

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

**CULTURAL CHARACTERIZATION, MOLECULAR DETECTION AND *IN-VITRO*
ANTIBIOTIC SUSCEPTIBILITY PROFILE OF *Streptococcus equi* subsp. *equi* FROM
DONKEYS SUSPECTED OF STRANGLES IN CENTRAL ETHIOPIA**

BY: MANYAHILISHAL ETANA KITAW

JUNE, 2012

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TABLE OF CONTENTS

	Pages
ACKNOWLEDGEMENTS.....	I
TABLE OF CONTENTS.....	II
LIST OF TABLES.....	IV
LIST OF FIGURES.....	V
LIST OF ANNEXES.....	VI
ABBREVIATIONS.....	VII
ABSTRACT.....	IX
1. INTRODUCTION.....	1
2. LITERATURE REVIEW.....	3
2.1. Strangles.....	3
2.1.1. Etiology.....	3
2.1.2. Epidemiology.....	4
2.2. Molecular basis of <i>Streptococcus equi</i> subsp. <i>equi</i> virulence.....	5
2.2.1. Adherence.....	5
2.2.2. Evasion of host defenses.....	8
2.2.3. Nutrient acquisition.....	11
2.3. Pathogenesis of Strangles.....	12
2.4. Diagnostic Approaches.....	14
2.4.1. Clinical, bacteriology and endoscopy.....	14
2.4.2. Serology.....	15
2.4.3. Molecular diagnosis.....	16
2.5. Treatment.....	17
2.6. Prevention and Control.....	18
2.7. Economic Importance of Strangles.....	19
2.8. Status of Strangles in Ethiopia.....	20
3. MATERIALS AND METHODS.....	21
3.1. Study area.....	21
3.2. Sampling method.....	21
3.3. Sample collection and transportation.....	21
3.4. Isolation and Identification of <i>Streptococcus equi</i> subsp. <i>equi</i>	22

3.5. Molecular detection	23
3.5.1. DNA extraction	23
3.5.2. Polymerase chain reaction (PCR)	23
3.5.3. Agarose gel electrophoresis	24
3.6. Antibiotic sensitivity testing.....	24
3.7. Data Collection and Analysis	26
4. RESULTS.....	27
4.1. Clinical presentation of donkeys with suspected strangles	27
4.2. Postmortem examination of guttural pouch.....	29
4.3. Isolation and identification of <i>Streptococcus equi</i> subsp. <i>equi</i>	30
4.4. PCR based identification of <i>Streptococcus equi</i> subsp. <i>equi</i>	34
4.5. Antibiotic sensitivity pattern of isolates.....	35
5. DISCUSSION.....	38
6. CONCLUSIONS AND RECOMMENDATIONS	44
7. REFERENCES	46
8. ANNEXES	56
9. CURRICULUM VITAE	67
10. SIGN OF DECLARATION.....	69

LIST OF TABLES

Table 1. Zone size interpretation chart (NCCL(1997))M31-A	25
Table 2. Clinical signs observed in different age groups of donkeys.....	29
Table 3. Bacterial species isolated from nasopharyngeal swabs, lymph node aspirates, guttural pouch abscesses and chondroids.	31
Table 4. Isolation rate of <i>Streptococcus</i> spp. from swab and aspirate samples.....	33
Table 5. Isolation frequency of <i>Streptococcus</i> spp. by age and sex from nasopharyngeal swab, aspirate and chondroid	33
Table 6. The comparison between culture and PCR for the detection of <i>Streptococcus</i> <i>equi</i> subsp. <i>equi</i> in suspected strangles cases.	35
Table 7. <i>In-vitro</i> Drug sensitivity pattern for isolates of <i>Streptococcus equi</i> subsp. <i>equi</i>	36

LIST OF FIGURES

Figure 1. A one year old female donkey suspected of strangles with guttural pouch empyema and purulent nasal discharge at DHWP clinic, Debre-Zeit, Ethiopia.....	28
Figure 2. A 3 years old male donkey suspected of strangles showing parotid lymph node rupture presented at DHWP clinic, Debre- Zeit, Ethiopia.....	28
Figure 3. (A) Chondroids in guttural pouch of a 12 years old female donkey; (B) Chondroids collected from the same donkey.	30
Figure 4. Gram's staining of guttural pouch aspirate characteristic chain cocci of <i>Streptococcus equi</i> subsp. <i>equi</i>	32
Figure 5. Typical isolate of <i>Streptococcus equi</i> subsp. <i>equi</i> with β -hemolytic 'honey dew' appearance	32
Figure 6. API 20 Strep results for isolates of <i>Streptococcus equi</i> subsp. <i>equi</i>	32
Figure 7. Typical PCR products of <i>Streptococcus equi</i> subsp. <i>equi</i> with SeM gene. Lane N is negative control and lane M showing 100bp gene marker.	34
Figure 8. Antibiotic disk sensitivity test for <i>Streptococcus equi</i> subsp. <i>equi</i> isolates on blood agar. The drugs used are 1- Gentamycin, 2-Norfloxacin, 3-Doxycycline, 4-vancomycin- and 5- sulphonamid.	37

LIST OF ANNEXES

Annex 1. Case record sheet 56

Annex 2. Laboratory data record sheet..... 57

Annex 3. Donkey suspected of strangles with characteristics nasal discharge, parotid lymph node enlargement with extended head and neck (Godino public veterinary clinic)..... 58

Annex 4. Donkey suspect of strangles with erupted sub mandibular lymph node (DHWP clinic) 58

Annex 5. Donkey ssuspect of strangles with retropharngeal lymph node enlargement (DHWP clinic) 59

Annex 6. Antibiotic susceptibility test for *S. equi* subsp. *equi* on blood agar plate. The drugs used are 1- Ampicillin, 2-Ceftriaxone, 3- Chloramphenicol, 4- Gentamycine 59

Annex 7. API 20 Strep test procedure 60

Annex 8. DNA Extraction Protocol 65

Annex 9. PCR Protocol 66

ABBREVIATIONS

AAU	Addis Ababa University
ABC	ATP-binding cassette
ADH	Arginine Dihydrolase
AHT	Animal Health Trust
AMD	Amidon
API	Analytical profile index
ARA	Arabinose
ATP	Adenine Tri Phosphate
CLS-TC	Cell Lysis/DNA Solubilizing Solution for Bacteria
CNE	Collagen Binding Proteins
DES	DNA elution solution
DHWP	Donkey Health and Welfare Project
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
ESC	Esculin (β -glucosidase hydrolysis)
GLYG	Glycogen
HAP	Hyaluronate Associated Protein
HAP-GST	Hyaluronate Associated Protein-Glutathione S-transferase
HIP	Hippuric acid
IgG	Immunoglobulin G
INU	Inulin
KDa	Kilo Dalton
LAC	Lactose
LAP	Leucine Amino Peptidase
MAN	Mannitol
MBL	Mannose Binding Lectin
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing

MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
NCCL	National committee for clinical laboratory Standards
NCTC	National Collection of Type Cultures
NMSA	National Metrological Services Agency
PAL	Alkaline Phosphatase
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PYRA	Pyrrolidonyl Arylamidase
RAF	Raffinose
RIB	Ribose
sAgs	Superantigens
SEWS-M	Salt/Ethanol Wash Solution
SLS	Streptolysin S
SOD	Super Oxide Dismutase
SOR	Sorbitol
SPSS	Statistical Package for the Social Sciences
TAE	Tris-Acetate-EDTA (Ethylenediaminetetraacetic acid)
TNF α	Tumor Necrosis Factor α
TRE	Trehalose
VP	Voges Proskauer
ZAG	Zinc Binding Alpha Globulin
α GAL),	α -Galactosidase
β GAL	β -Galactosidase
β GUR),	β -Gulcuronidase

ABSTRACT

Cultural characterization and molecular detection of *Streptococcus equi* subsp. *equi* from strangles suspected naturally infected donkeys was conducted in central Ethiopia from September 2011 to April, 2012. Antibiotic susceptibility profile of the isolates was also studied. Basic bacteriology for isolation and characterization, PCR for genome detection and disc method for antibiotic susceptibility were used. A total of 87 donkeys were sampled. Out of these 21 (24.1%) donkeys had only nasal discharge, 47 (54%) had both nasal discharge and swelling of either submandibular or retropharyngeal lymph nodes, 2 (2.3%) had parotid lymph node involvement, 12 (13.8%) had severe guttural pouch empyema and extended head and neck with strider breathing and also 5 (5.8%) donkeys chondroid positive at postmortem. Young donkeys were affected with severe form of the disease, swelling of lymph nodes of the head region and guttural pouch empyema, than adults ($p<0.05$). Culturally, a total of 189 bacterial isolates belonging to 11 genera were obtained. These were *Streptococcus* spp. (42.3%), *Staphylococcus* spp. (18%), *Neisseria* spp. (1.6%), *Enterococcus* spp. (10.1%), *Bacillus* spp. (7.9%), *Corynebacterium* spp. (4.8%), *Rhodococcus* spp. (2.1%), *Pasteurella* spp. (5.8%), *Actinomycetes* spp. (3.7%), *Pseudomonas* spp. (2.1%) and *Proteus* spp. (1.6%). Swab samples had contributed for the majority of the isolates (87.8%). The isolation rate of *S. equi* subsp. *equi* from aspirate samples was 40% being significantly ($p<0.05$) higher than swab samples 14%. The detection rate by PCR was 72% and 87% from swabs and aspirates samples, respectively. The molecular method was more sensitive ($p<0.05$) compared to cultural method. which was significantly ($p<0.05$) higher compared to cultural method. The *in-vitro* antibiotic susceptibility test of *S. equi* subsp. *equi* revealed that all isolates were found to be susceptible to ampicillin, gentamicin, chloramphenicol, norfloxacin, erythromycin, vancomycin, cephalothin, doxycycline, ceftriaxone and tetracycline; however, all of the isolates were resistant to sulphonamide, kanamycin and streptomycin. Despite its lack of sensitivity relative to PCR, culture is of value because it definitively establishes infection. Routine use of both microbial culture and PCR analysis of samples maximize detection of *S. equi* subsp. *equi*. Aspirates, when possible, are the preferred sample to increase the sensitivity of both culture and PCR assays.

Keywords: Antibiotics, Central Ethiopia; Donkey; *S. equi* subsp. *equi*; Strangles

1. INTRODUCTION

Strangles caused by infection with the bacterium *Streptococcus equi* subsp. *equi*, remains one of the most commonly diagnosed and important infectious diseases of equids. The disease occurs worldwide and causes heavy economic losses in terms of the cost of treatment, quarantine measures and, occasionally, the death of animals. It is characterized by pyrexia, followed by profuse nasal discharge and abscessation of lymph nodes of the head and neck, which subsequently burst, discharging highly infectious pus. Rarely, this lymph node enlargement can cause inspiratory stridor and dyspnoea and it is this clinical feature that gave the disease its name (Timoney, 1993; Sweeney *et al.*, 2005; Slater, 2007).

A particular problem in the management of strangles outbreaks is the lack of a suitable assay to differentiate between *S. equi* strains and so to determine if an outbreak is likely to be due to the recurrence of an earlier infection or the introduction of a new *S. equi* strain. Furthermore, in the absence of methods for sub-typing strains, it has not been possible to investigate whether *S. equi* strain variation may contribute to the different presentations of disease seen in different outbreaks. Subclinical carriers that intermittently shed bacteria may act as sources of infection for naïve animals for up to several months or even years and are believed to be key to the epidemiology of the disease through persistence of the infection between outbreaks (Timoney *et al.*, 1998; Newton *et al.*, 2000;). Approximately 10% of horses that recover from strangles become persistent carriers of *S. equi*, harboring the infectious agent in chondroids located in the guttural pouch (Dalglish *et al.*, 1993; Timoney, 1993).

Although the status of strangles in the equine population of Ethiopia is not well known, attempts were made to isolate normal floras of the upper respiratory tract of donkeys (Desissa *et al.*, 2009) and horses (Tesfaye, 2010). Strangles is also one of important health problems affecting donkeys in Ethiopia (Getachew *et al.*, 2002). Base line data on the epidemiology of the disease in the donkeys and the causative bacteria, *Streptococcus equi* subsp. *equi*, involved in causing the disease is not well studied and documented. Questionnaire survey and bacteriological findings from studies made by Alemnesh (2004) indicated that strangles is one of the major health

problems of donkeys in the study areas. According to the observations made and the 2010/11 annual reports by the donkey health and welfare project, at the School of veterinary medicine, AAU, Debre Zeit, upper respiratory diseases, of which suspected strangles was the main one, were the major problems in the donkey population. A participatory need assessment by the project also revealed similar findings, in which communities rated suspected strangles, locally called 'furro', as one of the major health problems of their donkeys.

This study was therefore conducted with the following objectives:

- To isolate and characterize *Streptococcus equi* subsp. *equi* organisms from suspected cases of strangles in donkeys
- To determine the antibiotic susceptibility profile of the *Streptococcus equi* subsp. *equi* isolates through drug sensitivity test

2. LITERATURE REVIEW

2.1. Strangles

2.1.1. Etiology

The highly host adapted *Streptococcus equi* subspecies *equi* (*S. equi* subsp. *equi*) of Lancefield group C causes equine strangles (Timoney, 2004). *S. equi* subsp. *equi* is a Gram-positive coccobacillus that produces a beta-hemolysin, evident as a zone of clear hemolysis surrounding colonies growing on blood agar. *S. equi* demonstrates no serological variation, although genetic analysis demonstrates the existence of clones that vary geographically (Al-Ghamdi *et al.*, 2000).

