IDENTIFICATION AND CHARACTERIZATION OF THE PRIMARY INFECTIOUS AGENTS ASSOCIATED WITH OVINE ULCERATIVE BALANOPOSTHITIS AND VULVOVAGINITIS IN SOUTH AFRICA

By

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A dissertation submitted in partial fulfilment of the requirements for the degree of Master in Veterinary Science (Microbiology)

Department Of Veterinary Tropical Diseases

Faculty Of Veterinary Science

University Of Pretoria

Pretoria

2003

DECLARATION

I declare that, apart from the assistance received, which has been duly acknowledged, this dissertation is the original work of the author and has not been presented by me for any other degree at any other University.

CANDIDATE DATE: March, 2003

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DEDICATION

To my wife Muluberhan, my children Michael, Bisrat and Kal for their love, commitment and whole-hearted encouragement that gave me the power and enthusiasm to complete my studies.

To my parents for their efforts and contribution to my academic achievements at schools and university.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and thanks to the following people and organizations:

My wife Muluberhan and my children Michael, Bisrat and Kal, for their love, support and encouragement, and for understanding my preoccupation over the last two years for my postgraduate studies

My promoter Professor Moritz van Vuuren, for giving direction and purpose to this project, for his sound advice and insight, and limitless effort to give the final shape to the dissertation.

My co-promoter Professor Bruce Gummow, for his guidance in the project design, valuable input regarding statistics and for the many discussions on some aspects of the project.

Mr. Johan Gouws, for his unreserved full support, sharing his knowledge and experience, and for his sincere friendship.

Dr. J. A. Picard and the late Mr. Johan Carstens, who provided me with invaluable assistance in bacteriology.

Dr. Jannie Crafford for assisting me with all my computer enquires.

Drs. M. P. van Aardt, J. Pienaar, A. J. van Rensburg, and Ms. E. van Aardt and Mr. F. C. Theron for their invaluable contribution in organising the field work for the project.

Professor Gareth Bath for his professional advice particularly at the initial stage of the project.

Professor J. A. W. Coetzer for his valuable comments and advice in the preparation of the protocol.

Professor P. Stadler for providing insight and information regarding the history of Dorper sheep in South Africa and their current status.

Professor G. E. Swan for his valuable comments on the pharmacokinetics of antimicrobial drugs.

Ms. Estelle Mayhew and Mrs. C. Vermeulen, for all the assistance with the scanner, computer graphics and photographs.

Ms. Daleen Josling, for many hours spent demonstrating the principles of electron microscopy.

The National Animal Health Research Centre, Sebeta, Ethiopia, for giving me the opportunity to study for a masters degree.

The Ethiopian Agricultural Research Organization for providing me the fellowship to complete my studies.

The Dorper Breeder's association, for their considerable financial support to the project.

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ABBREVIATIONS

%	Percentage
&	And
<	Less than
>	Greater than
API	Analytical profile index
ccu	Colour changing unit
CI	Confidence interval
CO_2	Carbon dioxide
CPD	Contagious pustular dermatitis
cpe	Cytopathic effect
DNA	Deoxyribonucleic acid
EL	East degree longitude
ELISA	Enzyme linked immunosorbent assay
et al	And others
FBS	Foetal bovine serum
Fig	Figure
g	Gram
i.e.	That is
ID	Identification
IFAT	Indirect immunofluorescent antibody test
IM	Intramuscular
IU	International unit
IV	Intravenous
LFK	Lamb foetal kidney
*Mmm*LC	*Mycoplasma mycoides mycoides* large colony
MEM	Minimum essential medium
MIC	Minimum inhibitory concentration
mg	Milligram
mℓ	Millilitre

mm	Millimetre
$MnSO_4$	Manganese sulphate
n	Number
NaOH	Sodium hydroxide
^oC	Degree Celsius
OR	Odds ratio
p	Probability
PBS	Phosphate buffered saline
PPLO	Pleuropneumonia-like organism
RMIB	Rabbit meat infusion broth
SL	South degree latitude
spp	Species
ub/vv	Ulcerative balanoposthitis and vulvovaginitis
µg	Microgram
µℓ	Microlitre
µm	Micrometre
v/v	Volume/volume
w/v	Weight/volume
x	Gravity
γ	Gamma

SUMMARY

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Ulcerative balanoposthitis and vulvovaginitis is a disease characterized by erosion and ulceration of the glans penis and muco-cutaneous junction of the vulval lips of sheep. The disease was first recognized in South Africa in 1979 in the Calvinia district, Northern Cape province, and its distribution has since extended to all major Dorper sheep farming areas of the country with serious economic consequences. It is now a major concern for Dorper sheep breeders and farmers due to the fact that the disease has a detrimental effect on

conception and subsequent lambing percentages. The aetiology and epidemiology of the disease are not well established.

During this study the microbial flora in the genital tract of both clinically healthy and infected sheep were compared. The aim was to isolate and identify the pathogenic microorganism/s that contribute to the disease in sheep and to determine the *in vitro* antimicrobial susceptibility of the isolates.

Flocks of sheep in the Northern Cape province showing clinical signs of ulcerative balanoposthitis and vulvovaginitis were examined. The microbiological flora of 116 clinically unaffected sheep and 104 affected sheep from 15 different farms, and with characteristic ulcerative lesions were examined. Swabs from rams and ewes were collected aseptically, and put into cryovials consisting of transport medium for bacterial, mycoplasmal and viral maintenance. Swabs for *Chlamydophila* species antigen detection were placed into tubes without transport medium.

All specimens were processed for virus isolation in cell culture, *Chlamydophila* antigen detection with ELISA, and bacteria and mycoplasmas were isolated on standard culture media. The latter were further identified with biochemical tests and indirect immunofluorescence antibody (IFA) test.

The IFA test was found to be useful when contamination with other microorganisms was prevalent, especially in genital tract specimens. This procedure had reduced the necessity for sub-culturing and cloning. The IFAT was found to be effective for the identification of *Mycoplasma* spp. growing on primary growth media in mixed cultures. The technique also helped to confirm the presence of mycoplasmas that did not produce typical colonies, and it was possible to identify mycoplasma colonies overgrown by bacterial contaminants.

Bacteriological examination of materials from affected and unaffected ewes and rams resulted in the isolation of a sizeable number of *Arcanobacterium*

pyogenes. It was isolated from 44.2 % affected sheep and 17.2 % healthy ones. This isolation difference was statistically significant ($p<0.01$). Seventy four per cent of the isolates came from severe clinical cases.

There were no significant differences in isolations of *Corynebacterium* species and *streptococcus* species between normal and clinically affected sheep.

The mollicutes isolated from the genito-urinary tract of the sampled sheep included *Mycoplasma mycoides mycoides* large colony, *Mycoplasma* species Group 7, *Mycoplasma capricolum*, *Mycoplasma capri*, *Mycoplasma bovigenitalium*, *Mycoplasma agalactiae*, *Mycoplasma arginini*, and unidentified *Mycoplasma* spp. and *Acholepalsma laidlawii* and *Ureaplasma* species.

Mycoplasma was isolated from 49.3 % of 116 clinically normal sheep and 78.2 % of 104 affected sheep. There were significant differences in rates of isolation among clinical groups ($p<0.05$). Of all the mycoplasma isolates, *Mycoplasma mycoides mycoides* LC was isolated from 61.5 % of clinically diseased sheep while 6.0 % of the isolates were from apparently healthy animals ($p<0.05$). There was a significant association between the degree of the severity of the lesion and the rate of isolation of *M mm* LC ($p<0.05$).

The extent of the isolation of *M mm* LC suggests a causal relationship with ulcerative balanitis and vulvitis in sheep in South Africa. The present findings, together with those of Trichard *et al*, (1993), showed that *M mm* LC is the major pathogenic mycoplasma incriminated in ulcerative balanitis and vulvitis in sheep in South Africa. However, results also point towards other pathogens such as *A. pyogenes* playing a role in the pathogenesis of the disease and predisposing the genital tract to infection with mycoplasma.

A number of other identified and unidentified strains of mycolasma were isolated from both clinically affected and healthy sheep. However, in genital tract infection is uncertain.

Virus isolation efforts in cell culture and *Chlamydophila* antigen capture ELISA yielded negative results in affected rams and ewes, and healthy sheep.

Current clinical observations during this project have shown that the typical ulceration appears to be confined to the glans penis and lips of the vulva and no ulceration has been observed on the shaft of the penis and vaginal vestibule. In uncomplicated cases inflammation of the prepuce and vaginal vestibule is not a regular feature of the disease. Therefore, the name ulcerative balanitis and vulvitis most accurately describe the clinical signs of the disease in South Africa.

Age-related susceptibility to the disease was observed. Young sheep are 2.5 times more likely to have the lesion than adult sheep ($p<0.05$). It was also observed that male sheep acquire severe lesions more often than female sheep.

Since, infection in both male and female sheep can spread by coitus, it may valuable to attempt detection of infected animals and follow a strict isolation policy. In addition, further studies to elucidate predisposing factors related to management and environment are required.

The *in vitro* activities of enrofloxacin, florfenicol, oxytetracycline and spiramycin were determined against field isolates of *Mmm*LC by means of the broth microdilution technique. The minimum inhibitory concentrations (MIC) of these antimicrobial drugs were determined for a representative number of 10 isolates and one type strain. The susceptibility of *A. pyogenes* to enrofloxacin, oxytetracycline and tilmicosin was determined by means of agar disk diffusion test.

The MICs of enrofloxacin, florfenicol, oxytetracycline and spiramycin were within the ranges of 0.125-0.5, 1.0-2.0, 2.0-4.0 and 4.0-8.0 μg $m\ell^{-1}$, respectively. This study has shown that resistance of *Mmm*LC against enrofloxacin, florfenicol, oxytetracycline and spiramycin was negligible. All tested field strains of *A. pyogenes* were susceptible to enrofloxacin,

oxytetracycline and tilmicosin with mean inhibition zones of 30.6, 42.3 & 35.8 mm, respectively.

Although, there is a lack of data on *in vivo* efficacy and *in vitro* MIC breakpoints of these antimicrobial drugs for *Mmm*LC, the MIC results indicate that these 4 classes of antimicrobial drugs should be effective in the treatment of ulcerative balanitis and vulvitis in sheep in South Africa.

CHAPTER I

1 INTRODUCTION

Sheep are believed to be one of the first mammals to be domesticated and are known to have been closely associated with man from a very early date (Shelton, 1995). Sheep make an important and continuing contribution to providing high quality food and fibre for the growing world population.

The original and native sheep of South Africa were the leggy, hair-bearing and fat tailed types indigenous to hot climates and a primitive husbandry system. The beginning of a modern sheep industry in this country dates back to the early part of the 19th century, when the first exotic breed, a flock of Merino sheep, were imported from Spain (Erasmus, 1976; Ensminger, 2002). The industry has grown significantly during the last century. Today, the Republic of South Africa is an acknowledged sheep-farming country particularly in terms of wool production. Wool production is one of the country's biggest agricultural industries and contributes a substantial amount of foreign currency earnings (Ensminger, 2002).

The majority sheep in South Africa are Merinos, although several meat breeds have been introduced. The best known mutton breed is the Dorper that was developed in South Africa in 1942 from crosses of Dorset Horn males and Black head Persian females (Wilson, 1991). There were two main reasons for this cross breeding. Firstly, to have a sheep breed, that can best adapt to the arid or semi-arid areas of the country. Secondly, a desire to have a mutton breed with a uniformly distributed fat cover all over the body and a high quality carcass (P. Stadler, personal communication, 2002). Over the years, there have been efforts to refine the breed to the highest degree of perfection. The Dorper is currently the second most numerous sheep breed in the country.

The major obstacles for sheep production in South Africa are, (1) prevalence of diseases, (2) unreliable labour, (3) predatory animals, especially jackal, and (4) frequent droughts (Ensminger, 2002; P. Stadler, personal communication, 2002).

Among the disease problems, ulcerative balanoposthitis and vulvovaginitis (ub/vv) is an economically important disease and receives a lot of attention from stockowners and veterinarians. There has recently been renewed interest and research effort to provide farmers with alternative approaches to control the disease. This has mainly been directed at increasing our knowledge of the epidemiology and the aetiology of the disease.

The information obtained from earlier investigations indicate that the cause of ub/vv has not clearly been established and the epidemiology, clinical features, immunopathology and control still require further investigation. Furthermore, confusion could arise with the different names used by researchers in different countries to describe the lesions. Various names given to the syndrome include, vulvovaginitis (Cottew, G. S., Lloyd, L. C. & Parsonson, I. M., 1974), balanitis and vulvovaginitis (Webb & Chick, 1976), granular vulvovaginitis (Doig & Ruhnke, 1977), vulvitis (Ball & McCaughey, 1982), ulcerative balanitis and vulvitis (Deas, 1983; Dunn, 1996; Greig, 2000), ulcerative balanoposthitis and vulvovaginits (Trichard, C., J., V., Jordan, P., Prozesky, L., Jacobsz, E. P. & Henton, M., 1993; Trichard & Van Tonder, 1994). For the purpose of this dissertation, all contagious ulcerative lesions of the genital tract of sheep, irrespective of the geographical location, will be referred to as ub/vv.

Ulcerative balanoposthitiss and vulvovaginitis of sheep is a venereal disease characterized by erosion and ulceration on the glans of the penis and vulval labia of sheep and has been described in several countries (Deas, 1983;Trichard *et al*, 1993; Trichard & Van Tonder, 1994; Dunn, 1996; Greig, 2000; Bath & De Wet, 2000). The disease was first encountered in South Africa in the Calvinia district in the Northern Cape province in 1979, and later spread and infected

sheep populations on numerous farms in the semi-arid parts of the Free State, Kwazulu Natal and Eastern and Western Cape provinces (Trichard *et al*, 1993; Trichard & Van Tonder, 1994). The disease appears most frequently in Dorper breeds (Trichard *et al*, 1993; Bath & De Wet, 2000; Gummow & Staley, 2000).

The introduction of ub/vv onto farms has hampered active participation of breeders in shows and auctions, contributing further to financial losses.

Although the aetiology of the disease has not been conclusively resolved, Trichard *et al*, (1993) isolated mollicutes from naturally infected ewes and rams with signs of vulvovaginitis and balanoposthitis. They further suggested that *Mycoplasma mycoides mycoides* large colony biotype (*Mmm*LC) could be incriminated as the major cause of ub/vv in Dorper flocks in South Africa. Although the disease was reproduced following the application of *Mmm*LC intravaginally, it is imperative to undertake additional research to isolate and identify the causative organism of this disease. Furthermore, a search of the literature has failed to reveal any references to the role of *Mmm*LC as the sole agent of the disease.

Clinical signs and microbiological examination forms the basis of the diagnosis of ub/vv. The gross clinical appearance of ub/vv may vary depending on secondary bacterial invasion, severity and age of the lesion. This has led to inconsistencies in the description of the clinical signs by various authors (Dent, 1971; Webb & Chick, 1976; Deas, 1983; Linklater & Smith, 1993; Trichard *et al*, 1993; Trichard & Van Tonder, 1994; Greig, 2000; Bath & De Wet, 2000). Furthermore, the difficulties of diagnosing the disease are compounded by the fact that a single causative pathogen up until now has not been consistently identified.

In South Africa, farmers endeavour to control the disease through treatment and selective breeding using clean rams. However, none of the options are efficient enough to meaningfully alleviate the problem. The antimicrobial drugs

readily available and administered to affected sheep are helpful for a temporary remission of the clinical signs, which at a later stage may reappear after mating. On the other hand the use of clean rams for breeding purposes may not be a panacea by virtue of the fact that the rams can develop clinical signs just after the start of breeding, due to the tendency of over-working that suppresses their resistance and succumb to infection either through endogenous source in the ram itself, or from carrier ewes.

It is clear from published and unpublished reports that ub/vv in sheep flocks in South Africa is reason for concern and requires serious attention. The dearth of reports of the occurrence of the disease in flocks of Dorper sheep in South Africa warrants further investigation. The most important elements that have not been conclusively determined are the specific organism or group of organisms associated with the disease, susceptibility of the latter to antimicrobial drugs, and predisposing factors.

The objectives of this study were to determine whether ulcerative balanoposthitis and vulvovaginits in South Africa is a multifactorial disease in which the major causative organism is a mollicute, whether the disease manifests mainly in sheep of both sexes under the age of 36 months, and to detrmine the antimicrobial sensitivities of the causative organisms.

CHAPTER II

2 REVIEW OF THE LITERATURE

2.1 ULCERATIVE DISEASES OF THE GENITAL TRACT OF SMALL RUMINANTS

2.1.1 Contagious pustular dermatitis (CPD): Synonyms; orf, contagious ecthyma, scabby mouth

Venereal orf occurs as one aspect of the disease CPD, the lesions of which are most commonly found on the lips, muzzle, ears and buccal cavity of sheep (Munz & Dumbell, 1994[a]). The disease is caused by a *Parapoxvirus* and is characterized by the formation of small pustules, which later develop into granulo-ulcerative lesions on the prepuce, penis and skin of the vulva (Linklater & Smith, 1993). The lesions are proliferative rather than ulcerative.

In the genital from of CPD, lesions occur on the scrotum, prepuce and penis, and on the vulval labiae at the mucosal-cutaneous junction (Linklater & Smith, 1993; Munz & Dumbell, 1994[a]). Venereal orf appears shortly after rams are turned out for mating. The disease is also expressed in pedal forms, where the coronet and interdigital spaces are involved.

CPD is common in South Africa and was reported for the first time by Theiler, in 1928 and cited by Munz & Dumbell (1994[a]).

2.1.2 Ulcerative dermatosis: Synonyms; ovine venereal disease, lip and leg ulceration

Ulcerative dermatosis is a contagious disease of sheep characterized by the formation of encrusted ulcers on the face, feet, 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 & Dumbell, 1994[b]). However, reports in the literature indicate that the viral agent causing ulcerative dermatosis is physically similar (Trueblood & Chow, 1963), but antigenically different (Radostits, O. M., Blood, D. C. & Gay, C. C., 1994) from CPD virus.

The term ulcerative dermatosis was originally given by Tunnicliff (1949) in the USA to an infectious condition with epidermal and subcutaneous tissue destruction resulting in granulating ulcers of the affected lips, legs, feet, lips of the vulva, the prepuce at the orifice and the glans penis. Recent publications have also supported the description given by Tunnicliff (Kimberling, 1988; Munz & Dumbell, 1994[b]). The lesions are ulcerative and destructive rather than proliferative as in CPD.

2.1.3 Sheath Rot: Synonyms; enzootic posthitis, urine scald, balanoposthitis

A disease variously termed as ulcerative posthitis, sheath rot and enzootic posthitis has been reported in Australia (Beveridge & Johnstone, 1953; Southcott, 1965[b]) and the UK (Roberts & Bolton, 1945; Doherty, 1985). Sheath rot, an enzootic inflammation of the prepuce and penis of mainly castrated male sheep, is caused by a urea-producing diphteroid organism, identified as *Corynebacterium renale*, (Southcott, 1965[a]; Barajas & Biberstein, 1974; Blood, D. C., Radostits, O. M., Arundel, J. H. & Gay, C. C., 1989; Linklater & Smith, 1993). However, Brightling (1988) pointed out that *C. renale* can also be found in healthy sheep as commensalistic bacteria.

In Australia, it is generally considered that Merinos are much more prone to the disease than other breeds and their crosses (Beveridge & Johnstone, 1953). The disease has also been recorded in wether goats in the USA (Shelton & Livingstone, 1975).

In South Africa, Steyn (1930) and Styen (1940) described a condition called 'pisgoed' or 'pisgras' affecting wethers, which resembled sheath rot clinically and was confirmed as infectious in nature. He was able to transmit the disease through pus, but not by pure culture from the lesion.

Until Southcott (1963) reported the involvement of a Gram-positive diphtheroid bacterium, the disease was considered for many years to be non-infectious and exclusively attributed to dietary factors. Excess dietary protein, which eventually leads to an increase in the urinary concentration of urea, probably predisposes to infection (Belschner, 1971; Blood *et al*, 1989; Linklater & Smith, 1993). *Corynebacterium renale* breaks down the urea to produce ammonia, that irritates and damages the inner lining of the prepuce, the penis, and skin surrounding the preputial orifice (Southcott, 1965[a]). The seasonal variation in the incidence of the disease results mainly from the seasonal variation in the amount of inciting factor in the pasture.

The possible role of anatomical features in predisposing sheep to the disease have also been studied and Beveridge & Johnstone (1953) have shown that there is no definitive relationship between the preputial pendancy and the diameter of the penile orifice with the occurrence of the disease. However, the work of Southcott (1965[a,b]) suggests that varying preputial pendancy and orifice diameter may predispose to disease. Furthermore, the high incidence of the disease in wethers and young rams could probably be related to the close adherence of the preputial and penile skins, which separate in mature entire animals (Belschner, 1971; Blood *et al*, 1989; Linklater & Smith, 1993).

The disease is characterized by a spreading superficial ulceration of the skin of the prepuce and may sometimes involve the preputial lining and the penis (Beveridge & Johnstone, 1953; Linklater & Smith, 1993). The most important manifestations of the disease are swelling of the sheath, dribbling of urine and in progressive conditions heavily stained wool around the sheath, which

contains urine, pus and necrotic material with characteristic foul odours (Ensminger 2002).

In very advanced cases the penis itself becomes ulcerated and the glans penis eventually erodes and it is common for the urethral process to slough off. Ulcers are small at first but they gradually enlarge, coalesce with others and become covered with a scab. The preputial opening may become blocked, and the sheath will become distended with foul-smelling urine and pus (Beveridge & Johnstone, 1953). The removal of the overlying material from the ulcers does not leave a bleeding surface but a smooth glistening one.

