indole and H₂S production. These species were able to reduce nitrate into nitrite and methyl blue.

M. haemolytica were isolated as non motile, positive for catalase and oxidase. They ferment glucose, sucrose, maltose, mannose, sorbitol and arabinose but they did not produce indole and H₂S and also unable to ferment trehalose like *P. multocida* and *B. trehalosi*.

B. trehalosi were recognized as non motile, positive for catalase but variable in oxidase reaction. They ferment manitol, glucose, sucrose and trehalose but did not ferment arabinose and were negative for urease, indole and H₂S production.

4.1.3. Isolation rate of bacteria from lung and trachea of ruminants from aerobic cultures

The data revealed that 12 different species of bacteria were isolated from 50 sheep lung tissue cultured aerobically, and the higher percentage were taken by *Staphylococcus* species 72 (42.4%), *M. haemolytica* 25 (14.7%) and *Bacillus* species 18 (10.6%) out of the total of 170 isolates. While from tracheal culture 6 species of bacteria were recognized on aerobically and

it is pointed out that 46 (66.7%) were *M. haemolytica*, 9 (13%) were *B. trehalosi* and 6 (8.7%) were *Bacillus* species out of the total 69 isolates (Table 3).

Out of 192 overall isolates 8 species of bacteria were identified from aerobic inoculation of goat lung and 87 (45.3%) were *Staphylococcus* species followed by *Bacillus* species, 38 (19.8%) and *Micrococcus* species, 24 (12.5%) and the rest included *M. haemolytica* which accounted for 12 (6.25%) of the total isolates. From the aerobic cultures of trachea 6 species of bacteria were recognized and the highest percentage were attributed to *Enterobacter* species, 25 (30.1%) followed by *M. haemolytica*, 23 (27.7%) and *Bacillus* species 22 (26.5%).

Staphylococcus species, 29 (31.2%) were the highest among the 12 different bacterial species isolated from aerobic cultures of cattle lung which was followed by *Bacillus* species, 17 (18.3%) and *M. haemolytica* 8 (8.6%) out of the entire 93 isolates. While *Staphylococcus*, *B. trehalosi*, *M. haemolytica* and *Bacillus* species were among the 8 bacterial species identified and accounted for 18 (31.6%), 14 (24.6%), 8 (14%) and 8 (14%) respectively from the total of 57 subcultures.

The data showed that there is a statistically significant difference between each species of bacteria and anatomical site at $p < 0.05$. And the statistical significance was also observed between the species of animal and bacterial species at $p < 0.05$.

Table 3: Bacterial species isolated from lung and trachea of ruminants cultured aerobically

Bacterial species	Animal species and Anatomical site examined						Total
	Sheep Lung	Sheep Trachea	Goat Lung	Goat Trachea	Cattle Lung	Cattle Trachea	
<i>Staphylococcus spp.</i>	72 (42.4%)	3 (4.3%)	87 (45.3%)	6 (7.2%)	29 (31.2%)	18 (31.6%)	215 (32.4%)
<i>Streptococcus spp.</i>	6 (3.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	5 (5.4%)	3 (5.3%)	14 (2.1%)
<i>Micrococcus spp.</i>	8 (4.7%)	1 (1.4%)	24 (12.5%)	0 (0.0%)	7 (7.5%)	0 (0.0%)	40 (6.0%)
<i>Bacillus Spp.</i>	18 (10.6%)	6 (8.7%)	38 (19.8%)	22 (26.5%)	17 (18.3%)	8 (14.0%)	109 (16.4%)
<i>M. haemolytica</i>	25 (14.7%)	46 (66.7%)	12 (6.3%)	23 (27.7%)	8 (8.6%)	8 (14.0%)	122 (18.4%)
<i>P. multocida</i>	0 (0.0%)	0 (0.0%)	3 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (0.5%)
<i>B. trehalosi</i>	7 (4.1%)	9 (13%)	0 (0.0%)	0 (0.0%)	5 (5.4%)	14 (24.6%)	35 (5.3%)
<i>Enterococcus spp.</i>	7 (4.1%)	0 (0.0%)	5 (2.6%)	0 (0.0%)	6 (6.5%)	0 (0.0%)	18 (2.7%)
<i>Pseudomonas spp.</i>	5 (2.9%)	0 (0.0%)	0 (0.0%)	1 (1.2%)	3 (3.2%)	0 (0.0%)	9 (1.4%)
<i>Bordetella spp.</i>	3 (1.8%)	0 (0.0%)	1 (0.5%)	0 (0.0%)	4 (4.3%)	0 (0.0%)	8 (1.2%)
<i>Enterobacter spp.</i>	4 (2.4%)	4 (5.8%)	10 (5.2%)	25 (30.1%)	4 (4.3%)	3 (5.3%)	50 (7.5%)
<i>Klebsiella pneumoniae</i>	2 (1.2%)	0 (0.0%)	0 (0.0%)	6 (7.2%)	0 (0.0%)	2 (3.5%)	10 (1.5%)
<i>Actinobacillus ligneresii</i>	2 (1.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (3.2%)	1 (1.8%)	6 (0.9%)
<i>Arcanobacterium pyogens</i>	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.1%)	0 (0.0%)	1 (0.2%)
Unidentified	11 (6.5%)	0 (0.0%)	12 (6.3%)	0 (0.0%)	1 (1.1%)	0 (0.0%)	23 (3.5%)
Total	170	69	192	83	93	57	664

4.1.4. Isolation rate of bacteria from lung and trachea of ruminants from anaerobic cultures

A total of 522 bacterial isolates were detected on blood agar plates cultured anaerobically; out of these 199 (38.12%), 186 (35.63%) and 137 (26.25%) were recovered from sheep, goats and cattle, respectively.