S. equi is described as being highly capsulated giving it a 'honey dew' appearance although approximately half of fresh clinical isolates appear indistinguishable from *S. zooepidemicus*. *S. equi* can survive for long periods either in the host (George *et al.*, 1983; Timoney, 1988; Wood *et al.*, 1993) or in the environment (Jorm, 1991). Jorm (1991) demonstrated that the survival of *S. equi* was affected by its substrate and the temperature. The organism can survive for 63 days on wood at 2°C and for 48 days on glass or wood at 20°C. Weese *et al.*, (2009) examined survival of saline or mucus-based inoculums of *S. equi* on wood, rubber and metal surfaces exposed to outdoor conditions, and found that mucus-based inoculums of *S. equi* persisted longer than saline-based inoculums and that rain, daily temperatures or surface type did not affect the persistence of *S. equi*. When exposed to sunny weather, none of the samples yielded organisms after 24 hours whereas approximately 20% of the samples exposed to partly cloudy or cloudy weather conditions remained positive for *S. equi*.

2.1.2. Epidemiology

Occurrence

Strangles occurs in horses, donkeys, and mules worldwide. Outbreaks are seen relatively frequently on breeding farms and racing stables, when the infection is introduced by new arrivals that are often asymptomatic. An incidence of 35% over a 3-year period is reported for horse in Australia (Jorm, 1990). Strangles can affect horses of any age. However, the morbidity rate is usually greater in younger equids. Older horses often exhibit a mild form of the disease characterized by a mild, transient fever, mild mucoid to mucopurulent nasal discharge and small lymph node abscessation. Age-specific attack rates of strangles of 18% for brood mares, 48% for 1-year-old horses and 38% for foals during an outbreak on a breeding farm are reported, although higher morbidity rates (100%) can occur, especially in young horses (Sweeney *et al.*, 2005).

According to the findings of Jorm, (1990), an outbreak of strangles increases with the size of the group of horses: farms with 100 or more horses have a 26 times greater risk of experiencing an outbreak than farms with fewer than 15 horses. The case-fatality rate without treatment is about 9%, but with adequate early treatment may be as low as 1-2 %.

Source of infection and transmission

S. equi is an obligate parasite of equids and all infections are attributable to transmission from infected equids, either directly or by fomites. Nasal and abscess discharge from infected animals that contaminates pasture, tack, stalls, feed and water troughs, grooming equipment, and hands and clothes of grooms and veterinarians is often the source of infection for susceptible horses. Direct transmission from infected animals to susceptible animals occurs through contact (Radostits *et al.*, 2007).

Nasal shedding of *S. equi* usually begins 4-7 days after infection, or 2 days after onset of fever, and persists for 2-3 weeks in most horses but up to years in exceptional horses. Cessation of shedding accompanies development of an effective serum and mucosal immune response (Galan

et al., 1986). Approximately 10-40% of horses that recover from the clinical disease has persistent infection of *S. equi* in the pharynx and guttural pouches for many months and are important source of infection (Newton *et al.*, 1997; Newton *et al.*, 2000). Horses with clinically inapparent disease, such as some cases of guttural pouch empyema, may shed the organism for over 3 years (Newton *et al.*, 1997).

Equids that previously had the disease are less likely than naive animals to develop the disease on subsequent exposure (Hamlen *et al.*, 1994). A proportion (approximately 25%) of horses that recover from the disease do not develop a protective immune response and are susceptible to reinfection and a second bout of strangles (Timoney, 1993; Hamlen *et al.*, 1994). Resistance to the disease is associated with the production of serum and mucosal IgG antibodies to the streptococcal M protein. Although strong immunity occurs immediately after an attack, this immunity wanes with time and a horse may suffer further disease if the organism is virulent (Hamlen *et al.*, 1994).

2.2. Molecular basis of *Streptococcus equi* subsp. *equi* virulence

Several factors produced by *Streptococcus equi* subsp. *equi* have been implicated in the molecular basis of its virulence. These factors are grouped into three broad categories based on their proposed functions; those that promote bacterial adherence to host surfaces; Fibronectin-binding proteins, Fibrinogen-binding proteins, Collagen binding proteins, Capsule, Lipoteichoic acid (LTA), those that contribute to immune evasion; Capsule, Hyaluronate-associated protein (HAP), M-like proteins, Mitogenic factors, Superoxide dismutase (SOD), Immunoglobulin-binding proteins and those that are involved in nutrient acquisition; Acid phosphatases, ABC transporters, Degradative enzymes, Streptolysin S (SLS)-like toxin (Harrington *et al.*, 2002).

2.2.1. Adherence

As with most microorganisms, the adherence of *Streptococcus equi* subsp. *equi* to host tissues is likely to involve multiple surface components. Moreover, the ability to interact with and adhere

to different substrates is likely to increase the organism's chances of surviving until it reaches surfaces where specific and irreversible interactions can occur and where conditions favour colonisation and growth. A number of host tissue and body fluid proteins have been shown to be ligands for streptococcal adhesins (Patti *et al.*, 1994; Chhatwal and Preissner, 2000). The most well studied group of such adhesins is the microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (Patti *et al.*, 1994). Extracellular matrix molecules are ideal ligands for bacterial adhesins since they are abundant, present on insoluble matrices and conserved between animal species. Thus bacteria possessing MSCRAMMs may bind to a range of animal tissues allowing access to a variety of nutrient sources (Patti *et al.*, 1994). Among the host ligands for MSCRAMMs are the proteins fibronectin and fibrinogen (Harrington *et al.*, 2002).

Fibronectin-binding proteins

Multiple fibronectin-binding (Fn-binding) proteins have been identified in various Gram-positive pathogens (Jenkinson and Lamont, 1997). The genes for at least two Fn-binding proteins have been demonstrated in *S. equi* (Lindmark *et al.*, 1999; Lindmark *et al.*, 2001). Lindmark *et al.* (1996) first identified the *fnz* gene that encodes a Fn-binding protein (designated FNZ) in *S. zooepidemicus*. FNZ was shown to have a similar organisation to previously described Fn-binding proteins and possessed at least two Fn-binding domains (Lindmark *et al.*, 1996).

Fibrinogen-binding proteins

Fibrinogen (Fg) is an abundant plasma protein that can serve as a substrate for MSCRAMMs since, at sites of trauma, it rapidly coats most naturally exposed body materials and foreign bodies. Furthermore blood clots and Platelet-fibrin thrombi are rich in fibrin (ogen) and may also act as attachment sites for Fg-binding proteins. Early studies on Fg-binding by *S. equi* indicated that one of two (Kronvall *et al.*, 1997) and none of five (Chhatwal *et al.*, 1985) *S. equi* strains tested could bind human Fg. However subsequent work indicated that the source of Fg has a significant effect on Fg-binding by *S. equi*, since binding to human Fg was 3-8% of that

observed with horse Fg (Meehan *et al.*, 2000). It has been long established that Fg-binding in *S. pyogenes* is associated with the organism's M protein (Harrington *et al.*, 2002).

Collagen binding proteins

Using bioinformatics a gene denoted CNE (gene encoding collagen-binding protein from *S. equi*) was identified coding for a novel potential virulence factor of *S. equi* called protein CNE. The protein is composed of 657 amino acids and has the typical features found in cell surface-anchored proteins in Gram-positive bacteria. The CNE gene was detected in all studied strains of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* by PCR (Lannergafird *et al.*, 2003).

Capsule

Most fresh clinical isolates of *S. equi* produce a hyaluronic acid capsule. Previous studies indicate that the hyaluronic acid capsule of *S. pyogenes* can serve as a ligand for protein receptors on both human epithelial and haematopoietic cells. The receptor was identified as CD44, a hyaluronic acid-binding glycoprotein (Wessels, 2000). The treatment of *S. equi* S-240 with hyaluronidase was shown to slightly increase its adherence to tongue cells from ponies (Srivastava and Darnum, 1983), suggesting that in this model the removal of the capsule exposes cell surface molecules that can then interact with host ligands. However, the same study suggested no significant difference in adherence to tongue, cheek or nasal epithelial cells between matt isolates (presumably with a smaller hyaluronate capsule) and mucoid isolates of the same *S. equi* strain. Adherence of encapsulated strains was reduced at least 20-fold following pre-treatment of bacterial cells with hyaluronidase but not pronase (Harrington *et al.*, 2002).

Lipoteichoic acid (LTA)

A model for bacterial adherence involving a two-stage process has been proposed by Hasty *et al.* (1992). In this model, specific, irreversible interactions such as those described above involving MSCRAMMs (e.g. Fn-binding) are preceded by non-specific, reversible interactions. Lipoteichoic acid (LTA) has been suggested as a good candidate for initiating these non-specific

interactions in streptococci. Harrington *et al.* (2002) investigated the adherence of two *S. equi* strangles isolates to tongue and cheek epithelia from normal adult ponies. Pre-incubation of cells with an antibody raised against *S. pyogenes* LTA reduced adherence to cheek cells but not tongue cells. Pre-incubation of epithelial cells with purified *S. pyogenes* LTA did not affect adherence of either strain to either cell preparation. These findings suggest that in contrast to other streptococci, LTA does not play a significant role in *S. equi* adherence (Harrington *et al.*, 2002).

2.2.2. Evasion of host defenses

The environment of the mammalian host provides significant challenges to bacterial survival. Consequently, if they are to be successful, pathogenic species need to have evolved mechanisms enabling persistence in this environment including those allowing bacterial resistance to innate and adaptive immune defenses of the host and those involved in nutrient acquisition (Harrington *et al.*, 2002).

Capsule

Streptococcus equi subsp. equi encapsulation was greatly influenced by the culture age. Young (4-6 h) cultures were highly encapsulated whereas the same cultures after 24 h incubation had barely detectable capsules. In both mouse and foal models of infection, young cultures were significantly more virulent than old cultures and they were also more resistant to phagocytic killing (Harrington *et al.*, 2002). In a horse infection study, the unencapsulated strain was shown to be capable of colonising and persisting in the guttural pouch of infected horses but unlike the encapsulated strains, was not associated with pathological changes. However, the unencapsulated strain was a natural isolate rather than an isogenic mutant, it remains possible that differences between these strains, other than in capsule production, could account for the observed differences in pathology. The importance of the capsule in resistance to phagocytosis was also demonstrated by Chanter *et al.* (1994) and Harrington *et al.* (2002).

Hyaluronate-associated protein (HAP)

It was demonstrated that in the presence of either high concentrations of extracellular ATP or antibody to the HAP protein, greater quantities of capsular hyaluronan were released into culture supernatants. *S. equi* produces a 53-kDa protein that is nearly identical (528/534 identical amino acids) to *S. equisimilis* HAP (Chanter *et al.*, 1999). Antibodies to the C-terminal half of the HAP protein expressed as a fusion protein (HAP-GST) reduced the level of *S. equi* encapsulation. Whether the HAP of *S. equi* possesses protein kinase activity and plays a role in capsule shedding remains to be established. The threonine kinase activity of *S. equisimilis* HAP was shown to be sensitive to endogenous phosphatases. The identification of at least two surface expressed phosphatase activities in *S. equi* suggests that the organism possesses surface-located enzymes that could potentially regulate such a protein kinase activity. This offers the intriguing possibility that in *S. equi* an extracellular kinase and its cognate phosphatase could regulate the degree of encapsulation of the organism, in response to the level of ATP in the host environment. However it is also notable that HAP exhibits lower but significant sequence homologies to members of the oligopeptide substrate binding protein family (Harrington *et al.*, 2002).

M-like proteins

It has now been shown that *S. equi* possesses genes encoding two M-like proteins, one of which (SeM) is unique to *S. equi* whilst the other (SzPSe) is a homologue of the M-like protein SzP produced by *S. zooepidemicus* (Timoney *et al.*, 1994; Timoney *et al.*, 1997). Both SeM and SzPSe show strong binding to equine Fg (Boschwitz and Timoney, 1994b; Timoney *et al.*, 1997). SzP and SzPSe are highly homologous (85% amino acid identity) but SeM and SzSPE are only distantly related and indeed only the signal peptide and cell wall anchor regions of these proteins share significant homology (Timoney *et al.*, 1997). SeM from different *S. equi* isolates was thought to be highly homogenous, but recent data identified a variable region of the gene immediately after the 5' end signal encoding sequence (Anzai *et al.*, 2005; Kelly *et al.*, 2006). Of the two M-like proteins, SeM plays a more dominant role in resistance of *S. equi* to phagocytosis in vitro (Kehoe, 1994; Timoney *et al.*, 1997).

The antiphagocytic activity of M-like proteins appears to be associated with two properties: (1) their ability to inhibit deposition of the complement component C3b on the bacterial surface and (2) their ability to bind Fg which then inhibits phagocytosis. Studies have shown that *S. equi* strains are capable of fixing the C3 component of complement and that C3 fixation is not inhibited by Fg (Chhatwal *et al.*, 1985; Traore *et al.*, 1991).

Mitogenic factors

The severe inflammation associated with a number of bacterial diseases appears to be associated with the release of high levels of pro-inflammatory cytokines such as TNF α (tumor necrosis factor- α) and interleukin-1 (IL-1). Massive T-cell proliferation such as that seen when bacterial super-antigens activate T cells in a non-MHC class II restricted fashion can lead to inappropriately high levels of cytokines causing capillary leakage and hypotension and in severe cases may lead to toxic shock-like syndromes (Romain *et al.*, 2010). Anzai *et al.* (1999) demonstrated that supernatant preparations of a number of clinical isolates of *S. equi* (but not *S. zooepidemicus*) elicited potent mitogenic responses from peripheral blood mononuclear cells (PBMCs). *S. equi* produces four recently acquired phage-associated bacterial superantigens (sAgs; SeeH, SeeI, SeeL, and SeeM) that share homology with the mitogenic toxins of *Streptococcus pyogenes*. Sera from naturally infected convalescent horses had only limited sAg-neutralizing activities. *S. equi* sAgs play an important role in *S. equi* pathogenicity by stimulating an overzealous and inappropriate Th1 response that may interfere with the development of an effective immune response (Romain *et al.*, 2010).