An ulcerative vulvitis occurs in the same flock in which posthitis occurs in wethers and is thought to be a venereal extension of that disease (Dent, 1971; Brightling, 1988; Blood *et al*, 1989). Ulcerative vulvitis begins as an inflammatory reddening of the vulvar lips associated with marked swelling and ulceration of the ventral vulvar commissure, clitoris and posterior parts of the vagina (Dent, 1971).

2.1.4 Ulcerative balanoposthitis and vulvovaginitis

Contagious ulcerative lesions of unknown aetiology are often observed on the penis of rams and the vulva of ewes during the breeding period (Deas, 1983; Linklater & Smith, 1993; Trichard & Van Tonder, 1994; Bath & De Wet, 2000; Greig, 2000). A deep ulcer is formed on the glans penis, which in most cases is filled with blood clots. The vulva of affected ewes show marked oedema and reddened erosions. Affected rams and ewes often refuse coitus and as a result the conception rate is reduced with ensuing economic implications. Poor conception rates associated with the disease have been reported elsewhere (Doig & Ruhnke, 1977; Livingstone & Gauer, 1982; Gummow & Staley, 2000).

Although no infectious agents have been consistently isolated (Webb & Chick, 1976; Linklater & Smith, 1993; Trichard & Van Tonder, 1994), there is some

evidence that bacteria (Ball, H. J., Kennedy, S. & Ellis, W. A., 1991; Trichard *et al*, 1993), *Ureaplasma* (Doig & Ruhnke, 1977; Ball & McCaughey, 1982; McCaughey & Ball, 1983) and *Mycoplasma* (Cottew *et al*, 1974; Doig & Ruhnke, 1977; Livingstone & Gauer, 1983; Kapoor, S. G., Pathak, R. C. & Singh, P. P., 1984; Trichard *et al*, 1993) may have been involved and have been recovered in some cases from clinical specimens. Isolation of *Acholeplasma laidlawii* and *A. axanthum* has been reported from sheep with ulcerative genital disease (Doig & Ruhnke, 1977; Jones *et al*, 1983). Herpesvirus antigenically related to infectious bovine rhinotracheitis virus (Rosadio, R. H., Evermann, J. F.& mueller, G. M., 1984) has also been isolated from an outbreak of vulvovaginitis in goats (Horner, G. W., Hunter, R. & Day, A. M., 1982; Grewal & Wells, 1986). In both reports, caprine herpesvirus was isolated from ulcerative vaginitis and vulvitis in does but no lesions were detected in the males. However, Tarigan, S, Webb, K.S. & Mcintosh, M. A (1987) have isolated a caprine herpesvirus from clinical cases of balanoposthitis in goats.

2.1.4.1 Global perspective

Ulcerative conditions of the genitalia of sheep have been described in Australia (Cottew *et al*, 1974; Webb & Chick, 1976), Canada (Doig & Ruhnke, 1977), Britain (Ball & McCaughey, 1982; Jones, G. E., Rae, A. E., Holmes, R. G., Lister, S. A., Jones, J. M. W. Grater, G. S. & Richards, N., 1983; Dunn, 1996), India (Kapoor *et al*, 1984), South Africa (Trichard *et al*, 1993) and in goats in India (Singh, N., Rajyan, B. S. & Mohanty, G. C., 1974), New Zealand (Horner *et al*, 1982), Australia (Grewal & Wells, 1986; Tarigan *et al*, 1987) and Nigeria (Chima, J. C., Ojo, M. O. & Adetosoye, A. I., 1992). Cottew and his associates (1974), Doig & Ruhnke (1977) and Ball & McCaughey (1982), in their reports, have shown that the disease was only seen in ewes and no mention was made of the simultaneous involvement of associated rams. On the other hand, Webb & Chick (1976), Deas (1983), Trichard *et al* (1993), Dunn (1996) and Bath & De Wet (2000) have described the occurrence of the disease in both ewes and rams running together.

Frequently the first sign of ulcerative balanoposthitis and vulvovaginitis in a flock is the presence of blood on or around the vulva of a number of ewes between two to three weeks after the start of mating, while associated rams have shown sharp-edged deep ulcers on the glans penis (Deas, 1983; Bath & De Wet, 2000; Greig, 2000). The ulceration can give rise to severe haemorrhage from the glans, which is recognized when blood appears on the wool around the vulva of the ewes.

Vulvitis, with swelling and ulceration of the vulva and posterior vagina, and ulcerative lesions on the penile tissues could be associated with *C. renale*, mycoplasma and ureaplasma infections (Greig, 2000). However, attempts to isolate infectious agents associated with the disease have failed to consistently yield specific organisms (Webb & Chick, 1976; Deas, 1983; Linklater & Smith, 1993; Trichard & Van Tonder, 1994; Greig, 2000). In goats, a similar condition has been ascribed to a herpesvirus (Horner *et al*, 1982; Grewal & Wells, 1986; Tarigan *et al*, 1987; Linklater & Smith, 1993).

Although *Mycoplasma* species *2D* was isolated from sheep with reproductive problems (Cottew *et al*, 1974; Livingstone & Gauer, 1983), isolations were also made from apparently normal flocks and to date the aetiological role of this species has not been established (Carmichael, L. E., St. George, T. D., Sullivan, N. D. & Horsfall, N., 1972; Livingstone & Gauer, 1983).

Ulcerative lesions of the vulva, penis and prepuce, associated with *Mycoplasma* and *Ureaplasma* serotypes, have been described in several countries worldwide (Jones *et al*, 1983). Mycoplasma and ureaplasma organisms have been isolated together, or separately, from rams and ewes and the diseases associated with this group of organisms include vulvovaginitis (Cottew *et al*, 1974), vulvovaginitis and granular vulvitis (Doig & Ruhnke, 1977), vulvitis (Ball & McCaughey, 1982) and ulcerative balanoposthitis and vulvovaginitis (Trichard *et al*, 1993).

Potentially pathogenic species involved in genital infections of small ruminants include *Mycoplasama capricolum* (Jones *et al*, 1983; Jones, 1983), *Mycoplasma arginini* (Jones, 1983), *Mycoplasma mycoides mycoides* (Cottew *et al*, 1974; Trichard *et al*, 1993), *Mycoplasma agalactiae* (Jones, 1983), *Acholeplasma laidlawii* and *Acholeplasma axantum* (Jones *et al*, 1983; Kapoor *et al*, 1984), *Ureaplasma* (Ball & McCaughey, 1982; Livingstone & 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.

Since the first report of ovine urogenital disease associated with ureaplasma (Doig & Ruhnke, 1977), there have been several similar reports from other parts of the world (McCaughey, W. J., Ball, H. J. & Irwin, D., 1979; McCaughey & Ball, 1981; Jones *et al*, 1983). Ureaplasma was also implicated in an outbreak of vulvitis in Northern Ireland, and vulvitis and vaginitis with ulceration of the vestibule was evident in some cases following experimental transmission (Ball & McCaughey, 1982).

Although, high isolation rates of ureaplasmas have been recorded from the genital tract of clinically normal ewes (Livingstone & Gauer, 1975; McCaughey *et al*, 1979; McCaughey & Ball, 1981), several serotypes have been shown to be associated with diseases in sheep (Cottew *et al*, 1974; Livingstone & Gauer, 1982).

Many workers who believed ub/vv is infectious, but failed to associate the disease with mycoplasma or bacteria, have suggested that a virus could be the cause. However, an investigation into a viral aetiology in tissue culture and electron microscopy, has failed to reveal the involvement of viruses in ulcerative balanitis and vulvitis in sheep (Webb & Chick, 1976; Deas, 1983; Trichard *et al*, 1993). Although viruses have consistently not been isolated concurrently with the disease, numerous pathogenic bacteria have been isolated from the lesions (Ball & McCaughey, 1982; Deas, 1983). The role of secondary bacteria in the

progression of the mycoplasma and ureaplasma infections is supported by reports that clinical lesions improve after broad-spectrum antibacterial treatment (Ball & McCaughey, 1982).

Ub/vv has been described as occurring before the mating season (Gummow & Staley, 2000), during the breeding season (Jones *et al*, 1983; Gummow & Staley, 2000) and before lambing (Jones *et al*, 1983; Cottew *et al*, 1974). An outbreak of the disease would affect up to 50 % of exposed flock (Jones *et al*, 1983) and a reduction of lambing percentage by not less than 50% in the affected flock has been reported (Bath & De Wet, 2000).

2.1.4.2 South African situation

Although ulcerative balanoposthitis and vulvovaginitis has been known in South Africa since 1979 (Trichard *et al*, 1993; Trichard & Van Tonder, 1994), it is only in the last decade that the disease underwent scientific investigation. The geographical distribution of the disease seems to follow the distribution of Dorper sheep in South Africa and thus the dry areas of the Northern, Western and Eastern Cape Provinces, Kwazulu Natal and Free State provinces are generally affected by the disease (Gummow & Staley, 2000). The disease appears most frequently in Dorper sheep (Trichard *et al*, 1993; Bath & De Wet, 2000; Gummow & Staley, 2000).

Since the first report on the occurrence of the disease in South Africa, issues relating to the epidemiology, aetiology and control have not been adequately addressed. However, Trichard *et al*, (1993) have carried out a field trial and laboratory experiments to determine the cause of the disease. They claimed that *M. mycoides mycoides* large colony biotype is the definitive cause of the disease, though some other invading bacteria have also been isolated from field specimens. They reported that the ewes experimentally infected with a field strain of *Mmm*LC and in which coitus was allowed, developed vulvovaginitis and the corresponding rams developed ulcerative balanoposthitis. Furthermore, the

disease was reproduced in another group of ewes by *Mmm*LC isolated from an earlier experiment. This later result attested to the fact that *Mmm*LC is likely to be the major aetiological agent responsible for ulcerative balanoposthitis and vulvovaginitis in Dorper sheep in South Africa. However, this finding remains the only one in the world and at present there is insufficient evidence to support this view and this needs to be verified. Bath & De Wet (2000) and Gummow & Staley (2000) were of the opinion that ulcerative balanoposthitis and vulvovaginitis could likely be a diseases associated with mycoplasmal aetiology.

Several electron microscopic investigations of ub/vv have failed to detect any viruses (Trichard *et al*, 1993).

Although the lesions can be observed during clinical examination of rams, which is a prerequisite prior to the start of breeding (G. van Aardt, personal communication, 2001), outbreaks of the disease are mostly observed a few days after the start of mating (G. van Aardt, personal communication, 2001; J. Pienaar, personal communication, 2002). The first sign noticed by shepherds or stockowners is blood on or around the vulva of ewes and on the wool around the preputial orifice of rams.

It is assumed that secondary bacterial infections could complicate the situation by causing damage to the soft tissues of the penis leading to the accumulation of pus and dead tissues on the penis and preputial cavity, and cause either phimosis or paraphimosis. Affected rams are often reluctant to mate, however, infected ewes can produce lambs if mated with a fertile ram. Low lambing percentages of about 50 % in Dorpers have been reported (Bath & De Wet, 2000).

The disease often appears to be self-limiting as evidenced by spontaneous recovery of infected sheep (G. van Aardt, personal communication, 2002). However, the disease may flare occasionally in affected flocks (Bath & De Wet,

2000) and the efforts of farmers to contain the disease through treatment are sometimes not successful. The drugs commonly used by farmers are tetracyclines and topical applications of acriflavine – glycerine mixtures or iodine solutions (Gummow & Staley, 2000).

2.1.4.3 Possible causes of ulcerative balanoposthitis and vulvovaginitis

2.1.4.3.1 *Mycoplasma* species

2.1.4.3.1.1 General

Mycoplasma species are members of the class mollicutes and are the smallest self-replicating free-living bacteria (Yamamoto, 1990; Coetzer, J. A. W., Thomson, G. R., Tustin, R. C. & Kreik, N. P. J., 1994). Mycoplasmas have correspondingly small genomes, and as a consequence of this limited genetic potential, they usually require intimate association with mammalian cell surfaces and manifest complex nutritional requirements for *in vitro* growth (Thomas; 1985; Coetzer *et al*, 1994).

Mycoplasma spp. can be identified with the aid of biochemical and serological tests. Tests for glucose fermentation, arginine utilization, tetrazolium HCl reduction, urea hydrolysis, sensitivity to digitonin, serum digestion, phosphatase activity and metabolism of some carbohydrates have been described by several investigators (Purcell, R. H., Taylor-Robinsin, D., Wong, D. & Chanock, R. M., 1966; Taylor-Robinson, D., Purcell, R. H., Wong, D. & Chanock, R. M., 1966; Alutto, B. B., Wittler, R. G., Williams, C. O. & Faber, J. E., 1970; AL-Aubaid & Fabricant, 1971[a]; Carmichael *et al*, 1972; Onoviran, O., Truscott, R. B., Fish, N. N. & Barker, C. A. & Ruhnke, H. L., 1975; Goll, 1994). A digitonin test, which measures the zone of growth inhibition surrounding a digitonin-containing disk, is used to differentiate between *Mycoplasma* and *Acholeplasma* species

(Freundit, E. A., Andrews, B. E., Ernø, H., Kunze, M. & Black, F. T., 1973; Thurmond, M. C., Holmberg, C. A. & Luiz, D. M., 1989).

The identification and classification of *Mycoplasma* spp. have become increasingly dependent on serological tests (Gois, M., Kuksa, F., Franz, J. & Taylor-Robinson, D., 1974), and the growth inhibition (GI), growth precipitation (GP), metabolic inhibition (MI), complement fixation, immunoperoxidase, immunofluorescent antibody (IFA) and enzyme linked immunosorbent assays (ELISA) are the most adapted and widely used serological techniques (Ernø & Jurmanova, 1973; Onoviran *et al*, 1975; Cottew, 1983; Goll, 1994). The use of DNA probes is also becoming increasingly important in the identification of *Mycoplasma* spp. (Taylor, M. A., Wise, K. S. & Mcintosh, M. A., 1985). The GI and MI tests have been found to be very specific (Clayde, 1964; Freundit *et al*, 1973) and therefore suitable for the demonstration of intra-species differences (Hollingdale & Lemcke, 1970; Haller, G. J., Boiarski, K. W. & Somerson, N. L., 1973 and Gois *et al*, 1974). Unlike the GI test, MI is highly sensitive (Goll, 1994).

The GI test is based on the principle that specific antisera inhibit the growth of homologous mycoplasmas (Edward & Fitzgerald, 1954; Clayde, 1964; Edward & Moore, 1975). The term GI applies to those techniques that measure a decrease in the number and/or size of mycoplasma colonies formed on agar medium.

The MI utilizes the ability of specific immune sera to inhibit the effects of such metabolic activities as glucose fermentation (Taylor-Robinson *et al*, 1966), arginine decarboxylation (Purcell *et al*, 1966) and reduction of 2, 3, 5-triphenyl tetrazolium chloride (Senterfit & Jensen 1966; Ernø, H., Jurmanova, K. & Leach, R. H., 1973) and thereby indirectly prevent the change of colour of the culture medium.

Immunofluorescence was first adapted to the identification of mycoplasmas by Liu *et al*, 1956 (cited by Potgieter & Ross, 1972). The IFA technique has proved to be a highly species-specific test for the identification of various *Mycoplasma* species (L'Ecuyer & Boulanger, 1970; Meyling, 1971; Al-Aubaid & Fabricant, 1971[b]) and sensitive like the MI test. Indirect FAT has the advantage that it allows mixed infections to be identified without the isolates having to be cloned (Del Giudice, R. A., Norman, F. R. & Carski, T. R., 1967). The IFAT is more sensitive than and as specific as the direct FAT and is a useful serological tool for the identification and characterization of *Mycoplasma* species (Rosendal & Black, 1972).

Although, the genetically closely related *Mycoplasma mycoides* groups have been difficult to classify on the basis of serological tests due to cross reactivity, extensive studies confirmed that *M. mm* LC and *M. m. capri* were inseparable by protein analysis, and showed that serological tests, in particular immunofluorescence, could usually distinguish them (Leach *et al*, 1989). However, since rRNA genes are highly conserved and since restriction enzyme maps of these genes have been constructed, parts of rRNA were used as probes for characterizatioin of each group of mycoplasmas, and used to determine the relative relatedness of corresponding organisms (Christiansen & Ernø, 1990). Comparison of rRNA sequences has been useful in studying phylogenetic relationships. The development of the polymerase chain reaction (PCR), with specific primers, has also provided a powerful tool that distinguishes the *M. mycoides* subspecies from other members of the cluster (Bashiruddin *et al*, 1994).

2.1.4.3.1.2 Major mycoplasmas of small stock

Mycoplasma agalactiae

This mycoplasma causes contagious agalactia, one of the most important diseases of sheep and goats (DaMassa, Wakenell, P. S. & Brooks, D. L., 1992). Abortion associated with *M. agalactiae* has been reported in sheep and goats in

Spain (Ramírez, A. S., Garcia, M., Díaz-bertarana, Fernández, A. & Poveda, J. B., 2001). Singh *et al* (1974) and DaMassa (1983) have described the involvement of *M. agalactiae* in a clinical case of granular vulvovaginitis in goats.

Mycoplasma mycoides **subspecies** ***mycoides*** **large colony variant**

M mm LC is a member of the *Mycoplasma mycoides* cluster, a group of mycoplasmas that share serological, genomic and antigenic characterstics (DaMassa *et al*, 1992).

Although the LC biotype is not associated with disease that are clinically and pathologically well defined, there are some indications that this mycoplasma could be involved in pathological conditions in small ruminants (Naglic, T., Hotzel, H., Ball, H. J., Seol, B. & Busch, K., 2001). This mycoplasma has also been isolated from goats with polyarthritis, conjunctivitis, keratitis, pneumonia and cervical abscess (Rosendal, S., Ernø, H., & Wyand, D. S., 1979). It has also been reported that *M mm* LC biotype is the cause of ulcerative genital diseases of sheep in South Africa (Trichard *et al*, 1993).

Mycoplasma mycoides **subspecies** ***capri***

It is one of the members of the *M. mycoides* cluster and was considered for many years to be the cause of contagious caprine pleuropneumonia (CCPP) in goats until MacOwan & Minette (1976) reported isolating a new mycoplasma, designated F38, from cases of fibrinous pneumonia. Although highly virulent under experimental conditions, it is not any more the primary cause of classical CCPP. The organism appears to be specific for goats and causes septicaemia and polyarthritis (Rosendal, 1994), but is not recognized as a cause of natural disease in sheep.

Mycoplasma capricolum **subspecies** ***capripneumoniae***

In 1976, MacOwan & Minette reported the isolation of a new mycoplasma (F38) from a CCPP outbreak in Kenya and demonstarted that it caused a highly

contagious form of pneumonia in goats. Later, Leach, R. H., Ernø, H. & MacOwan, K. J. (1993) proposed the designation of F38 type caprine mycoplasma as *Mycoplasma capricolum capripneumoniae*, and since then the name has been widely in use for the casuative agent of classical contagious caprine pleuropneumonia.

Mycoplasma capricolum

Outbreaks of ovine arthritis (Yamamoto, 1990), pneumonia, conjunctivitis and arthritis (Taoudi, A., Johnson, D. W. & Kheyyali, D., 1987) caused by *M. capricolum* have been reported. There is evidence that *M. capricolum* is present in the ear canal of sheep and goats (Cottew & Yeats, 1982), respiratory and genital mucosa of goats (DaMassa, A. J., Brooks, D. L. & Holmberg, C. A., 1984) and has been isolated from cases of vulvovaginitis and balanoposthitis in sheep (Jones, 1983). DaMassa, A. J., Brooks, D. L. & Adler, H. E. (1983) described the involvement of this mycoplasma in severe pneumonia with high mortality in kids.

Mycoplasma arginini

Although the species is not specific for sheep and goats (Leach, 1970), reports indicate that it has been recovered from the genital tracts (Jones, 1983) and respiratory and genital tracts of small ruminants (Rosendal, 1994).

Mycoplasma ovipneumoniae

It affects both sheep and goats and is one of the many agents associated with the pneumonia complex in both species. It has been proven that *M. ovipneumoniae* causes proliferative exudative pneumonia in sheep together with *Mannheimia haemolytica* (Rosendal, 1994)

Mycoplasma conjunctivae

It has been isolated from clinical cases of ovine keratoconjunctivitis together with other bacteria and was suggested as the primary agent of the disease

(Jones, G. E., Foggie, A., Sutherland, A. & Harker, D. B., 1976). It has been shown to induce the disease experimentally (Greig, 1989).

Acholeplasma laidlawii

The species is not host specific and may frequently be isolated from both sheep and goats (Jones *et al*, 1983; Kapoor *et al*, 1984), but is generally regarded as non-pathogenic (Rosendal, 1994).

Ureaplasma

There are only two species, *U. diversum* and *U. urealyticum*, found in animals and humans, respectively. *U. diversum* has been shown to contain several strains that can commonly be found on the mucosa of urogenital and respiratory tracts of animals (Timoney, J. F., Gillespie, J. H., Scott, F. W. & Barlough, J. E., 1988)

2.1.4.3.1.3 Pathogenicity of mollicutes for the genital tract of sheep and goats

Mycoplasmas have a predilection for mucous/serous membranes of the urogenital tract, respiratory tract, joints, conjunctiva and air sacs of animals, and cause diseases like vulvovaginitis, vesiculitis, pneumonia, arthritis, mastitis and air sacculitis (Clayde, 1983; Chima *et al*, 1992).

Several studies carried out in sheep and goats have shown that *M. agalactiae, M. arginini, M. capricolum, M. mycoides mycoides* LC*, A. laidlawii* and *Ureaplasma* species can be isolated from genital tract specimens and illustrate their potential impact on genital infections (Carmichael *et al*, 1972; Cottew *et al,* 1974; Singh *et al*, 1974; Doig & Ruhnke, 1977; Ball & MacCaughey, 1982; Livingstone & Gauer 1983; Jones *et al*, 1983; Chima *et al*, 1992; Trichard *et al*, 1993).

