MOLECULAR CHARACTERIZATION OF MYCOPLASMAS SPECIES ISOLATED FROM THE GENITAL TRACT OF DORPER SHEEP IN SOUTH AFRICA

BY

HABU ALI

A thesis submitted in partial fulfilment of the requirements for the degree of

MAGISTER SCIENTIAE (VETERINARY SCIENCE)

In the

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science University of Pretoria
South Africa

Supervisor: Prof Moritz van Vuuren
Co-supervisor: Ms Anna-Mari Bosman

APRIL 2012

DEDICATION

To my Lord, the almighty the supreme, the sustainer, the all knowing, the provider to whom I seek for guidance.

ACKNOWLEDGEMENTS

I wish to express my appreciation and gratitude to the following:

Professor Moritz van Vuuren my supervisor, for his guidance, advice, criticism and encouragement throughout this study and the writing of this dissertation, and without whom this thesis would not have been completed.

The Executive Director, Management and the staff of the National Veterinary Research Institute, Vom, Nigeria, for the opportunity given to me to do my postgraduate studies in South Africa.

The Dorper sheep Breeders Association of South Africa, for giving their financial support to this research.

I acknowledge the research grant from the department of Veterinary Tropical Diseases and the Research Committee of the Faculty of Veterinary Science, University of Pretoria.

My Co-supervisors Ms Anna-Mari Bosman and Dr Jackie Picard for the laboratory training and encouragement for this research.

The bacteriology laboratory staff Mr Johan Gouws and Janita Greyling for the isolates and training for this research.

To my beloved wife hajiya Jummai Mohammed kwairanga and my lovely kids, Sabir, Sabiat, Salim, Sudais, Sahir and Samir for their patience and support during the period of my absence to pursue this research.

My entire family for giving me the support and encouragement to complete my studies.

To the entire staff and postgraduate students in the Department of Veterinary Tropical Diseases (DVTD), I wish to express my appreciation for all the help and support showed to me during my stay.

TABLE OF CONTENTS

TABLES

FIGURES

LIST OF ABBREVIATIONS

A	Acholeplasma
BLAST	Basic local alignment search tool
CCPP	Contagious caprine pleuropneumonia
CPD	Contagious pustular dermatitis
EB	Elution buffer
GI	Growth inhibition
LC	large colony
LB	Laurie broth
M	Metabolic inhibition
*Mmm*LC	*Mycoplasma mycoides mycoides* Large Colony
M	*Mycoplasma*
MCC	*Mycoplasma capricolum* capripneumonia
*Mmm*SC	*Mycoplasma mycoides mycoides* Small Colony
MAKEPS	Mastitis, arthritis, keratitis, pneumonia and septicaemia
NCBI	National Center for Bioinformatics
OvHV-2	Ovine herpes virus type 2
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real time polymerase chain reaction
Subsp	Subspecies
SC	Small colony
UK	United Kingdom
USA	United State of America

ABSTRACT

Mycoplasmas are prokaryotic micro-organisms belonging to the class *Mollicutes*, which lacks rigid cell walls. Their genomic size ranges from 500-1500 bp. It causes a wide variety of different diseases in small ruminants and in particular ulcerative balanitis and vulvitis that affects Dorper Sheep in South Africa. The disease causes high economic losses to the Dorper sheep breeders in South Africa. The presence of the disease has been known in South Africa since 1979. Earlier publications have identified the causative agent of this disease as *Mycoplasma mycoides mycoides* LC (*Mmm*LC). However, several *Mycoplasma* organisms isolated from cases of ulcerative balanitis have been shown not to be *Mmm*LC. There is a need to characterize the organisms isolated from sheep suffering from this disease using conventional and genetic molecular methods. In this study, 16SrRNA gene-based PCR assays and gene sequencing was used for the detection and characterization of *Mycoplasma* species from cases of ulcerative vulvovaginitis and balanoposthitis in Dorper sheep in South Africa. This investigation was conducted on 34 stored field isolates of mycoplasmas collected between 2003-2009 from 15 different farms in the Northern and Western Cape provinces of South Africa.

The isolates were screened and characterized by means of microbiological culture and biochemical methods and confirmed by PCR and sequencing. Evidence of involvement of these *Mcoplasma* idolates in ulcerative vulvovaginitis and balanoposthitis was obtained from the submission histories.

All 34 isolates were analysed by means of PCR, cloning and sequencing of a 1 078 bp fragment length of 16S a rRNA gene and identified as *Mycoplasma* species.

BLAST searches for sequence similarity from Genbank data revealed 18 isolates out of 34 four are 99 % similar to *M. arginini*, six out of 34 are 99 % similar to *M. bovigenitalium,* and two out of 34 were found to be 99 % similar to *M.* sp. *ovine/caprine* serogroup II. Two isolates out of 34 are 99 % similar to *A. Laidlawii*, and BLAST searches of two isolates gave 99 % similarity to *M.* sp*. USP120.* Two isolates were found to be 99 % similar to synthetic *M. mycoides*

mycoides Jvc1. A last isolate gave 99 % similarity to *M. canadense.* Phylogenetic trees were drawn using the neighbour joining method and maximum parsimony analysis to compare the South African isolates with other GenBank reference strains to determine relationships between South African isolates with isolates in other parts of the world.

This thesis is composed of five chapters. The first chapter deals with the historical background of ulcerative vulvovaginitis and balanoposthitis in Dorper sheep in South Africa and comparisons with findings from previous research. The chapter ends with the aims and objective of this research project.

Chapter two contains a literature review that deals with ulcerative vulvovaginitis and balanoposthitis in various parts of the world and controversy about the views of researchers about the aetiology of ulcerative vulvovaginitis and balanoposthitis in sheep.

Chapter three presents the first research on molecular characterization of mycoplasmas species isolated from cases of ulcerative vulvovaginitis and balanoposthitis in Dorper sheep in South Africa by means of PCR and gene sequencing.

Chapter four provides the findings of the analyses of the various *Mycoplasma* species that were involved in ulcerative vulvovaginitis and balanoposthitis in Dorper sheep in South Africa. The chapter also gives the results of phylogenetic analysis of the various *Mycoplasma* species with their relationship to sequences from all over the world deposited by researchers in Genbank.

Chapter five summarizes the research findings and provides conclusions.

CHAPTER 1
INTRODUCTION

Mycoplasmas are prokaryotic micro-organisms belonging to the Class *Mollicutes*, which lack rigid cell walls. Their genomic sizes range from 580-1350 base pairs. It causes a wide variety of diseases in small ruminants, including ulcerative balanitis and vulvitis that affects Dorper sheep in South Africa. The latter disease is the cause of severe economic losses for Dorper sheep breeders in South Africa.

Ulcerative balanoposthitis and vulvovaginitis is a venereal disease characterized by erosion and ulceration of the glans penis and vulval labia of sheep and has been described in several countries (Kidanemariam, Gouws, Van Vuuren and Gummow, 2005). In South Africa, the disease was first diagnosed in the Calvinia district of the Northern Cape Province in 1979, and later spread to other parts of the country such as the Free State, KwaZulu Natal, Eastern Cape, and Western Cape Provinces (Trichard, Jordaan, Prozesky, Jacobsz and Henton, 1993; Bath and De Wet, 2000; Gummow and Staley, 2000). A high prevalence of the disease in Dorper sheep in South Africa has been reported (Gummow and Staley, 2000).

In the UK, a similar disease in ewes with clinical signs such as swollen, oedematous, congested vulvas, and blood stained fluid or reddish stringy mucus oozing from the external orifices were reported. Other signs also reported were vulval scabs on the lower commissure with small vesicles and plaques on the posterior floor of the vagina (Martin and Aitken, 2000).

Similar disease syndromes such as vulvovaginitis, granular vaginitis, vulvitis and balanophosthitis has been reported in countries like Australia (Cottew, Lloyd and Parsonson, 1974), Canada (Doig and Ruhnke, 1977), USA (Livingstone and Gauer, 1983), and India (Kapoor, Pathak and Singh, 1984). It was reported from New South Wales that the pathogen only affects Border Leicester rams with severe ulcerative balanitis, and ewes which were allowed to mate with these rams showed a degree of vulvovaginitis (Webb and Chick, 1976).

Greig (2007) divided the causative agents of ulcerative balanitis and vulvitis in sheep flocks in the UK into four main entities: venereal parapoxvirus (orf) infection, enzootic posthitis (pizzle rot) caused by *Corynebactrium renale* or other diptheroid organisms; *Mycoplasma*-associated vulvovaginitis and a condition of unknown aetiology. Other organisms associated with the disease which have been isolated from the lesions include *Streptococcus zooepidemicus* (Dunn, 1996), *Histophilus ovis, Arcanobacterium pyogenes*, the mycoplasmas *M. fermentans*, and *M. bovigenitalium* (formerly *Mycoplasma ovine/caprine* sero group11) (Nicholas, Greig, Baker, Ayling, Heldtander, Johansson, Houshaymi and Miles, 1998). There was also a report of severe outbreaks in the UK during the Autumn and winter of 2006, including a particularly severe outbreak in a lowland flock in East Anglia from which mycoplasmas were not isolated, but ovine herpesvirus type 2 (OvHV-2) was detected in the blood of two ewes, from the vulval ulcer of one of them and from the penis of an affected ram (Pritchard, Scholes, Foster, Mitchell, Lawes, Ibata and Bank, 2008).

Although, the causative agent of ulcerative balanitis and vulvitis has not explicitly been identified, the aetiology of the disease has been ascribed to multifactorial agents by several researchers. A number of mollicutes such as *M. bovigenitalium, M. arginini,*

M. mycoides subsp. *mycoides* large colony variant (*Mmm*LC), *M. mycoides* subsp. *capri, M. agalactiae, M. capricolum, Acholeplasma Laidlawii*, and Ureaplasma species have been isolated from penile, preputial, vestibulovaginal, and vulvar specimens (Kidanemariam *et al.*, 2005). Although, it has been postulated that bacteria (Ball, Kennedy and Ellis, 1991; Trichard, *et al.*, 1993), caprine herpesvirus (Horner, Hunter and Day, 1982*;* Tarigan Webb and Kirkland, 1987), and parapoxvirus (Linklater and Smith, 1993) could possibly cause vulvovaginitis in sheep and goats, their involvement in the pathogenesis of the disease needs to be established.

Mycoplasma mycoides subsp. *mycoides* large colony variant (*Mmm*LC) was isolated from several infected ewes and rams with vulvitis and balanitis in South Africa. The inoculation of a field isolate in healthy animals reproduced the disease, which suggested that it may be the major cause (Trichard *et al.*, 1993). However, the aetiology of the disease has not been conclusively resolved because other organisms (*M. bovigenitalium, M. arginini, M. capricolum, Acholeplasma Laidlawii*, and *Ureaplasma*) have been isolated from sheep with the same clinical signs (Kidanemariam *et al.*, 2005). The latter organisms may well be important contributors to the development of the clinical signs.

*Mmm*LC is a member of the *Mycoplasma mycoides* cluster, a group of mycoplasmas that share serological, genomic and antigenic characteristics (DaMassa, Wakenell and Brooks, 1992). Although the *Mmm*LC biotype is not associated with disease that is clinically and pathologically well defined, there are some indications that this *Mycoplasma* could be involved in pathological conditions in small ruminants (Naglic, Hotzel, Ball, Seol and Busch, 2001). It has also been isolated from goats with polyarthritis, conjunctivitis, keratitis, pneumonia and cervical abscesses (Rosendal,

Ernø and Wyand, 1979). The disease only started to receive serious scientific attention in South Africa during the last three decades. However, several mycoplasma organisms recently isolated from cases of ulcerative balanitis in South Africa have been shown not to be *Mmm*LC. Previously, biochemical and serological methods were used in the identification and characterization of the isolates obtained from affected sheep. These methods suffered some limitations such as serological cross-reactions between various mycoplasmas. The tests are laborious, often difficult to interpret and sometimes contradictory, making it difficult to differentiate the mycoplasmas to species level. Currently, there are new developments in the identification and characterization of mycoplasmas using molecular techniques, which allow reliable identification and classification of the organisms. The aim of this study was to identify and characterize recent and stored isolates from Dorper sheep suffering from ulcerative vulvitis and balanitis in South Africa with the aid of the polymerase chain reaction (PCR) assay and gene sequencing.

CHAPTER 2
LITERATURE REVIEW

2.1 ULCERATIVE DISEASES OF THE GENITAL TRACT OF SHEEP AND GOATS

2.1.1 Introduction

Ulcerative balanoposthitis and vulvovaginitis (ub/uv) has been described in several countries in small stock and may have different aetiologies. Information obtained from earlier investigations indicates that the cause of ub/uv has not clearly been established. Furthermore, confusion could arise from the different names used by researchers in different countries to describe the lesions. Different names given to the syndrome include vulvovaginitis (Cottew *et al.*, 1974); balanitis and vulvovaginitis (Webb and Chick, 1976); granular vulvovaginitis (Doig and Rhunke, 1977); vulvitis (Ball and McCaughey, 1982); ulcerative balanitis and vulvitis (Deas, 1983; Dunn, 1996, Greig, 2000); ulcerative balanophosthitis and vulvovaginitis (Trichard *et al.*, 1993, Trichard and Van Tonder, 1994).

2.1.2 Sheath rot: synonyms, enzootic phostitis, urine scald, balanophostitis or, pizzle rot

Sheath rot is an enzootic inflammation of the prepuce and glans penis of castrated rams (wethers) and vulvovaginitis in ewes respectively. The disease is mainly caused by a urea-producing diphteroid organism, identified as *Corynebacterium renale* (Linklater and Smith, 1993). The occurrence of the disease has been reported in Australia (Beveridge and Johnstone, 1953; Southcott, 1965) and the UK (Roberts and Bolton, 1945; Doherty, 1985). For many years, the disease was considered to be non-infectious and

exclusively attributed to dietary factors until a Gram-positive diphteroid bacterium was isolated in an outbreak of the disease (Southcott, 1963). The disease manifests by spread of a superficial ulceration on the skin of the prepuce, and may sometimes involve the preputial lining and the penis (Linklater and Smith, 1993).

Merino sheep are considered to be more susceptible to the disease than their crosses and other breeds, particularly in Australia, while in the USA the disease has been identified in goats (Beveridge and Johnstone, 1953; Shelton and Livingstone, 1975). In South Africa, Steyn (1930) and Steyn (1940) described a condition affecting wethers as “pisgoed” or “pisgras”, which resembled sheathrot clinically and was confirmed as infectious in nature. He was able to transmit the disease by inoculation of exudates from affected sheep into other hosts.

2.1.3 Contagious pustular dermatitis (CPD)

Contagious pustular dermatitis (CPD), often referred to as orf, contagious ecthyma or scabby mouth is a contagious disease that can also be transmitted venerealy. The lesions are commonly found on the lips, muzzle, ears, and buccal cavity of sheep (Munz and Dumbell, 1994[a]). The disease is caused by a parapox virus and is characterized by small pustules which can subsequently develop into granulo-ulcerative lesions on the prepuce, penis and skin of the vulva (Linklater and Smith, 1993). The lesions are proliferative rather than ulcerative. In the genital form of CPD, lesions occur on the prepuce and penis, and on the vulval labiae at the mucosal-cutaneous junction (Munz and Dumbell, 1994[b]). CPD appears shortly after rams are put out for mating. The disease may also occur in pedal forms, where the coronet and interdigital spaces are involved.

2.1.4 Ulcerative dermatosis

Ulcerative dermatosis, also referred to as ovine venereal disease (lip and leg ulceration) is a contagious disease of sheep characterized by the formation of encrusted ulcers on the face, prepuce, penis and vulva. The disease is considered to be of viral aetiology, but the virus has not yet been classified (Tunnicliff, 1949; Kimberling 1988, Munz and Dumbell, 1994[b]). Reports have indicated that the viral agent causing ulcerative dermatosis is physically similar, but antigenically different from CPD virus (Trueblood and Chow, 1963; Radostits, Blood, and Gay, 1994). An infectious condition with epidermal and subcutaneous tissue destruction causing granulated ulcers of the lips, legs, feet, lips of the vulva, the prepuce at the orifice and glans penis of infected animal was described as CPD in the USA by Tunnicliff in 1949. This description is still valid as recent reports supported the claim that the lesions in ulcerative dermatosis are generally ulcerative rather than proliferative similar to CPD (Kimberling, 1988; Munz and Dumbell, 1994[b]).

2.1.5 Ulcerative balanitis and vulvitis

Contagious ulcerative lesions of unknown aetiology are often observed on the penis of rams and the vulva of ewes during the breeding period (Greig, 2000). A deep ulcer is formed on the tip of the gland penis, which in most cases is filled with blood clots. The vulva of affected ewes often showed marked oedema and reddened erosions. Affected rams and ewes often refuse coitus, and as a result the conception rate is reduced with serious economic implications.

In ewes, ulcerative vulvovaginitis begins as an inflammatory reddening of the vulval lips associated with marked swelling and ulceration of the ventral vulval commissure,

clitoris, and the posterior part of the vagina (Dent, 1971). Ulcerative vulvovaginitis and balanoposthitis were noticed to co-exist in the same flocks, and were assumed to be venereal diseases (Dent, 1971; Blood, Radostits, Arundel and Gay, 1989).

Although, no infectious agent has consistently been isolated and regarded to be the major causative agent of the disease, the following organisms have been isolated and described in the literature: *Acholeplasma laidlawii* and *Acholeplasma axanthum* (ulcerative genital disease) (Jones, Rae, Holmes, Lister, Jones, Grater and Richards, 1983); a herpesvirus antigenitically related to infectious bovine rhinotracheitis virus from an outbreak of vulvovaginitis in goats (Rosadio, Evermann and Mueller, 1984; Grewal and Wells, 1986); bacterial organisms (Ball, Kennedy and Ellis, 1991; Trichard *et al.*, 1993; Kidanemariam *et al.*, 2005); *Ureaplasma* spp. (Doig and Ruhnke, 1977; McCaughey and Ball, 1983), and *Mycoplasma* species (Cottew *et al.*, 1974; Doig and Ruhnke, 1977; Livingstone and Gauer, 1983; Kapoor *et al.*, 1984; Trichard *et al.*; 1993; Kidanemariam *et al.*, 2005). Caprine herpesvirus was reported to be present in ulcerative vaginal and vulval lesions of ewes but absent in rams (Horner *et al.*, 1982; Grewal and Wells, 1986). These reports contradict that of Tarigan *et al.* (1987) who had isolated caprine herpesvirus from a clinical case of balanoposthitis in a male goat. There was also a severe outbreak of ulcerative vulvovaginitis and balanophosthitis in a lowland flock in East Anglia from which ovine herpesvirus type 2 (OvHV-2) was detected by PCR from blood of two acutely affected ewes, from vulval ulcers of one of them and from the penis of an affected ram (Pritchard, *et al.*, 2008).