Fifty-two (39.7%) of the isolates observed from sheep lung were *Staphylococcus* species; 33 (25.2%) were *M. haemolytica*, 8 (6.1%) were *Bacillus* species, while *B. trehalosi*, and *Micrococcus* species constituted 7 (5.3%) each from the total isolates and taken as a whole 10 bacterial species were recognized. Whereas, 43 (60.6%) *M. haemolytica*, 14 (19.7%), *B. trehalosi* and 9 (12.7%) *Enterobacter* species were the majorly isolated species among the 5 isolated bacterial species which contribute for a total of 71 isolates of anaerobic sheep trachea culture (Table 4).

Among those bacteria cultured from goat lung tissue anaerobically 8 species of bacteria were determined and the majority of them were taken by *Staphylococcus* species which was 45 (36%) followed by *Bacillus* species, 32 (25.6%) and *M. haemolytica* 14 (11.2%) out of the total 125 isolates. But in the case of tracheal culture only 5 species were recorded and of which *M. haemolytica* accounts for 33 (54.1%) followed by *Bacillus* species, 14 (23%) and *Enterobacter* species, 7 (11.4%) from the total of 61 isolates.

Eleven different species of bacteria were known from anaerobic culture of lung of cattle. The most frequently isolated species were *Staphylococcus* 33 (42.9%), *Bacillus* species 18 (23.4%) and *B. trehalosi* 6 (7.8%), while the most prevalent species in the trachea of cattle were *M. haemolytica* and *Staphylococcus* 15 (25.0%) each followed by *Bacillus* species 12 (20.0%) and *Enterobacter* species 10 (16.7%) from the total of 7 identified bacterial species. The other species isolated and identified from pneumonic cattle lung and their respective trachea is shown in Table 4.

There was statistically significant difference ($p < 0.05$) between anatomical site and species of animal in terms of the species of bacteria.

Table 4: Bacterial species isolated from lung and trachea of ruminants cultured anaerobically

Bacterial species	Animal species and Anatomical Site						Total
	Sheep Lung	Sheep Trachea	Goat Lung	Goat Trachea	Cattle Lung	Cattle Trachea	
<i>Staphylococcus spp.</i>	52 (40%)	2 (2.8%)	45 (36.0%)	6 (9.8%)	33 (42.9%)	15 (25.0%)	153 (29.3%)
<i>Streptococcus spp.</i>	1 (0.8%)	0 (0.0%)	2 (1.6%)	0 (0.0%)	3 (3.9%)	1 (1.7%)	7 (1.3%)
<i>Micrococcus spp.</i>	7 (5.4%)	0 (0.0%)	9 (7.2%)	0 (0.0%)	3 (3.9%)	0 (0.0%)	19 (3.6%)
<i>Bacillus spp.</i>	8 (6.2%)	0 (0.0%)	32 (25.6%)	14 (23.0%)	18 (23.4%)	12 (20.0%)	84 (16.1%)
<i>M. haemolytica</i>	33 (25.4%)	43 (60.6%)	14 (11.2%)	33 (54.1%)	1 (1.3%)	15 (25.0%)	139 (26.6%)
<i>P. multocida</i>	0 (0.0%)	0 (0.0%)	3 (2.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (0.6%)
<i>B. trehalosi</i>	7 (5.4%)	14 (19.7%)	0 (0.0%)	0 (0.0%)	6 (7.8%)	5 (8.3%)	32 (6.1%)
<i>Enterococcus spp.</i>	1 (0.8%)	0 (0.0%)	1 (0.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.4%)
<i>Pseudomonas spp.</i>	1 (0.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.2%)
<i>Enterobacter spp.</i>	5 (3.8%)	9 (12.7%)	6 (4.8%)	7 (11.5%)	2 (2.6%)	10 (16.7%)	39 (7.5%)
<i>Klebsiella pneumoniae</i>	3 (2.3%)	3 (4.2%)	0 (0.0%)	1 (1.6%)	1 (1.3%)	0 (0.0%)	8 (1.5%)
<i>Actinobacillus lignieresii</i>	3 (2.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	6 (7.8%)	2 (3.3%)	11 (2.1%)
<i>Arcanobacterium pyogens</i>	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (2.6%)	0 (0.0%)	2 (0.4%)
Unidentified	9 (6.9%)	0 (0.0%)	13 (10.4%)	0 (0.0%)	2 (2.6%)	0 (0.0%)	22 (4.2%)
Total	131	71	125	61	77	60	522