Superoxide dismutase (SOD)

The reactive oxygen intermediates that are generated during the respiratory burst associated with phagocytosis are toxic to many microorganisms. Bacterial enzymes that convert reactive oxygen intermediates into less toxic forms may contribute to an organism's ability to survive inside phagocytic cells. Superoxide dismutase (SOD) catalyses the conversion of superoxide anions to hydrogen peroxides and water. *S. equi* has been shown to possess a gene that encodes a

manganese-dependent SOD although its role in combating oxidative stress has not been investigated (Poyart *et al.*, 1998; Yesilkaya *et al.*, 2000).

Immunoglobulin-binding proteins

Despite demonstrations showing that *S. equi* strains do not bind high levels of IgG (Lindmark *et al.*, 1999), DNA from 98/98 strains of *S. equi* was shown to hybridise to a probe based upon the *zag* gene of *S. zooepidemicus*. The ZAG protein has been shown to bind IgG, α 2-macroglobulin and albumin. Binding of IgG could interfere with both antibody-mediated phagocytosis and complement activation (Harrington *et al.*, 2002).

2.2.3. Nutrient acquisition

Acid phosphatases

Acid phosphatases hydrolyze phosphomonoesters at acidic pH and are thought to participate in a wide variety of functions including nutrient acquisition. *S. equi* NCTC 9682 was shown to produce two cell-associated acid phosphatase activities with pH optima of pH 5 and pH 6.5 (Hamilton *et al.*, 2000).

ABC transporters

ATP-binding cassette (ABC) importer systems are crucial for the scavenging of a wide range of nutrient substrates. Typically the substrate-binding protein components of Gram-positive bacterial ABC transport systems are lipoproteins. A range of clinical isolates of *S. equi* were shown to possess a lipoprotein homologue (designated MBL) of the PsaA protein of *S. pneumonia* (Harrington *et al.*, 2000). Mutants deficient in these latter proteins have been shown to be less virulent than wild-type strains in animal models of disease, suggesting a role for these proteins in virulence (Kitten *et al.*, 2000). PsaA is the substrate-binding component of a manganese import system and is one of a group of metal-binding lipoproteins known as the LraI family which also includes FimA of *S. parasanguis*, SloC of *S. mutans* and MtsA of *S. pyogenes*

(Harrington *et al.*, 2000). In addition to MBL, analysis of the *S. equi* genome has revealed the presence of putative solute binding lipoproteins (suggestive of the presence of ABC transporters) for a wide variety of substrates including amino acids, other metals, phosphate and sugars (Harrington *et al.*, 2002).

Degradative enzymes

Degradative enzymes are likely to play important roles in the utilisation of available peptide and carbohydrate substrates in the host and may also be involved in other aspects of virulence such as immune evasion and tissue invasion. Clinical isolates of *S. equi* have been shown to produce extracellular enzymes capable of degrading a number of protein substrates including fibrinogen, gelatin and casein. The caseinolytic activity of *S. equi* was associated with a 29-kDa putative cysteine protease that could be detected in both cell-associated and supernatant fractions. Hyaluronidase activity may be required for penetration of mucous membranes and tissue spread but it is also likely to be important in the utilisation of host hyaluronic acid, an abundant carbon source and possibly in the recycling of released capsular hyaluronic acid (Collin *et al.*, 1998).

Streptolysin S (SLS)-like toxin

Extracellular cytolytic toxins that can damage host immune cells such as macrophages and PMNs may be important in immune evasion. Moreover, cell lysis is also likely to result in the release of essential nutrients. Young cultures of *S. equi* produce a toxin that damages PMNs and makes them unable to phagocytose opsonised bacteria (Flanagan *et al.*, 1998).

2.3. Pathogenesis of Strangles

Although very small numbers of *S. equi* entered the lingual and nasopharyngeal tonsils, carriage to regional lymph nodes occurred within hours of inoculation. This observation, together with visual evidence of intracellular and extracellular multiplication of *S. equi* in tonsillar lymphoid

tissue and lymph nodes over the following days, indicates involvement of potent antiphagocytic activity and failure of innate immune defenses (Timoney and Kumar, 2008).

Virulence of *S. equi* is attributable to the presence of M proteins on the surface of the bacteria, a hyaluronic acid capsule and the production of a leukocidal toxin (Al-Ghamdi *et al.*, 2000). M proteins are associated with *S. equi* adhesion to oral, nasal and pharyngeal tissues, invasion of pharyngeal tonsils and associated lymphoid structures, and evasion of the innate host immune response (Chanter *et al.*, 2000). *S. equi* produces two M proteins - SeM and SzPSe. SeM is unique to *S. equi* and plays a dominant role in resistance of the organism to phagocytosis (Harrington *et al.*, 2002). Variations in structure of M protein are associated with decreased virulence (Chanter, 1997). The M proteins interfere with the deposition of complement component 3b on the surface of the bacteria and bind fibrinogen, both of which reduce the susceptibility of the bacteria to phagocytosis by neutrophils (Boschwitz and Timoney, 1994a). The antiphagocytic activity of *S. equi* reduces the efficacy of neutrophils in engulfing and destroying the bacteria. The capsule of *S. equi* is associated with resistance to non-immune phagocytosis and pathogenicity. Strains of *S. equi* that do not produce a capsule do not induce disease, although they are able to infect guttural pouches and cause seroconversion in experimental studies (Chanter, 1997). Following exposure of the oral and nasopharyngeal mucosal surfaces to *S. equi*, bacteria lodge in the pharyngeal and tonsillar lymphoid tissues where they multiply rapidly. There is no evidence of colonization of mucosal surfaces and streptococci can be detected in pharyngeal tonsils within hours of exposure (Sweeney *et al.*, 2005). The binding of *S. equi* to pharyngeal cells is caused by fibrinogen binding proteins associated with M protein (Harrington *et al.*, 2002). The resistance of *S. equi* to non-immune phagocytosis results in accumulation of large numbers of organisms surrounded by degenerating neutrophils. Release of streptolysin S and streptokinase may contribute to tissue damage by directly injuring cell membranes and indirectly through activation of plasminogen (Sweeney *et al.*, 2005).

Death is usually due to pneumonia caused by aspiration of infected material, although other causes of death include asphyxiation secondary to upper airway swelling and impairment of organ function by metastatic infection, which is called bastard strangle. Rare deaths also occur as a result of infarctive purpura hemorrhagica in horses infected with *S. equi*. Metastatic infections

of the heart valves, brain, eyes, joints, and tendon sheaths or other vital organs may occur and cause a chronic illness and eventual death (Ainsworth and Cheetham, 2010). Metastatic infection may occur because of bacteremia or extension of infection along chains of lymph nodes. Purpura hemorrhagica may occur as a sequela to *S. equi* infection and is associated with high serum antibody titers to SeM (Radostits *et al.*, 2007).

2.4. Diagnostic Approaches

2.4.1. Clinical, bacteriology and endoscopy

The clinical diagnosis of classical strangles is usually straight forward because there are often abscesses on the submandibular lymph nodes. Diagnostic problems may arise in early or atypical disease when there may be little or no nasal discharge and no discharging lymph nodes. Culture of *S. equi* has been difficult in some classical cases of strangles possibly owing to overgrowth with *S. equi* subsp. *zooepidemicus* (Dalglish *et al.*, 1993).

Confirmation of strangles depends on the isolation of *S. equi* from nasopharyngeal swabs and discharges from abscesses. Shedding of *S. equi* in nasal discharges begins 1-4 days after the onset of fever, and ruptured abscesses often become contaminated with *Streptococcus zooepidemicus* and *S. equisimilis* (Radostits *et al.*, 2007).

Swabs and nasal wash pellets are cultured on Columbia CAN horse blood agar and incubated for 18 hours at 37°C. β haemolytic colonies are subcultured and their fermentation behaviour tested in lactose, sorbitol and trehalose broths. Mucoid β -haemolytic colonies that do not ferment any of these sugars were identified as *S. equi* (Timoney and Artiushin, 1997). Wood *et al.* (1993) isolated *S. equi* from the nasopharynx of affected horses more than one month after nasal swabs were negative, and so it is likely that the nasal sampling used was less sensitive than nasopharyngeal swabs. Newton *et al.* (1997) reported an even higher recovery of *S. equi* from the culture of guttural pouch lavages than from nasopharyngeal swabs (88% vs 45% sensitivity).

The detection of asymptomatic carriers is difficult, as haematological and biochemical screening methods are poorly sensitive and non-specific. Bacteria may be shed intermittently, and the sensitivity of bacteriological culture of a nasopharyngeal swab is only approximately 45 per cent (Newton *et al.*, 1997). Endoscopic assessment of both guttural pouches by culture and PCR analysis of lavage samples is significantly more sensitive (Newton *et al.*, 2000). There was a good correlation between the endoscopic findings and the results of cultures from the guttural pouches, although some horses retained small pouch lesions after becoming culturally negative. However, the converse was not true, in that all the horses with positive cultures had pouch abnormalities by endoscopy (Fintl *et al.*, 2000). The current codes of practice on equine diseases state that 'no convalescent horse or in-contact can be considered free from infection until three negative nasopharyngeal swabs have been obtained and the horse has been tested negative by guttural pouch endoscopic examination and lavage. These techniques are costly, and impractical for routine screening of large numbers of horses at low risk of being infected with *S. equi* (Knowles *et al.*, 2010).

2.4.2. Serology

Serological testing for *S. equi* antibodies using a SeM-specific ELISA has been advocated for diagnosing recent *S. equi* infection, determining the need for booster vaccination against strangles, and as an aid in the diagnosis of purpura haemorrhagica and metastatic abscesses (Timoney *et al.*, 1997). Despite the small number of horses tested, Pusterla *et al.* (2007) suggested the usefulness of the SeM-specific ELISA as a diagnostic aid for an internal abscess. However, considerable variation in the immune response of individual horses should be kept in mind when interpreting serology results.

Recently a new serological test, developed by the AHT, has become available in the UK. This ELISA detects IgG antibodies to two novel *S. equi*-specific antigens, and reveals recent exposure to *S. equi* with a reported sensitivity of 91.5% and specificity of 90.5%. The test is offered as a means to screen asymptomatic horses before they are introduced to a disease-free population. The test primarily detects recent exposure to *S. equi* but is also claimed to detect asymptomatic carriers with a sensitivity of 90.9% and a specificity of 82.6% (AHT, 2008). Positive ELISA

results should be interpreted with caution as an indication for the isolation of horses and endoscopy and further diagnostic procedures (Knowles *et al.*, 2010). Importantly, this test is primarily offered to screen for exposure to strangles, rather than to test specifically for carriers. Endoscopy and guttural pouch lavage remain the ‘gold standard’ techniques for detecting strangles carriers and this serological test should aid in the selection of cases to examine endoscopically (Newton *et al.*, 2000; Davidson *et al.*, 2008).

Serology is potentially a quick and easy way to determine recent exposure to *S. equi*. However, a commercially available ELISA (IDEXX Laboratories) for measuring antibody to the *S. equi*-specific protein SeM does not discriminate between vaccinated and infected horses, and interpretation of the results can also be complicated by cross reaction with antibodies to *Streptococcus zooepidemicus* (Timoney *et al.*, 1997). An ELISA blood test recently developed at the Animal Health Trust (AHT) uses two antigens (referred to as antigen A and B) that are unique to *S. equi* and targeted by the equine immune system following exposure to this agent. A positive result for either or both antigens suggests recent or past exposure to *S. equi*, as a result of either acute infection, carrier status or immunity following a full recovery (Ling *et al.*, 2011).

2.4.3. Molecular diagnosis

Highly sensitive *S. equi* detection is now feasible with the use of polymerase chain reaction (PCR) to detect living or dead organisms draining into the pharynx from the guttural pouches as an aid to presumptively identifying carriers. A PCR assay based on the M-like protein gene (Timoney and Artiushin, 1997) in conjunction with bacterial culture of nasopharyngeal swabs, presumptively detected over 90% of guttural pouch carriers whereas culture alone would have detected less than 60%. Unfortunately this improved rate of detection still depended on the results from a series of three nasopharyngeal swabs (Newton *et al.*, 2000).

The *S. equi* genome is largely homogeneous, and little variation of housekeeping genes is found between isolates when analysed by multilocus enzyme electrophoresis (Jorm, 1991) or multilocus sequence typing (MLST) (Webb *et al.*, 2008). To date, *S. equi* strains have been differentiated only by restriction fragment length polymorphism typing or repetitive PCR (Al-Ghamdi *et al.*,

2000; Takai *et al.*, 2000). However, these techniques are poorly portable, and the variation that is indexed tends to change rapidly for unknown reasons. Multilocus enzyme electrophoresis (MLEE) analysis of 70 *S. equi* isolates placed 69 of them in a single MLEE type (Jorm, 1990). Recently, the availability of the *S. equi* genome sequence enabled us to identify a number of housekeeping genes for the application of multilocus sequence typing (MLST) techniques. The sequence typing of virulence genes or hypervariable genes has been utilized to enhance the discriminatory power of some MLST schemes (Reid *et al.*, 2000; Meinersmann *et al.*, 2004; Zadoks *et al.*, 2005). In addition, the *S. equi* M-protein SeM contains a variable N-terminal region and has been proposed as a target gene to distinguish between different strains of *S. equi* and determine the source of an outbreak. Sequencing of the SeM gene generally agreed with pulsed-field gel electrophoresis profiles. SeM sequencing as a epidemiological tool is supported by the agreement between SeM and PFGE and that sequencing of the SeM protein gene is more sensitive than PFGE in discriminating strains of *S. equi* (Susanne *et al.*, 2011)

2.5. Treatment

The goal of treating strangles is to control transmission and eliminate infection while providing future immunity to the disease. Uncomplicated cases of strangles are often left to run their course with supportive care providing lasting immunity. Affected horses should be isolated in a clean, dry stall and fed moist palatable feed. Non-steroidal anti-inflammatory drugs should be used judiciously to decrease swelling and promote feeding. Hot compresses or 20% ichthammole can be used to accelerate maturation of abscessation. Mature external abscesses should be lanced to allow drainage followed by daily lavage of open abscesses using 3% to 5% povidone iodine solution. This expedites resolution of abscessation as well as alleviation of compression of surrounding structures (Sweeney *et al.*, 2005).