Although experimental inoculation of the organism did not reproduce the disease fully, strong circumstantial evidence suggested involvement of the *2D Mycoplasma* biotype, apparently related to *Mmm*LC (Rosendal, 1994), with an outbreak of vulvovaginitis in ewes in Australia (Cottew *et al*, 1974) and the USA (Livingstone & Gauer, 1983). *M. mycoides* subspecies *mycoides* has been recovered from colostrum, mastitic milk, aborted foetuses and amniotic fluids in goats (DaMassa *et al*, 1983). An outbreak of vulvovaginitis and balanoposthitis caused by *M. capricolum* has also been reported from a sheep flock in England (Jones, 1983).

*Mmm*LC type was assumed to occur almost exclusively in goats (DaMassa *et al*, 1983), however, evidence is emerging to establish its important role in genital diseases of sheep. For example, work conducted in South Africa has shown that *Mmm*LC plays an important role in ovine genital tract infections causing ulcerative balanoposthitis and vulvovaginitis, and reproduction of the disease was possible following experimental inoculation with a field isolate (Trichard *et al*, 1993).

Although the role of *Acholeplasma* spp. as definitive pathogens have not been adequately confirmed (Rosendal, 1994), Tiwana & Singh (1982) reported the isolation of *Acholeplasma oculi* from material from the vulvovaginal canal and uterus of 22 ewes presenting with vulvovaginitis lesions.

The first report of ovine granular vulvitis suggested that certain strains of *Ureaplasma* were associated with the disease (Doig & Ruhnke, 1977). Ureaplasmas have been transmitted to ewes by natural insemination from infected rams (McCaughey & Ball, 1981; Livingstone & Gauer, 1982). Furthermore, the ability of ureaplasmas to induce genital diseases has been substantiated by artificial inoculation of *Ureaplasma* strains, isolated from sheep with vulvitis, into healthy ewes and resulted in mild granularity and hyperaemia of the vulva (Doig & Ruhnke, 1977; Ball & McCaughey, 1982).

Studies with ureaplasmas also showed that infection occurs more frequently in older sheep. As they tend to remain carriers for long periods after primary infection, the nature of the vulvitis produced might be mild due to immunity resulting from a previous infection (McCaughey & Ball, 1981). The prevalence of genital mycoplasmas and ureaplasmas is related to the number of times the animals had been bred (Langford, 1975). It appears that ureaplasmas are not responsible for serious ulcerative genital diseases in sheep (H. J. Ball, personal communication, 2002).

2.1.4.3.2 Bacteria

Prior to its recent re-classification on the basis of 16s rRNA gene sequences as *Arcanobacterium pyogenes* (Ramos, C. P., Foster, G. & Collins, M. D., 1997), this bacterium was formerly called *Corynebacterium pyogenes* and was renamed *Actinomyces pyogenes* (Reddy, C. A., Cornell, C. P. & Fraga, A. M., 1982). However, the names *Actinomyces pyogenes* and even *C. pyogenes* are still frequently used in clinical veterinary medicine. It is a Gram-positive, pleomorphic, rod shaped bacterium associated with a wide variety of pyogenic diseases of animals, including mastitis, abortion, pyometra, arthritis, foot abscess and lameness (Beverley & Watson, 1962; Dennis & Bamford, 1966; Smith, R. E., Reynolds, I. M., Clark, G. W. & Milbury, J. A., 1971; Hinton, 1972; Kasari, T. R., Marquis, H. & Scanlan, C. M., 1988; Gardner, I. A., Hird, D. W. & Sullivan, N. M., 1990; Noakes, D. E., Wallace, L. M. & Smith, G. R., 1990; Semambo, D. K., Ayliffe, T. R. & Boyd, J. S., 1991; Chauhan & Kaushik, 1992).

Ihemelandu (1972) reported the isolation of *A. pyogenes* in Nigeria from pus specimens taken from does affected with ulcerative vulvitis.

Although this organism is a normal inhabitant of the upper respiratory and urogenital tracts of ruminants, swine and other domestic animals (Carter & Chengappa, 1991), it is an important opportunistic pathogen responsible for

suppurative infections of any sort in a wide range of domestic animals (Addo & Dennis, 1977; Lechtenberg, K. F., Nagaraja, T. G., Leipold, H. W. & Chengappa, M. M., 1988). The involvement of *A. pyogenes* in economically significant diseases ranks it as one of the most important bacterial pathogens of domestic animals. Although the transition from a commensal to a pathogenic state is not well described for *A. pyogenes*, it appears that some predisposing conditions such as concurrent microbial invasion or physical trauma to the underlying tissue could allow the dissemination of the organism (Billington, S. J., Songer, J. G. & Jost, B. H., 2001). Furthermore, the development of genetic techniques to analyse the virulence factors (haemolytic exotoxin, pyolysin) of *A. pyogenes* has given additional evidence about the pathogenesis of this organism in a wide spectrum of infections (Billington *et al*, 2001; Trinh, H. T., Billington, S. J., Field, A. C., Songer, J. G. & Jost, B. H., 2002)

Haemophilus species have been isolated from outbreaks of vulvitis in sheep flocks in Northern Ireland and experimental reproduction of vulvitis identical to the field condition was possible (Ball *et al*, 1991). In Canada, *Histophilus ovis* was recovered in vaginal fluids of three ewes from a flock in the province of Quebec (Higgins, R., Delasalle, F. C. & Messier, S., 1982). *H. ovis* is culturally and morphologically similar to *Actinobacillus seminis*, which has been isolated from sheep in Australia (Baynes & Simmons, 1960; Watt, D. A., Banford, V. & Nairn, M. E., 1970), in the United States (Livingstone & Hardy, 1964) and in South Africa (Van Tonder, 1973; Van Tonder, 1979).

2.1.4.3.3 Chlamydiae

Chlamydial organisms are members of the Order *Chlamydiales* and Family *Chlamydiaceae*. Until recently, the family *Chlamydiaceae* comprised only one genus, *Chlamydia*, with four species. However, Everett, K. D. E., Bush, R. M. & Andersen, A. A. (1999) subdivided the family *Chlamydiaceae* into two genera,

Chlamydia and *Chlamydophila*, based on the phylogenetic analysis of rRNA and gene sequences.

Chlamydophila abortus (formerly the ovine subtype of *Chlamydia psittaci*) is the parasite that causes enzootic ovine abortion in ewes and genital infections in rams and bulls resulting in infertility and sterility. Epididymitis, orchitis, seminal vesiculitis and infections of other accessory glands have been identified in bulls and rams with natural and artificial genital chlamydia infections (Ball, L., Griner, L. A. & Caroll, E. J., 1964; McKercher, D. G., Wada, E. M., Robinson, E. A. & Howarth, J. A., 1966; Storz, J., Carroll, E. J. Ball, L. & Faulkner, L. C., 1968). Evidence suggests that experimentally inoculated bulls and rams excrete organisms in semen and the semen was found to be of unsatisfactory quality for fertilization (Eugster, A. K., Ball, L. Carroll, E. J. & Storz, J., 1970; Storz, J. Carroll, E. J., Stephenson, E. H., Ball, L. & Eugster, A. K., 1976).

For the diagnosis of chlamydiosis in small ruminants, the ELISA has been used to detect antigens in vaginal (Souriau & Rodolakis, 1986) and placental (Thomas, R., Daison, H. C. & Wilsmore, A. J., 1990) specimens.

2.1.4.3.4 Viruses

Vulvovaginitis and balanoposthitis in cattle (Saxegaard, 1970) and goats (Saito, J. K., Gribble, D. H., Berrios, P. E., Knight, H. D. & McKercher, D. G., 1974) caused by a herpesvirus have been reported.

An outbreak of genital disease in goats associated with infection by a herpesvirus that was isolated from vulval and vaginal lesions of affected does has been reported in New South Wales (Grewal & Wells, 1986) and in New Zealand (Horner *et al*, 1982). It was characterized by a vulvovaginitis, return to service and a vaginal discharge. Recovery was, however, spontaneous. Tarigan *et al* (1987) identified herpesvirus in two feral goats from central New South Wales by culturing penile lesions obtained from the abattoir.

Although, bovine herpesvirus type 1 (BHV-1) is primarily a pathogen of the respiratory tract, manifestations of infections such as vulvovaginitis, balanoposthitis, conjunctivitis, encephalitis and abortion have been described in sheep and goats (Whetstone & Evermann, 1988). Bovine herpesvirus 6 (BHV-6) is a caprine herpesvirus that causes a generalized and often fatal infection in goat kids and can cause respiratory tract disease, vulvovaginitis and abortion in adult goats (Berrios, P. E., McKercher, D. G. & Knight, H. D., 1975; Horner *et al*, 1982).

2.1.4.4 General approach to the treatment and prevention of ulcerative genital disease of small stock induced by mollicutes

The treatment of infected sheep includes the topical application of aerosolised antibiotics, followed by parenteral administration of antibiotics effective against mycoplasma. Lotagen (metacresol-sulphonic acid and methanol) or 1-2 % acriflavine solution in glycerine administered topically could be useful (Bath & De Wet, 2000).

It is generally agreed that tetracyclines (oxytetracycline), macrolide antibiotics (erythromycin, tylosin, tilmicosin), lincosamides (lincomycin) and quinolones (danofloxacin, enrofloxacin) have a growth inhibiting activity upon mycoplasmas of animals (Kishma & Hashimoto, 1979; Lloyd Reeve-Johnson, 1999).

A report on the *in vitro* results of antimicrobial drugs danofloxacin, florfenicol, oxytetracycline, spectinomycin and tilmicosin (Ayling, R. D., Baker, S. E., Nicholas, R. A. J., Peek, M. L. & Simon, A. J., 2000) and tiamulin and tylosin (Allan & Pirie, 1981) showed mycoplasmacidal activity against *Mycoplasma* strains. A combination of lincomycin - spectinomycin - tylosin was also reported to be quite active in an *in vitro* trial against several strains of mycoplasmas and ureaplasmas (Truscott & Ruhnke, 1984). The same study furthermore indicated

the effectiveness of minocin, rosaramicin, tiamulin and declomycin against several isolates of *Ureaplasma* while rosoxacin and gentamycin were less active.

It has been reported that tiamulin possesses marked *in vitro* activity against *Mycoplasma* species and showed inhibition of growth of mycoplasmas at lower concentration than oxytetracycline hydrochloride and tylosin tartrate (Goodwin, 1979). Kishma & Hashimoto (1979) demonstrated that among the tested drugs, furamizole, tiamulin and erythromycin were the most active while kanamycin sulphate showed weak activity against all strains of *Ureaplasma* species tested.

Although, an *in vivo* therapeutic trial of enrofloxacin and oxytetracycline LA against mycoplasma infection didn't show promising results (Trichard *et al*, 1993), oxytetracycline is the most commonly used antibiotic for the treatment of ulcerative balanoposthitis and vulvovaginitis in sheep in South Africa with limited success.

Ball & McCaughey (1984) have shown that treatment with injectable oxytetracycline solution can result in clinical improvement in the majority of animals infected with *Ureaplasma* spp. Investigations into treatment of ureaplasma-associated vulvitis in sheep have demonstrated that oxytetracycline and tiamulin may be effective, but neither antibiotic eliminates the organism from all infected animals (Ball & McCaughey, 1984). However, the elimination of urogenital ureaplasma infection by prolonged administration of tiamulin has been suggested (Ball & McCaughey, 1986[a]). Ball & McCaughey (1986[b]) further recommended the combined use of oxytetracycline and tiamulin to eliminate chronic infection of ureaplasma from the genital tract of sheep.

An investigation into the inhibitory effect of flurofamide (urease inhibitor) on animal ureaplasmas lead to the successful elimination of genital ureaplasma infection in sheep after intramuscular injection at a dose rate of 5-20 mg/kg body weight (Ball & MacCaughey, 1986[c]).

Experimental intramuscular administration of tylosin in the treatment of ureaplasma infection in ewes did not completely eradicate the infection from all animals (Ball & McCaughey, 1987).

To prevent the spread of infection into clean flocks, it is important to avoid the introduction of rams or ewes from infected areas or farms. In infected flocks, further spread into clean sheep can be avoided initially by segregating the clinically infected sheep as soon as they are detected.

CHAPTER III

3 IDENTIFICATION OF THE PRIMARY INFECTIOUS AGENTS ASSOCIATED WITH ULCERATIVE BALANOPOSTHITIS AND VULVOVAGINITIS

3.1 MATERIALS AND METHODS

3.1.1 STUDY AREA

The study was conducted in the Northern Cape province and included four districts, namely Namakwaland, Hay, Berkley West and Hopetown. Beaufort West, an adjacent district in the Western Cape, was also included in the study. A total of 15 sheep farms in these five districts were visited to collect specimens.

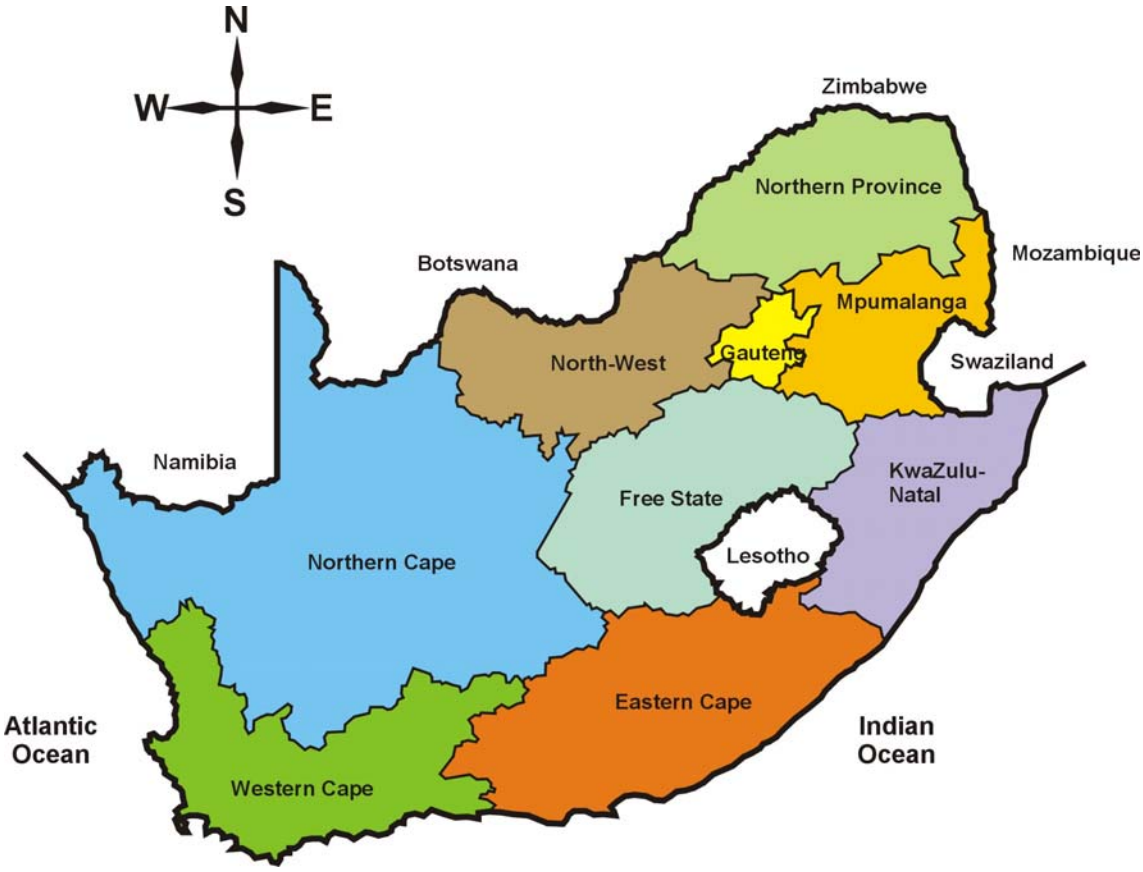

Figure 3.1 Map of The Republic of South Africa indicating the nine provinces

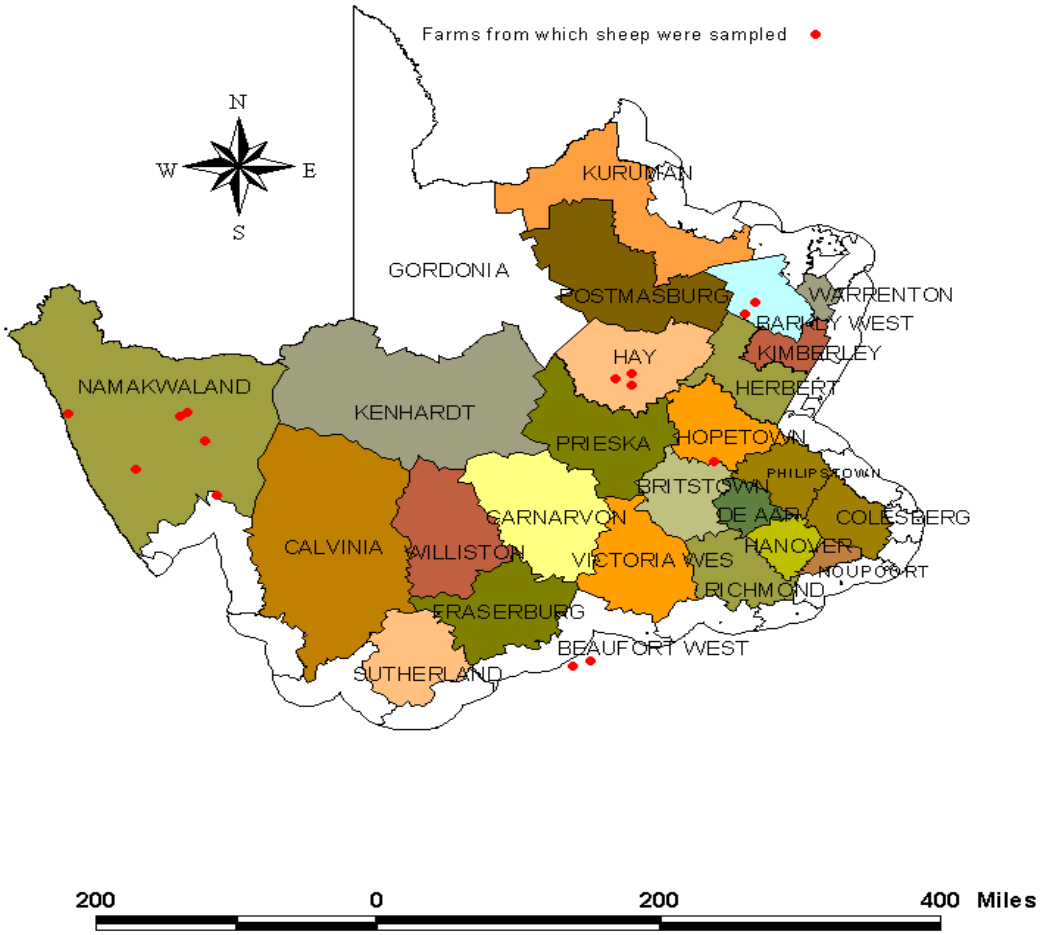

Figure 3.2 Map of the Northern Cape province of The Republic of South Africa indicating study sites

Table 3.1 Grid-reference of the selected study sites in each district

District	Farm	SL†	EL‡
Namakwaland			
	Grootvlei	30· 12' 41''	17· 47' 34''
	Matjiesfontein	30· 31' 41"	18· 37' 58"
	De Beers	29· 35' 00"	17· 05' 58"
	Smorgenskadu	29· 30'	18· 15'
	Grootkou	29· 34' 19"	18· 18' 48"
	Dooddrink	30· 35'	18· 45'
	Koppieskraal	29· 55'	18· 25'
Hay			
	Tolo	22· 43'	29· 08'
	Dam	22· 54'	29· 05'
	Gladiam	22·54'	29· 12'
Barkley West			
	Melkvlei	28· 24'	24· 21'
	Hondevlei	28· 17'	24· 09'
Strydenburg			
	Kortkop	30· 07' 45.2"	23· 43' 32. 4"
Beaufort West			
	Bellevue	22· 24' 35.6"	32· 28' 10"
	Springfontein	32· 30'17. 8"	22· 17' 4.01"

† South latitude

‡ East longitude

3.1.2 STUDY ANIMALS

The study focused exclusively on Dorper sheep, as this breed is the one primarily affected by the disease. The Dorper is white with a black head (Fig.3.3). Fat deposits on the rump, tail and brisket are absent. Under good management conditions mature rams can reach a weight of 82 – 91 kg and ewes 54 – 63 kg. Lambs can reach a live weight of 34 kg at about 6 months of age (Devendra & McLeroy, 1982).

Figure 3.3 A flock of Dorper sheep photographed near Beaufort West

For the purpose of description of clinical signs and specimen collection, animals were divided into 3 groups on the basis of clinical examination:

0 Normal – no clinical signs

1 Mild to moderate hyperaemia

2 Varying degree of ulceration

The age of the animals was determined on the appearance of their teeth as follows:

0 tooth (Milk tooth)	< 12 month
2 teeth	12 – 14 months
4 teeth	18 – 20 months
6 teeth	26 – 30 months
Full mouth	> 36 months

3.1.3 STUDY DESIGN

The study was designed as a case-control study in which diseased animals (cases) and non-diseased animals (controls) were selected and compared in terms of the presence of the risk factor (Thrusfield, 1995).