2.1.6 Global perspective

Ulcerative conditions of the genital tract of sheep have been described in many countries such as South Africa (Trichard *et al.*, 1993; Bath and De Wet, 2000;

Kidanemariam *et al.*, 2005); Australia (Cottew *et al.*, 1974; Webb and Chick, 1976; Grewal and Wells, 1986; Tarigan *et al.*, 1987); Canada (Doig and Ruhnke, 1977); India (Kapoor *et al.*, 1984; Singh, Rajyan, and Mohanty, 1974); New Zealand (Horner *et al.*, 1982); Spain (Loste *et al.*, 2005); Nigeria (Chima, Ojo, and Adetosoye, 1992) and the United Kingdom (Greig *et al.*, 2007; Pritchard *et al.*, 2008). While Cottew *et al.* (1974), Doig and Ruhnke (1977), Ball and McCaughey (1982) reported cases only in ewes, other researchers such as Trichard *et al.* (1993), Bath and De Wet (2000), Kidanemariam *et al.* (2005), Greig *et al.* (2007), Pritchard *et al.* (2008) reported the disease in both rams and ewes. So far, these are the only countries where the disease has been identified with some scientific evidence. Although the disease may be present in other countries, there are no reports to confirm it.

However, attempts to isolate the actual pathogen involved in the disease had failed to consistently identify specific organisms (Webb and Chick, 1976; Deas 1983; Linklater and Smith, 1993; Trichard and Van Tonder, 1994; Greig, 2000). The first isolate to be associated with the disease was *Mycoplasma* species 2D which was isolated from sheep with reproductive problems (Cottew *et al.*, 1974; Livingstone and Gauer, 1983). However, the aetiological role of this species has not been proved as it has also been isolated from animals in a healthy flock (Carmichael, St George, Sullivan, and Horsfall, 1972; Livingstone and Gauer, 1983).

Other species of *Mycoplasma* suspected as pathogens in genital infections of small stock include *Mycoplasma capricolum* (Jones, *et al.*, 1983), *Mycoplasma arginini* (Jones, *et al.*, 1983), *Mycoplasma mycoides mycoides* (Cottew *et al.*, 1974; Trichard *et al.*, 1993), *Mycoplasma agalactiae* (Jones *et al.*, 1983), *Mycoplasma fermentans* and *Mycoplasma bovigenitalium* (formerly *Mycoplasma* ovine group 11) (Nicholas, Greig,

Baker, Ayling, Heldtander, Johansson, Houshaymi and Miles, 1998; Ayling, Bashiruddin and Nicholas, 2004). *Acholeplasma laidlawii* and *Acholeplasma axanthum* (Jones *et al.*, 1983; Kapoor *et al.*, 1984), and *Ureaplasma* (Ball and McCaughey, 1982; Livingstone and Gauer, 1982). Although, *Acholeplasma* species have been isolated from animals with vulvovaginitis (Kapoor *et al.*, 1984), their potential role as disease causing agents is still uncertain.

Ulcerative balanoposthitis and vulvovaginitis have been described as viral infections by many authors who could not associate the disease with mycoplasmas or bacteria. Recently, Greig *et al.* (2007), divided the disease ulcerative balanoposthitis and vulvovaginitis into four main disease entities: venereal parapox-virus (orf) infection, enzootic posthitis (pizzle rot) caused by *Corynebacterium renale* or other diptheroid organisms, a mycoplasma associated vulvovaginitis (related to *Mycoplasma mycoides* subsp. *mycoides*) and a condition of unknown aetiology.

An investigation into a viral aetiology using tissue cultures and electron microscopy has failed to reveal the involvement of any virus in ulcerative balanitis and vulvitis in sheep (Webb and Chick, 1976; Deas, 1983; Trichard *et al.*, 1993), although viruses have been isolated in some cases of the disease (Pritchard *et al.*, 2008). Numerous pathogenic bacteria such as *Corynebacterium renale*, *Arcanobacterium pyogenes*, *Enterococcus faecalis* and *Streptococcus zooepidimicus* have been isolated from the lesions (Ball and McCaughey, 1982; Deas, 1983; Dunn, 1996; Kidanemariam *et al.*, 2005). Some opportunistic bacteria always aggravate the progression of the *Mycoplasma* and *Ureaplasma* infection. This is evidenced by a report that application of broad-spectrum antibacterial treatment can improve the clinical condition (Ball and McCaughey, 1982).

Ulcerative balanoposthitis and vulvovaginitis can occur usually after mating especially during the breeding season (Gummow and Staley, 2000; Jones *et al.*, 1983; Cottew *et al.*, 1974). An outbreak of the disease could affect up to 50 % of the exposed flocks (Jones *et al.*, 1983) and lead to a reduction of lambing percentages by more than 50 % in the affected flocks (Bath and De Wet, 2000).

2.1.7 Features of the disease seen in Dorper sheep in South Africa

The clinical expression of the disease in South Africa justifies the statement that the terms ulcerative balanitis and vulvitis (ub/uv) better describe the clinical signs of this condition (Kidanemariam, 2003). When referring to the disease in the South African context in this dissertation, the terms balanitis and vulvitis will be used.

The first sign indicating the presence of the disease is the appearance of blood around the vulva of ewes, and also on the wool around the preputial orifice of rams. Other clinical signs of the disease described in South Africa include swollen and reddened vulvae. In ewes the swelling is also accompanied by discrete mucosal ulcers at the mucocutaneous junction of the vulval labia. Close examination of the vulval labia may reveal shallow, blistering-like lesions covered with scabs. In the case of infected rams, ulcerative lesions can be observed on the soft glans of the penis. Hyperaemia of the glans penis can also be a sign in rams while other parts of the penile tissue and preputial mucosa remain unaffected (Kidanemariam *et al.*, 2005).

The affected rams are often reluctant to mate, but when they do mate, blood oozes from the preputial opening. Preputial haemorrhages also occur with frequent urination, which is often accompanied by straining. Because of the constant irritation caused by the infection, coupled with invasion of secondary pathogens, the lesions are aggravated.

The affected areas become swollen resulting in erosions of the preputial mucous membrane. The refusal to coitus leads to reduced conception rates with ensuing serious economic implications. Almost 10-20 % reduction in lambing may be caused by the infection (Gummow and Staley, 2000).

2.1.8 Pattern of disease distribution in South Africa

The occurrence of ulcerative balanoposthitis and vulvitis in South African Dorper sheep has been known since 1979, but the first scientific report describing the disease was published in 1993 (Trichard *et al.*, 1993). The distribution pattern of the disease seems to follow the geographical location of Dorper sheep in South Africa indicating that the disease affected mostly Dorper sheep (Trichard *et al.*, 1993; Bath and De Wet, 2000; Gummow and Staley, 2000). Since Dorper sheep are predominantly reared in dry areas of South Africa, the disease generally affected regions such as Northern, Western and Eastern Cape Provinces, KwaZulu Natal and the Free State Province (Gummow and Staley, 2000; Kidanemariam, *et al.*, 2005).

The epidemiology, aetiology and control of the disease in South Africa are still unresolved. However, Trichard *et al.* (1993) have carried out a field trial and laboratory experiments to determine the cause of the disease. They reported that ewes experimentally infected with a field strain of *Mycoplasma mycoides mycoides* LC and in which coitus was allowed, developed vulvovaginitis and the corresponding rams developed ulcerative balanoposthitis. The disease was also reproduced in another group of ewes during experimental infections. These results have provided support for *Mycoplasma mycoides mycoides* LC as the causative organism of ulcerative balanoposthitis and vulvovaginitis of Dorper sheep in South Africa. However, other invading bacteria have also been isolated from field samples. This claim is still the only

valid one associating the disease with *Mycoplasma mycoides mycoides* LC, hence the need to verify this claim with more data. This claim is also supported by other researchers such as Bath and De Wet (2000), Gummow and Staley (2000) and Kidanemariam *et al.,* (2005), although they reported other bacteria in association with *Mycoplasma mycoides mycoides* LC.

There are reports of the isolation of viruses from animals suffering from the disease in other parts of the world but several investigations of ulcerative balanitis and vulvitis have failed to detect any viruses associated with the disease in South Africa (Trichard *et al*., 1993; Kidanemariam *et al*., 2005).

Although the lesions can be observed during clinical examination of the rams (which is a pre-requisite prior to the start of the breeding season in South Africa), the outbreaks of the disease are mostly observed a few days after the start of mating. The first sign noticed by shepherds or stock owners is blood on or around the vulva of the ewes and on the wool around the preputial orifices of the rams.

It is assumed that secondary bacterial infection can cause more complications by causing damage to the soft tissues of the penis. This leads to accumulation of pus and formation of dead tissue on the penis and preputial cavity, thus causing either phimosis or paraphimosis. Affected ewes can produce lambs if mated with a fertile ram. The disease often appears to be self-limiting as evidenced by spontaneous recovery of infected sheep. However the disease may flare up occasionally in affected flocks (Bath and De Wet 2000) and the effort of farmers to contain the disease through treatment have not always been successful. The drugs commonly used by farmers are

tetracyclines and topical application of acriflavine-glycerine mixtures or iodine solutions (Gummow and Staley, 2000).

2.2 MOLLICUTES AS CONTRIBUTING CAUSES OF ULCERATIVE BALANOPOSTHITIS AND VULVOVAGINITIS

2.2.1 General introduction

The class Mollicutes belongs to the order Mycoplasmatales (Family: Mycoplasmataceae). There are eight genera in the class Mollicutes namely *Mycoplasma, Ureaplasma, Spiroplasma, Acholeplasma, Anaeroplasma, Asteroleplasma, Entomoplasma* and *Mesoplasma*. The last two genera were recently identified. Some species of *Mycoplasma* and *Acholeplasma* are associated with insects and plants and have been re-classified under these two new genera including some species from the *Spiroplasma* genus. The differences in their generic characteristics are based on molecular data, morphology, genome sizes, nutrition and ecological habitat (Razin, and Freundit, 1984; Tully, 1989). More than 100 species have been isolated from vertebrates, plants and insects and the largest group is from the genus Mycoplasma with more than 90 species.

Mollicutes possess small genomes, indicating that they might have evolved from the clostridia-bacilli branch of the phylum Firmicutes many years ago by losing considerable regions in their genome (Dandekar, Snel, Schmidt, Lathe, Suyama, Huynen and Bork, 2002). This group of organisms was erroneously classified as viruses because they passed through filters which normally block the passage of bacteria. The class Mollicutes consists of wall-less prokaryotes which are small in size, with genome sizes ranging from 580 bp (*Mycoplasma genitalium*) to 1 358 bp (*Mycoplasma penetrans*) (Sasaki, Ishikawa, Yamashita, Oshima, Kenri, Furuya, Yoshino, Horino, Shiba, Sasaki,

and Hattori, 2002), and have a single circular chromosome of double stranded DNA. The DNA contains 24-33 mol % of guanidine and cytosine, which is low compared to other Gram-positive prokaryotes. The distribution of guanidine and cytosine in the genome are uneven (Razin, Yogev and Naot, 1998). The variable genome sizes cut across genera and strains of the same species. One of the reasons attributed to this variability is the frequent occurrence of the repetitive monomers, which consist of segments of protein-coding genes differing in the size and number of insertion sequences (Mrazek, 2006).

Species of some genera of the *Mollicutes* have been known to be pathogenic in ruminants most notably mycoplasmas, which cause pneumonia, arthritis, conjunctivitis, vulvovaginitis and balanitis. Mycoplasma was first identified from a case of pleuropnemonia in cows; the organism was designated as “pleuropneumonia-like organism”, or PPLO. This term is still used today, particularly for certain mycoplasmas (Eaton, Meiklejohn and Herick, 1944; Eaton and Low, 1967). Mycoplasmas are the smallest self-replicating organisms, and highly pleomorphic because they lack definite cell walls which also render them completely resistant to β-lactam and other cell wall targeting antibiotics (Razin, 1992). Mycoplasmas have limited biosynthetic capabilities as a consequence of small genomes; they therefore require intimate association with mammalian or plant cell surfaces as ubiquitous parasites (Razin *et al.*, 1998). They are frequently implicated in respiratory and urogenital tract infections in a variety of mammalian and avian species (Baseman and Tully, 1997). In contrast to other pathogenic bacteria where virulence is mostly determined by toxins, invasins, and cytolysins, pathogenic *Mycoplasma* species appear to have no such primary virulence factors, as revealed by the genomic sequence analysis of eight species that have been

completely characterized (Wang, Wilkinson, Nicol, Nusbaum, Birren, Berg and Church, 2004).

2.2.2 Identification of the Genus *Mycoplasma*

During *in vitro* growth under laboratory conditions, most of the species require complex, undefined media and it may take several days to weeks of incubation because of their limited biosynthetic capacities. The optimum growth conditions are temperatures of 36-37 ºC in an atmosphere of air with 5 % CO_2 and pH of 7.0 (Razin *et al.*, 1998). *Mycoplasma* spp. can be identified with the aid of biochemical and serological tests. Tests used include glucose fermentation, arginine utilization, tetrazolium-HCl reduction, urea hydrolysis, sensitivity to digitonin, serum digestion, phosphatase activity, and metabolism of carbohydrates (Goll, 1994). In the digitonin test, the zone of inhibition surrounding a digitonin-containing disk is used to differentiate between *Mycoplasma* and *Acholeplasma* species (Thurmond, Holmberg and Luiz, 1989). However, results from biochemical and serological tests are sometimes contradictory. Identification and classification of *Mycoplasma* spp became increasingly dependent on serological tests (Gois, Kuksa, Franz and Taylor-Robinson, 1974), using techniques such as growth inhibition (GI), metabolic inhibition (MI), complement fixation and enzyme linked immunoabsorbent assays (ELISA) (Goll, 1994). The use of DNA probes has also become increasingly important in the identification of *Mycoplasma* spp. (Taylor, Wise and Mcintosh, 1985). The GI and MI tests have been found to be very specific (Freundit Andrews, Ernø, Kunze and Black, 1973) and it is therefore suitable for the demonstration of intra-species differences (Gois *et al.*, 1974). However, MI is more sensitive than GI (Goll, 1994).

Previously, standardized methods for identification of mycoplasmas were based on serological procedures. However, serological cross-reactivity between the species and strains often hinder the identification of mycoplasma isolates, particularly in the mycoides cluster. The mycoides cluster of bovine, caprine and ovine pathogenic mycoplasmas contain six glucose fermenting groups (Rawadi, Lemercier and Roulland-Dussoix, 1995) namely, *M. mycoides* subsp. *mycoides* small and large colony biotypes; *M. mycoides* subsp. *capri; M. capricolum; M.* sp. type F38 and *M.* sp. *bovine* serogroup 7 (Cottew, Breard, Damassa, Ernø, Leach, Lefevre, Rodwell and Smith, 1987). Each species is a significant animal pathogen, and is closely related, showing serological cross-reaction, with similar biochemical features (Cottew *et al.*, 1987; Ernø *et al.,* 1987).

Comparison of the nucleotide sequences of the 16S rRNA genome among the 15 prototype strains of human mycoplasmas and ureaplasmas demonstrated that the flanking regions of 5' V3 are highly conserved among prokaryotes and that the flanking regions of 3' V3 are conserved among mycoplasmas and ureaplasmas. Based on the 16S ribosomal RNA sequences, mycoplasmas have been divided into five groups (Homonis group, Pneumoniae group, Spiroplasma group, Anaeroplasma group and the mycoides cluster) (Weisburg, Tully, Rose, Petzel, Oyaizu, Yang, Mandelco Sechrest, Lawrence, Van Etten, Maniloff and Woese, 1989).

Other methods such as protein fingerprints (Rodwell, 1982; Costas, Leach and Mitchelmore, 1987) or genomic DNA analysis (Bonnet, Saillard, Bove, Leach, Rose, Cottew and Tully, 1993) have been used in order to redefine the taxonomy within the mycoides cluster. However, these remain difficult because mycoplasmas included in

this cluster are closely related and have similar biochemical features, and show serological cross-reactions (Cottew *et al.*, 1987).

The common traits exhibited by the *Mycoplasma mycoides* cluster often cause confusion both in diagnosis and taxonomy (Cottew *et al.*, 1987). Classification of mycoplasmas belonging to the mycoides cluster has always been problematic, because there is only a few biochemical or physiological properties which can be used for differentiation of these species. A diversity of other characteristics such as morphology, growth rate, host spectrum, and pathogenicity should also be considered in the classification.

Serological methods have been extensively used for the definition of species within the genus *Mycoplasma.* Based on growth inhibition and immunofluorescent tests, *Mycoplasma mycoides* subsp. *capri* was found to be serologically different from *Mycoplasma mycoides* subsp. *mycoides* isolated from cattle (Al-Aubaid and Fabricant, 1971). Further studies have confirmed that *Mycoplasma mycoides mycoides* large colony and *Mycoplasma mycoides capri* were inseparable by protein analysis. Mycoplasma isolates from goats were also found to be serologically indistinguishable from *Mycoplasma mycoides* subsp. *mycoides* isolated from cattle. However, they differ in several physiological and biochemical features, and therefore were provisionally designated as small colony (SC) and large colony (LC) types, respectively (Cottew and Yeats, 1978).

The results of serological analyses are often difficult to interpret for members of the *Mycoplasma mycoides* cluster because of immunological cross-reactions notably between *Mycoplasma capricolum* subsp. *capripneumoniae* and *Mycoplasma* sp. strain

PG50, as well as some strains of *Mycoplasma capricolum* subsp. *capricolum* (Bolske, Msami, Humlesjo, Erno and Johansson, 1988). The close relationship between these species has been confirmed by one or two dimensional polyacrylamide gel electrophoresis, which has been used to classify the organisms on the basis of their protein profiles (Thiaucourt, Lorenzon, David and Breard, 1994).