4.2. Isolation rate of bacteria from the nasal cavity of sheep

Fourteen various types of bacterial species were isolated from a total of 960 nasal swabs which accredited for 1556 bacterial isolates from each of the three different highland areas of the country, namely, Debre Berhan, Gimba and Asella. In this case all the swabs were cultured with 5-10% of CO₂. In Debre Berhan *M. haemolytica* was isolated in higher proportion 140 (25.1%) followed by *H. somni* 81 (14.5%), *Staphylococcus* species 57 (10.2%) and *B. trehalosi* 52 (9.3%). Nasal swabs collected from Gimba showed that there is a high prevalence of *M. haemolytica* which accounted for 163 (25.2%) of the total 647 bacterial isolate count and this was followed by *Staphylococcus*, *Streptococcus* and *H. somni* in which each contributed for 103 (15.9%), 74 (11.4%) and 38 (5.9%) of the total bacterial count, respectively. In Asella out of the 352 bacterial species isolated 93 (26.4%) of them were identified as *M. haemolytica*, 48 (13.6%) were *Staphylococcus* species, 26 (7.4%) were *B. trehalosi* and 17 (4.8%) were recognized as *H. somni* (Table 5).

Table 5: Species of bacteria isolated from nasal cavity of highland sheep

Bacterial Species	Area			
	Debre Berhan	Gimba	Asella	Total
<i>H. somni</i>	81 (14.5%)	38 (5.9%)	17 (4.8%)	136 (8.7%)
<i>M. haemolytica</i>	140 (25.1%)	163 (25.2%)	93 (26.4%)	396 (25.4%)
<i>P. multocida</i>	11 (2.0%)	18 (2.8%)	8 (2.3%)	37 (2.4%)
<i>B. trehalosi</i>	52 (9.3%)	27 (4.2%)	26 (7.4%)	105 (6.7%)
<i>A. ligneresii</i>	29 (5.2%)	42 (6.5%)	4 (1.1%)	75 (4.8%)
<i>Staphylococcus spp.</i>	57 (10.2%)	103 (15.9%)	48 (13.6%)	208 (13.4%)
<i>Streptococcus spp.</i>	41 (7.4%)	74 (11.4%)	6 (1.7%)	121 (7.8%)
<i>Enterococcus spp.</i>	14 (2.5%)	15 (2.3%)	2 (0.6%)	31 (2.0%)
<i>Micrococcus spp.</i>	14 (2.5%)	18 (2.8%)	2 (0.6%)	34 (2.2%)
<i>A. pyogens</i>	6 (1.1%)	6 (0.9%)	14 (4.0%)	26 (1.7%)
<i>Enterobacter spp.</i>	19 (3.4%)	15 (2.3%)	6 (1.7%)	40 (2.6%)
<i>K. pneumonie</i>	6 (1.1%)	11 (1.7%)	8 (2.3%)	25 (1.6%)
<i>Pseudomonas spp.</i>	17 (3.1%)	12 (1.9%)	4 (1.1%)	33 (2.1%)
<i>Bacillus spp.</i>	25 (4.5%)	54 (8.3%)	86 (24.4%)	165 (10.6%)
Unidentified	45 (8.1%)	51 (7.9%)	28 (7.9%)	124 (8.0%)
Total	557	647	352	1556

On the overall study it was observed that the isolation rate of pathogenic bacteria from animals which showed respiratory clinical signs was higher than the rate from animals which did not exhibit any clinical signs but in most of the cases the normal flora bacteria are more predominant in apparently healthy animals than animals with clinical signs and there is a statistically significant difference between animals with clinical sign and apparently healthy animals with a specific bacterial species at $P < 0.05$ (Fig 2).