The study areas were selected on the basis of reported outbreaks of the disease during the study period. The study population was defined as all Dorper sheep that were available for clinical examination on farms at the time of survey. The inclusion criteria therefore included sheep of both sexes and ages that showed genital ulcerative lesions, for cases, and their healthy counterparts for controls. The sampled animals were selected on the basis of clinical observation, and the appearance of genital lesions for cases and no genital lesions for controls. The number of sampled animals at each farm was at least five diseased and five healthy sheep for each sex group. However, the limitation in case-control studies is the lack of estimation of the incidence of the disease in exposed and unexposed individuals to the risk factor. It is also known that the control of some extraneous factors may not be complete (Thrusfield, 1995).

The survey took place between September 2001 and March 2002. The data were recorded on a standard data sheet before they were entered into the computer database.

The variables analyzed included temporal and spatial data, population variables such as age, sex and disease status and microbiological data. A dichotomous variable with either yes (Y) or no (N) as an entry was used to denote the disease status of each sampled animal and describe if certain microorganisms were isolated from the genital swabs examined.

3.1.4 SPECIMEN COLLECTION

Genital swab specimens were collected from both clinically normal and diseased Dorper sheep using commercial sterile cotton swabs. A total of 220 vulval and penile swabs consisting of 116 and 104 swabs from the healthy and clinically infected sheep, respectively, were examined (Table 3.2).

Specimens were obtained from the vulvae by spreading the vulval lips, inserting a swab and rubbing back and fourth gently before removing. Swab samples from rams were obtained by gentle rubbing of the glans penis and preputial mucosa after manual extrusion of the penis from the prepuce.

Swabs were placed into cryovials containing transport medium appropriate for different selected microorganisms. The transport media included Hayflick's broth for mycoplasma, brain heart infusion broth for bacteria and phosphate buffered saline for viruses. Transport medium was not used for swabs to be used for *Chlamydophila* antigen detection. The cryovials were placed in liquid nitrogen and transported to the laboratory, where they were stored at –85 ^{0}C until processed.

Table 3.2 Summary of the number of specimens obtained from affected and unaffected sheep at each study site

District	Farms	No. of examined animals		Total
		Affected	Unaffected	
Beaufort West	Bellevue	9	4	13
	Springfontein	3	6	9
Barkley West	Hondevlei	8	9	17
	Melkvlei	10	9	19
Hay	Dam	10	8	18
	Gladiam	3	3	6
	Tolo	5	4	9
Hopetown	Kortkop	9	9	18
Namakwaland	Dooddrink	11	7	18
	Grootvlei	4	20	24
	Grootkou	4	11	15
	De Beers	10	10	20
	Koppieskraal	3	0	3
	Matjiesfontein	5	7	12
	Smorgenskadu	10	9	19
Total	15	104	116	220

3.1.5 ISOLATION AND IDENTIFICATION OF BACTERIA

3.1.5.1 Isolation of bacteria

Swabs were streaked onto Columbia blood agar (Difco) enriched with 5 – 6 % horse blood and incubated in an atmosphere of 5 % CO_2 at 37 ^{0}C for 24 hours, with further re-incubation for 36 - 72 hours if no growth was observed. Single

colonies of the different colony types recognized on each plate were picked and re-inoculated on blood and MacConkey (Difco) agar. This was repeated until pure growth was obtained. Bacterial cultures were then subjected to Gram's stain, the catalase and oxidase tests and oxidation - fermentation (O - F) reactions. Cultures that conformed to certain criteria were subjected to additional biochemical tests.

3.1.5.2 Biochemical tests

Biochemical characterization of isolates was done using conventional methods that included a panel of sugars, and three commercial analytical systems, API 10S (bioMérieux), Microbact 12A and 12B (Medvet Science Pty. Ltd.) and API Coryne (bioMérieux). Colonies were picked and inoculated into the solutions of test sugars in test tubes and incubated for 24 – 48 hours. The results of the conventional sugar tests were interpreted according to the biochemical profiles described by Quinn, P. J., Carter, M. E., Markey, B. & Carter, G. R. (1994).

Colonies were suspended in saline for inoculation of API 10S or in saline with 1% serum for inoculation of Microbact or in the suspension medium provided with the kit for API Coryne. The suspensions were then inoculated into the test kits according to the manufacturer's instructions and incubated for 24 – 48 hours. Results were read after adding the reagents and the interpretation done with the aid of the manufacturer's colour chart. *Staphylococcus* and *Streptococcus* colonies were tested using a Staph kit (Oxoid) and Strep kit (Oxoid), respectively, and identified by their ability to ferment different carbohydrates.

3.1.6 ISOLATION AND IDENTIFICATION OF MYCOPLASMA

3.1.6.1 Growth media for mollicutes

The standard growth media used for the propagation of field isolates and reference strains of *Mycoplasma* were Hayflick's agar and broth, Chalquest agar and broth, ureaplasma agar and broth and rabbit meat infusion broth. Rabbbit meat infusion broth was used to grow *Mycoplasma* reference strains that were used as antigens to inoculate rabbits for the production of hyperimmune sera.

3.1.6.1.1 Hayflick's medium

The basal medium includes PPLO agar (Difco) and PPLO broth (Difco) enriched with horse serum, yeast extract (Oxoid) and Deoxyribonucleic acid (DNA) (Sigma). The media were prepared by dissolving 23.3 g of dehydrated agar or 14.7 g of dehydrated broth in 800 mℓ of deionized distilled water and 10 % (w/v) yeast extract solution was added before sterilizing at 121 ^{0}C for 15 minutes.

Two hundred mℓ filter-sterilized horse serum was dispensed in a separate container to which 0.0025 % (w/v) DNA (Sigma) was added. The DNA was sterilized by filtration through a 0.45 µm Millipore membrane filter (Millex® - HV). Penicillin G (1 million IU) was added into the solution as bacterial inhibitor, and the pH adjusted to 7.8 with 5N NaOH. The latter solution was added aseptically into the basal medium, which was allowed to cool to 52 – 56 ^{0}C.

3.1.6.1.2 Chalquest medium

The basal medium includes PPLO agar (Difco) and PPLO broth (Difco) enriched with swine serum inactivated at 56 ^{0}C for 30 minutes, 5 % trypticase (w/v), 0.5 % starch (w/v) dissolved in 100 mℓ of distilled water under heat and 1 % β-Nicotinamide Adenine Di-nucleotide (NADH) (Sigma). The media were prepared

by dissolving 25 g of dehydrated agar or 19.3 g of dehydrated broth in 900 mℓ of deionized distilled water. Five per cent trypticase (w/v) and separately dissolved 0.5 % starch (w/v) solutions were added before sterilizing at 121 ^{0}C for 15 minutes.

One hundred mℓ filter-sterilized and inactivated swine serum plus 1 % NADH (w/v) aqueous solution sterilized by filtration through a 0.45 µm Millipore membrane filter (Millex® - HV) was dispensed in a separate container. Penicillin G (200 000 IU) was added into the solution as bacterial inhibitor, and the pH adjusted to 7.8 with 5N NaOH. The latter solution was added aseptically into the basal medium, which was allowed to cool to 52 – 56 ^{0}C.

3.1.6.1.3 Ureaplasma medium

The ureaplasma agar medium was prepared by dissolving 10 g of bacto agar in 1000 mℓ of deionized distilled water and sterilized by autoclaving at 121 ^{0}C for 15 minutes. The following solutions were prepared separately and sterilized by filtration using a 142 mm diameter filter housing and a membrane filter (Millex® - HV) with pore size of 0.22 µm, and added to the basal medium. These were: Heart infusion broth, 200 mℓ; 10 x medium 199, 45 mℓ; yeast extract, 5 % (w/v); urea 20 % (w/v); dithiothreitol 10 % (w/v); DNA 0.2 % (w/v); $MnSO_4$ 1.5 % (w/v) and phenol red 0.4 % (w/v). Penicillin G (1 million IU) was added to the solution, and finally the pH was adjusted to 6.0 – 6.2 with 5N NaOH.

The composition of the broth medium was essentially that of the agar without the agar itself.

Approximately 15 mℓ of agar media was poured into a 90 mm petridish in a laminar flow cabinet. Plates were then left to solidify at room temperature for several hours. Plates were placed in plastic bags, sealed and stored at 4 ^{0}C for later use. The liquid media were dispensed into 7 - 8 mℓ screw-cap bottles and stored at 4 ^{0}C until used.

3.1.6.1.4 Rabbit meat infusion broth

The antigens for production of hyperimmune sera in rabbits were prepared from organisms grown in rabbit meat infusion broth (RMIB). The method of preparation of RMIB was according to the standard operating procedure of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. Briefly, infusions were prepared by adding 500 g finely ground rabbit meat to 1000 mℓ deionized distilled water. The infusion was serially filtered through gauze, cotton wool and then through Whatman filter paper. Peptone (1%, Difco) and 0.5 % sodium chloride were added. After dissolving the peptone and salt, the pH was adjusted to about 7.8 and the solution was sterilized by filtration through a panel of Millipore filters (Sartorius) with pore sizes of 1.2, 0.8, 0.45 and 0.2 µm. A cholesterol solution was prepared by dissolving 100 mg of cholesterol in a minimal quantity of absolute ethanol, and then the dissolved steroid was added to 100 mℓ hot de-ionized distilled water. Two mℓ of this solution was added to each 100 mℓ of rabbit meat infusion broth.

3.1.6.2 Isolation of mycoplasma

Thawed swab specimens were streaked on mycoplasma and ureaplasma media. The cultures were incubated in a moist chamber at 37 ^{0}C with 5 % CO_2. All plates were examined daily or on alternate days, using a dissection microscope at a magnification of 20 – 40 X. The cultures were incubated for a minimum of 7 days and considered negative if there were no growth after 15 days of incubation.

Evidence of colony growth in broth medium was monitored by turbidity of the medium. When satisfactory growth was obtained, aliquots of broth culture were inoculated onto mycoplasma agar plates and incubated until well-defined colonies formed. These colonies were later identified with the indirect immunofluorescent antibody test.

3.1.6.3 Production of control sera

3.1.6.3.1 Reference *Mycoplasma* strains

Reference *Mycoplasma* species were used as antigens for the production of positive serological controls. Eight reference *Mycoplasma* spp. supplied by National Collection of Type Cultures, Central Public Health laboratory, 61 Colindale Avenue, London, NW9 5HT, UK, were used.

The strains were the following:

Acholeplamsa laidlawii (10116 PG8)

Mycoplasma bovigenitalium (10122 PG11)

Mycoplasma arginini (10129 G230)

Mycoplasma spp. group 7 (10133 N29)

Mycoplasma mycoides capri (10137 PG3)

Mycoplasma capricolum (10154 California kid)

Mycoplasma mycoides mycoides LC type (11706 Y-goat)

Mycoplasma agalactiae (101223 PG2)

3.1.6.3.2 Preparation of immunizing antigen

The reference *Mycoplasma* spp. were adapted to rabbit meat infusion broth. The volume of the growth medium used for the production of mycoplasmas was increased through three passages at 72-hour intervals of incubation until 800 mℓ volumes of growth medium were reached. The growth medium was dispensed in 9 mℓ, 70 mℓ, and 700 mℓ quantities. Initially one milliliter of each mycoplasma seed culture at its logarithmic phase of growth was transferred to 9 mℓ of rabbit meat infusion broth. This was incubated aerobically at 37 ^{0}C with 5 % CO_2 for 3 days, then the whole contents were aseptically poured into the 70 mℓ broth. After 3 days of incubation, the contents of each flask containing 70 mℓ of broth were transferred to flasks with 700 mℓ of growth media. A routine check for bacterial contamination was carried out on blood agar plates with each transfer.

Harvesting of mycoplasmas was accomplished by centrifugation at 20 000 x g for 45 minutes in a refrigerated centrifuge. The supernatant fluid was discarded and the precipitated cells were re-suspended in sterile buffered saline solution. The suspended mycoplasma cells were thoroughly washed thrice in buffered saline solution at the same speed and time. The collected cells were finally suspended in 10 mℓ PBS (i.e. 100-fold concentration), pH 7.8. These concentrated mycoplasma cells were dispensed in 1 mℓ volumes, and stored at – 85 ^{0}C until used.

3.1.6.3.3 Production of antiserum

Antisera to reference *Mycoplasma* spp. were produced in adult white New Zealand rabbits. Two rabbits were used for each reference strain. Briefly, the suspensions of mycoplasma cells were thawed and emulsified in an equal amount of Freund's incomplete adjuvant and then used as antigen for inoculation of rabbits. Each rabbit was initially given 4 mℓ of the adjuvanted antigen intramuscularly in eight different sites, 0.5 mℓ at each site, i.e. 2 sites on each hind leg and 2 sites on each shoulder. A second injection with 0.1 mℓ of antigen was administered intravenously (IV) on day 21, and three other successive IV inoculations at four day intervals were given at dose rates of 0.2 mℓ, 0.3 mℓ and 0.4 mℓ, respectively. A week after the last injection rabbits were bled by cardiac puncture and the serum portion was harvested from clotted blood by centrifugation at 200 x g for 10 minutes, sterilized by filtration and stored frozen at –20 ^{0}C.

3.1.6.3.4 Preparation of serum for the indirect immunofluorescent antibody test

The immunoglobulins in the sera were precipitated by the addition of twice the volume of saturated ammonium sulfate solution drop by drop, slowly, with continuous stirring on a magnetic stirrer. The mixture was stirred overnight at 4^{0}C. The refrigerated mixture was then centrifuged at 1 500 x g for 10 minutes

at 4 ^{0}C. The supernatant fluid was discarded and an equal volume of distilled water added to dissolve the precipitate. The mixture was again added slowly, drop-wise with continuous stirring, into saturated ammonium sulfate, the volume equal to half the volume of the mixture. Stirring was continued for 90 minutes at room temperature and the mixture centrifuged as mentioned earlier. After the last centrifugation, the precipitate was re-suspended in distilled water, approximately one third the volume of the serum. It was put in a dialyzing bag securely tied at both ends and was allowed to be dialyzed overnight with 0.85 % NaCl solution at 4 ^{0}C. This dialyzation was conducted for 48 hours with changes of saline until the colour of the globulins became ice-white. Finally, the volume was measured and adjusted to the original volume with PBS, pH 7.5 and stored at –20 ^{0}C.

3.1.6.4 Indirect immunofluorescent antibody test (IFAT)

3.1.6.4.1 Standardization of reagents

The potency of each antiserum against homologus antigen, and non-specific or cross-reactions with heterologus antigens was determined with the agar growth inhibition test. The growth inhibition test was used as described by Clyde (1964) with modification. Test strains in the broth were inoculated onto agar plates and allowed to dry. Eight wells, each approximately 5 mm in diameter, were cut from the inoculated agar and filled with the antisera raised to the reference strains, avoiding overflowing. Plates were incubated at 37 ^{0}C for 3 - 7 days and examined for a zone of growth inhibition.

3.1.6.4.2 Working dilutions of conjugate and antisera

Goat anti-rabbit globulins conjugated with fluorescein isothiocyanate were obtained from a commercial source (The Binding Site, Birmingham, UK). Phosphate buffered saline (PBS) at pH 7.2 was used as a diluent for the conjugate, antisera and Evans' blue, and PBS with 0.5 % Tween twenty was

used for washing. The working dilution of the anti-rabbit globulin was determined by a box-titration against a potent rabbit antiserum determined in the growth inhibition test. The conjugate was diluted in serial two-fold dilution from 1:10 and 1:640. A working dilution of 1:50 was determined to be the optimal dilution for the FA conjugate.

Titration of rabbit antisera raised against the eight reference strains of mycoplasma was done solely against the dilution of the conjugate. A two-fold serial dilution ranging from 1:10 to 1:10 240 was prepared for each antiserum and checked for optimal fluorescence. The working titre was taken 2-4 dilutions lower than the end titre since some strains required a higher concentration of antibody than that needed by the strain used in the production of the antiserum (Del Giudice, 1967) (Table 3.3).

Table 3.3 Reciprocals of end and working titres of each antiserum against the homologus antigens

Reference strains	End titre	Working titre
M. bovigenitalium	80	40
M. arginini	160	40
M. agalactiae	320	80
M. species Group 7	320	80
M. mm LC (Y-goat)	640	160
M. capricolum (California kid)	640	160
A. laidlawii	2560	320
M. m capri	10240	640

Serum titre end-points were defined as the reciprocal of the highest dilution giving clear positive reactions (bright-green fluorescence). All tests included positive and negative control sera to ensure that the reagents were working properly.

3.1.6.4.3 FA Staining procedure

For the indirect staining procedure employed in this study, agar blocks with the colonies *in situ* were used. Rabbit antiserum was used as the primary antibody, followed by addition of fluorescein-conjugated goat anti-rabbit IgG as secondary antibody.

A marking pen was used to circumscribe each agar block with growing mycoplasma colonies. This served to pick agar blocks with sufficient mycoplamsa colonies. The agar surfaces were initially rinsed with PBS, pH 7.2, to remove the surface growing mycoplasma colonies. Agar blocks, with mycoplasma colonies, were cut at a width of approximately 4 - 6 mm and transferred to a glass chamber with moist filter paper (Fig. 3.4). Excess buffer was removed by gently touching the rim of each block with clean blotting paper. A drop of antiserum was placed on the surface of each block and incubated for at least 30 minutes in a moist chamber at room temperature. Following incubation, the blocks were rinsed in PBS twice for ten minutes each. After the blocks were dried, they were flooded with fluorescein conjugated goat anti-rabbit γ- globulin diluted 1:50 in PBS. The blocks were incubated as before and then rinsed as previously described. Excess water was allowed to drain. To eliminate background fluorescence and provide a contrast, the blocks were counterstained with a drop of 0.75 % Evans' blue and incubated for another 30 minutes at room temperature in a moist chamber. The blocks were finally rinsed twice in PBS for 10 minutes each. A thin slice of each agar block was made and mounted onto a microscope slide. The preparation was ready for fluorescence microscopy after the addition of a drop of mounting fluid and a cover slip.

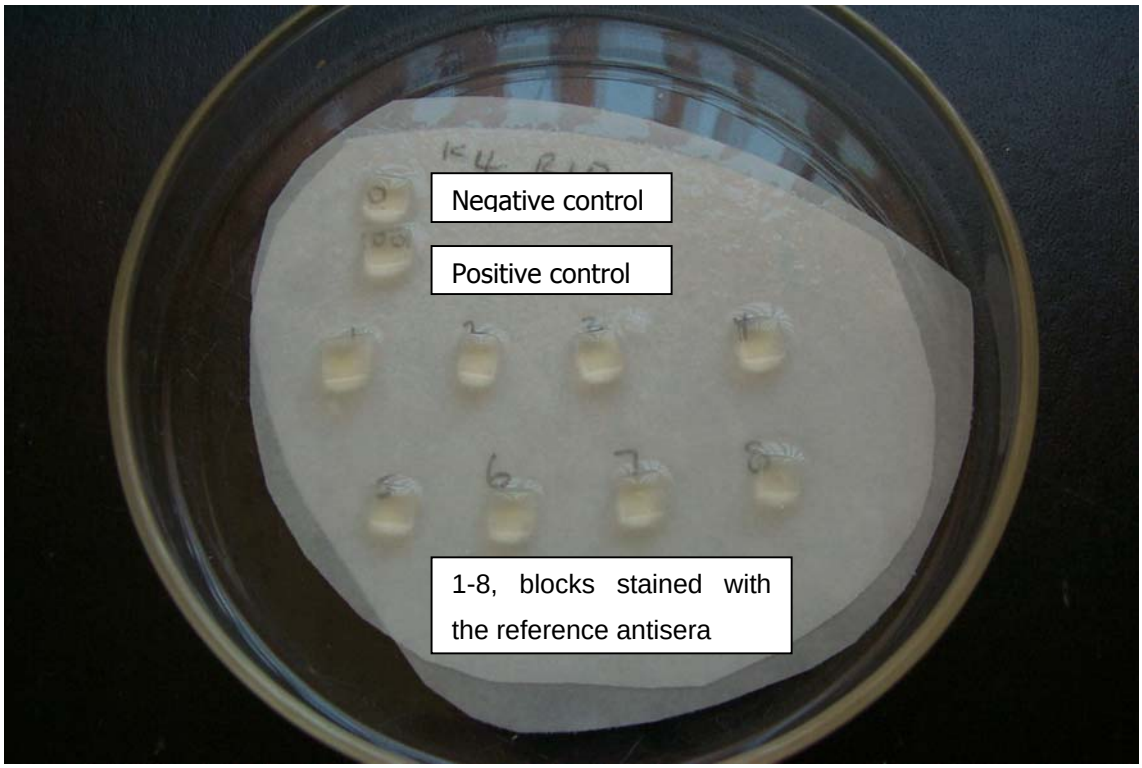

Fig. 3.4 Moist chamber with agar blocks for use in IFAT

3.1.6.4.4 Microscopy

Fluorescence microscopy was achieved with a binocular microscope equipped with epi-fluorescent attachment and Osram HBO 100 W/2 high-pressure mercury vapor lamp (Nikon corporation, Japan). A green excitatory filter (G 2A) and yellow barrier filter were used. The microscope was fitted with a photomicrographic apparatus (Model UFX-DX).

3.1.7 VIRUS ISOLATION

Primary and secondary lamb fetal kidney (LFK) cell cultures were used for the isolation of viruses. The LFK cells were grown in Eagles' Minimal Essential Medium (MEM) with Earle's salts supplemented with 5 % heat-inactivated foetal bovine serum (FBS). Gentamicin was added to the medium at a final concentration of 50 µg per mℓ.