DNA hybridization has also been used to study the relatedness of the genomes of the members of the *Mycoplasma mycoides* cluster (Bonnet, Saillard, Bove, Leach, Rose, Cottew and Tully, 1993). *M. capricolum* subsp. *capripneumoniae,* was found to be closely related to *Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma* species strain PG50. *M. mycoides* subsp. *capri* and the *M. mycoides mycoides* LC types were more distantly related to *M. capricolum* subsp. *capricolum.* The relatedness between strains from the two different species was about 70 %.

DNA-DNA hybridization studies revealed variable values for DNA homology between *Mmm*LC and *Mmm*SC (88-93 %), and between Mmc and *Mmm*SC (75-93 %) depending on the experimental conditions (Christiansen and Ernø, 1982). However, DNA hybridization only gives a rough estimate of the relatedness between organisms, and the method is prone to variability due to difficulties in controlling the experimental conditions.

Different DNA homology tests have been used in order to resolve the taxonomic discrepancies and the phylogenetic relationships (Taylor, Bashiruddin and Gould, 1992). One of the most widely used and precise methods for phylogenetic analysis is the sequence comparison of 16S ribosomal RNA genes (Olsen and Woese, 1993). Phylogenetic studies of mycoplasmas belonging to the *mycoides* cluster based on 16S

ribosomal RNA sequences showed ambiguities (Ros-Bascunana, Mattson, Bölske and Johansson, 1994). Furthermore, this kind of approach entails considerable time and effort. In the last few years the progress of PCR technology has allowed the development of finger-print techniques to differentiate similar DNA templates. In recent years a series of reports have described the use of arbitrary designed primers to type closely related mycoplasma strains (Caetano-Anollies, 1993). The arbitrary-primed PCR technique has shown to be more sensitive than multilocus enzyme electrophoresis for distinguishing related mycoplasma strains (Wang, Whitman, Berg and Berg, 1994).

Phylogenetic studies based on the sequence analysis of 16S rRNA genes revealed 99 % similarity between *Mmm*LC and *Mmc*. These results suggest that the two mycoplasmas could be grouped into a single subspecies, one distinct from *Mmm*SC (Pettersson, leitner, Ronaghi, Bolske, Uhlén and Johansson, 1996). Consequently, it is difficult to identify any strains of mycoplasma in this group by conventional methods as this has lead to erroneous diagnoses. Identification of new isolates and diagnosis of diseases caused by members of the *Mycoplasma mycoides* cluster are difficult, and improved methods are surely needed. Sequence analysis of certain genes is therefore an extremely useful complement or alternative to conventional methods for identification, and for phylogenetic studies. Complete and partial sequences of the 16S rRNA genes from the rrnA and rrnB operons have been determined for some members of the *M. mycoides* cluster (Pettersson, Johansson and Uhlén, 1994).

The phylogeny of some members of the *M. mycoides* cluster has been studied by sequence analysis of complete 16S rRNA sequences (Ros-Bascunana *et al.*, 1994), or sequences of PCR products of genomic segments of unknown function (Taylor, Bashiruddin and Gould, 1992). The rRNA genes are highly conserved and a restriction

enzyme map of this gene has been constructed partly as probes for characterization of each group of mycoplasmas, and is used to determined relatedness of the corresponding organisms (Christiansen and Ernø, 1990).

Diagnostic testing for the members of the mycoides cluster proves difficult because of the similarities in clinical signs caused by each species and the high degree of similarity between them (phenotypically and genetically). In addition, intra-species heterogeneity has been observed in *Mmm*LC, *Mmc* and *Mcc*, while *Mmm*SC and *Mccp* appear to be homogeneous (Thiaucourt, Lorenzon, David and Breard, 2000). As a result of the diagnostic challenge, numerous PCR assays have been developed based on various gene targets such as CAP-21 (Bashiruddin, Taylor and Gould, 1994), *Mycoplasma mycoides* cluster (Rawadi *et al.*, 1995), 16S rRNA (Bolske, Mattsson, Bergström, Ros-Bascunana, Wesonga and Johansson, 1996), lipoprotein gene (Monnerate, Thiaucourt, Poveda, Nicolet and Frey, 1999b) and insertion element (Vilei, Nicolet and Frey, 1999).

A new molecular method, namely denaturing gradient gel electrophoresis of the 16S rDNA, offers a rapid method for detecting *Mycoplasma* species. It can also detect multiple mycoplasma infections in mixed cultures for both sheep and goats (McAuliffe, Ellis, Lawes, Ayling and Nicholas, 2005). Real time PCR (RT-PCR) assays have been developed and are highly sensitive and specific and it provides accurate detection and differentiation of the members of the mycoides cluster (Fitzmaurice, Sewell, Manson sylvan, Thiaucourt, McDonald and O'Keefe, 2008). Several of these assays require further analysis using restriction enzyme digestion or DNA sequencing and also post PCR processing such as gel electrophoresis or Southern blotting.

2.2.3 Major mycoplasmas of sheep and goats

2.2.3.1 *Mycoplasma agalactiae*

One of the most important diseases of sheep and goats caused by *Mycoplasma agalactiae* is contagious agalactia. This disease is characterized by mastitis, arthritis, keratitis, pneumonia and septicaemia (MAKePS) (DaMassa, Wakenell and Brooks, 1992). Because of its economic importance, the disease is listed by the World organization for Animal Health (OIE) as a notifiable disease. Cases of abortion have also been associated with *M. agalactiae* infection of sheep and goats in Spain (Ramirez, Garcia, Diaz-bertarana, Fernandez and Poveda, 2001). The organism has been reported to be involved in the pathogenesis of other diseases such as granular vulvovaginitis in goats (Singh *et al.*, 1974; DaMassa, 1983), pneumonic lungs (Loria, Summation, Nicholas and Ayling, 2003), and non purulent encephalitis (Loria, Caracappa, Monteverde and Nicholas, 2007).

2.2.3.2 *Mycoplasma mycoides mycoides* large colony variant (*Mmm*LC)

*Mmm*LC is a member of the mycoides cluster; a group that share serological, genomic and antigenic characteristics (DaMassa *et al.*, 1992). However, the involvement of *Mmm*LC in any pathological disease is still not clearly defined but it is suspected to cause disease in small ruminants (Nagalic, Hotzel, Ball, Seol and Busch, 2001). This mycoplasma has been isolated from goats with polyarthritis, conjunctivitis, keratitis, pneumonia, and cervical abscesses (Rosendal, Erno and Wyand, 1979; Singh Vijendra, Srivastava, manoj Kumar, Jai Sunder and Varshney, 2004). *Mmm*LC biotype has been reported as a pathogen causing ulcerative genital disease of sheep in South Africa (Trichard *et al.*, 1993; Kidanemariam, *et al.*, 2005).

2.2.3.3 *Mycoplasma mycoides* subsp. *capri*

Mycoplasma mycoides subsp. *capri* is a member of the *Mycoplasma mycoides* cluster, and it is considered as the pathogen involved in contagious caprine pleuropneumonia (CCPP) of goats for many years until MacOwan and Minette (1976) reported the isolation of a new *Mycoplasma*, designated as F38, from a case of fibrinous pneumonia. Although, the new isolate (F38) exhibits a high virulence under experimental conditions, further work is still required to establish whether it is one of the primary causes of classical CCPP. *Mycoplasma mycoides* subsp. *capri* was reported to be specific for goats only, and it causes septicaemia, polyarthritis, and agalactia (Rosendal, 1994). Recent findings indicated that it is also present in sheep, and causes agalactia (Waleed Al-Momani, Mahmoud, Halablab, Mahmoud, Abo-Shehada, Katie, Miles, Laura McAuliffe and Nicholas, 2005). However, the inoculation of Nigerian goats with *mycoplasma mycoides* subsp. *capri* did not induce either arthritis or mastitis (DaMassa *et al*., 1992).

2.2.3.4 *Mycoplasma capricolum* subspecies *capripneumoniae*

The isolation of a new mycoplasma (F38) from a CCPP outbreak in Kenya as a pathogen of a highly contagious form of pneumonia in goats was reported by MacOwan and Minnett in 1976. The organism was designated as *Mycoplasma capricolum capripneumoniae* (Leach, Erno and MacOwan, 1993). Since then the name has been widely used for the causative agent of classical contagious capripneumonia. It is also classified as a member of *Mycoplasma mycoides* cluster and is the causative agent of contagious caprine pleuropneumonia (CCPP) primarily in goats, causing caprine pneumonia, although it has been found in sheep and cows. In goats this organism is highly virulent, causing significant mortality and morbidity (Wesonga *et al*., 2004).

2.2.3.5 *Mycoplasma capricolum*

Mycoplasma capricolum has been reported in many diseases such as ovine arthritis (Yamamoto, 1990), pneumonia, conjunctivitis, and arthritis (Taoudi, Johnson and Kheyyali, 1987). The *Mycoplasma capricolum* infection normally progresses with septicaemia and severe lesions in the joints leading to permanent lameness. The organism also causes fever in young goats (Wesonga *et al.*, 2004). There is evidence that *Mycoplasma capricolum* is present in the ear canal of sheep and goats (Cottew and Yeats, 1982), and the respiratory and genital mucosa of goats (DaMassa, Brooks and Holmberg, 1984). The organism has been isolated from cases of vulvovaginitis and balanophosthitis in sheep (Jones, 1983), and severe pneumonia in a goat kid (DaMassa, Brooks and Adler, 1983).

2.2.3.6 *Mycoplasma arginini*

Mycoplasma arginini occurs in goats and sheep, and has been isolated from various anatomical sites of other hosts including cows and horses (Jones, Rae and Holmes, 1983) and is considered to be a low pathogenic organism. The organism has been isolated from the genital tract of small ruminants (Rosendal *et al.*, 1994) and ovine keratoconjunctivitis (Leach *et al.*, 1970) but the pathogenicity of the organism is still in doubt (Chima, Erno and Ojo, 1986).

2.2.3.7 *Mycoplasma ovipneumoniae*

Mycoplasma ovipneumoniae plays an important role in diseases of goats and sheep. It has been isolated from the lungs, trachea, nose, and eyes of sheep. It has been proven

that *Mycoplasma ovipneumoniae* causes proliferative exudative pneumonia in sheep together with *Mannheimia haemolytica* (Rosendal *et al.*, 1994).

2.2.3.8 *Mycoplasma conjunctivae*

Mycoplasma conjunctivae were isolated from clinical cases of keratoconjunctivitis together with other bacteria. It was suggested as the primary agent of the disease (Jones, Foggie, Sutherland and Harker, 1976) and has been proven to induce the disease experimentally (Greig, 1989).

2.2.3.9 *Acholeplasma laidlawii*

Acholeplasma laidlawii is a common organism, and it has been isolated from cases of vulvovaginitis in goats in Nigeria (Chima, Erno and Ojo, 1986) and in Dorper sheep in South Africa (Kidanemariam *et al.*, 2005).

2.2.3.10 *Acholeplasma axanthum*

A. axanthum has been isolated from only one case of vulvar scabs of ewes in the United Kingdom, representing the only known isolation of this type of mycoplasma from sheep or goats. There is no report of pathogenic effects of the organism in goats or sheep (Jones *et al.,* 1983).

2.2.4 *Ureaplasma* spp.

Many *Ureaplasma* species have been isolated from both sheep and goats. Although vulvitis was reported to be reproduced in ewes following inoculation of *Ureaplasma* (Ball and McCaughey, 1982), there is generally little or no specific information on the role of

these organisms in their hosts as disease pathogens. Several strains of these organisms have been isolated from the urogenital tract or urine of sheep and goats (Livingston and Gauer, 1975).

CHAPTER 3
MATERIALS AND METHODS

3.1 *MYCOPLASMA* STRAINS

Thirty four strains of *Mycoplasma* species isolated from swabs and scrapings taken from the genital tract of Dorper sheep with clinical signs of ulcerative balanitis and vulvitis, and from clinically normal Dorper sheep were included in this study. The samples were collected in 2003 from animals from fifteen different farms covering five districts of the Northern Cape and Western Cape Provinces of South Africa. Several additional strains that were isolated in recent years from diagnostic submissions to the Faculty of Veterinary Science, University of Pretoria were also included. The original samples collected during 2003 were retrieved from storage and once again subjected to mycoplasmal isolation and purification procedures.

Samples were catalogued and stored at -85 ºC in Hayflick's medium as described by Ruhnke and Rosendal (1994), in the bacteriology laboratory of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

A *Mycoplasma* reference strain (*Mycoplasma mycoides* subsp. *mycoides* Y-goat 11706) and *Escherichia coli* strain S4 (09:K30) were obtained from the Bacteriology Section of the Department of Veterinary Tropical Diseases. The *Mycoplasma* strain was used as a positive control, and the *E. coli* strain and water were used as negative controls.

3.2 *MYCOPLASMA* GROWTH CONDITIONS

Mycoplasma strains were cultivated on Hayflick's agar medium (Ruhnke and Rosendal, 1994) and then subcultured on corresponding broth medium. The presence of L-form bacteria was determined by inoculation of the broth cultures onto blood agar plates (Simecka *et al.*, 1992). Bacterial colonies on blood agar plates were stained with Gram's stain and additional biochemical tests such as growth on McConkey agar, catalase, oxidase, glucose fermentation and arginine utilization was performed on all the samples, as described by Ernø and Stipkovits (1973).

3.3 NUCLEIC ACID-BASED ANALYSIS

3.3.1 DNA extraction

Three millilitres of cultured broth were centrifuged at 8 000 g for 10 min. These pellets were subjected to two extraction methods, a boiling method and a kit method (Qiagen QiaAmp® DNA mini kit, Whitehead South Africa).

The boiling method entailed suspension of the pellet in 200 µl phosphate buffered saline. This suspension was boiled at 96 ºC for 10 min, cooled on ice and centrifuged at 20 000 g for 2 min. The supernatant was collected and stored at -20 ºC until use.

The kit method (Qiagen QiaAmp® DNA mini kit, Whitehead Scientific, South Africa) was applied to the bacterial pellets. The pellet was suspended in 180 µl ATL buffer. The suspension was incubated at 50 ºC for 10 min. 180 µl AL buffer and 20 µl Proteinase K were added and mixed by vortexing. The mixture was incubated for 10 min at 56 ºC. After incubation 200 µl ethanol (96-100 %) was added followed by pulse-vortexing for 15 sec. The mixture was applied to a spin column supplied with the Qiagen kit. After

centrifugation the column was washed respectively with 750 µl of buffers AW1 and AW2. An additional centrifugation step for 1 min at 20 000g, to remove excess washing buffer, was applied to the column. DNA was eluted from the spin column using 50 µl AE buffer (Qiagen QiaAmp® DNA mini kit). Concentration determinations were done with a spectrophotometer (NanoDrop(R) ND-1000, Thermo Fisher Scientific, Inqaba Biotechnical, Industries (Pty) Ltd South Africa), and agarose gel electrophoresis (Sambrook *et al.*, 1989). Extracted DNA was stored at -20 ºC until use.

3.3.2 The polymerase chain reaction

PCR amplification was conducted by using an upstream primer specific for the 16S rRNA (Robertson *et al.*, 1993), and a downstream primer specific for the genus *Mycoplasma* (Van Kupperveld *et al.*, 1992) (**Table 1**). An additional set of primers that has been developed by Dr J Picard (Department of Microbiology, James Cook University, Australia) and designated croc primers (Croc 1 and Croc 2) (**Table 1**), were also used. Both primer sets amplified a product of ~1 078 base pairs (bp) in the 16S rRNA genome. The PCR was performed in a 25 µl reaction volume containing 12.5 µl Takara EX Taq™ Premix (Takara Ex Taq™1.25 units/µl, dNTP mixture, 2x concentration each 0.4 Mm, EX Taq™ buffer 2x including 4 mM Mg^{2+} (Separations, South Africa); 0.5 µl of each oligonucleotide primer (Myco-forward and Myco-reverse; Croc 1 and Croc 2) (20 pM/µl) (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) and 1 µl of the DNA (50-70 ng). The mixtures were subjected to 10 min initial denaturation at 94 ºC, followed by 35 cycles of amplification involving denaturation at 94 ºC for 30 sec, primer annealing at 59 ºC for 45 sec, and primer extension at 72 ºC for 45 sec; a final primer extension at 72 ºC for 7 min, using a DNA thermal cycler (Gene^{Amp} PCR system 9700, Applied Biosystems, South Africa). Amplified products were analyzed together with a DNA ladder (O' Gene ruler™, Fermentas Life Sciences,

Inqaba Biotechnical, Industries (Pty) Ltd Pretoria South Africa) on a 1.5 % agarose gel (Celtic Molecular Diagnostics, South Africa). Gels were stained with ethidium bromide, visualized and documented with Kodak Electrophoresis documentation (EDAS, 290) (Eastman Kodak Company, New York).