While the use of antimicrobial for treating strangles is controversial, cases with complications such as metastatic disease or purpura definitely require the use of systemic antimicrobials for extended period. Antibiotics are not always useful as they cannot penetrate the centre of an abscess where there is no blood supply. However, early treatment with antibiotics may be helpful

if lymph nodes have not become enlarged. Each case should be assessed individually (Sweeney *et al.*, 2005; Pusterla *et al.*, 2007).

Penicillin is considered the antimicrobial drug of choice for the treatment of streptococcal disease. Alternative drugs, such as cephalosporins, rifampin, macrolides, chloramphenicol, doxycycline and trimethoprim-sulphadiazine are used depending on ease of administration, the site of infection and treatment costs. Study done by Pusterla *et al.* (2007) showed that long-term administration of antimicrobials is necessary for the resolution of internal abscesses; duration of treatment ranged from 30 to 131 days (mean 72 days) among individual horses. Pusterla *et al.* (2007) also suggested that the preferred antimicrobial combination for long-term treatment of internal abscesses is rifampin and ceftiofur.

2.6. Prevention and Control

Most outbreaks are thought to originate from introduction of an infected horse into a naïve population. All new introductions should be isolated for three weeks and monitored for signs of disease. Equids should be screened for *S. equi* subsp. *equi* infection using nasopharyngeal washes or swabs. All movement of equids to and from the farm should be stopped until they are determined to be noninfectious (Newton *et al.*, 2000).

Most vaccines available on the market have incorporated inactivated whole cells of *S. equi* or M-protein extracts. However, such vaccines are notorious for their adverse reactions and induce hardly any protection against natural or experimental infections (Srivastava and Darnum, 1983; Jorm, 1990).

A live attenuated deletion mutant administered intramuscularly, induced complete protection but also induced unacceptable local reactions at the site of vaccination. Submucosal vaccination in the inner side of the upper lip with the live attenuated mutant at 10^8 colony-forming units per dose appeared to be safe and efficacious in foals as young as four months of age. The submucosal vaccinations caused small transient swellings that resolved completely within two weeks, and

postmortem no vaccine remnants or other abnormalities were found at the site of vaccination (Jacobs *et al.*, 2000).

Although vaccination with recombinant SeM enhances opsonisation *in vitro* (Timoney *et al.*, 1997) and has protective effects in a mouse model of *S. equi* infection (Meehan *et al.*, 1998), intranasal vaccination of ponies with SeM protein coupled to cholera toxin did not confer clinical protection (Sheoran *et al.*, 2002). No vaccine against strangles is currently marketed in the UK, but the previously available Equilis StrepE (Intervet) is based on a strain (TW928) with the uncommon SeM allele 1 (Kelly *et al.*, 2006), whereas a new developmental multicomponent protein vaccine is based on a SeM-9-containing strain (Guss *et al.*, 2009). Preferential protection against individual strains may alter the balance of strain prevalence, an effect that may become apparent if either of these vaccines becomes available (Parkinson *et al.*, 2011).

According to AHT, since strangles is able to establish in carrier horses that appear healthy, an important factor in the prevention of further outbreaks is the early identification and treatment of animals that could initiate new outbreaks of the disease. The AHT in conjunction with Forsite Diagnostics, is being developing blood test that can produce results within 10 minutes (AHT, 2011).

2.7. Economic Importance of Strangles

Strangles is one of the most important diseases of horses in developed countries, accounting for up to 30% of reported infectious disease episodes (Spoormakers *et al.*, 2003). Morbidity with strangles may be high but mortality is usually low with rare sequelae including dysphagia, metastatic abscessation (Spoormakers *et al.*, 2003), myositis (Sweeney *et al.*, 2005) and *purpura haemorrhagica* (Pusterla *et al.*, 2003). As many as 10% of cases may die from disseminated abscessation or *purpura haemorrhagica*. The disease is important not only because of the deaths that it causes but more importantly because of the disruption of the management of commercial horse establishments, the time necessary to treat affected horses and the esthetic unpleasantness of the running noses and draining abscesses (Chanter *et al.*, 2000).

2.8. Status of Strangles in Ethiopia

Although the status of strangles in the equine population of Ethiopia is not well known, attempts were made to isolate normal floras of the upper respiratory tract of donkeys (Desissa *et al.*, 2009) and horses (Tesfaye, 2010). Strangles is also one of important health problems affecting donkeys in Ethiopia (Getachew *et al.*, 2002). Base line data on the epidemiology of the disease in the donkeys and the strain of the bacteria, *S. equi*, involved in causing the disease is not well studied and documented. Questionnaire survey and bacteriological findings from studies made by Alemnesh (2004) indicated that strangles is one of the major health problems of donkeys in the study areas. According to the observation made and the 2010/11 annual reports by the Debre Zeit based donkey health and welfare project, upper respiratory diseases, of which suspected strangles was the main, were the major problems in the donkey population. A participatory need assessment by the project also revealed similar findings, in which communities rated suspected strangles, locally called ‘furro’, as one of the major health problems of their donkeys.

Yigezu *et al.* (1997) isolated *S. equi var equi* from the lungs of Ethiopian camel at an outbreak of respiratory disease problem and donkeys were suggested as possible source of infection to camels.

2.8.1. Sample collection and transportation

Thoroughbred and crossbred horses, Friesian and Arabian horses, and camels (from Addis Ababa, Dire Dawa, and Harar) were collected and transported to the laboratory for the purpose of the study. The animals were kept in separate pens and the samples were collected and transported to the laboratory for the purpose of the study.

3. MATERIALS AND METHODS

3.1. Study area

This study was conducted in purposively selected areas of central Ethiopia where the Debre-Zeit Donkey Health and Welfare Project operates; namely, Ada'a and Berh woredas of Oromiya regional state. Adaa is located 45 Km south east of Addis Ababa at an altitude of 1900m above sea level. It receives an annual rain fall of 850mm with a mean maximum and minimum temperature of 30.7°C and 8.5°C, respectively. Berh is located 44 -66 Km north of Addis Ababa at an altitude range of 2300-3000m above sea level. The mean annual rain fall is 3000mm the average daily maximum and minimum temperature are 28°C and 15°C, respectively (NMSA, 2004).

3.2. Sampling method

Sampling strategy was purposive where donkeys showing signs of respiratory problem; more specifically of strangles, were included in the study. Sampling was at both stationary and mobile clinics of the Donkey Health and Welfare Project between September 2011 and April, 2012. Donkeys were carefully and systematically examined to check for the presence of symptoms of respiratory system like nasal discharge, guttural pouch empyema, enlargement of lymph nodes in head and cervical region. Those that are suspected of acquiring strangles were included in the study and sampled accordingly.

3.3. Sample collection and transportation

Nasopharyngeal swabs, lymph node and guttural pouch aspirates and chondroid (from dead donkey due to other cause) were collected depending on the clinical manifestation of the disease. Nasopharyngeal swabs were taken from donkeys suspected of strangles using a sterilized swab

(15cm long) inserted through the ventral nasal meatus to the level of the pharynx and rotated gently over the pharyngeal tissue. Two separate swabs were taken from the same animal, one for microbial culture and the other for direct testing by PCR. The swabs were kept in sterile test tubes containing 3 ml tryptose soya broth and transported under cold condition, in ice box, to AAU, SVM microbiology laboratory for immediate processing. Similarly, aspirate samples were taken by using sterile 10 ml syringe after shaving and careful disinfection of the skin overlaying the swollen lymph node or guttural pouch. Up on arrival in the laboratory, samples were immediately inoculated on to culture media.

Furthermore, postmortem examination of guttural pouch was conducted on 13 donkeys that died of any reason to check for the presence of chondroid in the guttural pouch. The skin overlaying the guttural pouch demarcated by the base of the ear to the retropharyngeal area was cleaned and shaved before incision. Chondroid was found in five out of thirteen adult donkeys examined in the above method. Chondroid was collected using sterile forceps from small membrane pouches, placed in sterile petridish and transported to the laboratory for immediate culture on bacteriological media as well as direct detection of *Streptococcus equi* subsp. *equi* by PCR.

3.4. Isolation and Identification of *Streptococcus equi* subsp. *equi*

The collected samples were processed for bacterial isolation as soon as possible, usually within 0.5 - 4 hours after collection. Samples were streaked onto blood agar with 7% (v/v) sheep blood. All cultures were incubated at 37°C for 24 - 48 hours. Typical β -haemolytic streptococci-like colonies were detected on blood agar and identified by characteristic colony morphology, Gram staining and biochemical tests(Sugar fermentation test and were passed to Edward's media for selective growth and aesculin hydrolysis test to exclude *Enterococcus* spp). API 20 Strep (BioMérieux, SA, France), readymade biochemical tests designed for *Streptococcus equi* subsp. *equi*, was also used on selected isolates of Streptococci organisms. The first ten micro tube tests are enzymatic activity (VP (Voges proskauer), HIP (Hippuric acid hydrolysis), ESC (β -Glucosidase hydrolysis), PYRA (Pyrrolidonyl arylamidase), α GAL (α -Galactosidase), β GUR (β -Glucuronidase), β GAL (β -Galactosidase), PAL (Alkaline phosphatase), LAP (Leucine amino

peptidase) and ADH (Arginine dihydrolase)) and the remaining ten micro tube tests are for sugar fermentation tests (RIB (Ribose), ARA (Arabinose), MAN(Mannitol), SOR(Sorbitol), LAC (Lactose), TRE (Trehalose), INU (Inuline), RAF (Raffinose), AMD (Amidon), GLYG (Glycogen)). Isolated colonies were suspended in 2ml of API suspension medium. The turbidity of the suspension was controlled not to exceed 4 MacFarland. One hundred μ l of the suspension was immediately dispensed into each cupule of the first half of the strip (test VP to ADH). The remaining API suspension was mixed with API GP Medium and distributed to the remaining cups RIB to GLYG (10 cups). Then, filled with mineral oil to form convex meniscus and incubated at 37 °C for 4 hours. Test reagents were applied in VP to LAP cupule for enzymatic reaction. Reactions were interpreted after 10 minutes by referring to the reading table. After 24 hours the reactions for ESC, ADH, and RIB to GLYG were interpreted according to the manufacturer instructions (BioMérieux, SA, France).

3.5. Molecular detection

3.5.1. DNA extraction

A single colony of *S. equi* was resuspended in 3 ml of tryptose soya broth and incubated at 37°C for 24 hours. Of this suspension, 2 ml were centrifuged at 14,000 *g* for 30 seconds to pellet *S. equi*. The pellet was resuspended in 1ml Cell Lysiss Solution (CLS-TC) (FastDNA[®] SPIN Kit, MP Biomedical, Germany). The DNA was extracted and purified using GENECLEAN[®] procedure using SPIN filters according to the manufacturer's instructions (MP Biomedical, Germany). DNA was also extracted directly from swabs, aspirates and chondroid prepared the same way described above.

3.5.2. Polymerase chain reaction (PCR)

PCR, using amplification of the SeM gene, was used to further identify *S. equi* subsp. *equi* isolates that had fermented sorbitol and hydrolyzed aesculin and direct detection of *S. equi* subsp. *equi* from nasopharyngeal swabs, aspirates and chondroids. The primers used for the PCR were:

the forward primer, ASW73, (5'-CAG AAAACT AAG TGC CGGTG-3') (255.9nM) and the reverse primer, ASW74, (5'-ATT CGG TAA GAGCTT GAC GC-3') (210.6nM) (Invitrogen, Germany); they were used to amplify 541 base pairs length of the 5' region of the SeM gene unique to *S. equi* using Taq DNA polymerase (Fermentas, Germany). In brief, PCR was conducted by mixing 5 µL of extracted DNA sample with 5µl 10xDreamTaq buffer, 1.5µl 25mM magnesium chloride, 1µl 10mM of dNTP (deoxynucleoside triphosphates) mix, 1µl of each primer and 0.5µl Taq DNA Polymearase (Fermentas, Germany) in a reaction volume of 50 µl. The reaction mixture was amplified with a 2720 Thermal cycler (Applied Biosystems, USA). Amplification reaction was started by heating the mixture at 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min followed by final elongation for 8 min at 72°C.

3.5.3. Agarose gel electrophoresis

1.5% agarose gel containing 10mg/ml of Ethidium bromide (Sigma, Germany) was prepared. Wells were prepared by gel comb. 20µl of each PCR products was mixed with 5µl of loading buffer in 0.5ml PCR tube. 25µl of each mixture were loaded to each wells of the gel. 25µl of negative control was also loaded to one well of the gel and 10µl of 100bp marker (Thermo Scientific, Germany) was loaded into the first and last lanes. The gel was run using 1x TAE buffer for 1 hour at 100 Volt. Finally gel picture was read using UV light (Uvitec Cambridge, UK) gel documentation. The band size was compared with ladder.