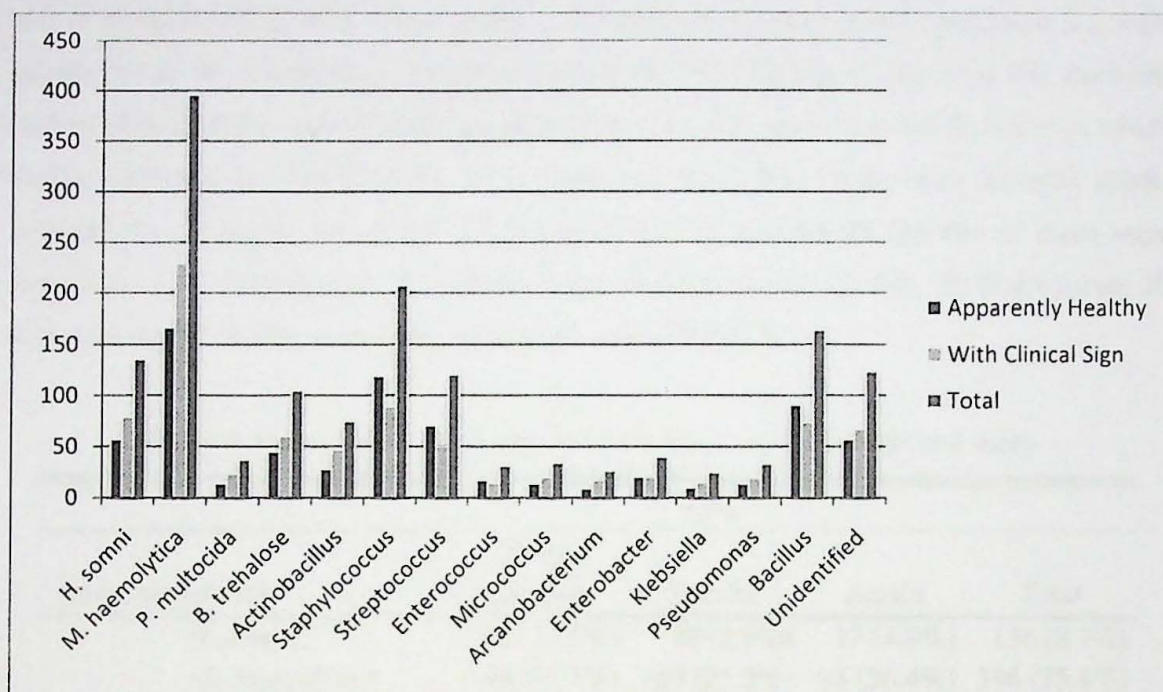


Figure 2: Isolation of Bacteria from Apparently Healthy and Diseased sheep

4.3. Identification of bacterial isolates using Biolog-Omnilog

Using OmniLog® two different bacterial species, *Enterococcus avium* and *Sphingomonas sanguinis* were identified at 100% probability, 0.74 and 0.537 similarity and 3.90 and 7.31 distance respectively. In addition also *H. somni* and *A. ligneresii*, were suggested by the machine at a different rate of similarity and difference.

5. DISCUSSION

Respiratory tract infections are of a common occurrence in ruminant species of animals. It is considered that the bacterial flora of the respiratory system includes both resident and transitory microflora (Quinn *et al.*, 2002). The causes of pneumonia in ruminants reflect a mixture of inter-related variables and stress factors that culminate in bacterial species (Boudreaux, 2004).

In Ethiopia, respiratory disease complex (RDC) is considered as the most important diseases of ruminants is known to cause a serious impact on the economy of the country given the size of the livestock population (Berhanu *et al.*, 2007).

This study has shown that a wide diversity of bacterial flora colonizes the respiratory passageways of apparently healthy and pneumonic ruminants. This is supported by several workers who previously isolated different bacterial species from pneumonic lungs of sheep (Al Sultan, 1995; Barbour *et al.*, 1997; Yimer and Asseged, 2007; Tilaye, 2010), and also from pneumonic caprine lungs (Almeida *et al.*, 1986; Mohammed, 1999; Megra *et al.*, 2006; Tilaye, 2010). In addition, Shemsedin (2002) isolated multiple bacteria from pneumonic lungs of camels. The invariable isolation of these organisms from the pneumonic lungs of various animal species might indicate their role in causing different respiratory syndromes especially when the immune system of the animals is compromised by some other external factor.

In this investigation the isolation and identification of multiple bacterial species from the respiratory tract of ruminants were achieved from field and abattoir survey. Even though, the proportion of *M. haemolytica* vary from one species of animal to the other and as well as from each anatomical site, the present study discovered that the isolation this bacteria is relatively in higher proportion in the tracheal culture of sheep when compared to the lung tissue culture. But the rate of isolation of this species was relatively decreased when it is isolated from the trachea and lung of goats. It is also considered that the rate of isolation of this species of bacteria is even less in cattle trachea and in the lungs of this species of animal. This agrees with the studies of multiple workers who describe *M. haemolytica* as a normal inhabitant of upper respiratory tract as well as a causative agent of secondary bronchopneumonia after

primary initiating agent has suppressed the host defense mechanism (Songer and Prescott, 1993; Baker, 1998).

The results of *M. haemolytica* obtained from lung and trachea of goat in this investigation was higher than the results of Megra and his coworkers (2006) which showed an isolation rate of 6% and 14% for trachea and lung of goats on their study carried out at Dire Dawa abattoir. But on the other hand, the result was lower than earlier reports of 35.5% by Almeida and his colleagues (1986), 47.5% by Mohammed (1999) and 18.8% by Tilaye (2010) of lung infection rates. This variation might be attributed to the difference in agro-ecological zones of the study areas and also the health status of the respective animal species.

The isolation of *M. haemolytica* from pneumonic lungs of sheep at a higher rate agrees with the reports of Tesfaye (1997) and Aschalew (1998) who reported an isolation rate of 67.6% and 63.8% respectively. But much higher than the report made by Tilaye (2010) who presents an isolation rate of only 28.4%. The rate of isolation of these bacteria is higher except those isolated from cattle lung (1.3%) when incubated with increment of CO₂ tension.

The isolation of *M. haemolytica* from the nasal cavity of sheep from three different highlands of the country at a total rate of 25.4% also indicated the significance of the bacteria in causing opportunistic disease in ruminants in general and in sheep in particular. The result obtained in this study was a little higher than the result described by Sisay and Zerihun (2003) who isolated *M. haemolytica* at a rate of 13% of the swabs. The variation might be attributed to that this study is a cumulative study of three different highlands of the country while the other study was conducted only on the highlands of Wollo.

In agreement with previous reports (Radostits *et al.*, 1994; Aschalew, 1998 and Sisay and Zerihun, 2003), both in terms of infection intensity and pathogenicity, it seemed that *M. haemolytica* assumed greater prominence in ruminant respiratory syndromes in this environment. In addition, the presence of *M. haemolytica* in higher proportion in clinically ill animals can make the bacteria to be considered as the major factor in causing RDC.