3.4 CLONING AND SEQUENCING OF PCR AMPLIFICATION PRODUCTS

Four PCR's were performed per sample and pooled. Pooled reactions were visualized on an agarose gel and purified before down-stream applications (cloning and sequencing) were performed. The QIAquick® PCR Purification Kit protocol (Qiagen, Whitehead Scientific, South Africa) was applied to the purified PCR products: 5 volumes of buffer PBI was added to one volume of amplification product and mixed. This mixture was applied to a spin column provided and centrifuged for 60 sec at 20 000 g. The flow through was discarded followed by a washing step using buffer PE. The flow through of the washing step was discarded and 50 µl of elution buffer (EB) was added to the column followed by a centrifugation step of 1 min at 20 000 g. Purified amplification products were used directly for down-stream applications or stored at -20 ºC until use. The concentration of purified products was determined by spectrophotometry and agarose gel electrophoresis

3.4.1 Cloning

Purified PCR products were cloned using the pJET® 1.2 cloning vector (Fermentas, Inqaba Biotechnical, Industries (Pty) Ltd Pretoria South Africa), and pGEM®T Easy vector system (Promega, Anatech, South Africa). The protocol of the pJET® 1.2 system has been adapted to be the same as the protocol for the pGEM® T Easy vector system. The ligation reactions were set up in 10 µl [(2x Ligation buffer; vector (50 ng), T4 DNA

Ligase (3 Weiss units/µl) and PCR amplification product (ratio: 1:3/vector:insert)]. The ligation reaction was performed in a dark room at room temperature overnight. 50 µl of competent cells (JM109, Promega, Anatech, South Africa) was added to 2 µl of the ligation reaction and incubated on ice for 20 min. The mixture was heat shocked for 45-50 sec at 42 ºC. After the heat shock the mixture was incubated on ice for 2 min. 1 ml of LB broth without antibiotics was added to the mixture and then incubated for 1.5 h at 37 ºC while shaking. Transformants were selected using Luria-broth (LB) agar plates containing 50 µg/ml of ampicillin, 200 mg/ml isopropylthio-ß-galactosidase (IPTG) and 20 mg/ml 5-bromo-4chloro-3-indolyl-ß-D-galactoside (X-Gal). (Fermentas life Sciences, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa). Recombinant colonies were selected after incubation at 37 ºC for 24 h. Selected colonies were grown in 5 ml LB broth containing 50 µg/ml of ampicillin (Fermentas Life Sciences, Inqaba Biotechnical, Industries (Pty) Ltd Pretoria South Africa). Plasmid DNA was purified using the High Pure plasmid purification kit (Roche diagnostics, South Africa). 1 ml *E. coli* culture was centrifuged at 6 000 g for 30 sec and the pellet was suspended in 250 µl suspension buffer containing RNase (High Pure Plasmid Purification kit, Roche Diagnostic, South Africa). 250 µl lysis buffer was added to the pellet suspension and the mixture was gently mixed by inverting the tube three to six times followed by incubation for 5 min at room temperature (between 15 ºC and 25 ºC). After incubation 350 µl chilled binding buffer was added followed by an incubation step of 5 min on ice. A centrifugation step at 13 000 g for 10 min followed the incubation. The supernatant was transferred into the upper buffer reservoir of a High Pure filter tube (High Pure Plasmid Purification Kit, Roche Diagnostic, South Africa). The column with the collection tube was centrifuged at 20 000 g for 1 min. After centrifugation, the flow through liquid was discarded and the filter tube was re-inserted in the same collection tube. Two washing steps followed using 500 µl wash buffer 1 (centrifugation at

20 000 g for 1 min) and 700 µl wash buffer 2 (centrifugation 20 000 g for 1 min). Plasmid DNA was eluted from the column by the addition of 100 µl elution buffer and centrifugation at 20 000 g for 1 min. Purified plasmids were tested by electrophoresis and PCR. The same PCR assay and primers as described in 3.3.2 in this chapter, was used.

3.5 SEQUENCING ANALYSIS

PCR amplification products and recombinant plasmid DNA were sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa for sequencing using the primer pairs as shown in **table 1**. Sequencing data obtained were assembled and edited to a total length of 1078 bp using Gap 4 of the Staden package (Staden, 1996). Sequencing data obtained was deposited in GenBank under Accession numbers shown in **table 4**.

Blast (Basic local alignment search tool) searches of the sequences were conducted using the National Centre of Bioinformatics website (http/www.ncbi.nih.gov) to determine the similarity between sequencing data obtained from local strains and those available in GenBank. Data was recorded as percentage similarity to related species.

Similarity matrices were constructed from six genera namely, *M. arginini, M. bovigenitalium, A. laidlawii M.* sp*. ovine/caprine* sero group II*, M. canadense and Mmm*LC, using the double parameter model of Kimura (1980) and the Jukes and Cantor correction model for multiple base changes (Jukes and Cantor, 1969). Phylogenetic trees were constructed using neighbor-joining (Saitou and Nei, 1987) and the maximum parsimony methods by using the Mega 3.0 software package (Kumar *et al.*, 2004). It was used in combination with the bootstrap method (Felsenstein, 1985) (1 000 replicates/tree for distance methods and 100 replicates/tree for parsimony methods).

Table 1 Primer sequences used for PCR and sequencing

No-	Primer pairs	Oligonucleotide sequences (5'-3')	Procedures performed	References
1	Myco-upstream (Croc1)	AGAGTTTGATCCTGGCTCAGGA	PCR Sequencing	Robertson *et al.*, 1993
2	Myco-downstream	TGCACCATCTGTCACTCTGTTAACCC	PCR Sequencing	Van Kupperveld *et al.*, 1992
3	FBAA5	GGAATATTGGACAATGGG	Sequencing	
4	RBAA5	GGAATATTGGACAATGGG	Sequencing	
5	FBAA6	GCGTGGGGAGCAAACAGG	Sequencing	
6	RBAA6	CCTGTTTGCTCCCCACGC	Sequencing	
7	FBAA7	ACGCGAAAAACCTTACC	Sequencing	Weisburg *et al.*, 1991
8	RBAA7	GGTAAGGTTTTTCGCGT	Sequencing	
9	FBAA8	GGAGGAAGGTGGGGA	Sequencing	
10	RBAA8	TCCCCACCTTCCTCC	Sequencing	
11	FBAA9	CGGTGAATACGTTCTCGGG	Sequencing	
12	RBAA9	CCCGAGAACGTATTCACCG	Sequencing	
13	pJET1.2/F	CGACTCACTATAGGGAGAGCGGC	Sequencing	pJET® 1.2 blunt cloning vector (Fermentas, Inqaba Biotechnology South Africa)
14	pJET1.2/R	AAGAACATCGATTTTCCATGGCAG	Sequencing	
15	Croc 2	GGTAGGGATACCTTGTTACGACT	PCR Sequencing	Dr J Picard, unpublished

CHAPTER 4
RESULTS

4.1 BACTERIOLOGICAL ANALYSIS

All 34 isolates obtained following culture on Hayflick's agar yielded negative results with Gram's staining method, and were negative with the catalase and oxidase tests. Eighteen isolates (18/34) hydrolysed arginine and 14 (14/34) were glucose positive (**Table 2**). Two (2/34) organisms (B1/01; B3/01) were not able to hydrolyse arginine or ferment glucose. All the isolates (n=34) were tested for L-forms of bacteria by growing them on blood agar, no bacterial growth was observed and biochemistry results were concordant with the results shown in **table 2**.

Table 2 Summary of the biochemical test results for the 34 isolates included in the study

No	Sample identity	Plate tests			Biochemistry tests				
		Hayflick's agar	Blood Agar	Mac-Conkey agar	Gram stain	Oxidase	Catalase	Arginine	Glucose
1	782/B567/10	+	-	-	-	-	-	+	-
2	B1197/08	+	-	-	-	-	-	+	-
3	B2639/07	+	-	-	-	-	-	+	-
4	B8973/06	+	-	-	-	-	-	-	+
5	D7/01	+	-	-	-	-	-	+	-
6	E3.5/01	+	-	-	-	-	-	+	-
7	KIR03/01	+	-	-	-	-	-	+	-
8	K5R01/01	+	-	-	-	-	-	-	+
9	K5R09/01	+	-	-	-	-	-	-	+
10	R3.6/01	+	-	-	-	-	-	-	+
11	K4E02/01	+	-	-	-	-	-	+	-
12	K6R06/01	+	-	-	-	-	-	+	-
13	8B/01	+	-	-	-	-	-	+	-
14	K4E03/05	+	-	-	-	-	-	-	+
15	K2E03/01	+	-	-	-	-	-	+	-
16	B8971/06	+	-	-	-	-	-	-	+
17	K1R04/01	+	-	-	-	-	-	+	-
18	B12294/09	+	-	-	-	-	-	+	-
19	B12296/09	+	-	-	-	-	-	-	+
20	B1/01	+	-	-	-	-	-	-	-
21	B3/01	+	-	-	-	-	-	-	-
22	B12291/09	+	-	-	-	-	-	-	+
23	D1/01	+	-	-	-	-	-	+	-
24	D5/01	+	-	-	-	-	-	+	-
25	E3.7/01	+	-	-	-	-	-	-	+
26	R3.2/01	+	-	-	-	-	-	-	+
27	E2.5/01	+	-	-	-	-	-	+	-
28	B1857/06	+	-	-	-	-	-	-	+
29	R3.4/02	+	-	-	-	-	-	-	+
30	787/B567/10	+	-	-	-	-	-	+	-
31	K2E01/02	+	-	-	-	-	-	_	+
32	B1179/sc/08	+	-	-	-	-	-	+	-
33	E3.6/02	+	-	-	-	-	-	-	+
34	C1/01	+	-	-	-	-	-	+	-
Total Positive		**34**	**0**	**0**	**0**	**0**	**0**	**18**	**14**

4.2 NUCLEIC ACID ANALYSIS

DNA was successfully extracted from all samples (n=34) and amplified using the conditions described in Chapter 3 (3.3.2). Extracted DNA was tested prior to amplification for purity and concentration by means of gel electrophoresis (**Figure 1**) and spectrophotometry. DNA concentrations ranged from 0.71 ng/µl to >266 ng/µl and 50-70 ng of DNA was subsequently used in 25 µl of the PCR mixture.

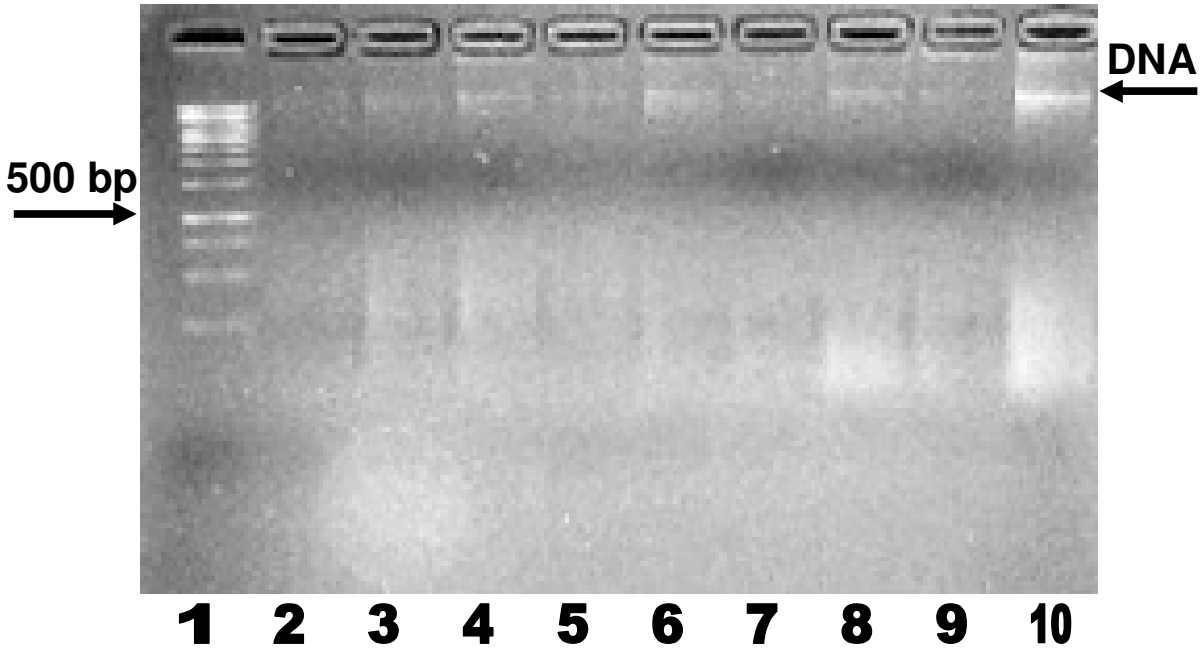

Figure 1 Electrophoretic analysis of unamplified DNA on a 1 % agarose gel. Lane 1 is the DNA ladder (Fermentas 1Kb 'O Gene ruler, Inqaba Biotechnical, industries (pty) Ltd Pretoria South Africa); Lanes 2-8 represent unamplified DNA from samples D7 (14.27 ng/µl); E3.5/01 (202.88 ng/µl); KIR03/01 (329.08 ng/µl); B3/01 (282.74 ng/µl); B12291/09 (282.74 ng/µl); D1/01 (2.43 ng/µl); D5/01 (7.01 ng/µl); E3.7/01 (266.61 ng/µl); Lanes 9-10 are extracted DNA from negative and positive controls namely *E. coli* strain S4 (09:K30) (17.08 ng/µl) and *Mycoplasma* reference strain (Y-goat 11706) (299.61 ng/1 µl).

The first amplification attempts using primer set Myco-upstream (Croc 1) and Myco-downstream (**Table 1**; **Figure 2**), resulted in low yields of amplification products. Primer set Myco-upstream Croc 1 and Croc 2 was applied (**Figure 3**), and resulted in better yields of amplification products. Products obtained using Croc 1 and Croc 2 were used in cloning and sequencing assays.

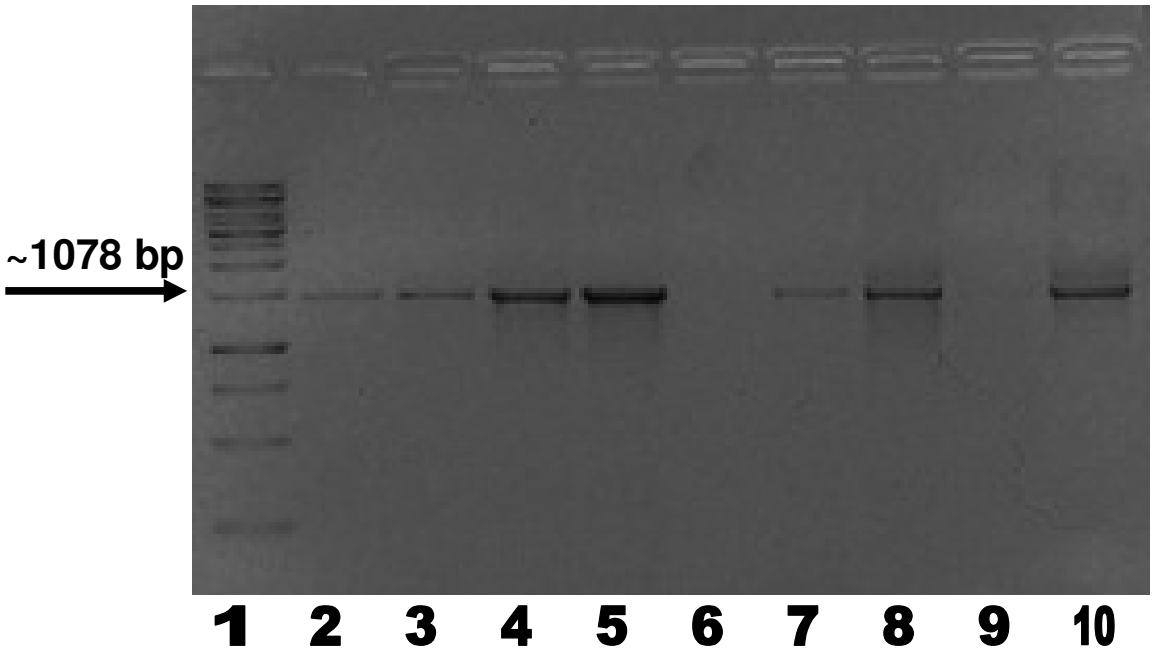

Figure 2 PCR products generated with Croc primers (Chapter 3, **Table 1**) on a 1 % agarose gel. Lane 1 is the DNA ladder (Fermentas 1 Kb 'O Gene ruler, Inqaba Biotechnical, industries (Pty) Ltd Pretoria South Africa); Lanes 2-8 represent isolates D7/01; E3.5/01; KIR03/01; B3/01; B12291/09; D1; D5/01; E3.7/01; lanes 9-10 represent the negative and positive controls respectively, namely *E. coli* strain S4 (09:K30) (17.08 ng/µl) and *Mycoplasma* reference strain (Y-goat 11706).

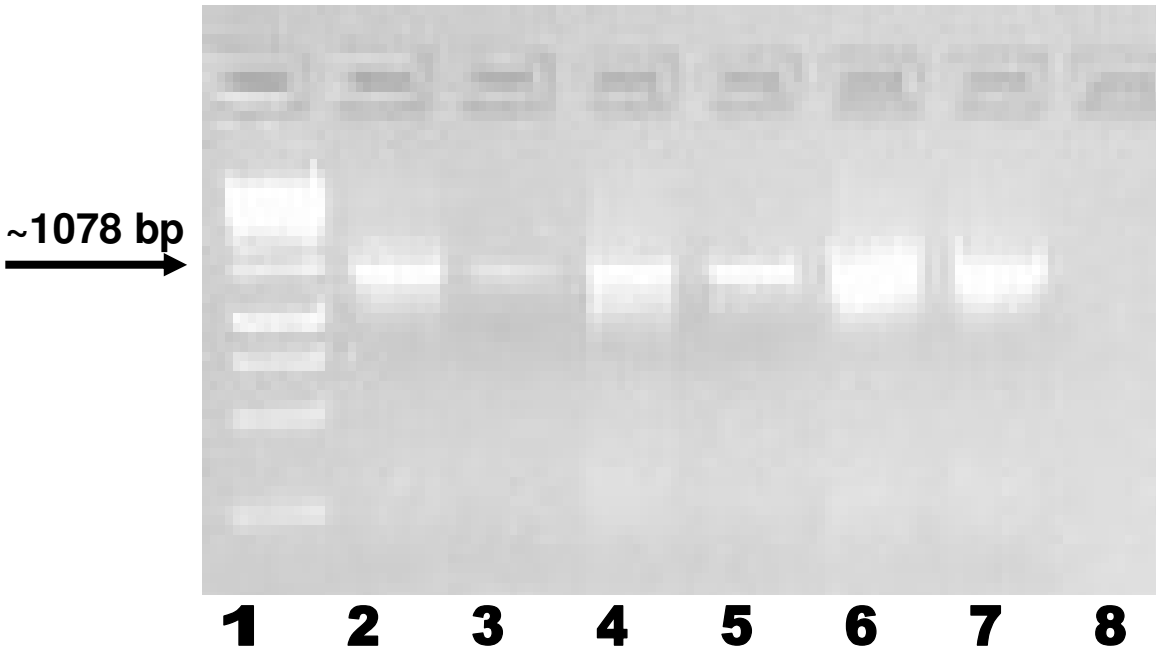

Figure 3 Illustrates the amplification product using Myco-upstream and Myco-downstream primers (**Table 1**). The amplification products of both primer sets are the same although they attach at different positions on the 16S rRNA genome.

Initially DNA from all 34 isolates was extracted, amplified and directly sequenced. The primer set Croc 1 and Croc 2 was used in the sequencing reaction. Good sequence data could only be obtained from 22 isolates and the decision was made to clone the PCR products for the remaining 12 isolates. The PCR products were cleaned and concentration determinations were done by means of gel electrophoresis (**Figures 4**) and spectrophotometry before cloning.