3.6. Antibiotic sensitivity testing

Selected colonies of *Streptococcus equi* subsp. *equi* were suspended on tryptose soya broth and incubated at 37°C for 2 - 4 hours. The turbidity of both pre-incubated broth and the suspension of bacteria were adjusted by comparison with a 0.5 McFarland turbidity standard. Blood agar media were smeared with standardized suspension and then were allowed to stand for 3-5 minutes, for any excess moisture from the inoculum to be absorbed before applying the antimicrobial discs. The antibiotic discs were placed at about 24 mm apart from center to center

and pressed on place by the tip of forceps to ensure complete contact with the agar surface. The plates were inverted upside down and incubated at 37°C overnight. After incubation, the diameters of the zones of inhibition were measured to the nearest millimeter using ruler over the surface of the agar. An interpretation of the size of the zones of inhibition was made based on the method described by Quinn *et al.* (1994). The antibiotics used were ampicillin, gentamicin, sulphonamid, doxycycline, tetracycline, vancomycin, chloramphenicol, erythromycin, ceftriaxone kanamycin, cephalothin, streptomycin, and Norfloxacin (Oxoid, UK).

Table 1. Zone size interpretation chart (NCCL(1997))M31-A

Disc content in µg	Antimicrobial agents	Diameter of zone of inhibition to the nearest millimeter			
		Resistant	Intermediate	Moderately Susceptible	Susceptible
10	Ampicillin	21	---	22-29	30
10	Gentamycine	12	13-14	---	15
300	Sulphonamides	12	---	13-16	17
30	Doxycycline*	≤12	13-15	---	≥16
30	Tetracycline	14	15-18	---	19
10	Norfloxacin	12	13-16	---	17
30	Vancomycin	9	10-11	---	12
30	Chloramphenicol	12	13-17	---	18
15	Erythromycin	13	14-22	---	23
30	Ceftriaxone*	≤14	14-20	---	≥21
30	Kanamycin	13	14-17	---	18
30	Cephalothin	14	---	15-17	18
10	Streptomycin	11	12-14	---	15

Source: Quinn *et al.* (1999); *Oxoid, DailyMed: Pfizer laboratories, (2010)

3.7. Data Collection and Analysis

The study areas were first coded and donkeys from each area were given the same code accordingly. Data were then entered into Microsoft Excel spread sheet and analyzed using SPSS version 15.0. statistical software. Descriptive statistics and chi square test statistics were used to check the role of the different risk factors on the occurrence of strangles. Level of significance (p) was 0.05 at all time in this analysis ($p < 0.05$).

4. RESULTS

4.1. Clinical presentation of donkeys with suspected strangles

During this study period, 87 donkeys suffering from suspected strangles were sampled either in the clinics or in the field before taking any treatment measures. Clinical signs considered for presumptive diagnosis of strangles were nasal discharge, guttural pouch empyema, enlargement of lymph nodes in head and cervical region. Donkeys were detected sick by their owners late in the course of the disease and hence all examined cases were not febrile at the time of sample collection. All suspected cases were included in the study and sampled accordingly.

Of all the total 87 donkeys sampled, 21 (24.1%) donkeys had showed only mucopurulent nasal discharge, 47 (54%) had showed both mucopurulent nasal discharge and swelling of either submandibular or retropharyngeal lymph nodes, 2 (2.2%) had showed parotid lymph node involvement and 12 (13.8%) had manifested severe guttural pouch empyema and extended head and neck with strider breathing and 5 (5.7%) donkeys with postmortem chondroid were also included in the study (Table 2). Therefore, in the study area the donkey suffering from strangles is most commonly presented clinically showing nasal discharge and swelling of either mandibular or retropharyngeal lymph nodes. The next common form of strangles is donkeys with only nasal discharge, followed by a donkey showing sever guttural pouch empyema.

The occurrence of strangles between the two sexes was statistically significant ($P < 0.05$). Clinically, females were seen more affected than males. There was also statistically significant difference ($p < 0.05$) in the occurrence of the diseases between young and adult age groups where young were highly affected than adult. Similarly, as shown in figure 1 and 2 severe clinical disease, lymph node abscessation and guttural pouch empyema, was observed in young donkeys (<5 years of age) than adult.



Figure 1. A one year old female donkey suspected of strangles with guttural pouch empyema and purulent nasal discharge at DHWP clinic, Debre-Zeit, Ethiopia.



Figure 2. A 3 years old male donkey suspected of strangles showing parotid lymph node rupture presented at DHWP clinic, Debre- Zeit, Ethiopia.

Table 2. Clinical signs observed in different age groups of donkeys

Age	Number of donkey sampled	Mucopurulent nasal discharge only	Mucopurulent nasal discharge and lymph node swelling	Guttural pouch empyema	Chondroid
Young (<5)	69(79.3%)	8(11.6%)	49(71%)	12(17.4%)	--
Adult (≥5)	18(20.7%)	13(72.2%)	--	--	5(27.8%)

The severe form of the disease where there was involvement of lymph nodes of the head region and guttural pouch empyema indicated in figure 1 and 2 above were observed in young than in adult. This variation in clinical signs between young and adult donkeys were statistically significant ($P<0.05$).

4.2. Postmortem examination of guttural pouch

Postmortem examination of guttural pouch was conducted on 13 donkeys died of any reason to check for the presence of chondroid in the guttural pouch. Five chondroid positive donkeys out of 13 examined were found at postmortem (Figure 3).

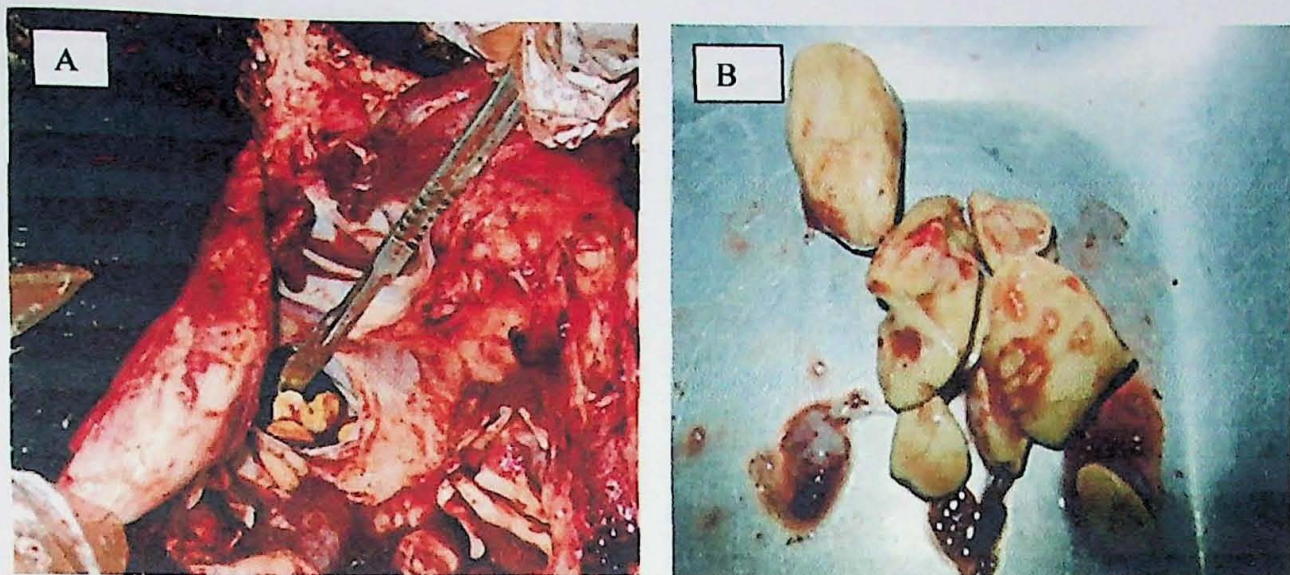


Figure 3. (A) Chondroids in guttural pouch of a 12 years old female donkey; (B) Chondroids collected from the same donkey.

4.3. Isolation and identification of *Streptococcus equi* subsp. *equi*

Samples were collected from 87 donkeys and processed bacteriologically as described in the materials and methods section. Accordingly, a total of 189 bacterial isolates were obtained upon growing on blood agar and using primary biochemical tests; they were further grouped into eleven different genera as presented in table 3.

The swab samples had contributed for the majority of the bacteria isolates (166 (87.8%)) (Table 3). Aspirate samples were taken from severe forms of the disease where there is involvement of guttural pouch and lymph nodes (Figure 1). As illustrated in table 2 the isolation of *Streptococcus* spp. were significantly ($P < 0.05$) higher in aspirates than from swabs and only *Staphylococcus* spp. were isolated from chondroid samples.

Table 3. Bacterial species isolated from nasopharyngeal swabs, lymph node aspirates, guttural pouch abscesses and chondroids.

Type of sample	Number of samples	Bacteria spp. isolated	Isolation rate Frequency (%)
Nasopharyngeal swab	66	<i>Streptococcus</i> spp.	60 (31.7)
		<i>Staphylococcus</i> spp.	31 (16.4)
		<i>Neisseria</i> spp.	3 (1.6)
		<i>Enterococcus</i> spp	19 (10.1)
		<i>Bacillus</i> spp.	15 (7.9)
		<i>Corynebacterium</i> spp.	9 (4.8)
		<i>Rhodococcus</i> spp.	4 (2.1)
		<i>Pasteurella</i> spp.	11 (5.8)
		<i>Actinomyces</i> spp	7 (3.7)
		<i>Pseudomonas</i> spp.	4 (2.1)
		<i>Proteus</i> spp.	3 (1.6)
Lymph node (11) and 23		<i>Streptococcus</i> spp.	20 (10.6)
guttural pouch (12)		<i>Staphylococcus</i> spp.	2 (1.1)
Aspirate			
Chondroid	5	<i>Staphylococcus</i> spp.	1 (0.5)
Total			189 (100)

Secondary biochemical tests using API 20 Strep and sugar tests for *Streptococcus* spp were conducted to differentiate them to the species level. From 25 isolates of *Streptococcus* spp tested using API20strep method, 8 were identified as *Streptococcus equi* subsp. *equi*, 13 as *Streptococcus equi* subsp. *zooepidemicus* and 2 as *Streptococcus dysgalactiae* subsp. *equisimilis*. The remaining isolates of *streptococcus* spp. were tested by conventional biochemical tests (sugar fermentation test: Glucose, sorbitol, trehalose, maltose and lactose). Isolates identified as *Streptococcus equi* subsp. *equi* in both API 20 Strep and conventional biochemical tests ferment glucose, maltose, glycogen and starch but not sorbitol, lactose, raffinose, inulin, trehalose, or mannitol. They do not hydrolyze aesculin. However, 7 isolates of *S. equi* subsp. *equi* fermented

sorbitol and hydrolyzed aesculin in API 20 Strep as shown in figure 6. The API 20 Strep results for *Streptococcus equi* subsp. *equi* isolates is demonstrated below in figure 6.

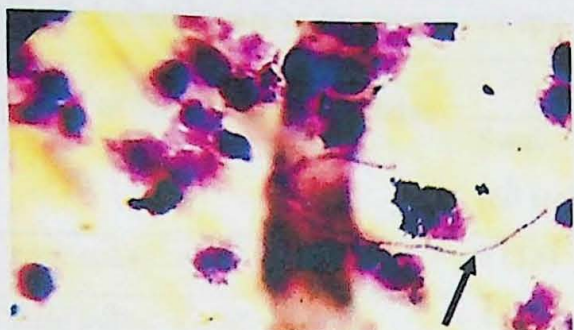


Figure 4. Gram's staining of guttural pouch aspirate characteristic chain cocci of *Streptococcus equi* subsp. *equi*



Figure 5. Typical isolate of *Streptococcus equi* subsp. *equi* with β -hemolytic 'honey dew' appearance

Figure 4 illustrated Gram's stained long chain *Streptococcus equi* subsp. *equi* directly from aspirate sample and it is observed that chain length to decrease from bacteriological media. As shown in figure 5 typical colonies of *Streptococcus equi* subsp. *equi* were β -hemolytic and honey-dew appearance on blood agar.

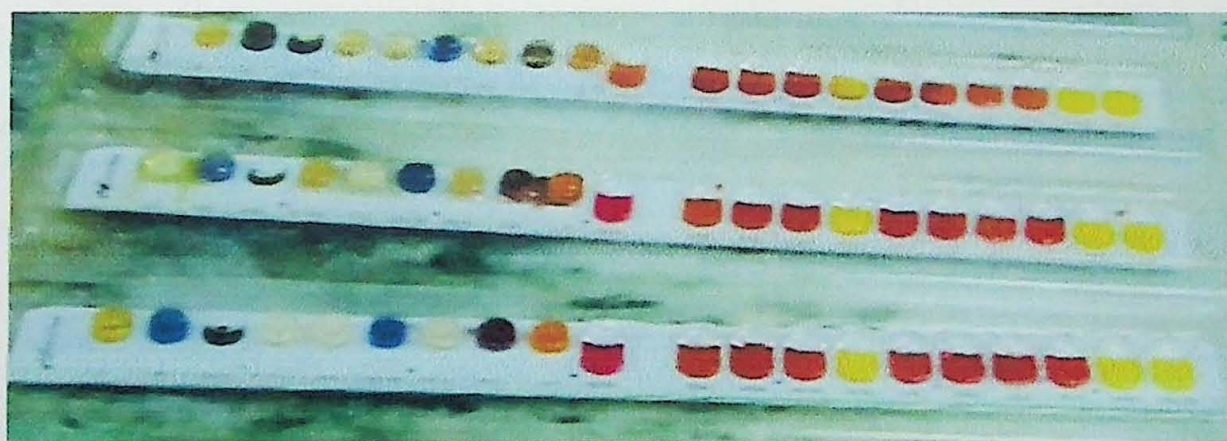


Figure 6. API 20 Strep results for isolates of *Streptococcus equi* subsp. *equi*.