The results of the abattoir survey also showed that the percentage of isolation of *B. trehalosi* from the trachea and lungs of cattle is higher when compared to the isolation rate from small ruminants. The isolation rate from the pneumonic sheep lung was closely related with the

reports of Tilaye (2010) who demonstrated a rate of 6.6%. The study also showed that the occurrence of these bacteria in the nasal cavity of sheep at a rate of 6.7% signifies its importance in contributing for presence of respiratory infection. In the present study *B. trehalosi* has not been isolated from either trachea or lung of goats. Unlike that of *M. haemolytica* anaerobic incubation does not improve the growth of *B. trehalosi* except in the case of cattle lung and this result was in agreement with the study of Sisay and Zerihun (2003) who presented isolation of *B. trehalosi* at 67% rate from nasal swabs collected from the highlands of Wollo. The decreased isolation rate of *B. trehalosi* in this study when compared to the previous works might be that these authors isolated the bacteria from tonsils in addition to lung and nasal swabs and *B. trehalosi* is generally isolated at higher rate from tonsils than from lung.

The current investigation also indicated that the isolation rate of *P. multocida* in all the three species were very low. Except for the lungs of goats the bacteria was not isolated from the rest sampled animal tissues. The isolation rate has increased when the lung tissue was cultured anaerobically revealing its presence at 2.4% rate in goat lung. This relatively agrees with Tilaye (2010) who isolated this bacterium from pneumonic lungs of goats and presents a 5.4% rate and failed to isolate the bacteria from pneumonic lungs of sheep. Similarly, *P. multocida* frequently inhabited nasal cavity and the result obtained from the nasal cavity of sheep from three sites indicated the importance of this bacterium in causing opportunistic infection in these animals. This is in accordance with previous reports (Almeida *et al.*, 1986; Mohammed, 1999), indicating that the organism lives as a commensal in the upper respiratory tract, and invading the lung under conditions of stress (Collier and Rossow, 1964).

In general, the highest frequency of isolation of *M. haemolytica* in comparison with *B. trehalosi* and *P. multocida* in this study coincided with the works reported by other workers. Most of the studies carried out on pneumonic lungs of sheep in Ethiopia revealed that *M. haemolytica* has higher rate of isolation than *P. multocida* and *B. trehalosi* (Tesfaye, 1997; Aschalew, 1998; Tilaye, 2010) and thus, has a much higher contribution in the occurrence of RDC.

The isolation of *Bordetella* species from the lungs of the three species of animals was the significance of this study. Even though, the isolation rate from sheep and goats is lower in comparison with cattle, its presence in the respiratory tract showed the importance of this

bacterium for the contribution of RDC. Even if, previous studies in our country did not indicate the significance of these bacteria, Ngom together with his colleagues (2006) reports an isolation rate of 3.3% and 4.8% from sheep and goats, respectively in their abattoir surveillance and indicate that the importance of *B. bronchiseptica* as a RDC is increasing from time to time in Senegal. The failure to isolate this bacterial species in previous reports from the country may be due to giving little consideration for possible role of the organism in respiratory tract health and disease so that they could not characterized suspected colonies.

Furthermore, us to our thoughtful, this is the first study ever that recovered *H. somni* from the respiratory tracts of ruminants in Ethiopia. The isolation of *H. somni* in this study from the nasal cavity of sheep from the three areas leads to a hypothesis of this bacterium might have some contribution in causing RDC. Although, this bacteria has not been reported from any of the studies carried out before in our country, its contribution for the RDC is paramount as indicated by many different literatures. Mostly the occurrence of this bacterial species was associated with cattle disease. But *H. somni* is also of great clinical importance for sheep worldwide (Humphrey and Stephens, 1983; Harris and Janzen, 1989; Kwiecien and Little, 1991). Diseases that have been associated with infection of sheep with *Haemophilus agni*, *Histophilus ovis* or *Haemophilus somnus* have been reported by several workers at different times and this concur with the current study.

Similar organisms, formerly identified as *Histophilus ovis*, were isolated from sheep in Australia and determined to be the causative agents of mastitis, epididymitis-orchitis, metritis, septicemia, meningoencephalitis, pneumonia, and polyarthritis (Philbey *et al.*, 1991). Another study conducted on herds of rams indicated an infection rate of 27.5% with *Haemophilus somnus* (Lees *et al.*, 1990); in addition, 12% of ewes in the infected herds failed to lamb, in comparison with 6% of ewes in non-infected herds, thus suggesting reduced fertility of ewes in infected herds abortion (Ward *et al.*, 1995), meningitis (Lees *et al.*, 1994), vulvitis and mastitis (Kennedy *et al.*, 1958). All these studies suggest that *H. somni* should be considered as a great health problem in causing opportunistic infection in sheep in addition to cattle population.