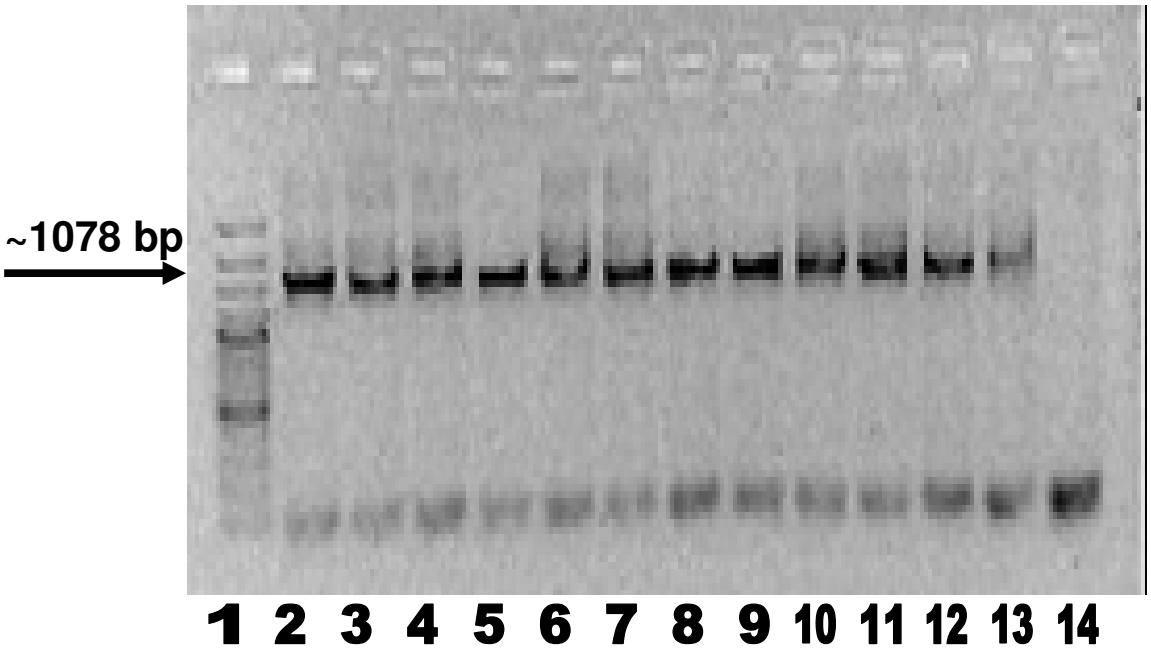

Figure 4 PCR amplification products (4 reactions per isolate) on a 1 % agarose gel for downstream applications. Lane 1 is the DNA ladder (fermentas 1Kb 'O Gene ruler, Inqaba Biotechnical Industries (Pty) Ltd, South Pretoria Africa); Lanes 2-12 represent isolates (B8971/06; D1/01, C1/01, K5R09/01, D7/01, KIR03/01, K2E03/01, 8B/01, 787/B567/10, E3.6/02, E2.5/01, K5R01/01. Lane 13 is the negative control (water).

A total of 120 plasmid colonies from 12 samples were screened for recombination by gel electrophoresis (**Figure 5**) and amplification using Croc 1 and Croc 2 primers (**Figure 6**). Only recombinant plasmids were further analysed. A total of 12 from 120 colonies were sequenced.

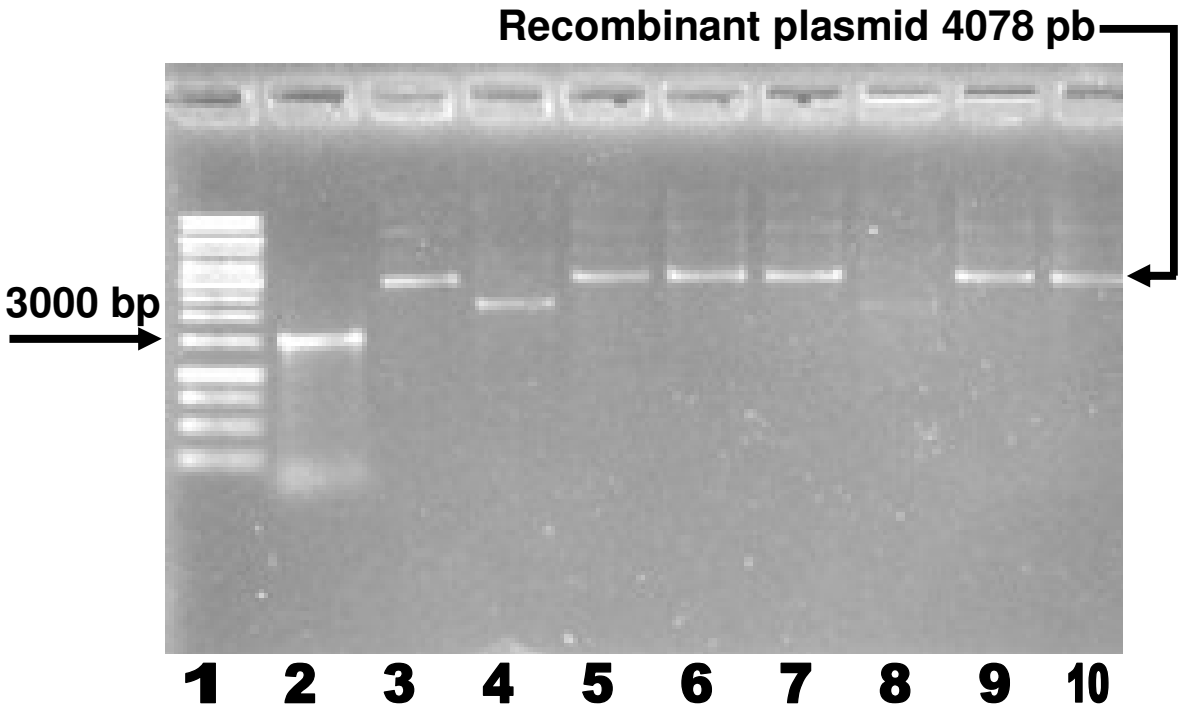

Figure 5 Purified plasmids. Lane 1 is the DNA ladder (Fermentas 1Kb 'O Gene ruler, Inqaba Biotechnology South Africa); Lane 2 is the vector without insert (pGEM-T, Promega, Anatech South Africa); Lanes 3, 5, 6, 7, 9 and 10 represent recombinant colonies (contains the PCR products: K2E03/01, K5R09/01, B8971/01, E2.5/01, E3.6/02, 8B/01, K4E03/05; Lanes 4 and 8 represent vectors without or with partial inserts (samples K2E03/02) and 787/B567/10. The number in bracket is the number assigned to the selected amplification product of interest) (samples, K5R09/01, B8971/01, E2.5/01, E3.6/02, 8B/01 K4E03/05).

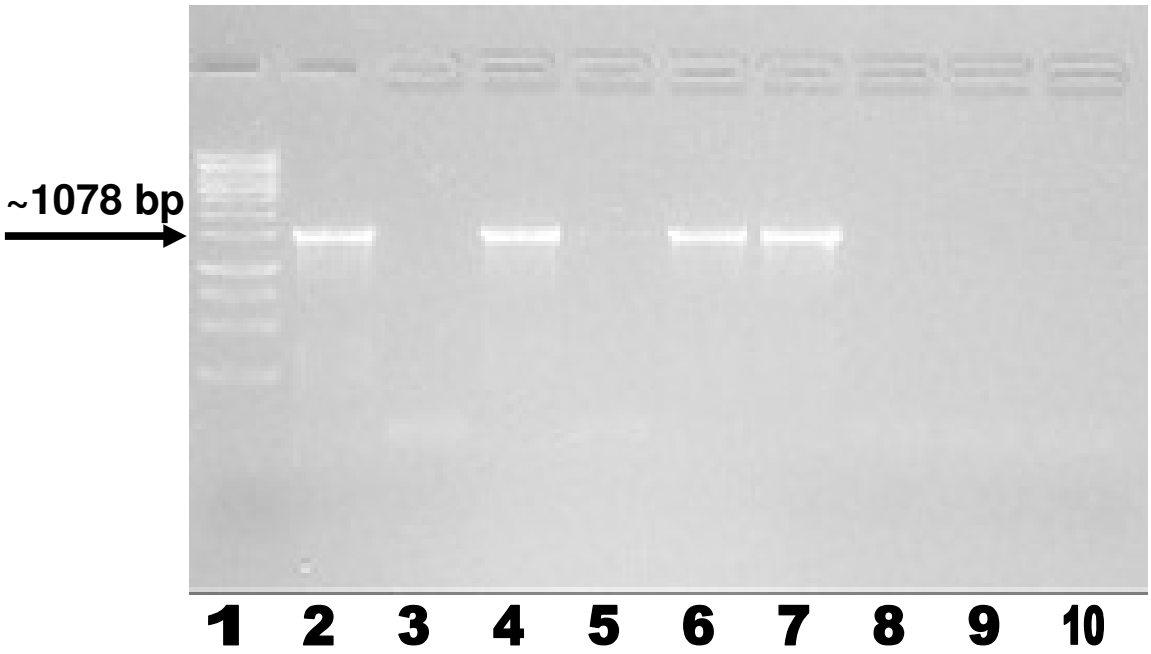

Figure 6 PCR reactions as confirmation of recombination. Lane 1 is the DNA ladder (Fermentas 1Kb 'O Gene ruler, Inqaba Biotechnology South Africa); Lanes 4, 6, 7 (samples, B8971/06, E2.5/01, E3.6/02), are plasmids isolated and amplified with Croc 1 and 2 primers; Lanes 3 and 5 (samples K2E03/01 and 787/B567/10) showed no amplification and confirmed that these plasmids did not contain the correct, or had no insert (PCR amplification product of interest). Lane 2 is positive control (Y-Goat 11706*)* and Lane 8 was a negative control (water).

4.3 PHYLOGENETIC ANALYSIS

BLAST results revealed six prominent *Mycoplasma* sp., namely M*. arginini*, *M. bovigenitalium*, *A. laidlawii*, *Mmm*LC, *M.* sp. *ovine*/*caprine* serogroup II and *M. canadense*. Sequence data representing each of the 34 isolates was further analysed using phylogenetic assays (**Figures 7-12**).

BLAST results revealed that 18 isolates (**Table 3**) out of 34 (50 %) were *M. arginini* with 99-100 % identity to previously published *M. arginini* 16S rRNA gene sequences from England and Sweden (GQ409971 and AF125581). Phylogenetic analysis from the *M. arginini* group of sequences showed that all the sequences obtained from studied isolates (D7/01, C1/01, E3.5/01, B2639/07, K1R03/01, B12294/01, E2.5/01, 787/B567/10, K2E03/01, 8b/01, K1R04/01 and D5/01) are highly similar to each other but branch separately from sequences obtained from GenBank (**Figure 7**).

Six isolates (K5R09/01; R3.2/01; B8971/06; B8973/06; E3.6/02, K4E03/05) showed a high similarity to *M. bovigenitalium* (99 % identity to *M. bovigenitalium* from Turkey and Germany AF464628 and AY121098). Two isolates (B1; B3) were found to have a 99 % identity to *M.* spp. ovine/caprine sero group II sequences (**Table 3**) from the United Kingdom (AY121104). Phylogenetic analysis of these BLAST results (**Figure 8**) showed that two isolates (E3.6 and K5R09) have a high similarity to GenBank sequence (AY12109, M24291, AF465628, AY121096, AY121097, AY121109, and AY121095), while isolates K4EO3, R3.2 and B89711 branched separately.

Blast results of two isolates (R3.6, K5R01) revealed a high similarity (99 %) to *A. laidlawii* from India (FJ655561). Phylogenetic analysis showed that these two isolates grouped separately from Genbank sequences (**Figure 9**).

BLAST results revealed that isolates E3.7/01 and B12291/09 have a similarity of 99 % to *Mycoplasma mycoides mycoides* Large Colony from Sweden (U26049). Isolates R3.4/02 and B12296/09 showed a similarity of 98 % to *M.* sp. usp120 partial 16S rRNA gene sequences from Brazil (GU227399). While, blast searches for K2E01 and B1857 showed similarity of 97-99 % to *Mmm.* Jcv1 partial 16S rRNA gene sequences from the

USA (CP002027). Phylogenetic analysis (**Figure 10**) revealed that E.3.7/01 cluster together with Genbank sequences U26049, U26044, GQ409970 and AF085350 from Sweden, England, Mexico and France all, *w*hile B12291/09 Branched on its own.

Phylogenetic analysis showed B1/01 in monophyletic group with Genbank sequences AY121094 from UK of *M.* sp. *ovine caprine sero* group II while the B3/01 branched on its own (**Figure 11**).

Blast results showed that isolate 782/B567/10 has a similarity of 99 % to *M. canadenses* partial 16S rRNA gene sequence data from Italy (NR025988). This isolate formed a monophyletic group with U44769 and NR025988 from USA and Sweden in the phylogenetic analysis (**Figure 12**).

Table 3 Summary of BLAST results

NO	Plasmid identification	Similar organism	% Similarity	% Identity
1	K6R06/01	*M. arginine* (GQ409971)	100	100
2	B2639/07 (HQ661825)	*M. arginine* (GQ409971)	100	100
3	E3.5/01 (HQ661820)	*M. arginine* (GQ409971)	100	100
4	B1179/08 (HQ661829)	*M. arginine* (GQ409971)	100	100
5	D7/01 (HQ661822)	*M. arginine* (GQ409971)	100	100
6	K2E03/01	*M. arginine* (GQ409971)	99	96
7	K1R04/01 (HQ661824)	*M. arginine* (GQ409971)	99	98
8	(K1R03/01) (HQ661821)	*M. arginine* (GQ409971)	100	99
9	D1/01 (HQ661819)	*M. arginine* (GQ409971)	99	99
10	D5/01 (HQ661816)	*M. arginine* (GQ409971)	100	98
11	B1179/SC/08 (HQ661830)	*M. arginine* (GQ409971)	100	99
12	K4E02/01	*M. arginine* (GQ409971)	100	99
13	B12294/09 (HQ661828)	*M. arginine* GQ409971	99	99
14	8B/01	*M. arginine* (GQ409971)	99	99
15	787/B567/10 (HQ661826)	*M. arginine* (GQ409971)	100	99
16	E2.5/01 (HQ661827)	*M. arginine* (GQ409971)	98	99
17	C1/01 (HQ661823)	*M. arginine* (GQ409971)	100	99
18	R3.2/01 (HQ661814)	*M. bovigenitalium* (AY121098)	99	99
19	B8971/06	*M. bovigenitalium* (AY121098)	99	99
20	K5R09/01 (HQ661817)	*M. bovigenitalium* (AY121098)	98	99
21	B8973/06 (HQ661813)	*M. bovigenitalium* (AF465628)	100	99
22	E3.6/0 (HQ6618151)	*M. bovigenitalium* (AY1211098)	98	99
23	K4E03/05 (HQ661816)	*M. bovigenitalium* (AY121098)	100	99
24	B1/01 (HQ661810)	*M. bovigenitalium* (*M. ovine/caprine* serogroup II) (AY121104)	100	99
25	B3/01 (HQ661809)	*M. bovigenitalium* (*M. ovine/caprine* serogroup II) (AY121104)	100	99
26	E3.7/01 (HQ661812)	*Mmm*LC (U26049)	100	99
27	B12291/09 (HQ661811)	*Mmm*LC (U26049)	100	99
28	R3.6/01	*A. laidlawii* (CPOOO896)	100	99
29	K5R01/01	*A. laidlawii* (CPOOO896)	100	99
30	K2E01/01	Synthetic *Mmm*. jvc1 (CPOO2027)	100	97
31	B1857/06	Synthetic *Mmm*. jvc1 (CPOO2027)	100	99
32	R3.4/02	*M.* sp. USP 120 (GU227399)	100	99
33	782/B56710 (HQ661808)	*M. canadense* (NR025988)	100	99
34	B12296/09	*M.* sp. USP 120 (GU227399)	100	98

Figure 7 Results of the neighbour-joining analysis of the 16S rRNA gene showing the phylogenetic relationship of *Mycoplasma* field isolate sequences with *M. arginini* sequences collected from GenBank. *Clostridium* spp. (AF154828) was used as an outgroup.

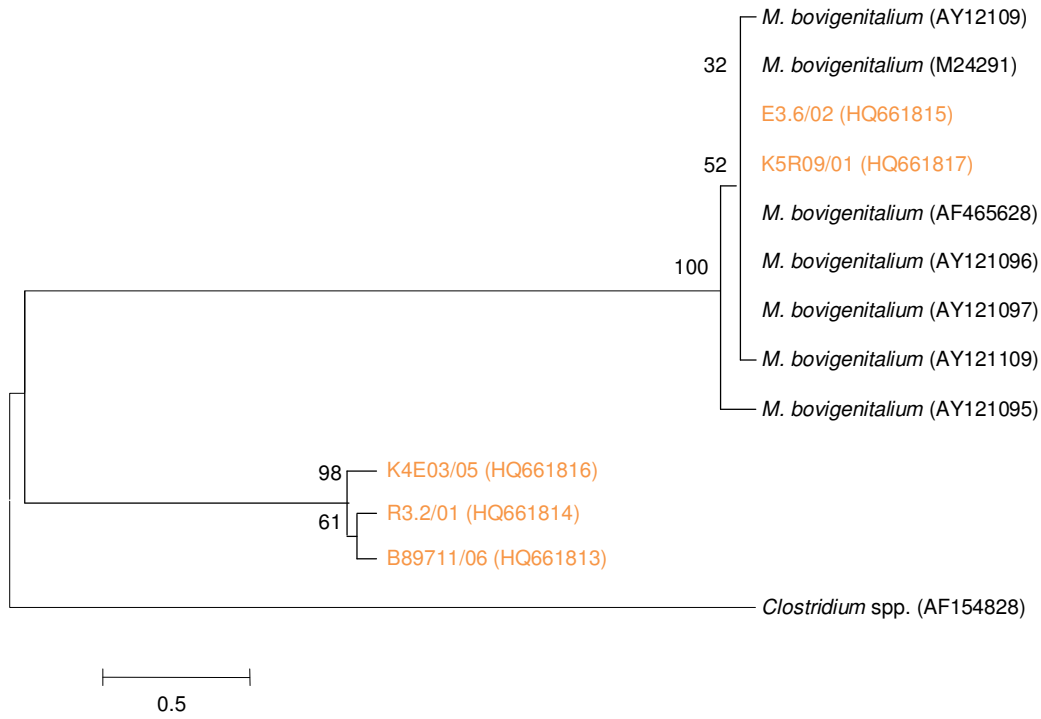

Figure 8 Phylogenetic tree based on the 16S rRNA gene sequences of five field isolates of *M. bovigenitalium* from South Africa highlighted in orange and seven reference strains of *M. bovigenitalium* from Genbank. AF154828, a Clostridium spp. *was* used as an outgroup.