The first ten micro tube tests are enzymatic activity (VP, HIP, ESC, PYRA, α GAL, β GUR, β GAL, PAL, and ADH) and the remaining ten micro tubes are for sugar fermentation tests (RIB,

ARA, MAN, SOR, LAC, TRE, INU, RAF, AMD and GLYG). Isolation rate of *Streptococcus equi* subsp. *equi*, as depicted in table 4 below, from guttural pouch and lymph node aspirate (pus) samples was significantly ($P<0.05$) higher than nasopharyngeal swab.

Table 4. Isolation rate of *Streptococcus* spp. from swab and aspirate samples

Species of <i>Streptococcus</i> isolates	Isolation rate (%)	
	Swab (60 isolates)	Aspirate (20 isolates)
<i>S. equi</i> subsp. <i>equi</i>	9 (15)	6 (30)
<i>S. equi</i> subsp. <i>Zooepidemicus</i>	45 (75)	14 (70)
<i>S. dysgalactia</i> subsp. <i>equismilis</i>	6 (10)	0 (0)

Table 5. Isolation frequency of *Streptococcus* spp. by age and sex from nasopharyngeal swab, aspirate and chondroid

Sample type				No. of <i>Streptococcus</i> spp. isolated		
Nasopharyngeal swab				<i>S. equi</i> subsp. <i>equi</i>	<i>S. equi</i> subsp. <i>zooepidemicus</i>	<i>S. dysgalactea</i> subsp. <i>equisimilis</i>
Female(54)						
Young	31	15		6	11	
Adult	6	--	2	3	24	5
Male(33)						
Young	22	8		4	7	
Adult	7	--	3	2	17	1
Total	66	23	5	15	59	6

The rate of isolation of *Streptococcus equi* subsp. *equi* from young donkeys was significantly ($P<0.05$) higher than adult animals (Table 5). Although there are differences in the recovery rate of *Streptococcus equi* subsp. *equi* between male and female, the difference was not statistically significant ($p>0.05$).

4.4. PCR based identification of *Streptococcus equi* subsp. *equi*

DNA was extracted from 5 Streptococcal isolates that had fermented sorbitol and hydrolyzed aesculin as well as directly from 65 nasopharyngeal swabs, 15 aspirates and 5 chondroid. Out of 90 samples tested by PCR, 67 were found to be positive for SeM gene. The size of the PCR product amplified by the SeM gene, specific for the *Streptococcus equi* subsp. *equi*, was 541 bp. The results of PCR products are illustrated in figure 7.

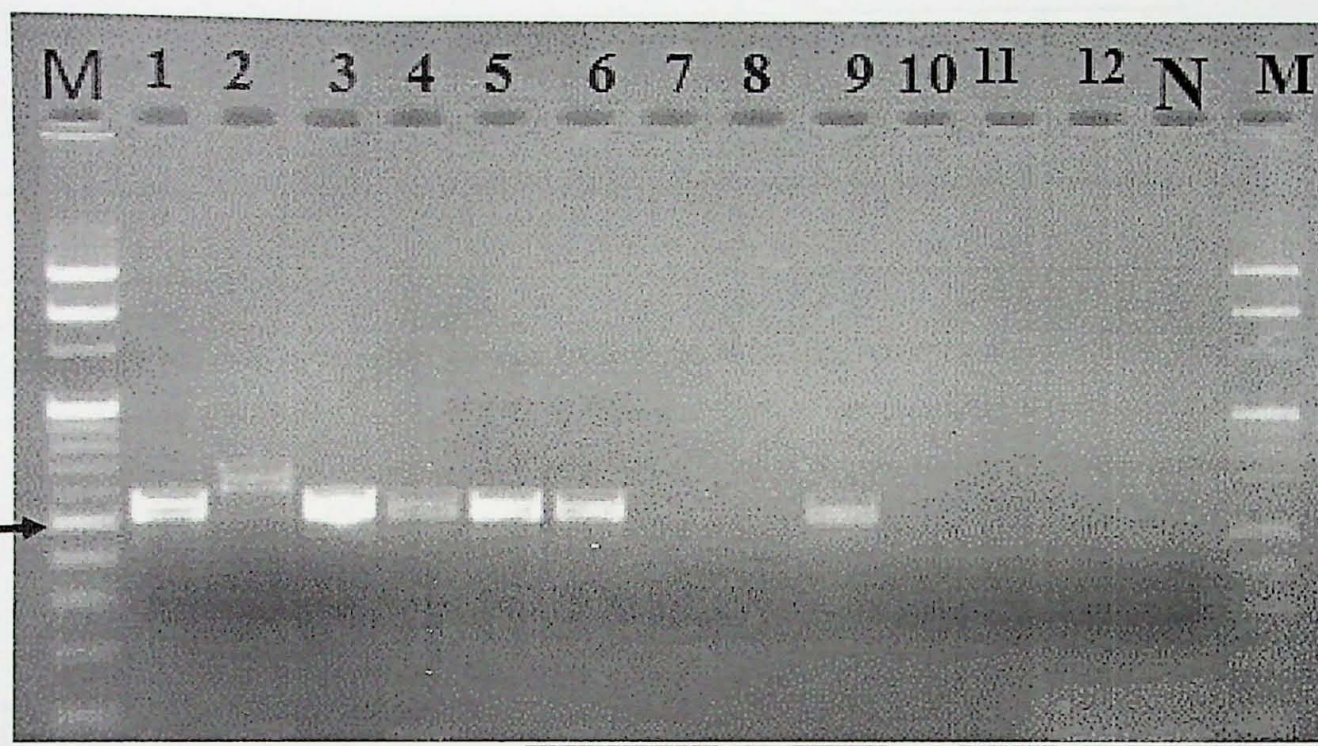


Figure 7. Typical PCR products of *Streptococcus equi* subsp. *equi* with SeM gene. Lane N is negative control and lane M showing 100bp gene marker.

Figure 7 shows that lanes 1-12 were samples, lane N was negative control and M was 100bp ladder (Thermo scientific, Germany) served as a size marker. Samples in lane 1, 3, 4, 5, 6 and 9 were positive PCR products with 541 bp using SeM primers. Sample in lane 2 could be mutant strain of *S. equi* subsp. *equi* or non-specific product since it has amplicon above 541 bp and the rest lanes revealed negative samples.

Table 6. The comparison between culture and PCR for the detection of *Streptococcus equi* subsp. *equi* in suspected strangles cases.

Type of samples	Number of samples	Culture positive	PCR positive	PCR positive culture positive	PCR negative culture positive
Cultures	5	5	5	5	0
Swabs	65	9 (13.9%)	47 (72.3%)	9	0
Aspirates	15	6 (40%)	13 (86.7%)	6	0
Chondroids	5	0	2 (40%)	0	0

All 67 PCR positive samples showed clear band at 541bp, whereas no amplicon was formed when testing the other 23 samples. 72%, 87% and 40% of samples from nasopharyngeal swabs, aspirates (pus) and chondroid, respectively, were found to be positive for PCR; the corresponding positivity for cultivation was 14% and 40% and 0% (Table 6). The diagnostic sensitivity was significantly ($p<0.05$) higher for PCR than bacterial cultivation in all three type of samples.

4.5. Antibiotic sensitivity pattern of isolates

Streptococcus equi subsp. *equi* isolates were subjected to different antibiotic discs to determine their *in vitro* susceptibility. Fifteen isolates of *Streptococcus equi* subsp. *equi* were used to test susceptibility to 13 antibiotics. All the isolates were found to be susceptible to ampicillin, gentamicin, chloramphenicol, norfloxacin, erythromycin, vancomycin, cephalothin, doxycycline, ceftriaxone and tetracycline. All the isolates were resistant to kanamycin, sulphonamide and streptomycin (Table 7).

Table 7. *In-vitro* Drug sensitivity pattern for isolates of *Streptococcus equi* subsp. *equi*

Drug discs used (μ g)	Resistance (%)	Intermediate (%)	Moderately Susceptible (%)	Susceptible (%)
Ampicillin (10)	0(0.0)	0(0.0)	1(6.7)	14(93.3)
Gentamicin (10)	0(0.0)	0(0.0)	0(0.0)	15(100)
Chloramphenicol (30)	0(0.0)	0(0.0)	0(0.0)	15(100)
Norfloxacin (10)	0(0.0)	2(13.3)	0(0.0)	13(86.7)
Erythromycin (15)	0(0.0)	0(0.0)	0(0.0)	15(100)
Vancomycin (30)	0(0.0)	0(0.0)	0(0.0)	15(100)
Kanamycin (30)	15(100)	0(0.0)	0(0.0)	0(0.0)
Cephalothin (30)	0(0.0)	0(0.0)	0(0.0)	15(100)
Tetracycline (30)	0(0.0)	0(0.0)	0(0.0)	15(100)
Sulphonamide (300)	15(100)	0(0.0)	0(0.0)	0(0.0)
Streptomycin (10)	15(100)	0(0.0)	0(0.0)	0(0.0)
Doxycycline (30)	0(0.0)	0(0.0)	0(0.0)	15(100)
Ceftriaxone (30)	0(0.0)	0(0.0)	0(0.0)	15(100)

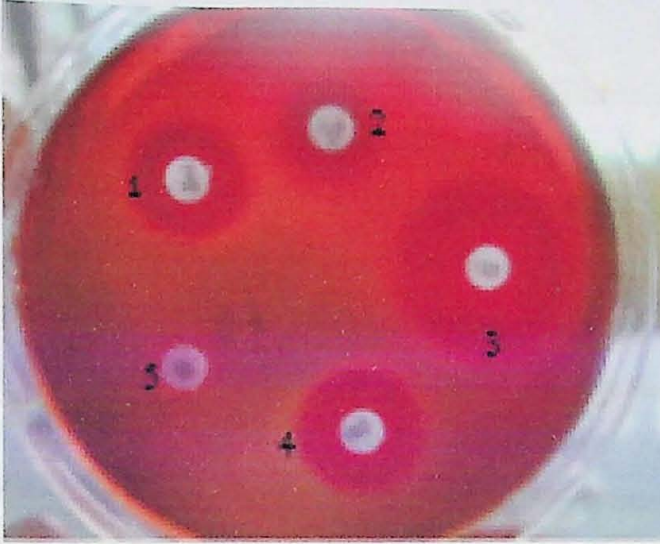


Figure 8. Antibiotic disk sensitivity test for *Streptococcus equi* subsp. *equi* isolates on blood agar. The drugs used are 1- Gentamycin, 2-Norfloxacin, 3-Doxycycline, 4-vancomycin- and 5- sulphonamid.

Figure 9 above depicted the susceptibility of *Streptococcus equi* subsp. *equi* isolate tested to 5 different antibiotic disks. The isolate was resistant to disk 5 (sulphonamide) but susceptible to antibiotic disks 1, 2, 3 and 4 designated for gentamycin, norfloxacin, doxycycline and vancomycin, respectively.

5. DISCUSSION

Strangles in donkeys has not been well studied in Ethiopia; hence, information specifically addressing the problem in donkeys is hardly available. Therefore, in this study the problem was studied clinically and by identifying the causative agent through cultural and PCR-based methods. In agreement with Alemnesh (2004) strangles in donkey is characterized by clinical signs of nasal discharge, swelling of submandibular, retropharyngeal and/or parotid lymph nodes, guttural pouch epyema and extended head and neck with strider breathing. Artiushin *et al.* (2002) described that one consequence of the acute phase is inappropriate immune response generated by *S. equi* is a rise in body temperature, which can be monitored and detected in the absence of bacterial shedding. However, in the present study fever was not detected in all clinical cases of strangles owing to the late stage detection of the disease by the owners.

Donkeys that have developed sever retropharyngeal and guttural pouch abscessation keep their head extended and ears erected and potentially can confuse with tetanus. A similar observation has been also made by Sweeny (1996) and Alemnesh (2004) and that occasionally horses and donkeys, respectively, may stand with their neck extended because of pain in the throat area. Head region lymph nodes abscessation and rupture along the rim of the mandible and at base of ears was observed in young donkeys (< 5 years). In agreement with Ainsworth (2012) donkeys of all ages are susceptible but infections typically occur in donkeys < 5 years of age.

In agreement with Newton *et al.* (2000) and Fintl *et al.* (2000) the severity of disease may vary widely between donkeys, and host factors appear to be highly significant. *S. equi* shows very little strain diversity (Galan and Timoney, 1988), although sequence analysis of the *SeM* gene has identified at least 66 strains of *S. equi* from cases of natural infection (Kelly *et al.*, 2006; Anon, 2008). To date, strain variations have not been linked with different disease presentations. The most significant host factor in natural outbreaks of strangles is likely to be pre-existing immunity, the levels of which have not been reported in the general equine population. The mechanism of resistance is not fully understood and may not correlate with serum levels of IgG (Galan and Timoney, 1985; Timoney *et al.*, 2007).