The isolation of high proportion of other bacterial species from the lung and trachea of ruminants in general as well as nasal swabs of sheep in particular in this study signifies that a

numbers of factors are involved for the contribution of respiratory infection in these animals. The findings of this study are in agreement with the previous study conducted. Yimer and Asseged (2007) isolated *E. coli*, *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *A. pyogens*, *Enterococcus*, *Micrococcus*, *Enterobacter*, *Citrobacter* and *Klebsiella* species with different proportion from the nasal cavity, tonsil, trachea and lung of sheep in addition to major pathogenic species. While Megra and his co-workers (2006) reports the presence of these bacteria from the respiratory tracts of goats. But the major difference observed from these studies was the presence of higher proportion of *E. coli* and *Corynebacterium* species in the above mentioned studies, which were not reported in the current study. Even though these groups of bacteria have not been considered as a major causative agent of RDC, their significance should not be disregarded.

Given the complex etiology of respiratory disease attributed to a particular species of bacteria, it is unlikely that any single strategy will be completely effective in preventing the disease. A combination of more definitive diagnostic methods, more efficacious vaccines, improved therapeutic agents and more rational management practices should be considered for preventing this highly devastating disease.

6. CONCLUSION AND RECOMMENDATIONS

It is evident from this study and previous works that respiratory infection is a highly complex multifactorial disease of a worldwide prevalence and distribution in cattle, sheep and goats. The disease primarily results from interaction of stress, immunity and the causative bacteria which is commensally resident in the respiratory tract of susceptible animals. The major factors leading to stress and compromised immunity are naturally created by adverse environmental and climatic conditions and also by previous or coinfection with certain respiratory viruses, *Mycoplasma* or some other types of bacteria.

Currently, detection of newly emerging and re-emerging pathogens has been increased due to improved methods of surveillance, epidemiological studies and the continuous development of more advanced methods of diagnosis. Hence, more species of bacteria in the future might be diagnosed as a cause of RDC. To know these pathogens initially it is compulsory to understand the prevalent commensal and pathogenic bacteria species harbouring the respiratory tract.

Bacterial groups belonging to the family *Pasteurellaceae* are known to cause a major respiratory disease in ruminants. *P. multocida*, *M. haemolytica* and *B. trehalosi* are among the most recognized bacterial species in contributing for RDC in these animals in addition to other stress factors. In this study it is shown that *M. haemolytica* plays a great role in causing respiratory infection among other bacterial species mentioned here. But, in addition, the involvement of other bacterial species in respiratory infection is obscured in many previous studies due to paying more attention to only these groups of bacteria.

To our understanding, this report is the first for the isolating and identifying of *H. somni* from sheep and *Bordetella* species ruminant respiratory tracts in Ethiopia. These bacterial species encountered in the present study from the lungs and nasal cavity of ruminants is an indicative of a threat for sheep, goat and cattle population in the country. Therefore, *H. somni* and *Bordetella* infection could and probably be one of the main pathogenic microorganisms involved in respiratory disease complex in Ethiopian livestock population where pneumonia is a major threat.

Therefore, based on the above concluding remarks the following points are forwarded:

In all of the cases, regarding diseases, prevention is a priority. Developing a polyvalent effective respiratory tract efficacious vaccine which includes antigenic components of *H. somni* in addition to other predominant causative agent should have to be developed and the search for solutions is likely to involve veterinary researchers for some time to come.

This study has been limited in time and also financial source and hence, it is not able to get more information on the disease aspect of *H. somni* as well as *Bordetella* species. Thus, further organized wide-scale research for better understanding of the magnitude and distribution of newly emerging respiratory pathogen including *H. somni* and *Bordetella* should have to be developed.

The progression of research currently using advanced molecular technologies is at its high point. Hence, both for assessing the presence of *H. somni* and *Bordetella* species in clinical samples and for identification of an isolate to the species of newly emerging respiratory pathogen, a genomic analysis should have to be implemented in our country.

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Figure 1. The first part of the manuscript, which is devoted to the description of the problem and the formulation of the research objectives. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the topic, followed by a detailed description of the problem. The research objectives are clearly stated, and the author provides a brief overview of the methodology used in the study.

Figure 2. The second part of the manuscript, which is devoted to the description of the methodology used in the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the methodology, followed by a detailed description of the methods used. The author provides a brief overview of the results of the study, and the conclusions are clearly stated.

Figure 3. The third part of the manuscript, which is devoted to the description of the results of the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the results, followed by a detailed description of the findings. The author provides a brief overview of the conclusions, and the conclusions are clearly stated.

Figure 4. The fourth part of the manuscript, which is devoted to the description of the conclusions of the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the conclusions, followed by a detailed description of the findings. The author provides a brief overview of the conclusions, and the conclusions are clearly stated.

Figure 5. The fifth part of the manuscript, which is devoted to the description of the conclusions of the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the conclusions, followed by a detailed description of the findings. The author provides a brief overview of the conclusions, and the conclusions are clearly stated.

Figure 6. The sixth part of the manuscript, which is devoted to the description of the conclusions of the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the conclusions, followed by a detailed description of the findings. The author provides a brief overview of the conclusions, and the conclusions are clearly stated.

Figure 7. The seventh part of the manuscript, which is devoted to the description of the conclusions of the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the conclusions, followed by a detailed description of the findings. The author provides a brief overview of the conclusions, and the conclusions are clearly stated.