The material used for virus isolation was collected in the virus transport medium in separate cryovials for each specimen. Suspensions were cleared by low speed centrifugation. Amounts of 0.5 mℓ of supernatant fluids were inoculated onto LFK monolayers, incubated at 37 ^{0}C and examined daily for cytopathic effects (cpe). When no cpe was observed after seven days of incubation, the tissue culture fluids were harvested and passaged blindly twice at an interval of seven days. Absence of cpe at the third passage was taken to indicate absence of virus. Where bacterial contamination was evident, the specimens were filtered through a 0.22 µm millipore filter (Millex® - HV).

3.1.8 *CHLAMYDOPHILA* ANTIGEN DETECTION

The Clearview® Chlamydia MF (Clearview®, Unipath Limited, Priory Park, Bedford, MK44 3UP, UK) test kit was used to detect *Chlamydophila* antigen from genital swabs collected from sheep. Although, the test was developed to detect *Chlamydia trachomatis* antigen from human genital swabs, it has been shown to detect antigens of *Chlamydophila psittaci* subspecies *ovis* (Unipath Limited). It provides a simple direct detection assay, which is highly sensitive, specific and rapid.

3.1.9 DATA ANALYSIS

All relevant data generated by the study were recorded in a data-capturing format and entered into a computer database for subsequent analysis (Fig.3.5). The statistical package used to store and analyze the data was EpiInfo 2000 version 1.0 (Centers for Disease Prevention and Control, Department of Health and Human Services, USA). The variables to be assessed were included in an EpiInfo questionnaire and were analysed using the ANALYSIS and STATCALC facilities of the EpiInfo software. Descriptive statistics was employed to designate the different types of microorganisms encountered from both healthy and clinically infected sheep. The association of each isolate (risk factor) with the disease process was assessed using an Odds Ratio. The differences were

analysed using the chi-square test, and the levels of significance were taken as $p<0.05$ and confidence intervals (CI) were set at 95 %.

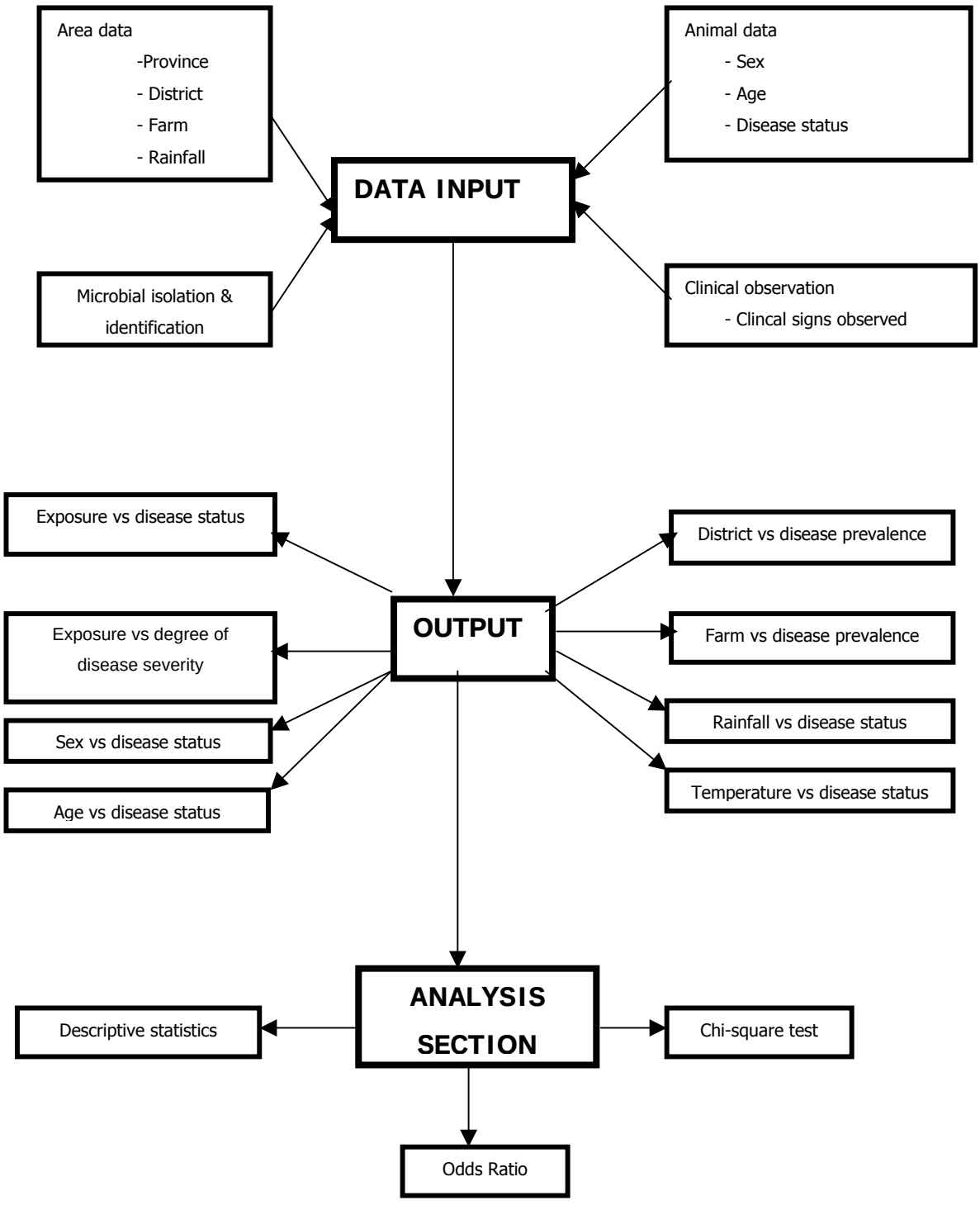

Fig. 3.5 Schematic presentation of data capture & analysis spreadsheet

3 2. RESULTS

3.2.1 CLINICAL OBSERVATIONS

3.2.1.1 Description of clinical signs

3.2.1.1.1 Ewes

Early signs of vulvitis were characterized by swollen and reddened vulvae visible at a distance in short docked Dorper ewes (Fig.3.6 & 3.7). In some ewes swelling was accompanied by the development of discrete mucosal ulcers at the mucocutaneous junction of the vulval labia.

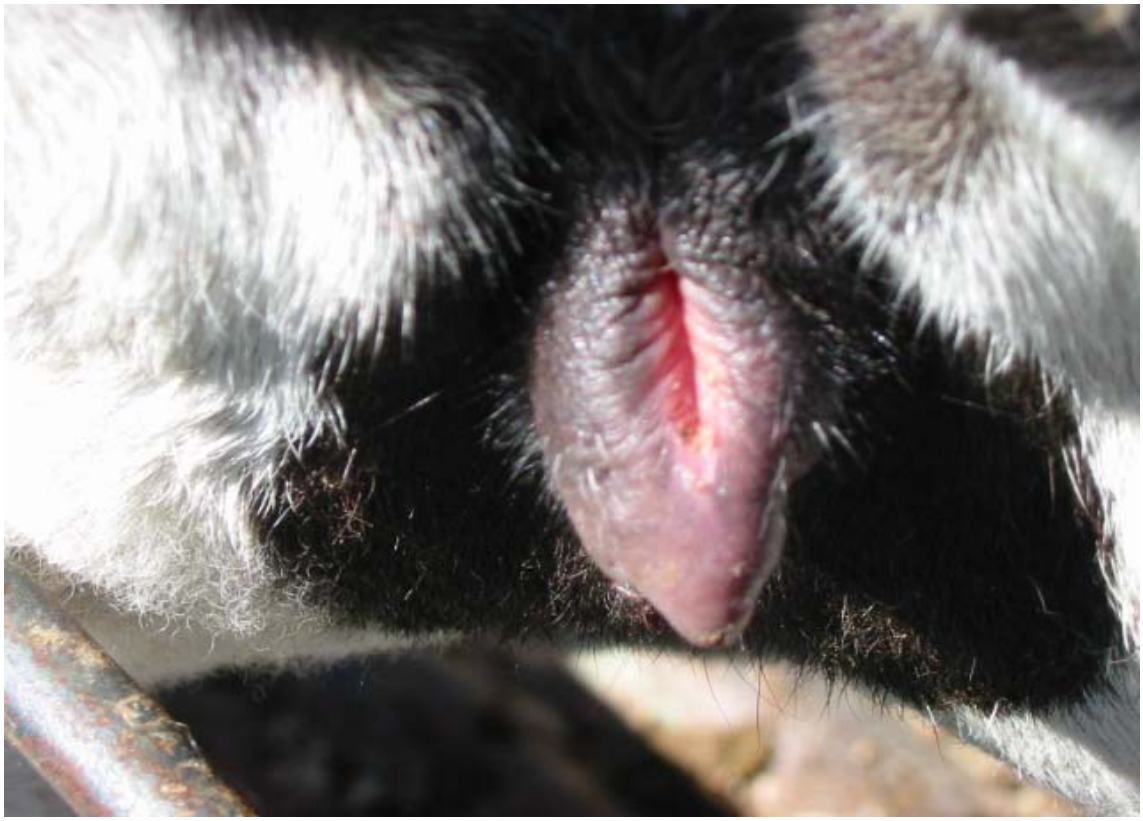

Fig.3.6 Swollen, oedematous and reddened vulva

On closer examination, shallow blister-like wounds covered with scabs could be found. No lesions were observed in the vestibule of the vulva/ vagina.

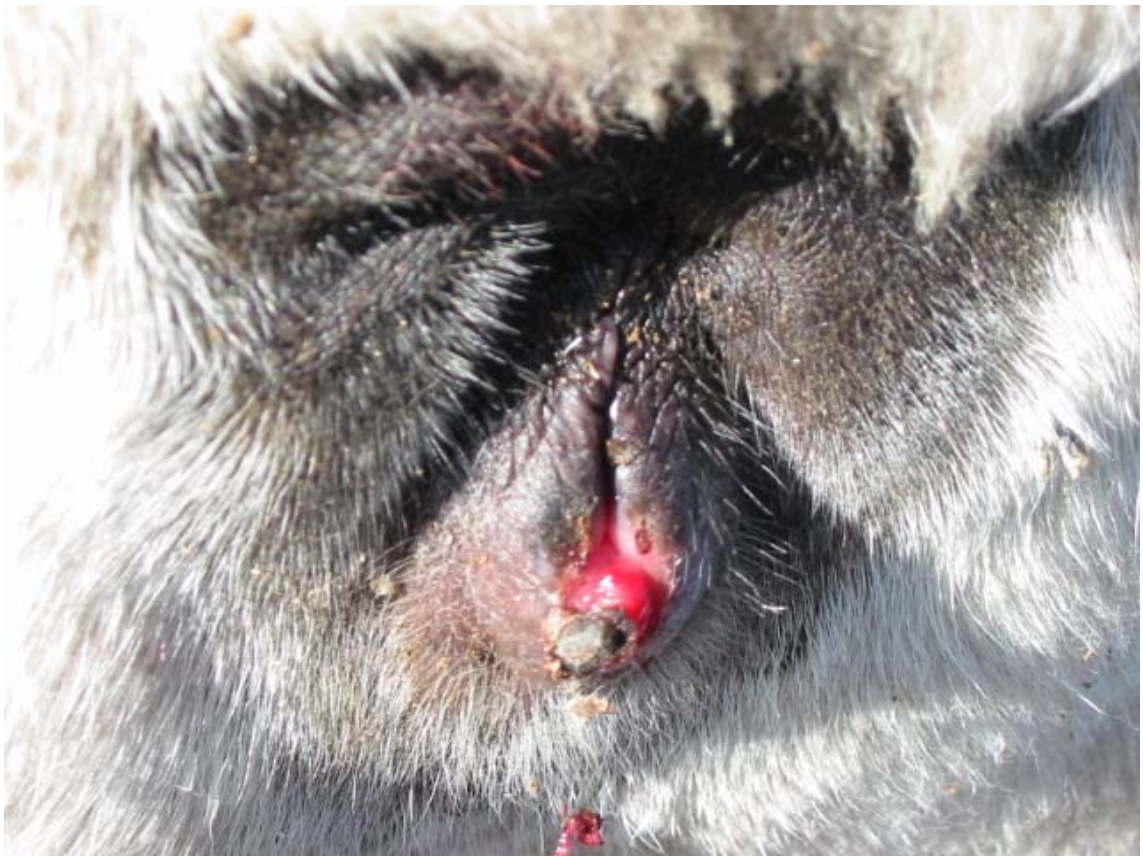

Fig. 3.7 Scabs and blister-like ulcers on the labia and heamorrhagic lesions on the ventral commissure

3.2.1.1.2 Rams

On manual withdrawal of the penis of affected rams, the characteristic lesions could readily be seen involving the soft glans of the penis (Figs. 3.8, 3.9, 3.10 & 3.11). The ulcers on the glans penis had a sharply defined edge and the rest of the penile tissue and preputial mucosa appeared normal. In some cases, probably the acute phase of the disease, the crater of the ulcer filled with a blood clot and gave a strawberry appearance to the lesion.

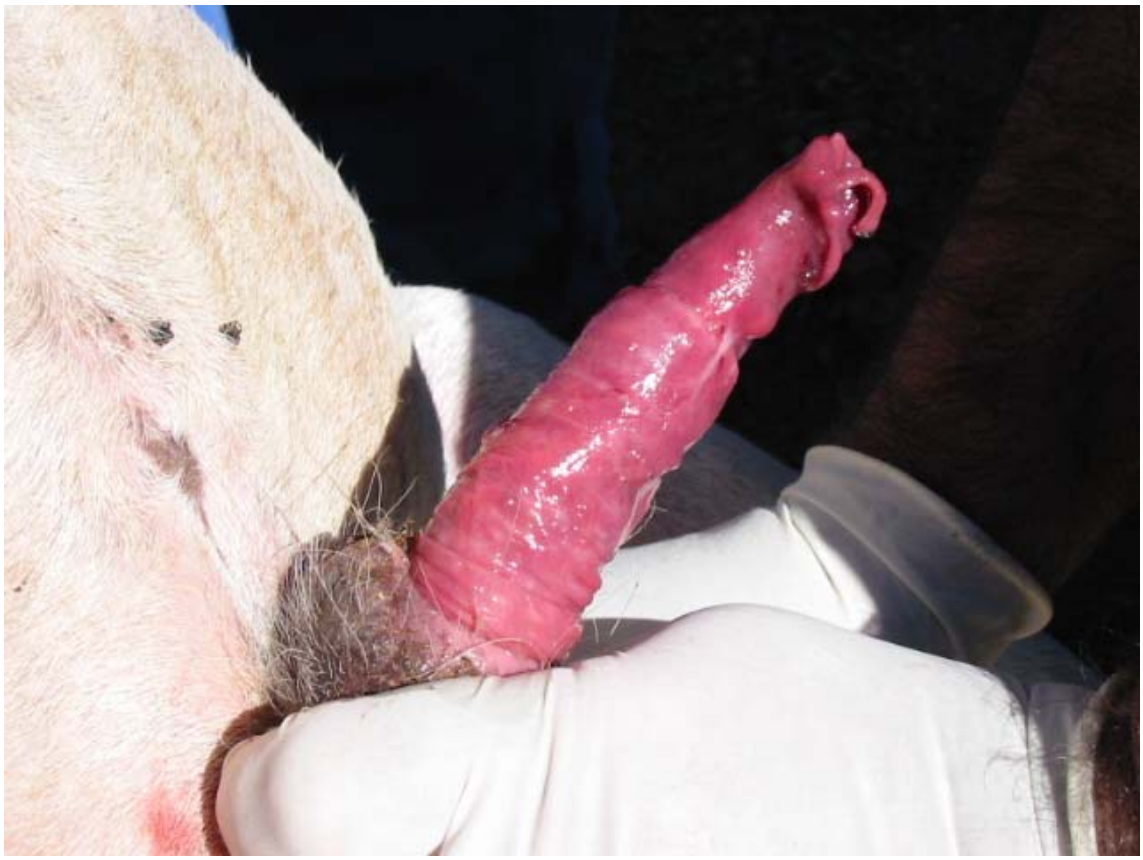

Fig. 3.8 Acute case showing hyperaemia, swelling and severe discomfort when palpated prior to the development of erosion or ulceration

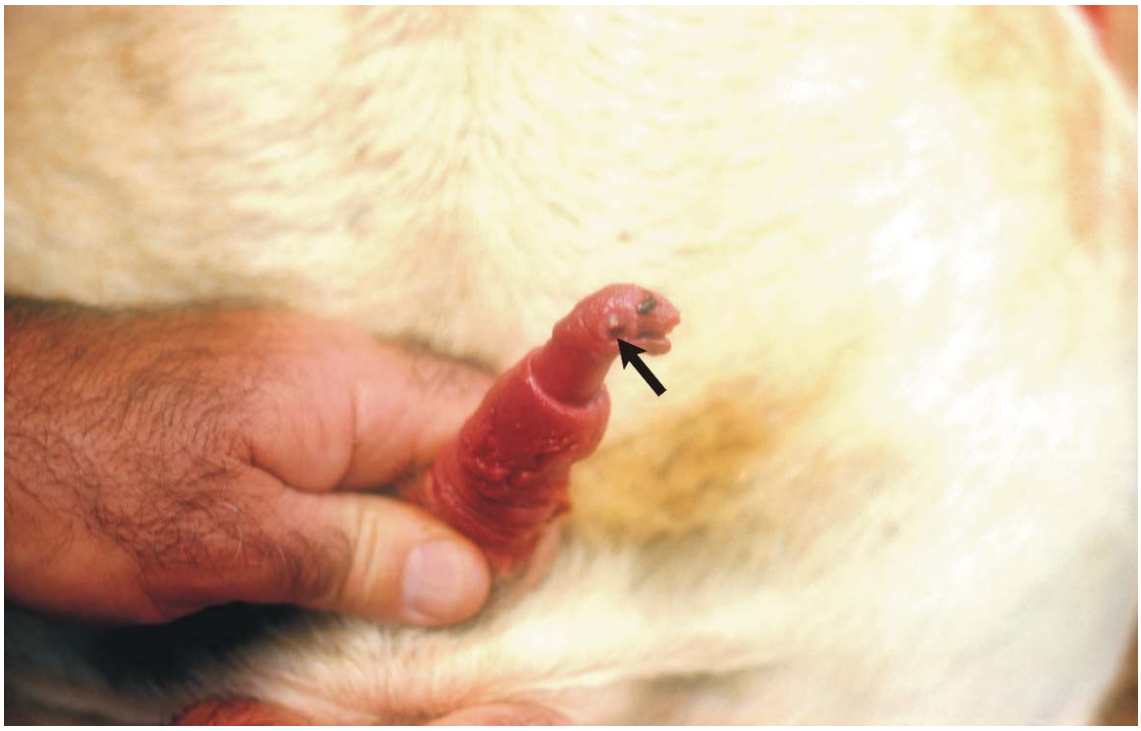

Fig. 3.9 Small circumscribed ulcer on the soft tissues of the glans penis

Fig. 3.10 Ulcer that damaged a large part of the glans penis

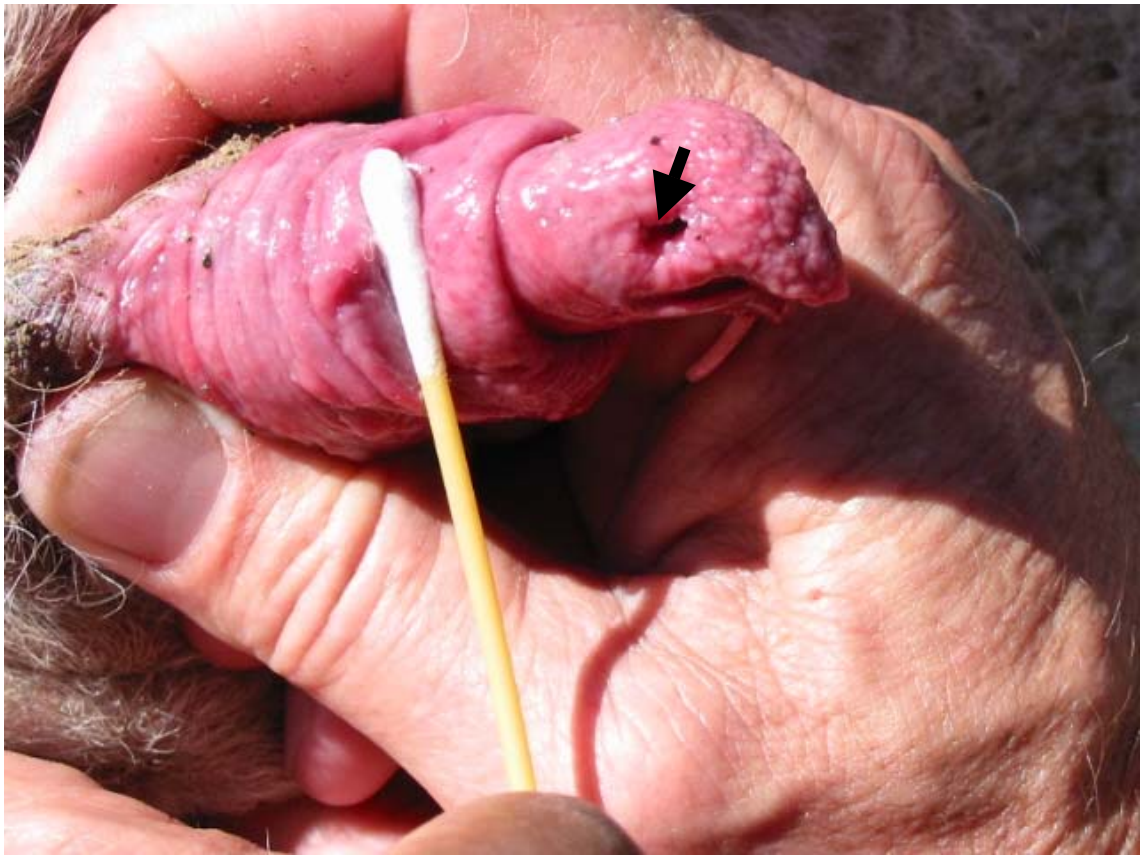

Fig.3.11 A perforated wound that can be filled with scar tissue when completely healed

3.2.1.2 Relationship between disease status and age

The age range of the study sample was 0 tooth to full mouth (Table 3.4), with 57.7 % of them under the age of full mouth. Twenty six percent of the sampled sheep were in the age group of 2 teeth. Most clinical cases were also observed among the 2 teeth age group (33.6 %) followed by the full mouth age group (30.7 %) (Fig.3.12).