Alemnesh (2004) reported that it was uncommon to find chondroid in the guttural pouches at postmortem of donkeys and also the fact that postmortem of dead donkeys for the purpose of investigating chondroid was not conducted in the donkey sanctuary, Ethiopia this information is obscured. However, in this study 5 (38%) out of 13 donkeys had chondroid in their guttural pouch. Approximately 10% of cases that recover from strangles become persistent carriers of *S. equi*, harboring the infectious agent in chondroids located in the guttural pouch (Dalglish *et al.*, 1993; Timoney, 1993; Newton *et al.*, 2000; Waller *et al.*, 2011). Although higher occurrence of chondroid is observed in this study, it suggests that these donkeys were either in contact or recovered from the disease. Therefore, those donkeys which had chondroids at postmortem were carriers and could be source for the intermittent occurrence of the disease in study areas and were important in interepizootic maintenance and initiating new outbreaks in the study areas. It would have been more appropriate in detecting carriers other than postmortem if endoscope-guided guttural pouch lavage were used as it remains the 'gold standard' techniques for detecting strangles carriers in control of the disease (Newton *et al.*, 2000; Davidson *et al.*, 2008).

Isolation of most bacterial species from the upper respiratory tract swabs has not been shown to be associated with respiratory disease in horses, with the exceptions of *S. equi* and possibly *Pasteurella* spp. and *S. zooepidemicus* which are generally considered to be normal upper respiratory tract commensal organisms (Jannatabadi *et al.*, 2008; Ainsworth, 2012). Similarly, in this study the following organisms were isolated from different samples: *Streptococcus* spp., *Staphylococcus* spp., *Neisseria* spp., *Enterococcus* spp., *Bacillus* spp., *Corynebacterium* spp., *Pasteurella* spp., *Actinomyces* spp., *Pseudomonas* spp., *Proteus* spp. This finding is in agreement with Jannatabadi *et al.* (2008) who have reported similar bacteria from horses suffering from respiratory problem except *Corynebacterium*, *Enterococcus* and *Actinomyces*. Dessisa *et al.* (2009) has also reported most of the organisms except *Neisseria* and *Enterococcus* from donkeys having respiratory illness.

Direct smear examination of pus samples made from guttural pouch and submandibular lymph nodes revealed clean chains of bacteria characteristics of *S. equi* subsp. *equi*. This explains the fact streptococci divide in only one plain and firmness of characteristics of the strain (Quinn *et al.*, 1999; Fintl *et al.*, 2000). Colony characteristics (size, shape, consistency and nature of

hemolysis) were observed on blood agar. Chain length was observed to decrease from direct smear to broth culture and agar culture and in subsequent subcultures in agreement with previous works (Hirsh and Zee, 1999; Quinn *et al.*, 1999; Alemnesh, 2004).

Results of the biochemical identification tests obtained using API 20 Strep in the present study were generally in agreement with Quinn *et al.* (1999). The variation between *Streptococcus equi* subsp. *equi* isolates reaction was not significant but some isolates fermented sorbitol and hydrolysed aesculin in agreement with Grant *et al.* (1993). This could be due to mutation or allelic difference as reported by Kelly *et al.* (2006) and Anon (2008). In most of the cases *Streptococcus equi* subsp. *equi* fails to ferment lactose, sorbitol, trehalose where as *S. equi* subsp. *zooepidemicus* was identified to ferment lactose and sorbitol (Newton *et al.*, 1998; Quinn *et al.*, 1999; Kumamoto *et al.*, 2001; Chanter, 2002).

The majority of isolates from nasopharyngeal swabs were *S. equi* subsp. *zooepidemicus* (75%), followed by *S. equi* subsp. *equi* (15%) and *Streptococcus dysgalactea* subsp. *equisimilis* (10%). The lower isolation rate of *S. equi* subsp. *equi* from nasopharyngeal swab might be due to obscuring during the incubation stage in early clinical phase of the disease or in guttural pouch carriage (Smith, 2002). *S. equi* subsp. *zooepidemicus* are opportunistic species in any of upper respiratory tract infections (Quinn *et al.*, 1999; Radostitis *et al.*, 2000). It is the commonest secondary bacterial invader following respiratory tract infection (Mair, 1996; Radostitis *et al.*, 2000; Timmony, 2004). Most Streptococci inhabit the upper respiratory tract and lower urogenital tract but recovery of *S. equi* subsp. *equi* is associated with strangles (Hirsh and Zee, 1999; Timmony, 2004).

There was higher isolation rate of *S. equi* subsp. *equi* from young than adult donkeys. The assessments of the isolation rate of *S. equi* subsp. *equi* from 87 animals sampled indicated that a statically significant ($p < 0.05$) difference was observed between young (< 5 years old) (11.5%) and adult donkeys (≥ 5) (5.6%). Like in horses, young donkeys are more susceptible to strangles. Foals older than 4 months and young (< 5 years) equines are most predisposed to strangles infection. Older horses are often immune to strangles due to prior exposure, or they may display only lethargy and nasal discharge without obvious lymph node swelling. However, these animals

can still spread the disease (Radostits *et al.*, 2000; Sweeney, 2005). Unlike Alemnesh (2004) who have reported higher occurrence of strangles in males, in this study females were seen more affected by strangles than males. This might be due to the close relation with their young foals which could potentially spread the pathogen.

The bacteriological identification systems based on sugar fermentation can sometimes cause diagnostic errors and it is possible to mistakenly identify *S. dysagalactia* subsp. *equisimilis* instead of *Streptococcus equi* subsp. *equi* (Grant *et al.*, 1993). Furthermore, the difficulty to isolate *S. equi* subsp. *equi* could be due to overgrowth of other beta-haemolytic C group streptococci. Nevertheless, the use of PCR test on the enrichment broth samples should have to rule out these hypotheses (Laus *et al.*, 2007).

Furthermore, PCR was used to detect *Streptococcus equi* subsp. *equi* from three sample types. There was significant difference ($p < 0.05$) in the recovery rate of *Streptococcus equi* subsp. *equi* from the three major samples: Nasopharyngeal swab and guttural pouch aspirate and chondroid. The proportion decreases in the order of guttural pouch aspirate, nasopharyngeal swab to chondroid. In this study *S. equi* subsp. *equi* was highly recovered from guttural pouch aspirate and this finding is in agreement with Newton *et al.* (2000) who have reported higher recovery of *Streptococcus equi* subsp. *equi* from the culture of endoscopic guttural pouch lavage (88%) than from nasopharyngeal swab (45%) from horse. The lower microbial yield from nasopharyngeal swabs may be the result of swabbing too early in the course of the disease; retrieving insufficient amount of secretions or having overgrowth of other organisms such as *S. equi* subsp. *zooepidemicus* (Waller *et al.*, 2011). The lower detection rate of *Streptococcus equi* subsp. *equi* from chondroid samples was probably due to large amounts of purulent material that contain high concentrations of DNA polymerase inhibitors and enzymatic degradation which removes DNA (Ainsworth, 2012).

The detection sensitivity was significantly ($p < 0.05$) higher in PCR than in bacterial cultivation which is in agreement with Newton *et al.* (2000) and Davidson *et al.* (2008). Based on the type of samples used in this study, the diagnostic sensitivity of both culture and PCR showed significant

difference ($P < 0.05$) and the sensitivity is higher in aspirate than nasopharyngeal swab and chondroid. Similar finding was also reported by Grønbaek *et al.* (2006).

The diagnostic sensitivity for PCR test to detect *S. equi* subsp. *equi* was significantly higher ($p < 0.05$) than the culture method. Although culture is the gold standard, it provides only a minimal estimate of the occurrence of *S. equi* subsp. *equi* in the nose because of inadequate numbers and failure of transfer from swab to culture medium or overgrowth by contaminants. The putative greater sensitivity of the PCR compared to culture is also explained by the fact that the entire swab is extracted for PCR, whereas only a portion of the swab surface is cultured. Despite its putative lack of sensitivity relative to PCR, culture is of value because it definitively establishes infection and may conveniently be performed on the same specimens used for PCR (Timoney and Artiushin, 1997). PCR analysis of samples for *S. equi* genes is considered to be 2-3 times more sensitive than microbial culture alone in detecting the organism (Newton *et al.*, 2000; Sweeney *et al.*, 2005; Waller and Jolley, 2007), however, PCR technique is not without its limitations. False negatives may occur in samples that have heavy growths of *S. equi* subsp. *zooepidemicus* and/or in samples with large amounts of purulent material that contain high concentrations of DNA polymerase inhibitors. False positives may occur when the organism has been killed but the PCR technology still detects residual DNA (Ainsworth, 2012).

There is little information available regarding appropriate antimicrobial selection in the horse. First line antimicrobial agents for suspected or proven infection should generally be broad spectrum and have known efficacy against the most likely pathogens. First line antimicrobials are those that are appropriate for use in the absence of, or pending, culture and sensitivity results (Hollis and Wilkins, 2009). In vitro susceptibility tests of the *streptococcus equi* subsp. *equi* isolates according to Qunin *et al.* (1994) indicated that all the isolates were found to be susceptible to ampicillin, gentamicin, chloramphenicol, norfloxacin, erythromycin, vancomycin, cephalothin, tetracycline, doxycycline and ceftriaxone. And these findings are in agreement with Ainsworth (2012), Pustelar *et al.* (2007) and Clercq *et al.* (2003) who have prescribing drugs to clinical cases of the diseases which need medical treatment.

In agreement with this study Clark *et al.* (2008) had reported that *S. equi* subsp. *equi* isolates were susceptible to ampicillin, cephalothin and erythromycin. In this study, however, two isolates of *S. equi* subsp. *equi* were found to be moderately susceptible to ampicillin indicating that wide use of this antibiotic needs attention. Isolates of *Streptococcus equi* subsp. *equi* were resistant to kanamycin, sulphonamide and streptomycin. In agreement with the present study Ensink *et al.* (2003) had tested sulphonamide in clinical cases treatment in comparison with penicillin both topically and systemically and he noticed that, sulphonamide has no effect when compared to penicillin in neither of the method of application. Therefore, sulphonamide should not be used to treat purulent infections. This report correlates with *in-vitro* test result of this study.

Ceftriaxone exhibits the widest antibacterial spectrum of third generation cephalosporins and this is reflected in clinical responses. Several pharmacoeconomic studies indicate that the once-daily dosing regimen required for ceftriaxone is the major factor responsible for its cost-effectiveness over third and fourth generation cephalosporin (Bijie *et al.*, 2005).

Therefore, based on the above conclusions the following points are recommended:

- Despite the lack of sensitivity relative to PCR, culture is of value because it definitively establishes infection. Therefore, routine use of both microbial culture and PCR analysis of swabs should be used to guarantee detection of *S. equi*.

6. CONCLUSIONS AND RECOMMENDATIONS

Strangles caused by the host adapted Lancefield group C *Streptococcus equi* subsp. *equi* is a frequently diagnosed infectious disease of equids worldwide. In this study identification of donkeys suffering from strangles was conducted based on clinical signs described in the literature. The diagnostic value of these signs was also statistically tested, and the association of these signs with age of donkeys was determined. Furthermore, bacteriological study was conducted on samples taken from donkeys suffering from strangles and also detection of the causative agent, i.e., *Streptococcus equi* subsp. *equi*, was conducted using PCR. Accordingly, *Streptococcus* spp. have contributed for the majority of the isolates from nasopharyngeal swabs and aspirates.. The isolation rate of *S. equi* subsp. *equi* is higher from aspirate samples than swabs. Similar result is also obtained in PCR from the above sample and chondroid. However, higher detection rate of *S. equi* subsp. *equi* is obtained in PCR compared to the conventional culture method. A reasonably higher percentage of donkeys with chondroids encountered in this study might serve as carrier and transmit the disease to naive equines.

There is little information available regarding appropriate antimicrobial selection in the donkeys. To date, penicillin is widely used in DHWP and other public vet clinics (Penstrep) to treat strangles when deemed necessary and the *in-vitro* susceptibility tests of *streptococcus equi* subsp. *equi* isolates indicated that it has still good value. Moreover, high susceptibility of *S. equi* subsp. *equi* isolates was observed to a wide range of antibiotics including ampicillin, gentamicin, chloramphenicol, norfloxacin, erythromycin, vancomycin, cephalothin, tetracycline, doxycycline and ceftriaxone and hence these drugs can potentially be used as an alternative to prevent the development of resistance associated with the use of single drug, penicillin. However, all isolates of *S. equi* subsp. *equi* were resistant to kanamycin, streptomycin and sulphonamide.

Therefore, based on the above conclusions the following points are recommended:

- Despite its lack of sensitivity relative to PCR, culture is of value because it definitively establishes infection. Therefore, routine use of both microbial culture and PCR analysis of samples should be used to maximize detection of *S. equi*.

- Owing to its high sensitivity and rapidness PCR should be used as an effective tool in the detection of clinical cases so as to conduct prompt management of sick animals.
- Aspirates, if possible, should be used as the preferred sample in diagnosing the disease to increase the sensitivity of both culture and PCR assays
- In addition to the judiciously administered procaine penicillin by DHWP to treat strangles alternative drugs pointed out in this study should be used to prevent possible resistance against penicillin.
- Further research on the role of carriers in maintaining the infection between outbreaks should be carried out by endoscope aided diagnosis.
- Further research to assess the circulating strain/s of the organism is necessary to craft informed control strategy against the disease.