Figure 8. The eighth part of the manuscript, which is devoted to the description of the conclusions of the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the conclusions, followed by a detailed description of the findings. The author provides a brief overview of the conclusions, and the conclusions are clearly stated.

Figure 9. The ninth part of the manuscript, which is devoted to the description of the conclusions of the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the conclusions, followed by a detailed description of the findings. The author provides a brief overview of the conclusions, and the conclusions are clearly stated.

Figure 10. The tenth part of the manuscript, which is devoted to the description of the conclusions of the study. The text is written in a clear and concise manner, and the structure is well organized. The author starts with a general introduction to the conclusions, followed by a detailed description of the findings. The author provides a brief overview of the conclusions, and the conclusions are clearly stated.

8. ANNEXES

Annex 1: Primary Biochemical Identification Test

Gram's stain (Quinn *et al.*, 2002)

Principle: Gram reaction is related to the structure of the cell wall. Some bacteria have a high lipid content in their cell wall and do not retain the crystal violet and are stained by the counter stain.

Procedure: A thin smear or film was made and allowed to dry on air. The film was fixed by passing through the Bunsen flame several times. The slide was flooded with crystal violet for 30 seconds. The stain was poured and the remaining stain was washed with iodine solution. The iodine was washed off and the excess water was shaken from the slide. Then, the slide was decolorized with 95% ethanol alcohol. The slides were counter stained with safranin for 30 seconds and washed with water.

Catalase test (Quinn *et al.*, 2002)

Principle: The breakdown of hydrogen peroxide into oxygen and water is mediated by the enzyme Catalase.

Procedure: A loop full of bacterial growth was taken from the top of the colonies avoiding the blood agar medium. The bacterial cells were placed on a clean microscope slide and a drop of 3% H₂O₂ was added. An effervescence of oxygen gas, within a few seconds, indicated a positive reaction.

Oxidase test (Quinn *et al.*, 2002)

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

Procedure: A solution of 1% tetramethyl-p- phenylenediamine dihydrochloride was used to moisten a piece of filter paper in a Petridish and the test bacteria was streaked firmly across the filter paper with a glass rod. A dark purple color along the streak line within 10 seconds indicated a positive reaction.

O-F test (Quinn *et al*, 2002)

Procedure: O-F base medium was Prepared and when the O-F base has cooled to 50 °C. 20 ml of sterile glucose solution was added into 200 ml of O-F base, for a final concentration of 1% glucose and dispensed into tubes. Two tubes of the O-F medium were heated in a beaker of boiling water immediately before use to drive off any dissolved oxygen and the tubes were then cooled rapidly under cold running water. Both tubes were stab inoculated with the bacterium and a layer of sterile paraffin oil was layered on top of one on the tubes (sealed tube) to a depth of about 1 cm and the tubes were incubated at 37 °C and examined in 24 hours and then daily for up to 14 days.

Motility test (Quinn *et al.*, 2002)

Procedure: SIM medium (BBL) was used to detect motility. The medium was stab inoculated using a straight wire. Then the tube was examined for motility after 24 and 48 hours. If there is a diffuse growth throughout the medium the bacterium is motile the growth of a non motile bacterium is confined to stab line. To interpret the results the tube was hold against a good light and the inoculated tubes were compared with uninoculated one.

Annex 2: Secondary Biochemical Identification Test

Methyl red (MR) test (Quinn *et al.*, 2002)

Principle: it is a quantitative test for acid production requiring positive organisms to produce strong acids (lactic acetic and formic).

Procedure: MR-VP broth was inoculated with pure culture of test organism and incubated at 37 °C for two days then 5 drops of MR solution was added in to the media. Production of red color indicated a positive result and yellow color negative in methyl red test.

Indole test (Quinn *et al.*, 2002)

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

Procedure: SIM medium was Stab inoculated with test bacterium and incubated at 37⁰C for 18 to 25 hours. Then add kovac's reagent (0.2 ml) was added to tube and stood for 10 minutes. The formation of dark red ring indicates positive reaction while in negative reaction a yellow ring was formed.

Citrate test (Quinn *et al.*, 2002)

Principle: Certain organisms are able to utilize citrate as a sole carbon source. This utilization is detected in citrate medium by the production of alkaline by products.

Procedure: inoculate Simmons citrate agar was inoculated slant surface was inoculated with a well isolated colony, and incubated at 37 ⁰C for 24 to 48 hours. A positive test was represented by the development of a deep blue color within 24 to 48 hours, indicating that the test organism was able to utilize the citrate contained in the medium with the production of alkaline products. In negative reaction the color remained green.

Annex 3: Media Used for Isolation and Identification

BLOOD AGAR BASE (Oxoid, Hampshire, England)

Formula in grams per liter: Heart Infusion.....10,00; Meat Peptone.....10,00; Sodium Chloride.....5,00; Bacteriological Agar..... 15,00 and the final pH is 7,3 ± 0,2 at 25°C.

Preparation: Suspend 40 grams of the medium in one litre of distilled water. Leave to stand for 5 minutes and mix well until a uniform suspension is obtained. Heat with gentle agitation and boil for one minute. Sterilize to 121°C (15 lbs. sp.) for 15 minutes. Cool to 45-50°C, and add 5 -10% sterile defibrinated blood, homogenize and pour into Petri plates.