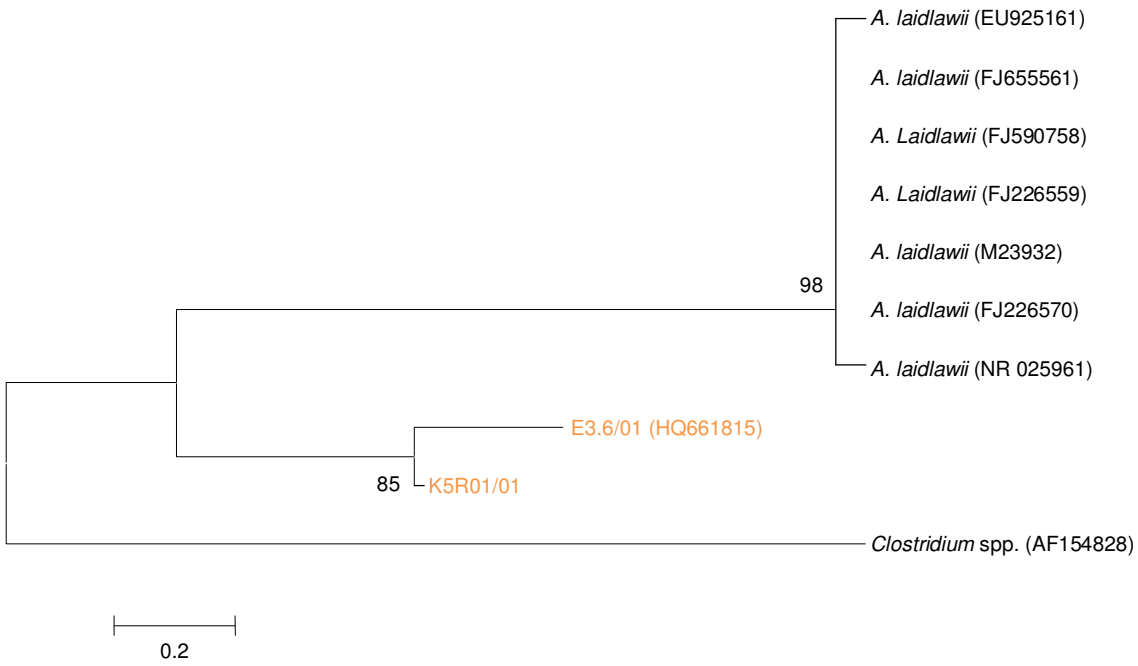

Figure 9 Phylogenetic tree based on the 16S rRNA gene sequences of two field isolates of *A. laidlawii* from South Africa highlighted in orange, seven reference strains of *A. laidlawii* from Genbank. AF154828, and a *Clostridium* spp. was used as an outgroup.

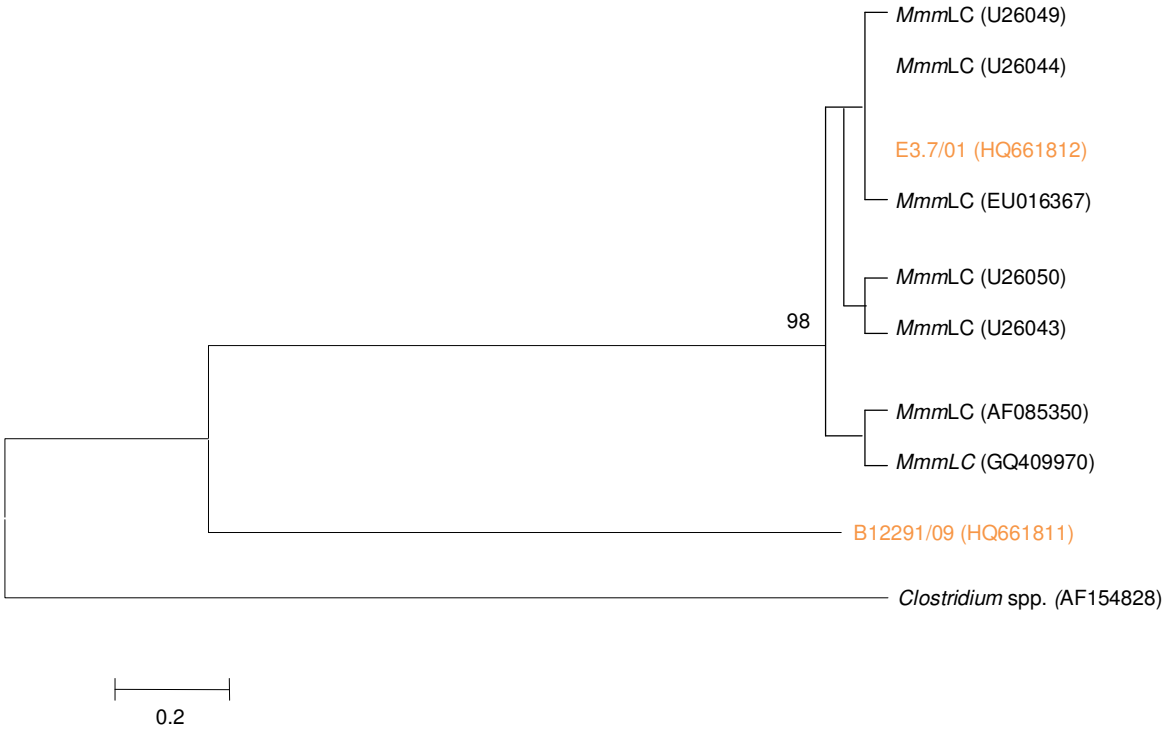

Figure 10 Phylogenetic tree based on the 16S rRNA gene sequences of two isolates of *Mmm*LC from South Africa highlighted in orange and seven *Mmm*LC reference strains from Genbank and *Clostridium* spp. as out group.

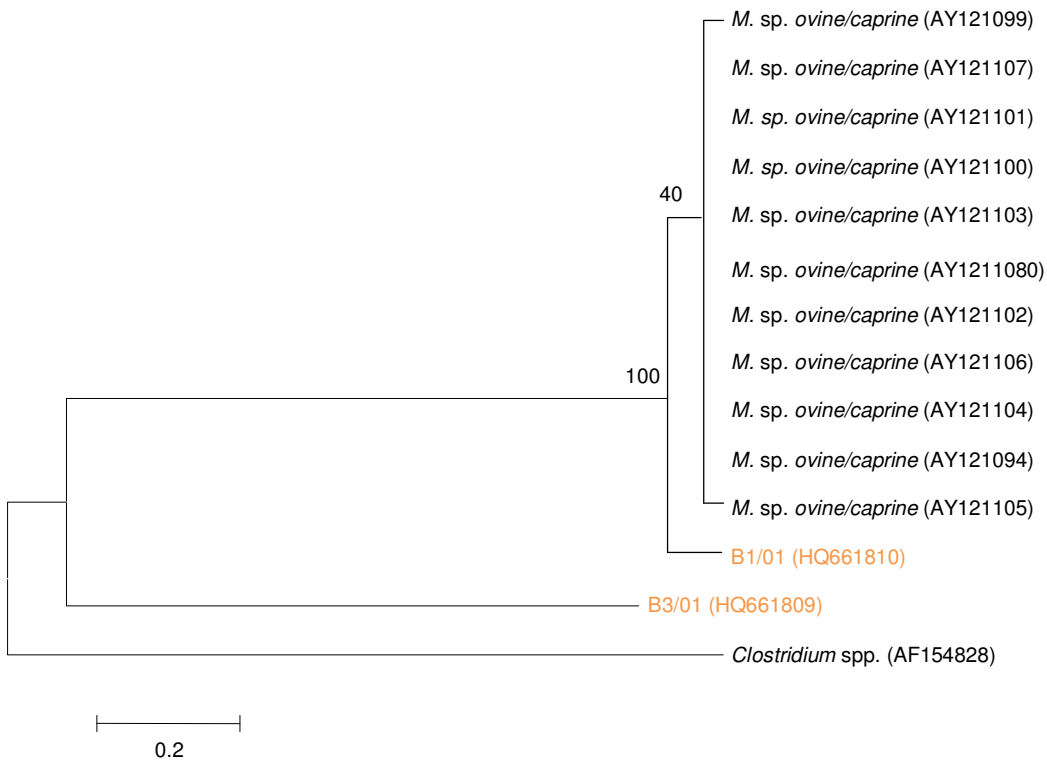

Figure 11 Phylogenetic tree based on partial 16S rRNA gene sequences of two field isolates of *M.* sp. *ovine*/*caprine* serogroup II from South Africa highlighted in orange, and 11 Genbank reference strains of M. sp. *ovine*/*caprine* serogroup II. AF154828, a *Clostridium* spp. was used as an outgroup.

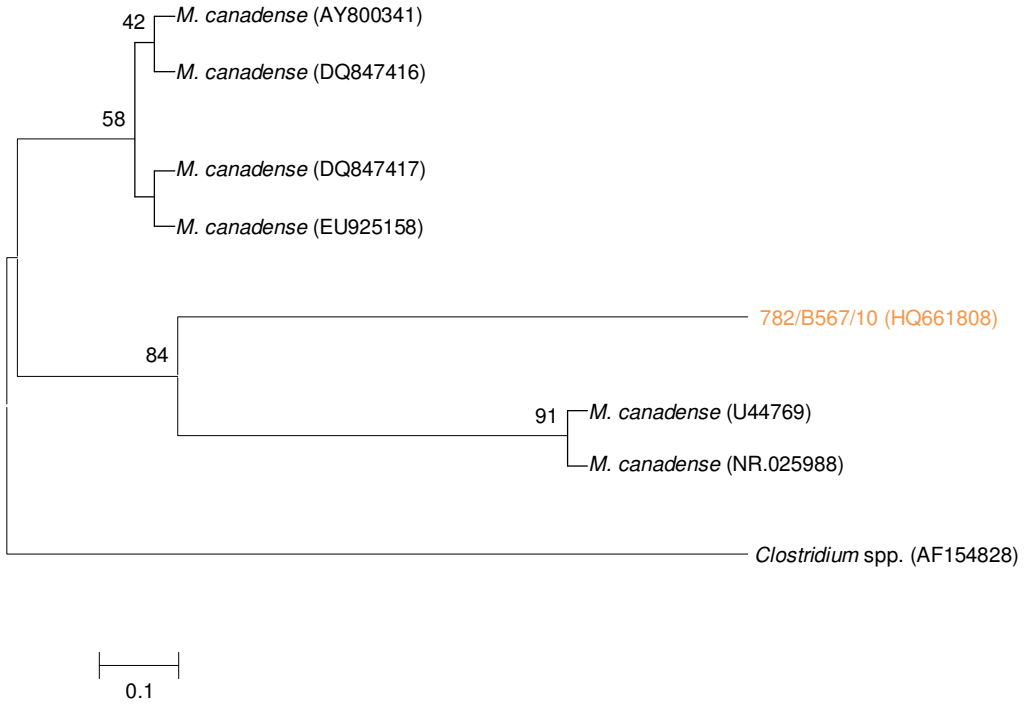

Figure 12 Phylogenetic tree based on the partial 16S rRNA gene sequence of one field isolate of *M. canadense* from South Africa highlighted in orange, and six *M. canadense* reference strains from Genbank. AF154828, a *Clostridium* spp. was used as an out group.

CHAPTER 5
DISCUSSION

Infections with mycoplasmas are commonly associated with mucous and serosal membranes of the respiratory tract, urogenital tract, mammary gland, joints and eyes. Although mycoplasmas are largely host specific, closely related animal species may share the same *Mycoplasma* species. *Mycoplasma, Ureaplasma and Acholeplasma,* species have been isolated from the urogenital tract and organs of healthy sheep and from sheep with clinical signs of balanoposthitis and vulvovaginitis. Although the aetiology of the latter still remains unresolved, it is considered to be multi-factorial. Evidence suggests that *Mycoplasma, Ureaplasma or Acholeplasma* infections are the primary causal factors in ulcerative balanitis and vulvitis while end-stage bacterial pathogens such as *Arcanobacterium pyogenes* are responsible for most of the lesions observed. Some authors have also incriminated viruses but little support for its role in the pathogenesis of ub/uv has been published.

In this study a total of 34 isolates from clinical cases of ulcerative vulvitis and balanitis were successfully cultivated on Hayfick's medium and identified and characterized. Bacteriological analysis of all the isolates was done by growth on Hayflick's agar, morphological appearance and biochemical tests. PCR and sequencing of the partial 16S rRNA gene was during this project used for the first time to characterize *Mycoplasma* species involved in ulcerative balanitis and vulvitis in sheep in South Africa. The PCR products that were sequenced did not yield good results with eight isolates with the identity numbers K2EO3/01, B8971/06, E3.6/01, K5R09/01, 8B/01, E2.5/01, 787/B567/01, K4E03/05. It was therefore decided to clone those samples, and

the recombinant plasmids with the inserts were sequenced after confirmation by PCR. Results obtained from the conventional methods of detection and characterization was in agreement with molecular methods of identification used.

The results showed that 18 isolates out of 34 (51 %) were strains of *Mycoplasma arginini*. Leach *et al*., 1970; Chima *et al*., 1986; and Kidanemariam, 2003 have all reported the isolation and identification of *Mycoplasma arginini* from cases of ulcerative vulvovaginitis and balanoposthitis in goats and sheep in South Africa and Nigeria respectively. It was also previously isolated from cases of ovine keratoconjunctivitis and mastitis.

Molecular analysis through blast searching of the 18 isolates showed 99-100 % similarity with previously published sequences of *M. arginini* 16S rRNA genes submitted to Genbank by Churchward, from England and Sweden (Weisburg *et al*., 1989).

Kidanemariam (2003) isolated four *M. arginini* strains from 104 sheep affected with ub/uv and 116 unaffected sheep. In this study we identified 18 *M. arginini* strains from 34 isolates from cases of ub/uv by both conventional and molecular methods. A high number of isolates of this organism does not confirm it as the causative agent for the disease since the organism is known to have a low pathogenicity in animals even though it has been isolated from cases of mastitis, pneumonia, arthritis and reproductive disease. Experimental animal studies done with this organism to prove it played a role in causing pneumonia have been published (Jones *et al*., 1985). The conclusion was that it was not capable of predisposing the lung to secondary invasion by *Mannheimia haemolytica,* nor of exacerbating the pneumonia. The current findings highlight the

need to re-examine the possible role that strains of *M. arginini* can play in diseases of ruminants.

Of the thirty four isolates characterized, two isolates (B12291/09 and E.3.7/01) tested negative for arginine and other biochemical tests but positive for glucose. It also showed sequence similarity of 99 % with a *Mmm*LC isolate that was isolated from *Capri hircus* in Sweden (strain UM32847). These two isolates were previously isolated from cases of ulcerative balanitis and vulvitis in Dorper sheep in South Africa. Trichard *et al*. (1993) inoculated sheep intravaginally using an *Mmm*LC isolate to try and establish the aetiology of ulcerative balanitis and vulvitis in Dorper sheep in South Africa. The authors re-isolated the same organism from the diseased animals and from their findings concluded that *Mmm*LC is the causative agent of ulcerative balanitis and vulvitis. This finding was supported by Kidanemeriam (2003) who obtained 104 strains of *Mmm*LC out of 220 samples collected from Dorper sheep on different farms in South Africa. Both groups only made use of conventional identification methods.

In view of the results of previous South African studies, it was surprising that so few strains of *Mmm*LC were detected even though the numbers of isolates were relatively small. It may suggest that it is not the primary cause of ub/uv. In addition, the high number of strains reported in a previous study that did not utilize molecular techniques, may point to an overestimation of the possible role of *Mmm*LC as a result of serological cross-reactions which have been documented for several *Mycoplasma* species and serological assays.

Five isolates gave positive reactions for glucose but were negative for all other biochemical tests. These were identified as *Mycoplasma bovigenitalium* and revealed a sequence similarity of 99 % with *Mycoplasma bovigenitalium* isolated from *Bos taurus* in Germany. The isolation of *Mycoplasma bovigenitalium* is in accordance with earlier isolations from goats and sheep that suffered from a similar disease (Chima *et al*, 1986; Kidanemariam, 2003). *Mycoplasma bovigenitalium* is also known to be the cause of a similar problem in cattle. Saed and Al-Aubaid (1983) in their work have confirmed the pathogenicity of *M. bovigenitalium* by experimentally inseminating 12 heifers with *M. bovigenitalium.* The entire group developed granular vulvovaginitis. Similarly Broughton and others (1983) have reported the isolation of *M. bovigenitalium* and *M. canadense* from an outbreak of granular vulvovaginitis in Israeli dairy herds. Afshar (1996) confirmed the pathogenicity of this organism in granular vulvovaginitis in cattle. These findings have further affirmed that *M. bovigenitalium* was a cause of genital mycoplasmosis in cattle.

Further characterization identified two isolates that were unable to ferment glucose or to hydrolyze arginine. These isolates were confirmed by sequence similarity to be 99 % similar to mycoplasma species ovine/caprine serogroup II. The first isolations of *Mycoplasma* species *ovine/caprine* serogroup II from cases of ulcerative balanitis and vulvovaginitis was reported from Australia in sheep (Rhunke, 1994) and goats induced experimentally (Rana *et al*., 1993). Davison and Stuart (1960) also reported the isolation of *M. bovigenitalium* which was very similar to *M*. sp. ovine/caprine serogroup II as neither fermented glucose nor hydrolyzed arginine or possessed phosphatase activity. According to Al-Aubaidi (1972) this isolate is biochemically similar to *M. bovigenitalium* thus they share close genetic and phenotypic characteristics with similar clinical manifestations observed in reproductive disorders in both cattle and

small stock (Rosendal, 1994; Nicholas *et al.*, 2008). These isolates shared serological relationships with the mycoides cluster. Appeals to classify these two organisms into a single species appeared in 2002 and were officially requested in a proposal published in 2008 (Nicholas, *et al.*).