Table 3.4 Number of sheep sampled during the project and divided according to clinical disease status and age groups

Age group	Disease Status				Total	
	Affected		Unaffected			
	(n)	(%)	(n)	(%)	(n)	(%
0 tooth (<12 months)	10	9.6	14	12.1	24	10.9
2 teeth (12-14 months)	35	33.6	23	19.8	58	26.4
4 teeth (18-20 months)	19	18.3	15	12.9	34	15.4
6 teeth (26-30 months)	8	7.7	3	2.6	11	5
Full mouth (> 36 months)	32	30.7	61	52.6	93	42.3
Total	104	47.3	116	52.7	220	100.0

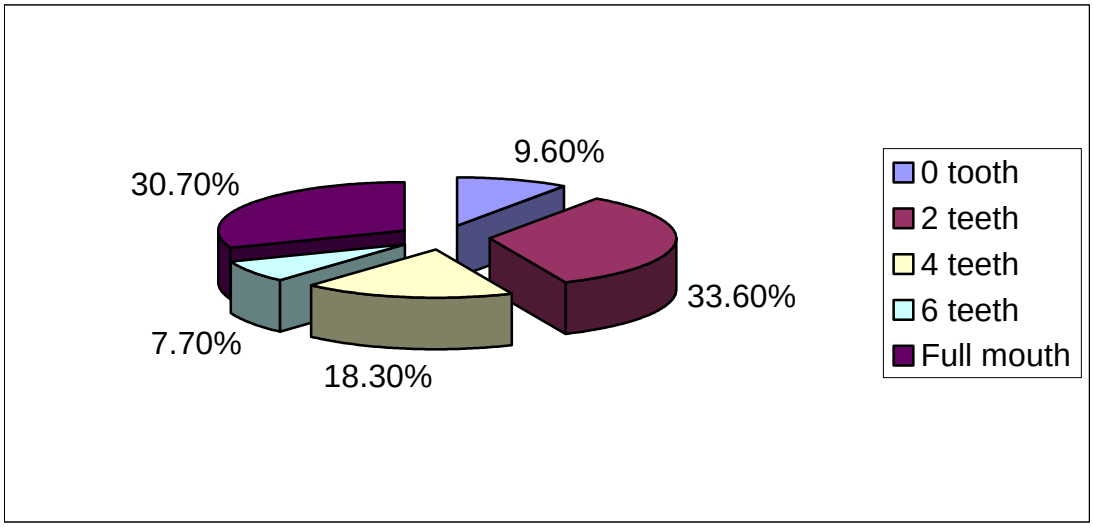

Fig. 3.12 Distribution of clinically affected sheep by age group

The association between age and clinical signs was statistically significant ($p<0.05$). Analysis of the odds ratio was performed after the age data was converted into a dichotomous variable where the 0 tooth to 6 teeth age group were sorted as one variable, young, and the full mouth age group as the second variable, adult. Young sheep (0-6 teeth) were 2.5 times more likely to have lesions than adult sheep (> 6 teeth) (Table 3.5).

Table 3.5 Odds ratio analysis to determine the association between age and exposure to the disease

Age	Disease status		Odds ratio	95 % CI	Chi-square	p value
	Affected (n)	Unaffected (n)				
Young	72	55	2.50	1.38<OR<4.51	10.70	0.001
Adult	32	61				

3.2.2 ISOLATION AND IDENTIFICATION OF BACTERIA

3.2.2.1 Bacteria isolated from healthy and affected sheep

The microbiological flora of 104 clinically affected genitals of both ewes and rams from sheep in 15 flocks was compared with the flora of 116 clinically unaffected genitals. The number and distribution of specimens taken from unaffected and affected sheep, and grouped according to age, are shown in Table 3.6.

Table 3.6 Number of samples taken from unaffected, mildly or severely affected sheep and grouped according to age

Age	Unaffected sheep	Sheep with mild lesions	Sheep with severe lesions	Total
0 tooth	14	2	8	24
2 teeth	23	12	23	58
4 teeth	15	6	13	34
6 teeth	3	4	4	11
Full mouth	61	9	23	93
Total	116	33	71	220

A total of 20 species of bacteria were identified from the 220 specimens. Only 6 (2.7 %) of the 220 samples showed no growth of bacterial organisms. The

isolates were usually mixed populations and only 8 specimens yielded pure cultures of *Arcanobacterium pyogenes.* A summary of the bacteria isolated from genital swabs of sheep is shown in Table 3.7.

Table 3.7 Summary of the bacterial species isolated from 104 clinically affected and 116 unaffected sheep

Bacterial isolates	Total isolates			
	Affected sheep (n=104)		Unaffected sheep (n=116)	
	(n)	(%)	(n)	(%)
Acinetobacter lwoffi	5	4.8	14	12.1
Actinobacillus actinomycetemcomitans	7	6.7	10	8.6
Actinobacillus seminis	2	1.9	4	3.4
Alcaligenes odorans	15	14.4	18	15.5
Arcanobacterium pyogenes	46	44.2	20	17.2
Corynebacterium pseudotuberculosis	12	11.5	14	12.1
Corynebacterium renale	3	2.9	2	1.7
Corynebacterium species	15	14.4	15	12.9
Enterococcus faecalis	9	8.6	26	22.4
Erysipelothrix rhusiopathiae	15	14.4	7	6.0
Eschericia coli	3	2.9	12	10.3
Flavobacterium multivorum	8	7.7	13	11.2
Lactobacillus species	6	5.8	11	9.5
Moraxella species	3	2.9	14	12.1
Pasteurella multocida	16	15.4	20	17.2
Rhodococcus equi	24	23.1	17	14.7
Staphylococcus aureus	11	10.6	7	6.0
Staphylococcus epidermidis	15	14.4	19	16.4
Streptococcus agalactiae	6	5.8	9	7.8
Streptococcus species	22	21.2	30	25.9
Total	243	100.0	282	100.0

Arcanobacterium pyogenes ($p<0.001$) and *Erysipelothrix rhusiopathiae* ($p<0.05$) were isolated from sheep affected with balanitis and vulvitis significantly more often than the other species of bacteria. The odds ratio analysis between the isolation of *A. pyogenes* and *E. rhusiopathiae* and the clinical occurrence of the disease is shown in Table 3.8.

Table 3.8 Odds ratio analysis of the isolation rate of *A. pyogenes* and *E. rhusiopathiae* from affected sheep compared to unaffected counterparts.

Species	Odds ratio	95 % CI	Chi-square	P value
A. pyogenes	3.8	2.05<OR<7.06	19.02	0.000
E. rhusiopathiae	2.6	1.01<OR<6.65	4.2	0.020

All strains of *A. pyogenes* showed similar phenotypic features, i.e., good growth on horse blood agar, Gram-positive coccobacilli (pleomorphic) and a positive CAMP test in the presence of *Staphylococcus aureus* (Fig. 3.13).

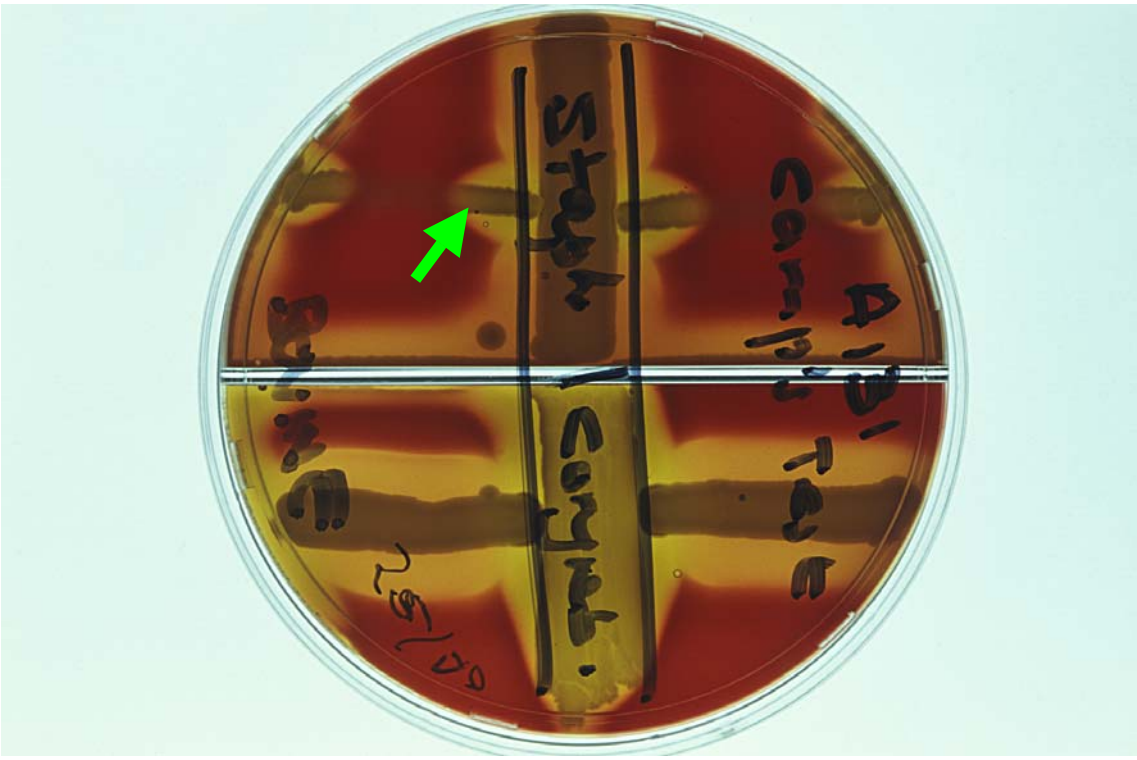

Fig. 3.13 CAMP test with *A. pyogenes* against *S. aureus* showing enhancement of the staphylococcal haemolysin (arrow)

The bacteria isolated most often from apparently healthy sheep were *Enterococus faecalis* (22.4 %), *Pasteurella multocida* (17.2 %), *Rhodococcus equi* (14.7 %), *Staphylococcus epidermidis* (16.4 %) and *Streptococcus species* (25.9 %).

3.2.2.2 Major bacterial isolates and their association with the clinical severity of the lesion

Of the isolated strains of *Arcanobacterium pyogenes*, 47.9 % (34/71) were from sheep showing severe balanitis and vulvitis whilst 36.4 % (12/33) were isolated from sheep that showed mild lesions (Table 3.9). This difference was statistically significant ($p<0.00001$) and the odds ratio analysis showed the association between *A. pyogenes* isolation and the degree of severity of the lesion (Table 3.10). There was, however, no association between other isolates listed in Table 3.9 and the degree of severity of lesions ($p>0.05$).

Table 3.9 Common bacterial isolates and their association with the degree of the severity of the disease

Bacterial isolates	Degree of disease severity				Total (n=104)	
	Mild (n=33)		Severe (n=71)			
	(n)	(%)	(n)	(%)	(n)	(%)
A. pyogenes	12	36.36	34	47.89	46	44.23
C. pseudotuberculosis	7	21.21	5	7.04	12	11.54
Corynebacterium species	6	18.18	9	12.68	15	14.42
E. rhusiopathiae	6	18.18	9	12.68	15	14.42
R. equi	7	21.21	17	23.94	24	23.08

Table 3.10 Odds ratio analysis to determine the association between *A. pyogenes* and the severity of the disease

Isolation status	Lesion status			Total
	Severe	Mild	Unaffected	
A. pyogenes	34	12	20	66
No *A. pyogenes*	37	21	96	154
Total	71	33	116	220

Odds Ratio 1.00 0.62 0.23

Chi-square for trend= 20.163; p <0.00001

Note: Severe is the comparison level hence by definition the odds ratio is 1.

3.2.2.3 Isolation rates of the most common bacterial species from different age and sex groups of the affected sheep

The isolation rate of *A. pyogenes* was significantly higher from young affected sheep as compared to unaffected ones (45.83 % versus 20.0 %; $p<0.05$). Similarly there was also a significant difference in the isolation of this species from affected and unaffected adult sheep ($p<0.05$). *Erysipelothrix rhusiopathiae* was also isolated from 15.3 % of young affected sheep compared to 3.6 % unaffected ones ($p<0.05$). There was no difference ($p>0.05$) in the isolation of *E. rhusiopathiae* from the adult affected and unaffected sheep (Table 3.11).

Table 3.11 The number and percentage of common bacterial isolates from affected and unaffected sheep by age group

Bacterial isolates	Young (n=127)				Adult (n=93)			
	Affected (n=72)		Unaffected (n=55)		Affected (n=32)		Adult (n=61)	
	(n)		(n)	(%)	(n)	(%)	(n)	(%)
A. pyogenes	33	45.83	11	20.00	13	40.63	9	14.75
C. pseudotuberculosis	6	8.33	5	9.09	6	18.75	9	14.75
Corynebacterium spp.	8	11.11	8	14.55	7	21.88	7	11.48
E. faecalis	6	8.33	15	27.27	3	9.38	11	18.03
E. rhusiopathiae	11	15.28	2	3.64	4	12.50	5	8.20
P. multocida	16	22.22	11	20.00	4	12.50	5	8.20
R. equi	13	18.06	6	10.91	11	34.38	11	18.03
S. aureus	9	12.50	0	0.00	2	6.25	7	11.48
Streptococcus species	17	23.61	13	23.64	5	15.63	17	27.87

Table 3.12 shows the association between isolation rates of *A. pyogenes*, the age and disease status of the examined sheep using Mantel-Haenszel weighted Odds Ratio. Mantel-Haenszel weighted Odds Ratio expresses the association between disease status and exposure to *A. pyogenes* corrected for the effects of age.

Table 3.12 Odds ratio to determine the association between the isolation rate of *A. pyogenes* , the disease status and age of the animal

Age stratum	Odds ratio	95 % CI	Chi-square	P vale
Young	3.38	1.41<OR<8.24	9.12	0.002
Adult	3.95	1.31<OR<12.14	7.7	0.005

The prevalence of *A. pyogenes* was higher in females (30/84; 35.7 %) as compared to males (36/136; 26.4 %). However, this difference was not statistically significant (Chi-square=2.10; p=0.147), and there was no significant association between sex and isolation of *A. pyogenes* (Odds ratio=1.74; 95 % CI=0.53<OR<5.72).

Table 3.13 The number and percentage of common bacterial isolates for affected and unaffected sheep by sex

Bacterial isolates	Female (n=84)				Male (n=136)			
	Affected (n=33)		Unaffected (n=51)		Affected (n=71)		Unaffected (n=65)	
	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)
A. pyogenes	19	57.58	11	21.57	27	38.03	9	13.85
C. pseudotuberculosis	0	0.00	5	9.80	12	16.90	9	13.85
Corynebacterium spp.	4	12.12	6	11.76	11	15.49	9	13.85
E. faecalis	4	12.12	18	35.29	5	7.04	8	12.31
E. rhusiopathiae	4	12.12	5	9.80	11	15.49	2	3.08
P. multocida	13	39.39	10	19.61	7	9.86	6	9.23
R. equi	10	30.30	5	9.80	14	19.72	12	18.46
S. aureus	5	15.15	2	3.92	6	8.45	5	7.69
Streptococcus species	3	9.09	11	21.57	19	26.76	19	29.23

3.2.3 ISOLATION AND IDENTIFICATION OF MOLLICUTES

As is customary, the term mollicutes in this study represent microorganisms in the genera *Mycoplasma*, *Acholeplasma* and *Ureaplasma*. A total of 222 mollicutes were isolated from genital swabs of 104 clinically affected and 116 unaffected sheep. Of the 222 isolates, *Mycoplasma* represents 153 (69.0 %), *Ureaplasma* 54 (24.3 %) and *Acholeplamsa* 15 (6.7 %). *Mycoplasma mycoides mycoides* large colony variant was the most frequently isolated mycoplasma (71 out of 153; 46.4 %). Thirteen specimens (18.3 %) yielded pure cultures of *Mmm*LC while the remaining 58 (81.7 %) were found in mixed cultures with one or more species of mycoplasma.

3.2.3.1 Association between the isolation rate of mollicutes and infection

The results of the isolation of mollicutes from vulval and penile swabs are shown in Table 3.14. A total of 130 genital swabs from the 220 sheep examined yielded mollicutes. Twenty-nine of the 33 vulval swabs from clinically affected

ewes and 23 from 51 apparently normal ewes yielded mollicutes. A total of 78 preputial swabs out of 136 yielded mollicutes. Fifty-five of those were from 71 clinically affected rams and the remaining 23 were from 65 apparently normal ones (Table 3.14).

Table 3.14 Results of attempted isolation of mollicutes from 220 genital swabs

Source	No. of animals examined			No. of positive for mollicutes		
	Unaffected	Affected	Total	Unaffected	Affected	Total
Vulval swab	51	33	84	23	29	52
Penile swab	65	71	136	23	55	78
Total	116	104	220	46	84	130

One hundred and thirty (59.1 %) of the 220 sampled animals yielded positive results for mollicutes. 2x2 tables (Tables 3.15 & 3.16) were used for chi-square analysis of the isolation rate of mollicutes from vulval and penile swabs of affected and healthy sheep. The isolation of mollicutes from affected animals was 6.39 times greater than from unaffected animals ($p<0.05$; Odds Ratio=6.39; 95 % CI=3.32<OR<12.40; Chi-square value=38.35; p value=0.000).

Table 3.15 A 2x2 contingency table showing the rate of isolation of mollicutes from vulval swabs

Isolation of mollicutes	Disease status		Total
	Yes	No	
Yes	29	23	52
No	4	28	32
Total	33	51	84

Table 3.16 A 2x2 contingency table showing the rate of isolation of mollicutes from penile swabs

Isolation of mollicutes	Disease status		Total
	Yes	No	
Yes	55	23	78
No	16	42	58
Total	71	65	136

No significant association could be found between the isolation of mollicutes from either vulval or penile swab specimens (Table 3.17).

Table 3.17 Analysis of the odds ratio to determine the association between the isolation rate of mollicutes and the origin of genital swab

Origin	Isolation of mollicutes		Odds ratio	95 % CI	Chi-square	p value
	Yes	No				
Vulval swab	52	32	0.83	0.46<OR<1.50	0.45	0.540
Penile swab	78	58				

3.2.3.2 Association between the isolation rates of *Acholeplasma, Mycoplasma* and *Ureaplasma* strains and infection

From a total of 222 mollicutes isolated from 130 genital swabs of both affected and healthy sheep, 153 isolates (69.0 %) were of *Mycoplasma* spp., while 54 (24.3 %) and 15 (6.7 %) were *Ureaplasma* and *Acholeplasma* species, respectively (Table 3.18).

Table 3.18 Summary of the isolation of members of the genera *Acholeplasma, Mycoplasma* and *Ureaplasma* from genital swabs of clinically unaffected and affected sheep

Genera	Isolates from unaffected sheep (n=71)		Isolates from affected sheep (n=151)		Total (n=222)	
	(n)	(%)	(n)	(%)	(n)	(%)
Acholeplasma	6	8.4	9	5.9	15	6.7
Mycoplasma	35	49.3	118	78.2	153	69.0
Ureaplasma	30	42.3	24	15.9	54	24.3

The isolation rates of mycoplasma from healthy and infected sheep varied significantly ($p<0.05$). The chance that mycooplasma would be isolated from affected sheep was 3.68 times greater than from apparently healthy sheep (Tables 3.19 & 3.20).

Table 3.19 A 2x2 table to determine the association between mycoplasma isolation and clinical signs

Mycoplasma isolation	Disease status		Total
	Yes	No	
Yes	118	35	153
No	33	36	69
Total	151	71	222

Table 3.20 Odds ratio analysis to show the association between the isolation rate of mycoplasma and clinical signs

Odds Ratio	95 % confidence interval	Chi-square value	p value
3.68	1.92<OR<7.05	18.76	0.000

Fifteen isolates of *A. laidlawii* were isolated from both healthy and affected sheep. Although *A. laidlawii* was isolated from 60% (9/15) of affected sheep as compared to 40% (6/15) from healthy sheep, the difference was not statistically significant ($p>0.05$; Odds Ratio=0.69; 95 % CI=0.21<OR<2.28; Chi-square value=0.48; p value=0.49) (Table 3.21).

Table 3.21 A 2x2 table to determine the association between acholeplasma isolation and clinical signs

Acholeplasma isolation	Disease status		Total
	Yes	No	
Yes	9	6	15
No	142	65	207
Total	151	71	222

Fifty-four ureaplasma strains were isolated of which 24 (45 %) were from infected and the remaining 30 (54 %) from healthy sheep. The difference in isolation of ureaplasma between healthy and infected sheep was statistically significant ($p<0.05$; Chi-square value=18.23; p value=0.000). However, the odds ratio of 0.26 (95 % CI=0.13<OR<0.51) indicated healthy sheep were more likely to have ureaplasma organisms than clinically affected sheep (Table 3.22).