MACCONKEY AGAR (Oxoid, Hampshire, England)

Formula in grams per liter: Pancreatic Digest of Gelatin..... 17,00; Lactose monohydrate..... 10,00; Sodium Chloride..... 5,00; Peptone Mixture 3,00; Bile Salts no 3..... 1,50; Neutral Red 0,03; Crystal Violet..... 0,001 Bacteriological Agar 13,50 and the final pH is $7,1 \pm 0,2$ at 25°C .

Preparation: Suspend 50 grams of the medium in one liter of distilled water. Mix well until a uniform suspension is obtained. Heat with frequent gentle agitation and boil for one minute. Sterilize in autoclave at 121°C (15 lbs. sp) for 15 minutes. Cool to 45°C , and pour into Petri dishes. Allow the plates to solidify and place them upside down to avoid excessive moisture in the surface of the medium.

Biolog Universal Growth Agar (BUG) (Biolog, California, USA)

Preparation: 57 grams BUG Agar was added into 1,000 ml distilled water then gently boiled while stirring to dissolve the agar and other components. Cooled and the pH is measured and adjusted to a final pH of 7.3 ± 0.1 . Finally sterilized by autoclaving at 15 pounds of pressure, at 121°C , for 15 minutes then cooled to $45-50^{\circ}\text{C}$ and dispensed into sterile petri dishes.

OF BASAL MEDIUM (Oxoid, Hampshire, England)

Formula in grams per liter: Casein Peptone 2,00; Sodium Chloride.....5,00; Dipotassium Phosphate 0,30 Bacteriological Agar2,50; Bromthymol Blue 0,03 and the final pH is $7,1 \pm 0,2$ at 25°C .

Preparation: Suspend 9,8 grams of medium in one litre of distilled water. Heat with frequent agitation until dissolved. Sterilize in an autoclave at 121°C (15 lbs. sp.) for 15 minutes. Add 10 ml. of 10% glucose (or any suitable sugar) solution sterilized by filtration to 100 ml. of liquid medium. Mix and dispense aseptically 5 ml. per tube. If preferred, add 1,0 grams of carbohydrate directly to 100 ml. of medium and sterilize in an autoclave at 118°C (12 lbs.) for 10 minutes to avoid the degradation of the sugar. The color of the prepared medium is green.

SIM MEDIUM (Oxoid, Hampshire, England)

Formula in grams per liter: Casein Peptone20,00; Meat Peptone6,10; Ferric Ammonium Sulfate..... 0,20; Sodium Thiosulfate.....0,20; Bacteriological Agar..... 3,50 and the final pH is $7,3 \pm 0,2$ at 25°C .

Preparation: Suspend 30 grams of the medium in one liter of distilled water. Leave to soak for 5 to 10 minutes. Mix well until a uniform suspension is obtained, heat agitating constantly and boil for one minute until completely dissolved. Dispense and sterilize by autoclaving at 121°C (15 lbs sp) for 15 minutes

TRYPTONE SOY AGAR (Oxoid, Hampshire, England)

Formula in grams per liter: Casein peptone.....15,00; Soy Peptone..... 5,00; Sodium Chloride.....5,00; Bacteriological Agar.....15,00 and the final pH is $7,2 \pm 0,1$ at 25°C .

Preparation: Suspend 40,0 grams of the medium in one liter of distilled water. Mix well and heat agitating frequently till boiling. Distribute into appropriate containers and sterilize at 121°C (15 lbs. sp.) for 15 minutes. Allow the medium to cool to 50°C and distribute in double layer plates, making the layers no less than 5 mm depth.

SIMMONS CITRATE AGAR (Oxoid, Hampshire, England)

Formula in grams per liter: Ammonium Dihydrogen Phosphate1,00; Dipotassium Phosphate.....1,00; Sodium Chloride..... 5,00; Sodium Citrate2,00; Magnesium Sulfate0,20 Bacteriological Agar..... 15,00; Bromthymol Blue.....0,08 and the final pH $6,9 \pm 0,2$ at 25°C .

Preparation: Suspend 24,3 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation until completely dissolved. Dispense in tubes and sterilize in the autoclave at 121°C (15 lbs sp.) for 15 minutes. Cool the tubes in a slanted position so that the base is short (1-1,5 cm. deep). Alternatively, the media can be poured into petri plates.

Annex 4: The OmniLog Microbe Identification Process

Step 1

Isolate a pure culture on Biolog media

Step 2

Prepare inoculum

Step 3

**Inoculate MicroPlates and load into
incubator/ reader**

Step 4

Obtain ID results from printer

9. DECLARATION

First, I declare that this thesis is the result of my own work and that all sources of materials used for this thesis have been duly acknowledged. This thesis is submitted in partial fulfillment of the requirements for MSc degree at Addis Ababa University and to be made available at the University's Library under the rules of the Library. I confidently declare that this thesis has not been submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

Name: Biruk Tesfaye

Signature: _____

Place: Addis Ababa University, School of Veterinary Medicine

Date of submission: June, 2011

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

Name of advisors:

Signature

Dr. Tesfaye Sisay (DVM, MSc, PhD, Asst. Prof)

Dr. Genene Tefera (DVM, PhD)