During this study two isolates which were previously isolated from cases of ulcerative balanitis and vulvovaginitis in sheep in South Africa were found to be *Acholeplasma Laidlawii*. It has been reported in sheep flocks in Australia and in free-living European bison (*Bison bonosus*) with balanophosthitis (Thiede *et al.*, 2002).

An interesting finding was the detection of two isolates identified as *Mycoplasma* sp. USP120, which is the first isolation and characterization of this organism from cases of ulcerative balanitis and vulvitis of Dorper sheep. These isolates were found to be 99 % similar to isolates from Brazil which was isolated from the urogenital tract of sheep (Unpublished results, Genbank).

Of the 34 isolates, two were found to be similar to the synthetic *Mycoplasma mycoides* JCVI-syn1.0 clone described by Gibson *et al.*, 2010. However, it must be borne in mind that it was demonstrated that the flanking regions of the 5' V3 region are highly conserved among prokaryotes while the 3' V3 are conserved amongst mycoplasmas and ureaplasmas (Yoshid *et al.*, 2002). This can explain why the plasmid sequences R3.4/02 and B12296 showed a similarity to a *Mycoplasma mycoides* JCVI-syn1.0. Gibson and co-workers created new *M. mycoides* cells that are controlled by a synthetic chromosome. These cells have predictable phenotypic properties and are capable of continuing self-replication. Although these authors used genetic material from *M. capricolum* and *M. mycoides,* no conclusions can be made in terms of the two South

African isolates that were identified as *M. mycoides* JCV1-syn1.0. To determine the true identity of the isolates, additional clones of those two specific isolates will have to be sequenced. Thus, the potential of these organisms to cause lesions in the genital tract of sheep is not known.

One field isolate out of 34 was identified as *Mycoplasma canadense* which has not previously been documented in sheep in South Africa. Petit and others (2008a, 2008b) have reported the isolation of *M. canadense* and *M. bovigenitalium* as the cause of outbreaks of ulcerative posthitis in cattle in Israel. *M. canadense* was also isolated from an unusual form of vulvitis in an outbreak affecting several heifers soon after introduction to a feedlot in South Africa (Gilbert and Oettle, 1990).

In conclusion the PCR amplification of the16S rRNA gene and cloning and sequencing applied during this study identified all 34 mycoplasmas species isolated from clinical cases of ulcerative balanitis and vulvitis in Dorper Sheep in South Africa. The techniques also enabled the identification of new species or strains of *Mycoplasma* that had not previously been described in the region. Species of *Mycoplasma* not previously described in South Africa included strains closely related to synthetic *Mycoplasma mycoides* JCVI-syn1.0 clone, and *Mycoplasma* sp. USP120.

The results from this study supported the findings of other researchers that ulcerative balanitis and vulvitus of sheep is a multifactorial disease that may involve different species of mycoplasmas. However, it does not provide strong support for the findings of Trichard *et al.* (1993) and Kidanemariam (2003) that *Mycoplasma mycoides mycoides LC* is the primary cause of ulcerative balanitis and vulvitis of Dorper sheep in South Africa. It would rather seem that several *Mycoplasma* species can act as primary

insults to the reproductive tract of small stock, and that the end-stage pathogens that are responsible for visible lesions are pathogenic bacteria, most notably *Arcanobacterium pyogenes* (Kidanemariam, 2003).

The implication of the findings of this study is that development of an anti-mycoplasmal vaccine for protection against ub/uv will be a complicated task as several species have been implicated in the pathogenesis of the disease. A further implication is that the control of the disease will for the foreseeable future be dependent on treatment with antimicrobial drugs that will necessitate regular bacterial isolation and testing for resistance to antimicrobial drugs.

REFERENCES

AFSHAR, A., STUART, P., HUCK, R.A., 1996. Granular vulvovaginitis (nodular venereal disease) of cattle associated with *Mycoplasma bovigenitalium. The Veterinary Record,* 78: 512-519.

AL-AUBAIDI, J.M. and FABRICANT, J., 1971. Characterization and classification of bovine mycoplasmas. *Cornell Veterinarian*, 61: 490-518.

AL-AUBAIDI, J.M. MCENTEE, KLEIN, D.H and ROBERT, S.J., 1972. Bovine seminal vesiculitis and epididymitis caused by *Mycoplasma bovigenitalium. Cornell Veterinarian*, 62, (4): 581-596.

AL-MOMANI, W., MAHMOUD, A., MAHMOUD, H., ABO-SHEHADA, M., MILES, K., McAULIFFE, L. and NICHOLAS, R.A.J., 2005. Isolation and molecular identification of small ruminant mycoplasmas in Jordan. *Small Ruminant Research*, 65: 106-112.

AYLING, R.D., BASHIRUDDIN, S.E. and NICHOLAS, R.A.J., 2004. Mycoplasmas and related organisms identified from ruminants 1990-2000. *The Veterinary Record*, 155: 413-416.

BALL, H.J. and McCAUGHEY, W.J., 1982. Experimental production of vulvitis in ewes with *Ureaplasma* isolates. *The Veterinary Record*, 110: 581.

BALL, H.J., KENNEDY, S. and ELLIS, W.A., 1991. Experimental reproduction of ovine vulvitis with bacteria of the *Haemophilus/Histophilus* group. *Research in Veterinary Science,* 50: 81-85.

BASEMAN, J.B. and TULLY, J.G., 1997. Mycoplasmas: Sophisticated, re-emerging, and burdened by their notoriety. *Emerging infectious Diseases*, 3: 1-14.

BASHIRUDDIN, J.B., TAYLOR, T.K. and GOULD, A.R, 1994. A PCR-based test for the specific identification of *M. mycoides* subspecies *mycoides SC*. *Journal of Veterinary Diagnostic Investigation*, 6: 428-434.

BATH, G. and DE WET, J., 2000. Pizzle Disease, In: *Sheep and Goat Diseases*. Tafelberg Publishers Limited, 28 Wale Street, Cape Town, 74-76.

BEVERIDGE, W.I.B. and JOHNSTONE, I.L., 1953. Sheath rot, non-contagious posthitis or chronic ulceration of the prepuce of sheep. *The Australian Veterinary Journal*, 29: 269-274.

BLOOD, D.C., RODOSTITIS, O.M., ARUNDEL, J.H. and GAY, C.C., 1989. Enzootic Posthitis (Pizzle Rot, Sheath Rot, Balanophosthitis). *Veterinary Medicine*. A Textbook of Diseases of Cattle, Sheep, Pigs and Horses. 7th Edition, Bailliere & Tindal, 418-419.

BOLSKE, G., MSAMI, H., HUMLESJO, N.E., ERNO, H. and JOHANSSON, L., 1988. *Mycoplasma capricolum* in an outbreak of polyarthritis and pneumonia in goats. *Acta Veterinaria Scandinavica*, 29: 331-338.

BOLSKE, G., MATTSSON, J.G., BERGSTROM, M.K., ROS-BASCUNANA, C.R., WESONGA, H. and JOHANSSON, K.E., 1996. Diagnosis of contagious caprine pleuropneumonia by detection and identification *of Mycoplasma capricolum* subsp. *capripneumoniae* by PCR and restriction enzyme analysis. *Journal of Clinical Microbiology*, 34: 785-791.

BONNET, F., SAILLARD, C., BOVE, J.M., LEACH, R.E., ROSE, D.L., COTTEW, G.S. and TULLY, J.G., 1993. DNA relatedness between field isolates of *Mycoplasma F38 group*, the agent of contagious caprine pleuropneumonia, and strains of *Mycoplasma capricolum*. *International Journal of Systematic Bacteriology*, 43: 597-602.

BROUGHTON, E., HOPPER, S.A. and GAYFORD, P.J.R., 1983. *Mycoplasma canadense* from bovine foetuses. *The Veterinary Record,* 112: 87.

CAETANO-ANOLLIES, G., 1993. Amplifying DNA with arbitrary oligonucleotide primers. *PCR Methods and Application*s, 3: 85-94.

CARMICHAEL, L.E., St. GEORGE, T.D., SULLIVAN, N.D. and HORSFALL, N., 1972. Isolation, propagation and characterization studies of an ovine *Mycoplasma* responsible for proliferative interstitial pneumonia. *Cornell Veterinarian*, 62: 654-679.

CHIMA, J.C., ERNØ, H. and OJO, M.O., 1986. Characterization and identification of caprine genital mycoplasmas. *Acta Veterinaria Scandinavical*, 27: 531-539.

CHIMA, J.C., OJO, M.O. and ADETOSOYE, A., 1992. Association of mycoplasmas with genital infection of goats in Nigeria. *Proceedings of the West African Commonwealth Veterinary Association/Ghana Veterinary Medical Association,* Accra International Conference Centre, Ghana, pp 87-100.

CHRISTIANSEN, C. and ERNØ, H., 1982. Classification of the F38 group of caprine mycoplasma strains by DNA hybridization. *Journal of General Microbiology*, 128: 2523-2526.

CHRISTIANSEN, G. and ERNØ, H., 1990. RFLP in rRNA genes of *Mycoplasma capricolum*, the caprine F38-like group and the Bovine sero-group 7. In: *Proceedings of the 7th Congress of the International Organization for Mycoplasmology*. Eds, G. Stanek, G.H. Cassell, J.G. Tully, and R.F. Whitcomb, pp 479-488.

COSTAS, M., LEACH, R.H. and MITCHELMORE, D.L., 1987. Numerical analysis of PAGE protein patterns and the taxonomic relationships within the *Mycoplasma mycoides cluster*. *Journal of General Microbiology,* 133: 3319-3329.

COTTEW, G.S., LLYOD, L.C. and PARSONSON, I.M., 1974. Isolation of *Mycoplasma* from vulvovaginitis in sheep. *Australian Veterinary Journal*, 50: 576-57.

COTTEW, G.S. and YEATS, F.R., 1978. Subdivision of *Mycoplasma mycoides subsp mycoides* from cattle and goats in two types. *Australian Veterinary Journal*, 54: 239-296.

COTTEW, G.S. and YEATS, F.R., 1982. *Mycoplasma* and mites in the ears of clinically normal goats. *Australian Veterinary Journal*, 59: 77-81.

COTTEW, G.S., BREARD, A., DAMASSA, A.J., ERNØ, H., LEACH, R.H., LEFEVRE, P.C., RODWELL, A.W. and SMITH, G.R., 1987. Taxonomy of the *Mycoplasma mycoides cluster*. *Israel Journal of Medical Science*, 23: 632-635.

DAMASSA A.J., BROOKS, D.L. and HOLMBERG, C.A, 1984. Pathogenicity of *M. capricolum* and *M. putrefaciens*. *Israel Journal of Medical Science*, 20: 975-978.

DAMASSA, A.J., BROOKS D.L. and ADLER, H.E., 1983. Caprine mycoplasmosis: Wide spread infection in goats with *Mycoplasma mycoides subspecies mycoides* (large colony type). *American Journal of Veterinary Research*, 44: 322-325.

DAMASSA, A.J., WAKENELL, P.S. and BROOKS, D.L., 1992. Mycoplasmas of goats and sheep. *Journal of Veterinary Diagnostic Investigation,* 4: 101-113.

DANDEKAR, T., SNEL, B., SCHMIDT, S., LATHE, W., SUYAMA, M., HUYNEN, M. and BORK, P., 2002. Comparative genome analysis of the *Mollicutes.* In: *Molecular Biology and Pathogenicity of Mycoplasmas*, ed. Razin and Herrmann, Kluwer. Academic/plenum Publishers, New York, pp 255-278.

DAVIDSON, I., STUART, P., 1960. Isolation of mycoplasma-like organisms from an out brake of bovine mastitis. *The Veterinary Record,* 72: 766.

DEAS, D.W., 1983. Ulcerative balanitis and ulcerative vulvitis. In: *Diseases of Sheep.* Ed. W.B. Martin, Oxford, Blackwell Scientific Publications, pp 144-146,

DENT, C.H.R., 1971. Ulcerative vulvitis and posthitis in Australian sheep and cattle. *The Veterinary Bulletin*, 41: 719-723.

DOHERTY, M.L., 1985. Outbreak of posthitis in grazing wethers in Scotland. *The Veterinary Record*, 116: 372-373.

DOIG, P.A. and RUHNKE, H.L., 1977. Isolation of *Ureaplasma* from sheep with granular vulvitis. *The Veterinary Record*, 100: 179-180.

DUNN, K., 1996. Vulvitis and balanitis in a lowland flock. *Proceedings of the Sheep Veterinary Society*, 20: 41-42.

EATON, M.D., MEIKLEJOHN, G. and HERICK, W., 1944. Study of the aetiology of primary atypical pneumonia. A filterable agent transmissible to cotton rats, hamster, and chicken embryos. *Journal of Experimental Medicine*, 79: 649-668.

EATON, M.D. and LOW, I.E., 1967. Propagation of *Mycoplasma pneumoniae* and other fastidious strains of PPLO. *Animal Biology*, 143: 375-383.

ERNØ, H, STIPKOVITS L., 1973. Bovine mycoplasmas: cultural and biochemical studies. *Acta Veterinaria Scandinavica,* 14, (3): 450-463.

ERNØ, H., 1987. Mycoplasmosis of ruminants: a general introduction. *Scientific and Technical Review - OIE,* 174: 8158-8152.

FELSENSTEIN, J., 1985. Confidence limits on phylogenies - an approach using the bootstrap. *Journal of Molecular Evolution,* 39: 783-791.

FITZMAURICE, J., SEWELL, M., MANSO-SYLVÁN, L., THIAUCOURT, F., MCDONALD, W.L. and O'KEEFE, J.S., 2008. Real-time polymerase chain reaction assays for the detection of members of the *Mycoplasma mycoides cluster*. *New Zealand Veterinary Journal*, 56, (1): 40-47.

FREUNDIT, E.A., ANDREWS, B.E., ERNØ, H., KUNZE, M. and BLACK, F.T., 1973. The sensitivity of *Mycoplasmatales* to sodium-polyanethiol-sulfonate and digitonin. *Zentralbl fur bacteriologies microbiologie und hygiene*, A225: 104-112.

GIBSON, D.G., GLASS, J., LARTINGUE, C., MOSKOV, V.N., CHUAG, R.U., ALGINE, M.A., BENDERS, G.A., MINTOGUE, M.G., MAL, L.M.M., MERYMAN, C., VASHEL, S., KRISH KUMAR, R., GARCIA A., NALGREW PUANKOCL, C., DANISOVA, R., YOUNG, L., 2010. Creation of a bacterial cell controlled by a chemically synthesized genome, *Science,* 329: 52-56.

GILBERT, R.O. and OETTLE, E.E., 1990. An outbreak of granulomatous vulvitis in feedlot heifers. *Journal of the South Africa Veterinary Association*, 61, (1): 41-43.

GOIS, M., KUKSA, F., FRANZ, J. and TAYLOR-ROBINSON, D., 1974. The antigenic differentiation of seven strains of *Mycoplasma hyorrhinis* by growth inhibition, metabolic inhibition, latex agglutination and polyacrylamide gel electrophoresis. *Journal of Medical Microbiology*, 7: 105-115.

GOLL, J.R.F., 1994. Identification of mycoplasma isolates from domestic animals. In: *Mycoplasmosis in Laboratory Diagnosis*. Eds, H.W. Whitford, R.F. Rosenbusch and L.H. Lauerman, . Iowa State University Press, Ames, pp 15-27

GREIG, A., 1989. Ovine keratoconjunctivitis: An update. In: *Practice*, May, 112-113.

GREIG, A., 2000. Ulcerative balanitis and vulvitis. In: *Diseases of Sheep.* Eds, W.B. Martin and I.D. Aitken, Blackwell Science, Ltd, 3rd edition, pp 1991-121,

GREIG, A., 2007. Ulcerative balanitis and vulvitis. In: *Diseases of Sheep*. Eds, I.D. Aitken. Blackwell Publishing, 4th edition, pp143-145.

GREWAL, A.S. and WELLS, R., 1986. Vulvovaginitis of goats due to herpesvirus. *Australian Veterinary Journal,* 63: 79-81.

GUMMOW, B. and STALEY, G.P., 2000. A retrospective survey of ulcerative balanoposthitis and vulvovaginitis in South African Dorper sheep. *Livestock Health and Production,* 12: 31-36.

HORNER G.W., HUNTER, R. and DAY, A.M., 1982. An outbreak of vulvovaginitis in goats caused by caprine herpesvirus. *New Zealand Veterinary Journal*, 30: 150-152.

JONES, G.E., FOGGIE, A., SOUTHERLAND, A., and HARKER, D.B., 1976. Mycoplasma and ovine keratoconjunctivitis. *The Veterinary Record*, 99: 137-141.

JONES, G.E., RAE, A.E., HOLMES, R.G., LISTER, S.A., JONES, J.M.W., GRATER, G.S. and RICHARDS, N., 1983. Isolation of exotic mycoplasmas from sheep in England. *The Veterinary Record*, 113: 619-620.

JONES, G.E., GILMOUR, J.S. and RAE, A.G., 1985. Investigations into the possible role of *Mycoplasma arginini* in ovine respiratory disease. *Research in Veterinary Science*, 38, (3): 368-72.

JUKES, T.H. and CANTOR, C.R., 1969. Evolution of protein molecules. In: H.N. MUNRO. Mammalian Protein Metabolism, Vol. 3, Academic Press. Inc, New York, pp 21-132.

KAPOOR, S.G., PATHAK, R.C. and SINGH, P.P., 1984. Prevalence of *Mycoplasma/Acholeplasma* in the genital tract of sheep. *Indian Journal of Animal Science,* 54: 653-656.

KIDANEMARIAM A., 2003. Identification and characterization of the primary infectious agent associated with ovine ulcerative balanoposthitis and vulvovaginitis in South Africa. MSc dissertation, University of Pretoria, South Africa.

KIDANEMARIAM, A., GOUWS, J., VAN VUUREN, M. and GUMMOW, B., 2005. Ulcerative balanitis and vulvitis of Dorper sheep in South Africa: a study on its aetiology and clinical features. *Journal of the South African Veterinary Association,* 76, (4): 197-203.

KIMBERLING, C.V., 1988. Ulcerative dermatosis. In: *Jensen and Swift's Diseases of Sheep*, Lea and Febiger, Philadelphia, pp 16-19.

KIMURA, M., 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16: 111-120.