Table 3.22 A 2x2 table to determine the association between ureaplasma isolation and clinical signs

Ureaplasma isolation	Disease status		Total
	Yes	No	
Yes	24	30	54
No	127	41	168
Total	151	71	222

Table 3.23 Summary of the isolated and identified *Mycoplasma*, *Acholeplasma* and *Ureaplasma* species by sex and clinical status of the host

Species	Unaffected (n=116)		Affected (n=104)		Total (n=220)
	Male (n)	Female (n)	Male (n)	Female (n)	
A. laidlawii	3	3	7	2	15
M. agalactiae	1	0	0	1	2
M. arginini	0	1	2	2	5
M. bovigenitalium	7	6	12	8	33
M. capri	2	2	0	1	5
M. capricolum	1	0	4	3	8
*M. mm*LC	2	5	40	24	71
M. species Group 7	3	2	15	5	25
Unidentified *Mycoplasma*	1	2	1	0	4
Ureaplasma	17	13	17	7	54

3.2.3.3 Association between the isolation rates of mycoplasma and clinical disease status

In this study, there were 153 mycoplasma isolates recovered from 130 of the 220 sheep (Table 3.23). Of the 153 isolates, 71 (46.4 %) were *Mmm*LC, 33 (21.6 %) were *M. bovigenitalium*, and 25 (16.3 %) were *M.* species Group 7. When the isolates from individual clinically affected sheep were compared, 64 (61.5 %) sheep had *Mmm*LC. There were 20 (19.2 %) sheep each with *Mycoplasma* species group 7 and *M. bovigenitalium*, 7 (6.7 %) sheep with *M. capricolum*, and 4 (3.8 %) sheep with *M. arginini* (Table 3.24).

Table 3.24 Summary of the isolated and identified *Mycoplasma* species from the genital swabs of 116 clinically unaffected and 104 affected sheep

Mycoplasma species	Unaffected		Affected		Total	
	(n)	(%)	(n)	(%)	(n)	(%)
M. agalactiae	1	0.86	1	0.96	2	0.9
M. arginini	1	0.86	4	3.8	5	2.3
M. bovigenitalium	13	11.2	20	19.2	33	15.0
M. capri	4	3.4	1	0.96	5	2.3
M. capricolum	1	0.86	7	6.7	8	3.6
*M. mm*LC	7	6.0	64	61.5	71	32.3
M. species Group 7	5	4.3	20	19.2	25	11.4
Unidentified *Mycoplasma* spp.	3	2.6	1	0.96	4	1.8

Table 3.25 shows that, of the isolated *Mmm*LC strains 64 (90.1 %) were from affected sheep while the rest 7 (9.9 %) were from healthy sheep. The difference in the isolation of this species between healthy and affected groups was statistically significant ($p<0.01$; Chi-square=77.29; p value=0.000). Sheep with clinical lesions are 24.46 times more likely to have *Mmm*LC than healthy sheep (95 % CI= 9.76–64.01).

Table 3.25 Isolated and identified *Mycoplasma* species from the genital swabs of 220 clinically unaffected and affected sheep

Mycoplasma species	Unaffected		Affected		Total	
	(n)	(%)	(n)	(%)	(n)	(%)
M. agalactiae	1	50.0	1	50.0	2	100.0
M. arginini	1	20.0	4	80.0	5	100.0
M. bovigenitalium	13	39.4	20	60.6	33	100.0
M. capri	4	80.0	1	20.0	5	100.0
M. capricolum	1	12.5	7	87.5	8	100.0
*M. mm*LC	7	9.9	64	90.1	71	100.0
M. species Group 7	5	20.0	20	80.0	25	100.0
Unidentified *Mycoplasma*	3	75.0	1	25	4	100.0

Fig. 3.14 72 hour colonies of a *Mmm*LC field isolate *in situ* on Hayflick's agar

The results show that 20 (60.6%) and 13 (39.4%) of *M. bovigenitalium* isolates were from affected and healthy sheep, respectively. This difference was, however, not statistically significant ($p>0.05$; Odds Ratio=1.87; 95 % CI= 0.83<OR<4.27; Chi-square=2.7; P value=0.100) (Tables 3.26).

Similarly *M.* species Group 7 comprised 20 (80.0 %) and 5 (20.0 %) of the mycoplasma isolates from affected and healthy sheep, respectively. The variation in colonization among the affected and healthy sheep with *M.* species Group 7 is statistically significant ($p<0.05$; Odds Ratio=5.29; 95 % CI= 1.74<OR<16.53; Chi-square=11.8; p value=0.000) (Tables 3.26).

No significant association was found between most mycoplasma isolates and clinical signs. However, a significant association was found between the isolation of *Mmm*LC, *M.* species Group 7 and *M. capricolum* and clinical signs (Table 3.26).

Table 3.26 Summary of the odds ratio analysis to determine the association between the isolation of *Mycoplasma* species and clinical signs

Mycoplasma species	Odds Ratio	95 % CI	Chi-square	p value
M. agalactiae	0.31	0.00<OR<41.45	0.01	0.930
M. arginini	4.57	0.47<OR<109.08	2.17	0.140
M. bovigenitalium	1.87	0.83<OR<4.27	2.70	0.100
M. capri	0.27	0.01<OR<2.59	1.57	0.209
M. capricolum	8.31	1.00<OR<182.93	5.40	0.020*
*M. mm*LC	24.46	9.76<OR<64.01	77.29	0.000**
M. species Group 7	5.29	1.74<OR<16.53	11.80	0.000**
Unidentified *Mycoplasma*	0.36	0.01<OR<3.95	0.84	0.358

* Statistically significant (p<0.05)

** Statistically significant (p<0.001)

3.2.3.4 Association between the isolation rate of *Mmm*LC and clinical disease in different age groups

A sizeable proportion of infected sheep at the age of 2 teeth (29.7 %), full mouth (26.6 %) and 4 teeth (20.3 %) had high isolation rates of *Mmm*LC. This difference in the prevalence of *Mmm*LC among different age groups is statistically significant (p<0.05) except for the 6 teeth age group (p>0.05) (Table 3.27). A significant association was found between clinical disease status and isolation of *Mmm*LC at most age groups (Table 3.28). Although healthy sheep in all the different age groups yielded *Mmm*LC isolates, the low number of positive specimens of this specific mycoplasma among the healthy groups was noticeable.

Table 3.27 Prevalence of *Mmm*LC in 104 affected and 116 unaffected sheep in different age groups

Age group	No. of examined animals		No. of positive for *Mmm*LC			
	Affected	Unaffected	Affected	(%)	Unaffected	(%)
0 tooth	10	13	8	12.5	1	14.3
2 teeth	35	23	19	29.7	3	42.8
4 teeth	19	17	13	20.3	0	0.0
6 teeth	8	2	7	10.9	2	28.6
Full mouth	32	61	17	26.6	1	14.3
Total	104	116	64	100.0	7	100.0

Table 3.28 Analysis of the odds ratio to show the association between *Mmm*LC isolation and clinical disease in different age groups

Age groups	Odds Ratio	95 % CI	Chi-square	p value
0 tooth	48.0	3.7<OR<622.02	12.4	0.000
2 teeth	7.9	1.74<OR<41.02	10.03	0.001
4 teeth	35.0	3.2<OR<890.92	14.86	0.000
6 teeth	1.75	0.00<OR<70.17	0.15	0.700
Full mouth	68.0	8.26<OR<1481.70	35.65	0.000

The analysis was repeated using the age data as a dichotomous variable where the age groups 0 tooth to 6 teeth were pooled to represent the young age group and compared to the adult age group (full mouth) in terms of the prevalence of *Mmm*LC (Table 3.29). The results showed a 75.8 % prevalence of *Mmm*LC in young affected sheep, whilst 53.1 % occurred in the affected adult age group. There was a significant difference in the prevalence of *Mmm*LC and the disease status in the two age groups ($p<0.001$) (Table 3.30). The prevalence of *Mmm*LC was 2.98 times higher in young animals than adult animals (Table 3.30).

Table 3.29 Prevalence of *Mmm*LC in 104 affected and 116 unaffected sheep in young and adult age groups

Age group	No. of examined animals		No. of positive for *Mmm*LC			
	Affected	Unaffected	Affected	(%)	Unaffected	(%)
Young	72	55	47	75.8	6	10.9
Adult	32	61	17	53.1	1	1.6
Total	104	116	64	61.5	7	6.03

Table 3.30 Odds ratio analysis to determine the association between the age of the host and isolation of *Mmm*LC

Age	*Mmm*LC isolates		Odds ratio	95 % CI	Chi-square	p value
	Yes (n)	No (n)				
Young	53	74	2.98	1.53<OR<5.85	12.30	0.000
Adult	18	75				

3.2.3.5 Association between the isolation rate of *Mmm*LC and the severity of the lesion

During this study, *Mmm*LC was isolated from 64 (61.53%) of 104 affected sheep. Of the 64 isolates, 46 (71.9%) were from sheep with severe clinical signs with a varying degree of ulceration and 18 (28.1%) were from mildly affected sheep (Table 3.31).

Table 3.31 Isolation rate of *Mmm*LC and stage of clinical disease severity

Status of isolation	Degree of disease severity				Total (n=104)	
	Mild infection (n=33)		Severe infection (n=71)			
	(n)	(%)	(n)	(%)	(n)	(%)
Yes	18	28.1	46	71.9	64	100.0
No	15	37.5	25	62.5	40	100.0

When the association between the isolate and degree of disease severity was evaluated, it appeared that there was a significant association ($p<0.05$) between the degree of the disease severity and the rate of isolation of *Mmm*LC (Odds ratio=2.89; 95 % CI=1.54<OR<5.43; Chi-square=12.9; p value=0.000). In other words, in sheep where *Mmm*LC was isolated, they were 2.89 times more likely to have severe lesions than mild lesions.

3.2.4 THE COMBINED ISOLATION OF *A. pyogenes* AND *Mmm*LC FROM CLINICALLY AFFECTED SHEEP

Arcanobacterium pyogenes and *Mmm*LC were the two most common isolates in sheep with clinical signs of the disease, and their possible synergistic role in the disease process was assessed. They were simultaneously isolated from 33 (31.7%) sheep out of 104 clinically affected sheep and from 1 (0.9%) sheep out of 116 healthy sheep (Table 3.32). Odds ratio analysis showed that when *A. pyogenes* and *Mmm*LC were present together they were 53.5 times more likely to occur in clinically affected sheep than unaffected sheep (Table 3.33).

Table 3.32 The combined isolation of *A. pyogenes* and *Mmm*LC from 104 clinically affected and 116 unaffected sheep

Isolates	Affected		Unaffected		Total	
	(n)	(%)	(n)	(%)	(n)	(%)
A. pyogenes alone	13	12.5	19	16.4	32	14.5
*Mmm*LC alone	31	29.8	6	5.2	37	16.8
A. pyogenes & *Mmm*LC	33	31.7	1	0.9	34	15.4

Table **3.33** Odds ratio analysis to determine the association between the mutual isolation of *A. pyogenes* and *Mmm*LC and clinical disease status

Disease status	Isolates		Odds ratio	95 % CI	Chi-square	p value
	Yes (n)	No (n)				
Affected	33	71	53.45	7.57<OR<1073	39.9	0.000
Unaffected	1	115				

3.2.5 *Chlamydophila* ANTIGEN DETECTION AND VIRUS ISOLATION

No *Chlamydophila* antigens were detected from 20 selected swabs each from affected and unaffected sheep, using the Clearview® chlamydia antigen detection ELISA kit.

No viruses were isolated from the 160 specimens from affected sheep in cell cultures.

3.3 DISCUSSION

3.3.1 CLINICAL DISEASE DESCRIPTION

Although, Trichard *et al* (1993) and Trichard & Van Tonder (1994) described posthitis and vaginitis as part of the disease syndrome, the involvement of the prepuce, vaginal and vulval vestibule was essentially absent in sheep examined in this study (Figs. 3.6 – 3.11). This observation is consistent with the description of the disease given by Dent (1971), Webb & Chick (1976) and Deas (1983). In almost all cases examined the tissues affected were the soft tissue of the glans penis of rams and the muco-cutaneous junction of the vulval lips of ewes. The fibro-elastic tissues of the shaft of the penis are less likely to be affected by such lesions than the soft tissues of the glans penis (Deas, 1983). In a few cases congestion of the vulval mucosa and inner lining of the prepuce and shaft of the penis were observed. Secondary bacterial infections

will in some rams lead to a swollen preputial orifice followed by either phimosis or paraphimosis. It is also not uncommon to find matted vulval lips as a result of inflammatory exudates. However, such signs may not be regarded as the typical inflammatory stages of the disease.

The name ulcerative balanitis and vulvitis as suggested by Deas (1983), Linklater & Smith (1993), Dunn (1996) and Greig (2000) is therefore a more appropriate description of the disease as seen in South Africa.

3.3.2 ASSOCIATION BETWEEN AGE AND SEX OF THE ANIMAL AND DISEASE OCCURRENCE

It is recognised that case control study design is generally prone to bias of several types particularly arising from the selection of cases and controls that are not representative in terms of confounders that may influence disease risk. Although the sources of selection bias in this particular study would be the age and sex category of the study groups, restrictive selection criteria was set in designing the study, and an effort was made to represent both age and sex categories of the two groups (cases and controls) while collecting specimens in the study flocks.

The results included in tables 3.4 and 3.5 demonstrated that young animals are more likely to have ulcerative balanitis and vulvitis infections than adults. There is no clear explanation for this finding, but more sexual activity was one explanation offered (Gummow & Staley, 2000). It is thought that vigorous mating causes abrasion of the surface of the glans penis, allowing microbial invasion to occur. Livingstone, C. W., Gauer, B. B. & Shelton, M. (1978) suggested that stress may influence the prevalence of ureaplasmas and mycoplasmas in genital infection of sheep.

This investigation has shown that adult animals experience a low exposure rate and less severe form of the disease. These observations are reinforced when

looking at the results on Tables 3.5. The odds ratio analysis confirmed that younger animals are 2.5 times more likely to have the lesins than adults. Deas (1983) indicated that some adult ewes and rams will not become re-infected. However, it has also been reported that the disease does not produce solid immunity and that animals can be repeatedly infected (Bath & De Wet, 2000). Although, information available about resistance to genital tract infections in sheep is insufficient (Taylor-Robinson & Furr, 1986), local production of immunoglobulin (Ig) A predominates in the reproductive tract, and would probably be protective (Winter, 1982). The findings of Ball, H. J., McCaughey, W. J. & Irwin, D. (1984) further support the conclusion that older sheep could develop a certain level of resistance to re-infection from their earlier exposures.

During this research project, it was demonstrated that, although the chance of developing lesions seemed low, adult sheep may have inapparent infection. Such animals probably remain asymptomatic carriers as suggested by Doig & Ruhnke (1977), and this may be the means by which infections flare-up and are transferred from sheep to sheep and from flock to flock. It is thought that clean, purchased rams are a source of infection for unaffected farms in South Africa (P. Stadler, personal communication, 2002).

The stress of oestrus, mating, pregnancy or lambing produces changes in the micro-environment of the genitalia (hormonal changes, pH changes, mucous production, increased in detached epithelial cells) that favours colonization by microorganisms (Winter, 1982). Once the disease is in a flock, mating facilitates further transmission of infection between sheep.

The severity of the lesions observed in rams is more alarming than in ewes. This is due to the fact that the ulcers become more lacerated during the act of erection and copulation. The severity of the lesion is also influenced by the pathogenicity of the particular aetiological agent and other predisposing factors. Host-adapted microorganisms such as mycoplasmas, are often responsible for multi-factorial diseases, in which factors such as inter-current infection,

crowding, bad climatic conditions and stress of any sort influence the final outcome of infection (Rosendal, 1986).

3.3.3 MAJOR MICROORGANISMS AS DETERMINANT FACTORS IN THE DISEASE PROCESS

It was mentioned previously that the causal organisms of ulcerative balanitis and vulvitis was often not isolated from vulval and penile lesions (Webb & Chick, 1976; Deas, 1983; Linklater & Smith, 1993; Dunn, 1996; Greig, 2000) and several possible explanations were advanced. In an attempt to identify the aetiology of ulcerative vulvitis and balanitis in sheep, a case-control study to observe the differences in microbial flora between apparently healthy and affected sheep was conducted. The results showed that Gram-positive, Gram-negative and mollicute organisms constituted the microbial flora of the genital tract of both healthy and affected sheep. Among the Gram-positive bacilli, *Arcanobacterium pyogenes* was the one predominantly isolated from clinically affected sheep, while Gram-negative cocco-bacilli such as *Pasteurella multocida*, among others, predominated in the genital flora of unaffected sheep.

There was also a difference in the isolation of mycoplasma organisms between healthy and affected sheep. Mycoplasma organisms were isolated 3.68 times more in affected sheep than unaffected sheep (Tables 3.19 & 3.20). This result points to an association of this group of microorganisms with ulcerative genital disease of sheep in South Africa. It confirms the suppositions from earlier publications that mycoplasma organisms are the primary aetiological agents of ulcerative balanoposthitis and vulvovaginitis in sheep (Trichard *et al*, 1993; Bath & De Wet, 2000; Gummow & Staley, 2000).

Although it has been shown that mollicutes are frequent inhabitants of the genital tract (Carmichael *et al*, 1972; Baseman & Tully, 1997), results from this project confirmed that the isolation of mycoplasmas was significantly greater in sheep with clinical infections than in those without lesions. These differences

may reflect the pathogenic role of the involved strains of mycoplasma rather than that of a normal inhabitant acting as a predisposing factor. These observations are consistent with earlier studies in which *Mycoplasma* spp were isolated frequently from clinically infected rams and ewes (Cottew *et al*, 1974; Jones *et al*, 1983; Trichard *et al*, 1993). Edward, D. G. FF., Hancock, J. L. & Hignett, S. L. (1947) were the first to isolate mycoplasmas from the bovine genital tract with a history of infertility that could not be ascribed to any recognized pathogen.

A large variety of microorganisms that include bacteria such as *A. pyogenes* (Ihemelandu, 1972), *Mmm*LC (Cottew, *et al*, 1974; Trichard *et al*, 1993), *M. capricolum*, *M. arginini* (Jones *et al*, 1983) and viruses (Horner *et al*, 1982), have been isolated from natural and artificial cases of balanitis and vulvitis. However, these results were difficult to interpret without parallel studies of clinically unaffected animals. Furthermore, evidence for the pathogenicity of isolated organisms in multifactorial diseases has mainly been based on the frequency of occurrence in diseased animals compared to healthy ones (Parsonson, I. M., Al-Aubaid, J. M. & McEntee, K., 1974; Doig, P. A., Ruhnke, H. L. & Palmer, N. C., 1980 [a, b]; Ball, H. J., McCaughey, W. J., Mackie, D. P. & Pearson, G. R., 1981; LaFaunce & McEntee, 1982).

Although causation cannot be established from a single case-control study, if exposure to the suspected cause is present more commonly in those with disease than in those without disease, it represents one proof of causality (Thrusfield, 1995). In the present case-control study mycoplasmas were isolated from 59.1 % (130/220) of the sheep, with *Mmm*LC being the predominant species (61.5%) (Table 3.24). The isolation of *Mmm*LC from 61.5% of the affected sheep was lower than that reported (83 %) by Trichard *et al* (1993). Possible reasons exist for this apparent difference. Longer intervals between the appearance of clinical disease and sampling may have an influence on the prevalence of mycoplasma isolation, as most of the time mycoplasma persists in sites of infection for only a few days (Cottral, 1978). McCaughey &

Ball (1981) have found a relatively lower prevalence of genital ureaplasma infection from swabs taken after a considerable time of mating. Thus it is less likely to isolate mycoplasmas from infection if the exact time at which mycoplasmas are abundant is not considered.

Secondly, the specimens taken from infected animals are always subject to adverse environmental challenges and thus pathogens may not be viable on arrival at the laboratory. This is particularly true for mycoplasmas, which are very sensitive to environmental effects.

Thirdly, pathogenic mycoplasmas are difficult microorganisms to grow *in vitro* from clinical specimens due to their intimate dependence upon host cells (Eaglesome, M. D., Garcia, M. M. & Stewart, R. B., 1992; Baseman & Tully, 1997).

Results from this study provided strong evidence for the association of *Mmm*LC with clinical genital tract infection in Dorper sheep in South Africa. This is demonstrated by the higher rate of isolation of *Mmm*LC from clinically infected sheep compared to the apparently normal flock mates (Table 3.25 & 3.26). Furthermore, the absence of viruses, *Chlamydophila* species and inconsistent isolation of different species of bacteria from clinically infected sheep indicate that these organisms are probably not responsible for the clinical signs observed. However, it is likely that bacteria may contribute to a more severe condition if present in association with mycoplasmas. This was further illustrated by the combined isolation of *A. pyogenes* and *Mmm*LC from 31.7 % of sheep with clinical signs of the disease (Table 3.32 & 3.33).

The study has shown, for the first time, the association of *Mmm*LC and *A. pyogenes* with genital tract infection of sheep in South Africa. Mycoplasmas are well-defined pathogens acting singly or in consort with other agents. Although one cannot be sure, it is possible that on the basis of the results from the current investigation, the isolation of *A. pyogenes* concurrent with *Mmm*LC

tends to support the concept that the bacterial pathogen is the primary factor that opens the door for further mycoplasmal invasion and subsequent pathological consequences. *A. pyogenes* is considered as the most pathogenic bacteria residing on the mucosal surfaces where it can cause tissue damage by means of its virulence factor, the exotoxin, pyolysin (Billington *et al*, 1997). Furthermore, *Mmm*LC has an endotoxic activity able to induce damage to the epithelial cells of the genital tract (Rosendal, 1986). These endotoxins are responsible for increased cellular damage in parts of the genital system of sheep colonized by this *Mycoplasma* species.