7. REFERENCES

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8. ANNEXES

Annex 1. Case record sheet

Case number	Owner address	sex	age	Nasal discharge	Lymph node swelling	Guttural pouch empyema	Temperature	Sample type

Annex 2. Laboratory data record sheet

Reference number	Sample type	Colony character	Hemolysis character	Catalase test	Gram's rxn and morphology characteristics	Oxidase test	Growth and character on MacConky agar	Oxidation Fermentation Test(O-F test)



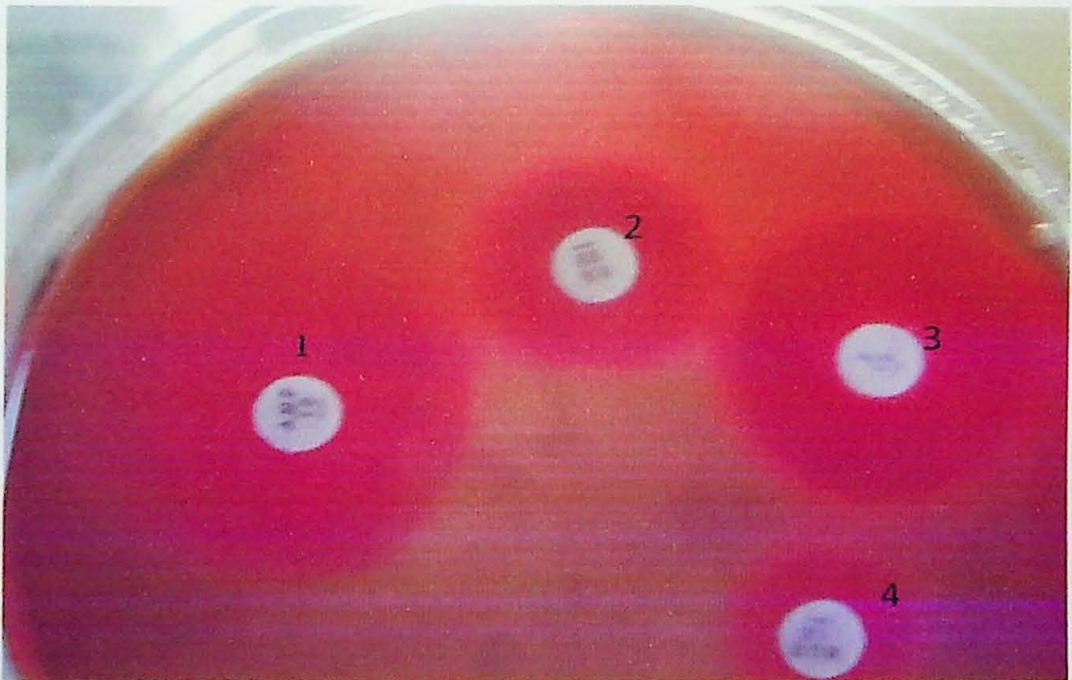
Annex 3. Donkey suspected of strangles with characteristics nasal discharge, parotid lymph node enlargement with extended head and neck (Godino public veterinary clinic)



Annex 4. Donkey suspect of strangles with erupted sub mandibular lymph node (DHWP clinic)



Annex 5. Donkey ssuspect of strangles with retropharngeal lymph node enlargement (DHWP clinic)



Annex 6. Antibiotic susceptibility test for *S. equi* subsp. *equi* on blood agar plate. The drugs used are 1- Ampicillin, 2-Ceftriaxone, 3- Chloramphenicol, 4- Gentamycine

Annex 7. API 20 Strep test procedure

Procedures of API 20 Strep identification system for *Streptococceae* (biomérieux SA)

i. Preparation of isolates

- A well isolated colony of the family *Streptococceae* (Gram positive, cocci, catalase negative on blood agar) was cultured on blood agar
- It was incubated aerobically at 37°C for 24 hrs
- The type of hemolysis (β -hemolysis) was noted on result sheet(21st test)
- A well isolated colony from blood agar was picked and suspended in 0.3 ml of sterile water and homogenized very well.
- The suspension was flooded on a sheep blood agar plate and incubated for 24 hrs at 37°C

ii. Preparation of strip impregnated with chemicals and/or reagents

- An incubation box (tray and lid) was prepared and about 5 ml of sterile distilled water was distributed in to the honey combed wells of the tray to create a humid atmosphere
- The isolate reference was recorded on elongated flap of the tray
- The strep was removed from its packaging and placed in the incubation box.

iii. Preparation of the inoculum

- An ampule of API suspension medium was opened
- Using a sterile swab the culture was harvested from the previously prepared subculture plate.
- A dense suspension was made with a reasonable turbidity

iv. Inoculation of the strip

- First half of the strip consisted of tests from Voges Proskeuer(VP) to Arginine DiHydrolase (ADH). For the tests from VP to Leucine Amino Peptidase (LAP) 100 μ l was distributed in each cupule. For the ADH test only the tube was filled.
- Second half of the strip was tests from RIBose (RIB) to GLYcogen(GLYG). An ampule of API GP medium was opened and the rest of the suspension was transferred in to it, and then mixed very well. This new suspension was distributed in to the tube only. The cupule of the underlined testes (ADH to GLYG) was filled with mineral oil to form a convex meniscus. The lid was placed on the tray.
- It was incubated at $36^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in aerobic conditions for 4 to 4 1/2 hrs to obtain a first reading and 24 ± 2 hours to obtain a second reading.

V. Reading the strip after 4 hours of incubation: Reagents were added;

- VP test: 1 drop to each of VP 1 and VP 2.
- HIPpuric acid (HIP) test: 2 drops of NIN.
- PYRrolidonyl Arylamidase(PYRA), α -GALactosidase(α GAL), β -GucuRonidase(β GUR), β -GALactosidase(β GAL), Alkaline Phosphatase(PAL) and LAP tests: 1 drop of each of ZYM A and ZYM B.
- After waiting for 10 minutes the reaction was read by referring to the reading table below. It was re incubated and then after 24 hours, the reaction ESC, ADH and RIB to GLYG were reread. The enzymatic reactions were recorded on result sheet and final identification was obtained by numerical profile.

Tests	Substrates	QTY (mg/c up)	Reactions/Enzyems	Results					
				Negative		Positives			
VP	Sodium pyruvate	1.9	Acetion production(Voges proskauer)	<u>VP1 +VP2/wait 10 min</u> <u>Colorless</u> <u>Pink-Red</u>					
HIP	Hippuric acid	0.4	Hydrolysis(hippuric acid)	<u>NIN/wait 10 min</u> Colorless/pale blue Bluish-grey				dark blue/violate	
ESC	Esculin	1.16	β -glucosidase hydrolysis	4 hrs	24 hrs	4 hrs	24 hrs		
	Ferric citrate	0.152	(Esculin)	Colorless pale yellow	Colorless pale yellow light grey	Black Grey	Black		
PYRA	Pyroglutamic acid β -naphthylamide	0.0256	Pyrarolidonyl Arylamidase	ZYM A+ZYM B/10 min(PYRA to LAP) Colorless or very pale orange				orange	
α GAL	6-bromo-2-naphathyl- α D-galactopyranoside	0.0375	α -Galactosidase	colorless		violate			
PAL	2-naphthyl phosphate	0.0244	Alkaline Phosphatase	Colorless or very pale violate		violate			
LAP	L-leucine- β -naphthylamide	0.0256	Leucine AminoPeptidase	colorless		orange			

<u>ADH</u>	L-arginine	1.9	Arginine Dihydrolase	Yellow		red	
<u>RIB</u>	D-ribose	1.4	acidification	4 hrs	24hrs	4hrs	24 hrs
				Red	Orange/red	Orange/yellow	yellow
<u>ARA</u>	L-arabinose	1.4	acidification	Red	Orange/Red	Orange/yellow	Yellow
<u>MAN</u>	D-mannitol	1.36	acidification	Red	Orange/Red	Orange/yellow	Yellow
<u>SOR</u>	D-sorbitol	1.36	acidification	Red	Orange/Red	Orange/yellow	Yellow
<u>LAC</u>	D-lactose(bovine origin)	1.4	acidification	Red	Orange/Red	Orange/Yellow w	Yellow
<u>TRE</u>	D-trehalose	1.32	acidification	Red	Orange/Red	Orange/Yellow w	Yellow
<u>INU</u>	inulin	5.12	acidification	Red	Orange/Red	Orange/yellow	yellow
<u>RAF</u>	D-raffinose	3.12	acidification	Red	Orange/Red	Orange/yellow	yellow
<u>AMD</u>	strach	2.56	acidification	Red	Orange/Red	Orange/Yellow w	yellow
<u>GLYG</u>	glycogen	1.28	acidification	Red or Orange		Bright yellow	
<u>HEM</u>							

Identification table: % of reactions positive after 4/24 hrs at 36°C±2°C

API 20 strep	VP	HI P	ES C	PYR A	AG AL	GUR	GA L	PA L	LA P	AD H	RI B	AR A	MA N	SOR	LAC	TRE	INU	RAF	AMD	GLYG	HEM
<i>S. equi</i> subsp. <i>equi</i>	1	0	1	0	0	100	1	100	100	100	0	0	0	0	0	1	0	0	100	100	100
<i>S. equi</i> Subsp. <i>zooepidemicus</i>	0	1	15	0	0	100	1	99	100	99	85	0	0	99	100	0	0	0	99	99	99
<i>S. dysgalactea</i> subsp. <i>equisimilis</i>	0	1	25	1	1	99	1	99	100	97	97	1	1	1	45	99	0	1	98	40	94

Annex 8. DNA Extraction Protocol (*source*: MP Biomedicals FastDNA® SPIN Kit)

1. Add sample to lysing Matrix A tube. Place up to 200 μ l suspension of cells suspended in water or isotonic saline solution. Centrifuge a sufficient volume to provide a pellet size of up to 10^9 bacteria. Resuspend pellets in water or isotonic saline to give a maximum suspension volume of 200 μ l.
2. Add 1.0 ml cell Lysis Solution (CLS-TC) to sample tubes
3. Homogenize in the FastPrep® Instrument for 40 seconds at a speed setting of 6.0
4. Centrifuge at 14,000xg for 5-10 minutes to pellet debris.
5. Transfer supernatant (700-800 μ l) to a 2.0 ml micro centrifuge tube and add an equal volume of Binding Matrix. Invert to mix.
6. Incubate with gentle agitation for 5 minutes at room temperature on a rotator.
7. Transfer half (approximately 800 μ l) of the suspension to a SPIN™ Filter and centrifuge at 14,000xg for 1 minute. Empty the catch tube and add the remaining suspension to the SPIN™ Filter and centrifuge as before. Empty the catch tube again.
8. Add 500 μ l prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip.
9. Centrifuge at 14,000 x g for 1 minute. Discard contents of catch tube and replace.
10. Without any addition of liquid, centrifuge a second time at 14,000 x g for 2 minute and replace the catch tube with a new, clean tube.
11. Elute DNA by gently resuspending Binding Matrix above the SPIN filter in 100 μ l of DES. Incubate for 5 minutes at 55°C in a heat block or water bath.
12. Centrifuge at 14,000 x g for 1 minute to bring eluted DNA in to the clean catch tube. Discard the SPIN FILTER. DNA is now ready for PCR.

Annex 9. PCR Protocol

Master Mix preparation (for one sample)

DNase free water = 35 μ l

10xDream Taq buffer = 5 μ l

25mM MgCl₂ = 1.5 μ l

10mMdNTPs= 1 μ l

Primer ASW73F= 1 μ l

Primer ASW74R= 1 μ l

Taq DNA polymerase = 0.5 μ l

➤ Add 5 μ l template (DNA) sample into tube containing 45 μ l of master mix

➤ Put the PCR tube into thermal cycler with setting of

- Initial denaturation at 95°C for 2 min
 - Denaturation at 94°C for 1 min, annealing at 55°C for 1 minute,
 - Elongation at 72°C for 1 min,
 - Final Elongation at 72°C for 8min and put at 4°C ∞
- } 35 Cycles

9. CURRICULUM VITAE

1. Personal information

Name	Manyahilishal Etana
Date of birth	13/03/72 E.C.
Place of Birth	Nathareth/ East shoa
Nationality	Ethiopian
Sex	Female
Marital status	Married

2. Educational background

<u>Level</u>	<u>Place</u>	<u>Award</u>
Primary education	Nazareth	Certificate
Secondary education	Nazareth	Certificate
Higher education	FVM, AAU	DVM Degree

3. Language skill

<u>Language</u>	<u>Speaking</u>	<u>Listening</u>	<u>Writing</u>
Amharic	Excellent	Excellent	Excellent
English	Very good	Very good	Very good
Oromifa	Very good	Very good	Very good

4. Seminar and research activity

- ❖ Seminar on ESAT-6 and CFT-10 Based Interferon Gamma Assay for the Diagnosis of Bovine Tuberculosis.
- ❖ Research on Determination of the Normal Physiological, Hematological and Serobiochemical Parameters of Normal Ethiopian Working Donkeys.
- ❖ Seminar on Review on Strangles with Emphasis on Virulence Factors of *S. equi* and Diagnosis

5. Publications

- **Manyahilishal** Etana, Bojia Endeabu, Shiferaw Jenberie and Haileleul Negussie (2011). Determination of reference physiological values for working donkeys of Ethiopia. Ethiopia Veterinary Journal, Vol. 15
- **Manyahilishal** Etana, Bojia Endeabu, Shiferaw Jenberie and Haileleul Negussie (2011). Determination of reference hematological and serum biochemical values for working donkeys of Ethiopia. Veterinary Research, Vol. 4

6. Work experience

- 2009 to date- team leader of the donkey health and welfare project at Debre-Zeit
- 2005- 2009- Instructor at Alage ATVET College, Ministry of Agriculture and Rural Development. In the college, I have been assigned and working in different positions as listed below:
 - ❖ Instructor of different professional courses like *Clinical diagnosis, Microbiology, Surgery and Reproductive Health, Meat inspection, Anatomy and Physiology and Infectious Diseases.*
 - ❖ Course and practical coordinator
 - ❖ Member of the grade approval and staff evaluation committee

7. Other skills

- ❖ Computer literate (Certified with Diploma).

8. Contact address

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Debre-Zeit, Ethiopia

10. SIGN OF DECLARATION

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

Name Manyahilishal Etana Kitaw

Signature _____

Date of submission _____

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

Dr. Tesfaye Sisay (DVM, MSc, PhD, Associate professor)

Dr. Bojia Endebu (DVM, MSc)