KUMAR, S. TAMURA, K. and NEI, M., 2004. Mega 3 integrated software for molecular evolution and genetic analysis and sequence alignment. *Brief Bioinformatics*, 5: 150-163.

LEACH, R.H., ERNØ, H. and MacOWAN, K.J., 1993. Proposal for designation of F38-type caprine mycoplasmas as *Mycoplasma capricolum* subsp. *capripneumoniae* subsp. *nov*. and consequently obligatory regulation of strains currently classified as *M. capricolum* (Tully, Barile, Edward, Theodore, and Ernø, 1974) to an additional new subspecies, *M. capricolum.* subsp. *capricolum* subsp. *nov*. *International Journal of Systemic Bacteriology*, 43: 603-605.

LEACH, R.H., 1970. The occurrence of *Mycoplasma arginini* in several animal hosts. *The Veterinary Record*, 87: 319-320.

LINKLATER, K.A. and SMITH, M.C., 1993. Diseases of the reproductive system and udder. In: *Color Atlas of Disease of the Sheep and Goat*. Eds, Karl A. Linklater and Mary C. Smith. WOLFE, pp 123-135.

LIVINGSTONE, C.W. and GAUER, B.B., 1975. Isolation of T-strains of *Mycoplasma* from sheep and goats in Texas. *American Journal of Veterinary Research*, 36: 313-314.

LIVINGSTONE, C.W. and GAUER B.B., 1983. Occurrence of *Mycoplasma* spp. 2D in Texas sheep flocks. *American Journal of Veterinary Research,* 44: 868-869.

LORIA, G.R., SAMMARTINO, C., NICHOLAS, R.A.J. and AYLING, R.D., 2003. In: vitro susceptibilities of field isolates of *Mycoplasma agalactiae* to oxytetracycline, tylosin, enrofloxacin, spiramycin, and lincomycin-spectinomycin. *Research in Veterinary Science*, 75: 3-5.

LOSTE, A., RAMOS, J.J., GARCIA, L., FERRER, L.M. and VERDE, M.T., 2005. High prevalence of ulcerative phosthitis in Rasa Aragonesa Rams associated with a legume-rich diet. *Journal of Veterinary Medicine*, A52: 176-179.

MARTIN, W.B. and AITKEN I.D., 2000. Mycoplasma associated vulvovaginitis. In: *Diseases of Sheep*. *Blackwell Science,* 3rd edition, pp 120-121.

McOWAN, K.J., MINETTE, J.E., 1976. The role of mycoplasma strain F38 in contagious acute caprine pleuropneumonia in Kenya. *The Veterinary Record,* 101: 380-381.

McAULIFFE, L., ELLIS, R. LAWES, J., AYLIG, R.D. and NICHOLAS, R.A.J., 2005. 16S rDNA and DGGE: a single generic text for detecting and differentiating *Mycoplasma* species. *Journal of Medical Microbiology*, 54: 1-9.

McCAUGHEY, W.J. and BALL, H.J., 1983. Ureaplasmas in ewes. *The Veterinary Record*, 12: 441.

MONNERATE, M.P., THIAUCOURT, F., POVEDA J.B., NICOLET, J. and FREY, J., 1999. Generic and serological analysis of lipoprotein LPPA in *Mycoplasma mycoides* subsp. *capri*. *Clinical and Diagnostic Laboratory, Immunology Journal,* 6: 224-230.

MRAZEK, J., 2006. Analysis of Distribution Indicates Diverse Functions of Simple Sequence Repeats in *Mycoplasma* Genomes. *Molecular Biology and Evolution*, 23: 1370-1385.

MUNZ, E. and DUMBELL, K., 1994a. Orf. In: Coetzer J.A.W., Thomson G.R., Tustin R.C., (eds). Infectious diseases of livestock with special reference to southern Africa. Vol. 1: 621-622. Oxford University Press. Cape Town, 621-622

MUNZ, E. and DUMBELL, K., 1994b. Ulcerative dermatosis. In: Coetzer J.A.W., Thomson G.R., Tustin R.C., (eds). Infectious diseases of livestock with special reference to southern Africa. Vol. 1: 621-622. Oxford University Press. Cape Town, 621-622

NAGLIC, T., HOTZEL, H., BALL, H.J., SEOL, B. and BUSCH, K., 2001. Studies on the aetiology of caprine mycoplasmosis in Croatia. *Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics.* Eds, J.A. Poveda, A. Fernandez, J. Frey and K. Johansson, Vol. 5, European Communities, Belgium, pp 137-140.

NICHOLAS, R.A.J., GREIG, A., BAKER, S.E, AYLING, R.D., HELDTANDER, M., JOHANSSON, K.E., HOUSHAYMI, B.M. and MILES, R.J., 1998. Isolation of *M. fermentans* from sheep. *The Veterinary Record*, 110: 581.

NICHOLAS, R.A.J., LIN, Y., SACHSE, K., HOTZEL, H., PARHARM, K., McAULIFFE, L., ROGER, J., MILLES, D, KELLY, P. and WOOD, A.P., 2008. Proposal that the strains of the *Mycoplasma ovine/caprine serogroup 11* be re classified as *Mycoplasma bovigenitalium*. *International Journal of Systematic and Evolutionary Microbiology*, 58: 308-312.

OLSEN, G.J. and WOESE, C.R., 1993. Ribosomal RNA: a key to phylogeny. *Federation of American Society for Experimental Biology* (*FASEB), Journal*, 7: 113-123.

PETTERSON, B., JOHANSSON, K.E. and UHL´EN, M., 1994. Phylogeny of some mycoplasmas from ruminants based on 16S rRNA by direct solid phase DNA sequencing. *Applied Environmental Microbiology,* 60: 2456-61.

PETTERSON, B., LEITNER, T., RONAGHI, M., BOLSKE, G., UHL´EN, M., and JOHANSSON, K.E., 1996. Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA gene from the two operons. *Journal of Bacteriology*, 178: 4131-4142.

PETIT, T., SPERGSER, J., AURICH, J., and ROSENGARTEN, R., 2008a. Prevalence of Chlamydiaceae and mollicutes on the genital mucosa and serological findings in dairy cattle, *Veterinary Microbiology*, 127: 325-333.

PETIT, T., SPERGSER, J., AURICH, J. and ROSENGARTEN, R., 2008b. Examination of semen from bulls at five Austrian artificial insemination centres for Chlamydiae and Mollicutes. *The Veterinary Record*, 162: 792-793.

PRITCHARD, G.C., SCHOLES, S.F.E., FOSTER, A.P., MITCHELL, E.S.E., LAWES, J., IBATA, G. and BANK, M., 2008. Ulcerative vulvitis and balanitis in sheep flocks. *The Veterinary Record,* 163: 86-89.

RADOSTITS, O.M., BLOOD, D.C., and GAY, C.C., 1994. Diseases caused by *Mycoplasma* species. In: *Veterinary Medicine*. A Textbook of the diseases of cattle, sheep pigs and Horses. 8th Edition, pp 908-909.

RAMIREZ, A.S., GARCIA, M., DIAZ-BERTARANA, FERNANDEZ, A. and POVEDA, J.B., 2001. Goat and sheep abortions associated with *M. agalactiae* in northern Spain. In: *Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics.* Eds, J.B. Poveda, A. Fernanàndez, J. Frey and K. Johansson. Vol. 5, European Communities, Belgium, pp 122.

RANA, J.S., GUPTA, P.P., BANGA, H.S., 1993. Pathology of the genital tract of goats experimentally infected with *Mycoplasma serogroup* 11. *Indian Journal of Animal Science*, 63: 706-709.

RAWADI, G., LEMERCIER, B., and ROULLAND-DUSSOIX, D., 1995. Application of arbitrarily-primed polymerase chain reaction to mycoplasma identification and typing within the *Mycoplasma mycoides cluster*. *Journal of Applied Bacteriology*, 78: 586-592.

RAZIN, S., 1992. Peculiar properties of *Mycoplasmas:* the smallest self-replicating prokaryotes. *Federation of European Microbiological societies*, (*FEMS*) *Microbiology. Letters*, 100: 423-432.

RAZIN, S., and FREUNDIT, E.A., 1984. The mycoplasmas. In: *Bergey's Manual of Systematic Bacteriology*. Eds, N.R. Krieg and J.G. Holt, Vol. 1, The Williams and Wilkins Co., Baltimore, pp 740-739.

RAZIN, S., YOGEV, D. and NAOT, Y., 1998. Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews*, 62: 1094-1156.

RUHNKE, H.L, ROSENDAL, S., 1994. Mycoplasmas associated with bovine genital tract infection. In: Mycoplasmas in animals: laboratory Diagnosis, Eds, Howards, W. Whitford, H., Ricardo, F., Rosenbush and Lloyd H. Lauerman, Iowa State. University Press, Ames, pp 56-63.

ROBERTS, R.S. and BOLTON, S.F., 1945. A venereal disease of sheep. *The Veterinary Record*, 57: 686-687.

ROBERTSON, J.A, VERKIS, A., BEBEAR, C. and STEMKE, G.W., 1993. Polymerase chain reaction using 16S rRNA gene sequences distinguishes the two biovars of *Ureaplasma urealyticum*. *Journal of Clinical Microbiology*, 13: 824-830.

RODWELL, A.W., 1982. The protein fingerprints of mycoplasmas. *Reviews of Infectious Diseases,* Vol. 4 supplement.

ROSADIO, R.H., EVERMANN, J.F. and MUELLER, G.M., 1984. Spectrum of naturally occurring diseases associated with herpesvirus infection. *Agri-practice*, 5: 20-26.

ROS-BASCUNANA, C., MATTSON, J.G., BÖLSKE, G. and JOHANSSON, K.E., 1994. Characterization of the 16S rRNA genes from *Mycoplasma* sp. strain F38 and development of an identification system based on the PCR. *Journal of Bacteriology*, 176: 2577-2586.

ROSENDAL, S., ERNØ, H. and WYAND, D.S., 1979. *Mycoplasma mycoides subspecies mycoides* as the cause of polyarthritis in goat. *Journal of the American Veterinary Association*, 175: 378-379.

ROSENDAL, S., 1994. Ovine and caprine mycoplasmas. In: *Mycoplasmas in animals: laboratory diagnosis*. Eds, H.W. Whitford, R.F. Rosenbusch and L.H. Lauerman, Iowa State University Press, Ames, pp 84-92.

SAED, O.M. and AL-AUBAIDI, J.M., 1983. Infertility in heifers caused by pathogenic strain of *Mycoplasma bovigenitalium*. *Cornell Veterinarian,* 73: 125-130.

SAITOU, N. and NEI, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolution*, 4: 406-425.

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T., 1989. Molecular Cloning: A Laboratory Manual, Vol. I, 2nd edition. Cold Spring Harbor Laboratory Press, New York.

SASAKI, Y., ISHIKAWA, J., YAMASHITA, A., OSHIMA, K., KENRI, T., FURUYA, K., YOSHINO, C., HORINO, A., SHIBA, T., SASAKI, T. and HATTORI, M., 2002. The complete genomic sequence of *Mycoplasma penetrans*, an intracellular bacterial pathogen in humans. *Nucleic Acid Research*, 30: 5293-530.

SHELTON, M. and LIVINGSTONE, C.W., 1975. Posthitis in angora goats. *Journal of American Veterinary Medical Association,* 167: 154-155.

SIMECKA, J.W., DAVIS, J.K., DAVISON, M.K., ROSS, S.E., STADTLANDER, C.T. and CASSELL, G.H., 1992. Mycoplasmal diseases of animals. In: J. Manilloff, R.N. Mccelhaney, L.R. Finch and J.B. Baseman (Eds.) Mycoplasma Molecular Biology, Microbiology and Pathogenesis, pp 391-415.

SINGH, N., RAJYAN, B.S. and MOHANTY, G.C., 1974. Granular vulvovaginitis (GVV) in goats associated with *Mycoplasma agalactiae*. *Cornell Veterinarian*, 64: 435-442.

SINGH VIJENDRA, P., SRIVASTAVA, N.C., MANOJ KUMAR, J.A.I., SUNDER, M., and VARSHNEY, J.P., 2004. Isolation and characterization of an Indian strain of *Mycoplasma mycoides* subsp. *mycoides* type LC from a case of caprine arthritis. *Comparative Immunology, Microbiology and Infectious Diseases*, 27: 273-284.

SOUTHCOTT, W.H., 1965. Epidemiology and control of ovine posthitis and vulvitis. *Australian Veterinary Journal*, 41: 225-234.

SOUTHCOTT, W.H., 1963. The aetiology of ovine posthitis. *Australian Veterinary Journal*, 39:212.

STADEN, R., 1996. The staden sequence analysis package. *Molecular Biotechnology*, 5: 233-241.

STEYN, D.G., 1930. "Pisgoed". *Report of the Director of Veterinary Services and Industry*, Union of South Africa,16:417-420.

STEYN, D.G., 1940. "Pisgoed". *Farming in South Africa*, 15: 27-39.

TAOUDI, A., JOHNSON, D.W. and KHEYYALI, D., 1987. Pathogenecity of *Mycoplasma capricolum* in sheep after experimental infection. *Veterinary Microbiology*, 14: 137-144.

TARIGAN, S., WEBB, R.F., KIRKLAND, D., 1987. Caprine herpes virus balanoposthitis. *Australian Veterinary Journal*, 64: 321.

TAYLOR, M.A. WISE, K.S. and MCINTOSH, M.A., 1985. Selective detection of *Mycoplasma hyorrhinis* using cloned genomic DNA fragments. *Infection and Immunity*, 47: 827-830.

TAYLOR, T.K., BASHIRUDDIN, J.B. and GOULD, A.R., 1992. Relationships between members of the *Mycoplasma mycoides* cluster as shown by DNA probes and sequence analysis. *International Journal of Systematic Bacteriology*, 42: 593-601.

THIAUCOURT, F., LORENZON, S., DAVID, A. and BREARD, A., 1994. Phylogeny of *Mycoplasma mycoides* cluster as shown by sequencing of a putative membrane protein gene. *Veterinary Microbiology*, 72: 251-268.

THIEDE, S., SPERGSER, J., ROSENGARTEN, R., JAKOB, W., STREICH, W.J., KRASINSKA, M., FROLICH, K., 2002. Antibodies against *Mycoplasma bovigenitalium* in free-living bison (*Bison bonasus*) with balanoposthitis. *Journal of Wild Life Diseases,* 38, (4): 760-3.

THURMOND, M.C., HOLMBERG, C.A. and LUIZ, D.M., 1989. Evaluation of a digitonin assay to discriminate between *Acholeplasma* and *Mycoplasma isolates* from bovine milk. *Cornell Veterinarian*, 79: 71-81.

TRICHARD, C.J.V. and Van TONDER, E.M., 1994. Ulcerative balanoposthitis and vulvovaginitis in sheep and goats. In: Coetzer J.A.W., Thomson G.R., Tustin R.C., (eds). Infectious diseases of livestock with special reference to southern Africa. Vol. 1, Oxford University Press, Cape Town, 1599-1602.

TRICHARD, C.J.V., PROZESKEY, L. JACOBSZ, E.P. and HENTON, M.M., 1994. The identification of *Mycoplasma mycoides subspecies mycoides* large colony as the aetiological agent of balanoposthitis and vulvovaginitis in sheep in South Africa. *Onderstepoort Journal of Veterinary Research*, 60: 29-37.

TRUEBLOOD, M.S. and CHOW, T.L., 1963. Characterization of the agents of ulcerative dermatosis and contagious ecthyma. *American Journal of Veterinary Research*.

TULLY, J.G., 1989. Class *mollicutes:* new perspectives from plant and arthropod studies, pp 1-31. In: R.F. Whitcomb and J.G. Tully eds. The mycoplasmas, Vol. 5, Academic press, Inc., New York.

TUNNICLIFF, E.A., 1949. Ulcerative dermatosis of sheep. *American Journal of Veterinary Research*, 10: 240-249.

VAN KUPPEVELD, F.J., VAN DER LOGT, J.T., ANGULO, A.F., VAN ZOOEST, J., QUINT, W.G., NIESTERS, H.G., GALAMA, J.M. and MELCHERS, W.J., 1992. Genus and species-specific identification of *Mycoplasma* 16S rRNA amplification. *Applied Environmental Microbiology,* 58: 2606-2615.

VEILEI, E.M., NICOLET, J. and FREY, J., 1999. A Novel insertion element creating long variable length direct repeat which is specific for *Mycoplasma mycoides* subsp. *mycoides* small colony type. *Journal of Bacteriology*, 181: 1319-13123.

WANG, G., WHITTMAN, T.S., BERG, C.M. and BERG, D.E., 1994. RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. *Nucleic Acid Research*, 21: 5930-5933.

WANG, J., WILKINSON, R., NICOL, C., NUSBAUM, B., BIRREN, H., BERG, C. and CHURCH, M.G., 2004. The complete genome and proteome of *Mycoplasma mobile*. *Genome Research*, 14: 1447-1461.

WEBB, R.F. and CHICK, B.F., 1976. Balanitis and vulvovaginitis in sheep. *Australian Veterinary Journal*, 52: 241-242.

WEISBURG, W.G., TULLY, J.G., ROSE, D.L., PETZEL, J.P., OYAIZU, H., YANG, D., MANDELCO, L., SECHREST, J., LAWRENCE, T.G., VAN ETTEN, J.,M., MANILOFF, J. and WOESE, C.R., 1989. A phylogenetic analysis of mycoplasmas: basis for their classification. *Journal of Bacteriology,* 171: 6455-6467.

WEISBURG, W.G., BARNS, S.M., PELLETIER, D.A., and LANE, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173: 697-703.

WESONGA, H.O., BÖLSKE, G., THIAUCOURT, F., WANJOHI, C. and LINDBERG, R., 2004. Pleuropneumonia: a long term study on the course of infection and pathology in a flock of goats infected with *Mycoplasma capricolum* subsp. *capripneumoniae. Acta Veterinaria Scandinavica*, 45: 167.

YAMATOTO, R., 1990. *Mollicutes*, In: *Review of Veterinary Microbiology*. Eds., E.L. Biberstein and Y.C. Zee, Blackwell Scientific Publications, pp 213-221.

YOSHID, T., MAEDA S., DEGUCHI, T. and ISHIKO, H., 2002. Phylogeny-Based Rapid Identification of Mycoplasmas and Ureaplasmas from Urethritis Patients. *Journal of Clinical Microbiology*, 40,(1): 105-110.