The 46 strains of *A. pyogenes* that were found among the genital isolates from clinically infected sheep (Tables 3.7 – 3.10), provides an important link in the epidemiology of ulcerative balanitis and vulvitis syndrome. *A. pyogenes* was detected in 44.2 % of the affected and 17.2 % of the unaffected sheep, suggesting a possible role of the organism in the disease process. Earlier workers (Beverley & Watson, 1962; Dennis & Bamford, 1966, Smith *et al*, 1971; Gardner *et al*, 1990; Nicholas, R. A. J., Greig, A., Baker, S. E., Ayling, R. D., Heldtander, M., Johansson, K. E., Houshaymi, B. M. & Miles R. J. 1998) have established the virulence of the bacteria and its associations with urogenital infections. *Arcanobacterium pyogenes* was for example isolated from three of the ten pus specimens taken from does affected with vulvitis in Nigeria (Ihemelandu, 1972).

It seemed likely that *A. pyogenes* had a synergistic effect with the primary pathogen, in this case *Mmm*LC, to exacerbate the clinical features of the disease. A synergistic interaction of *A. pyogenes* with some other organisms in various clinical conditions has been described (Roberts, 1967; Takeuchi, S., Nakajima, Y. & Hashimoto, K., 1983).

In this study, *Mycoplasma capricolum* was isolated from 7 sheep with clinical signs of ulcerative balanitis and vulvitis (Table 3.25 & 3.26). *Mycoplasma capricolum* is usually associated with polyarthritis in sheep and goats (Jones,

1983), and has also been isolated from the genital tract of sheep in England associated with an outbreak of vulvovaginitis and balanoposthitis (Jones *et al*, 1983). However, from the low isolation rate detected in this investigation, it appears unlikely that *M. capricolum* is a primary pathogen for the genital system of sheep. The role of *M capricolum* in the pathogenesis of genital tract disease is therefore unclear.

Although *Mycoplasma* species Group 7 was isolated from 23 per cent of affected sheep with an odds ratio of 5.19 (Table 3.25 & 3.26), it is difficult to conclude whether or not it has any significant role in the clinical signs of ulcerative balanitis and vulvitis. Moreover, there are no other reports incriminating this organism as a causal agent.

Nine of the 15 strains of *A. laidlawii* isolated were from animals with clinical cases of balanitis and vulvitis (Table 3.23). Although, *A. laidlawii* is usually regarded as a non-pathogenic organism, there are reports associating the organism with reproductive disease conditions (Ball, H. J., Neil, W. A., O'brien, J. J. & Ferguson, H. L., 1978; Tiwana & Singh, 1982; Kapoor *et al*, 1984). In the light of increasing evidence of association of this organism with disease conditions there is need for further assessment of the pathogenic potential of *A. laidlawii*.

Comparison of the rates of isolation of ureaplasma from clinically infected sheep and apparently normal ones showed that 54 % of the isolates came from apparently normal sheep (Tables 3.23). The prevalence of ureaplasmas in apparently healthy sheep in this survey was high. This may have been due to this organism being a common member of the normal flora of the genital system. These results were in agreement with earlier reports that many apparently healthy sheep carry ureaplasmas in their urogenital tract (Doig & Ruhnke, 1977; Jones & Rae, 1979; McCaughey *et al*, 1981). Therefore, it is possible that ureaplasmas were either commensals within the vagina and prepuce of sheep as suggested by Jones & Rae (1979), or alternatively, they

did not include pathogenic serotypes (Livingstone *et al*, 1978; H. J. Ball, personal communication, 2002).

3.3.4 SEROLOGICAL IDENTIFICATION OF MYCOPLASMA

When attempts are made to isolate mycoplasmas from genital mucous membranes, a large percentage of the isolates consist of different species that require identification. Eight species of mycoplasma were identified by this method in this study (Table 3.23). When the IFAT is applied to agar blocks with mixed colonies of different species satisfactory specific immunofluorescence of *Mmm*LC cultures is achieved.

In the immunofluorescent antibody staining procedure employed in this study, there was a clear distinction between the heterologus and homologus reactions (Fig. 3.15 & 3.16). The heterologus mycoplasma colonies absorbed the counter stain and appeared reddish brown, while the homologus colonies showed a bright yellowish-green fluorescence.

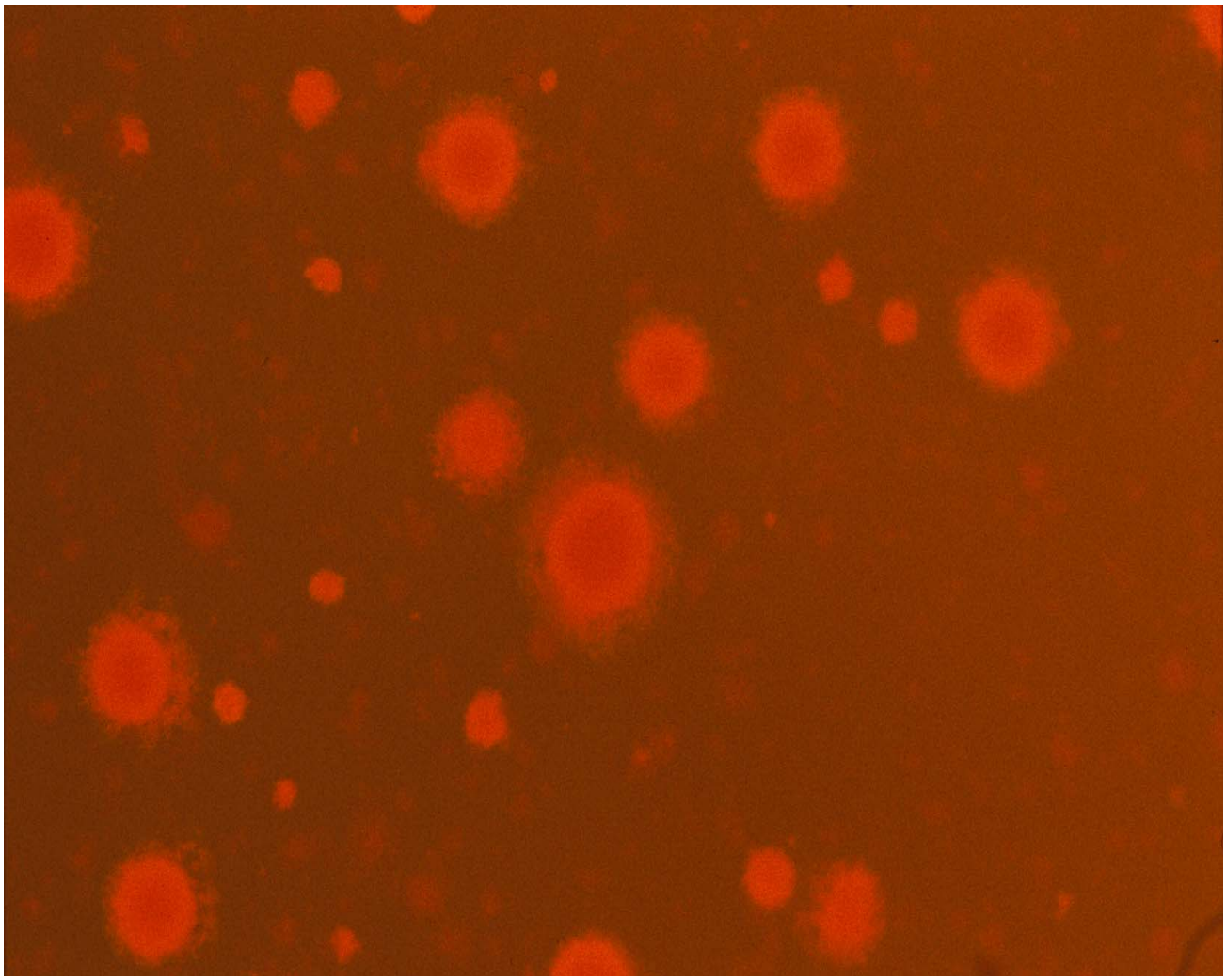

Fig. 3.15 Heterologus reaction in the IFA test: The capture antigen consisted of *Mmm*LC. The primary antibody used was the antiserum raised against the *M. bovigenitalium* reference strain, and the secondary antibody (conjugate) was an anti-rabbit γ globulin.

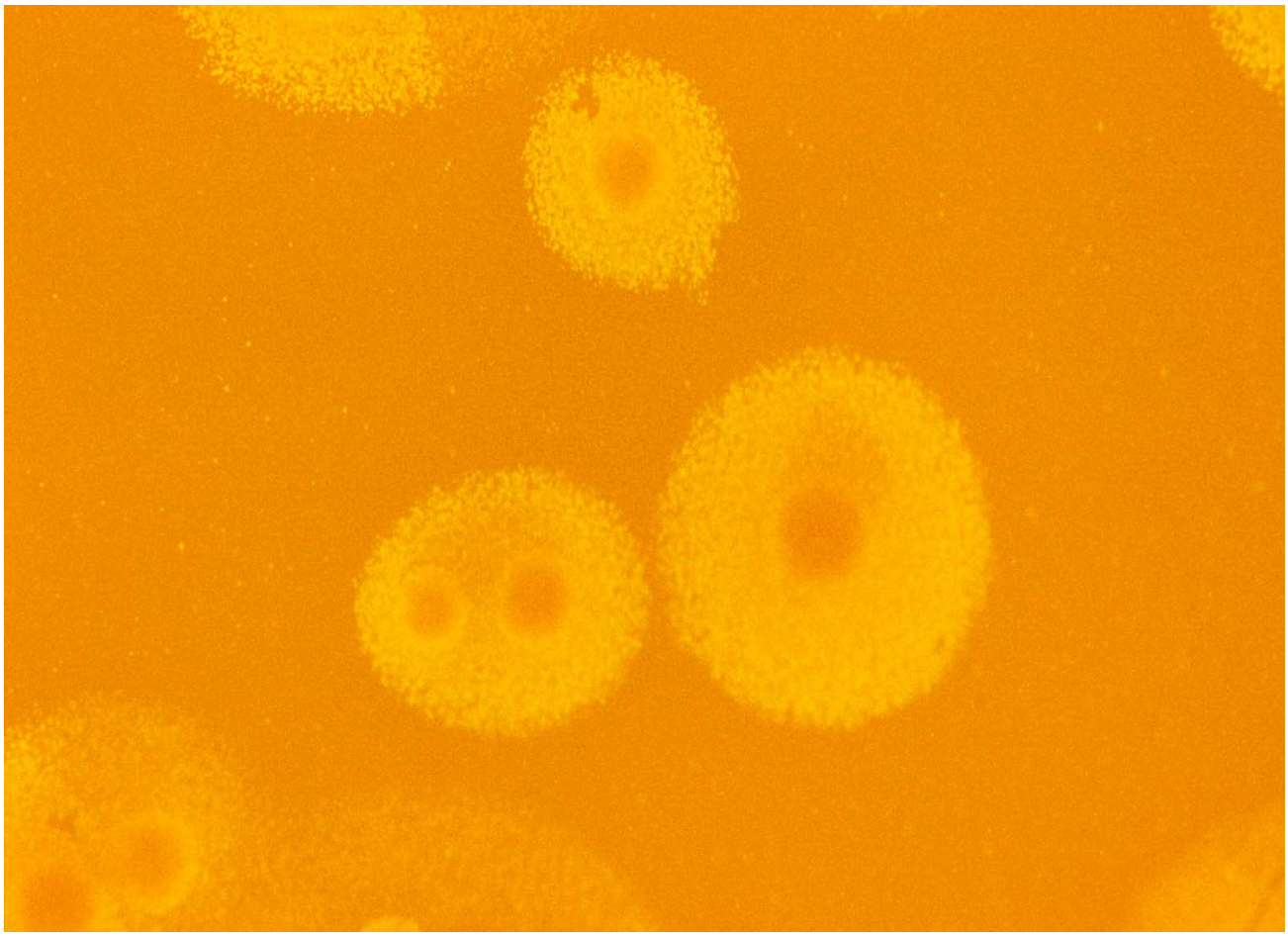

Fig. 3.16 Homologus reaction: The capture antigen consisted of *Mmm*LC. The primary antibody used was the antiserum raised against the *Mmm*LC reference strain, and the secondary antibody (conjugate) was an anti-rabbit γ globulin.

3.3.5 FUTURE RESEARCH

Further research is required on the pathogenesis of *Mmm*LC in the ovine genital system. The immune response induced by *Mmm*LC needs to be established, to better understand the development of the disease in different age groups of animals. Cell marker as indicator of the inflammatory and immune response in the mucous membrane of the genital system of infected sheep can be used for such studies. Furthermore, the significance of subclinical carriers in disseminating the infection before developing the disease, and the period of

time animals continue to shed organisms after recovery from infection needs to be addressed.

There should be a substantial increase in research efforts to curtail the deleterious effect of the disease through better disease management strategies. One aspect would be to develop a nucleic acid based test for improved diagnosis of the disease. A better understanding of the antigenic and immunogenic characteristics of the microorganism can form the basis of the development of a vaccine.

Investigation of treatments revealed that farmers based their choice of antibiotics on what is available on the market. Pharmacokinetic and efficacy studies to establish the most appropriate drugs for balanitis and vulvitis in sheep are needed.

Apparent breed differences in the susceptibility of ulcerative balanitis and vulvitis have been reported, the highest incidence being amongst the Dorper sheep (Trichard *et al*, 1993; Gummow & Staley, 2000). However, objective data are lacking on the question of breed susceptibility. Moreover, a case of balanitis in a Damara ram, a flock mate of Dorper sheep, at Smorgenskadu farm, Namakwaland district, Northern Cape, provides evidence that other breeds could also be infected if they are exposed to the disease. In other parts of the world, Border Leicester (Webb & Chick, 1976), Corriedale (Cottew *et al*, 1974) and Merino (Dent, 1971; Jones *et al*, 1983) sheep breeds have been shown to be susceptible. Studies to address breed susceptibility to ulcerative balanitis and vulvitis should become a focus area of research in the future.

CHAPTER IV

4. *IN VITRO* ANTIMICROBIAL SUSCEPTIBILTY OF *Mycoplasma Mycoides Mycoides* LARGE COLONY AND *Arcanobacterium pyogenes* FIELD STRAINS

4.1 INTRODUCTION

Mycoplasma mycoides mycoides large colony biotype (*Mmm*LC) is an economically important pathogen of sheep that has been isolated and identified as the major cause of ulcerative balanitis and vulvitis in South Africa.

Control of genital mycoplasma infections, such as ulcerative balanitis and vulvitis, by means of vaccination is currently not practiced. Control of these infections with antimicrobial drugs is necessary in conjunction with biosecurity measures to minimise economic losses. Notwithstanding dependence on antimicrobial drugs to minimize these losses, clinical efficacy has not yet been determined. Furthermore, the empirical use of antimicrobial drugs can contribute to selection pressure on the microorganisms that may lead to the development of resistance. Several antibiotic classes such as the macrolides, tetracyclines and fluoroquinolones have been shown to posses *in vitro* activity against various animal mycoplasmas (Bradbury, J. M., Yavari, C. A. & Gils, C. J., 1994; Hannan P. C. T., Windsor, G. d., DeJong, A., Schmeer, N. & Stegemann, M., 1997).

Rational antimicrobial drug use requires the *in vitro* testing of the susceptibility of mycoplasma isolates to selected antimicrobials (Tanner, A. C., Erickson, B. Z. & Ross, R. F., 1993), ideally by means of determination of their minimal inhibitory concentrations. At present no internationally accepted protocol for susceptibility testing of mycoplasmas is available and different methods and media are used in different laboratories. However, Hannan (2000) in his review article described the general principles and guidelines for antimicrobial

susceptibility tests to be done with mycoplasma isolates. Although MIC data for some veterinary *Mycoplasma* species have been published by various workers (Hannan, P. C. T., O'Hanlon, P. J. & Rogers, N. H., 1989; Cooper, A. C., Fuller, J. R., Fuller, M. K., Whittllestone, P. & Wise, D. R., 1993; Bradbury *et al*, 1994; Ball, H. J., Craig-Reilly, G. A. & Bryson, D. G. , 1995; Ayling, *et al*, 2000), standardised MIC breakpoints are still lacking.

In mycoplasmology the MIC is defined as the lowest concentration of an antimicrobial drug that will inhibit visible growth of the mycoplasma as judged by a colour change of the medium due to the metabolism of the substrate, glucose, after optimal incubation (Hannan, 2000).

Although *A. pyogenes* is considered a common inhabitant of the upper respiratory and genital tract of domestic animals (Jost, B. H., Songer, J. G. & Billington, S. J., 1999), results from this study suggest that it has a significant involvement in the pathogenesis of ulcerative balanitis and vulvitis in sheep in South Africa. Knowledge of the susceptibility of field strains of *A. pyogenes*, to the antimicrobial drugs indicated for use against mycoplasmas, is required to assist with the choice of the most appropriate drug against both *Mmm*LC and *A. pyogenes*. The susceptibility of both species for the same antimicrobial drugs was therefore deermined.

The objective of this part of the study was to determine the MICs of antibiotics with known anti-mycoplasma activity using the broth microdilution assay, and to determine the antimicrobial drug susceptibility of *A. pyogenes* using the agar disc diffusion.

4.2 MATERIALS AND METHODS

4.2.1 MYCOPLASMA CULTURES

Ten mycoplasma strains tested were selected from the field isolates obtained during the investigation of balanitis and vulvitis in Dorper sheep. The type strain, *Mmm*LC (Y-Goat) (NCTC 11706) was also included. Purification of cultures was based on the descriptions of Al-Aubaid & Fabricant (1971) and Carmichael *et al*, (1972). Mycoplasma colonies with morphological differences were located, and a block of agar containing what appeared to be a single colony was transferred into separate tubes of Hayflick's broth. Cultures were incubated for 3 days at 37 ^{0}C, 10-fold dilutions were made and loopfuls streaked onto Hayflick's agar. After incubating all transferred colonies, single colony picks were repeated from plates showing consistent morphological resemblance. Purification of strains was necessary to ensure that only pure cultures were used as inocula in the microdilution tests. The cloned colonies were confirmed as *Mmm*LC using the IFAT.

4.2.2 BACTERIA CULTURE

The *A. pyogenes* strains used in this study were field isolates obtained from sheep genital swabs. Nine representative isolates were selected for the test, and grown on horse blood agar at 37 ^{0}C for 24 hour.

4.2.3 MEDIA FOR BROTH MICRODILUTION TESTS

For the microdilution test, *Mmm*LC were grown at 37 ^{0}C aerobically with 5 % CO_2 in Hayflick's broth, pH 7.6, containing glucose (1 %, w/v) and 2 mℓ of 1 % (v/v) phenol red. Incubation was continued until a colour change from pink to orange-yellow was evident as a result of the fermentation of glucose during mycoplasma growth.

4.2.4 STANDARDIZATION OF INOCULA

Isolates were removed from cryostorage and allowed to thaw at room temperature. A panel of 9 tubes each containing 3.6 mℓ of glucose-Hayflick's broth (pH 7.6) were prepared for each isolate, and 0.4 mℓ of the thawed aliquot was added to the first tube. A ten-fold dilution was made up to the ninth tube by transferring 0.4 mℓ of the suspension at each step, and incubated at 37 ^{0}C until an acidic reaction (colour change from pink to yellow) was observed. The time taken for this reaction to occur was recorded, and the lowest dilution to show a colour change represented the reciprocal of the number of colour changing units (ccu) in the undiluted mycoplasma culture. The inoculum size was determined to be 10^4 ccu mℓ^{-1} for all strains tested. The acceptable number of organisms for MIC tests is 10^3 to 10^5 ccu per mℓ (Hannan, 2000).

4.2.5 ANTIMICROBIAL DRUGS

The antimicrobials tested and the classes to which they belong are indicated in the table below. They were included on the basis of their known activity against *Mycoplasma* spp., their market authorisation for local use and clinical considerations.

Table 4.1 Antimicrobial drugs tested and the class to which they belong

Antimicrobial drug	Class
Enrofloxacin	Fluoroquinolones
Florfenicol	Amphenicols
Oxytetracycline	Tetracyclines
Spiramycin	Macrolides

The choice of spiramycin as a representative for macrolide drugs was based on practical considerations. Apart from the fact that it is included in the VetMIC™ microwell plates, it can be used as a representative of the macrolides by virtue

of the fact that the antimicrobial spectrum of spiramycin is similar to tylosin, erythromycin and tilmicosin (A. Franklin, 2002, Personal communication).

Representative drugs of all 4 classes are registered in South Africa. The commercially produced microtitre plates, VetMIC™ were obtained from the National Veterinary Institute, 75189 Uppsala, Sweden. The plates are provided with the drugs coated on the bottom of the wells at specific concentrations. The plates were designed to provide doubling dilutions of the antimicrobials when 50 µℓ of inoculum was added. The concentration ranges of the antimicrobial agents, after the addition of 50 µℓ inoculum, are listed in Table 4.2. Two wells of the microplates to which 50 µℓ of inoculum and 50 µℓ of sterile broth were added, respectively, were used for control of growth and sterility.

Table 4.2 Concentration ranges of antimicrobials in the VetMIC™ microwell plates (µg $mℓ^{-1}$)

Antimicrobial drugs	Concentration ranges (µg $mℓ^{-1}$)
Enrofloxacin	1.0 - 0.125
Florfenicol	16.0 – 2.0
Oxytetracycline	8.0 – 1.0
Spiramycin	32.0 – 4.0

4.2.6 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS

The MICs were determined by a glucose metabolism inhibition method performed in 96-well microtitre plates. Two-fold dilutions of each drug was made. To each well of the microtitre plate was added 50 µℓ of diluted culture containing 10^4 ccu $mℓ^{-1}$. The plates were sealed with transparent self-adhesive tape to prevent evaporation, and incubated at 37 ^{0}C. The incubation time was controlled by observing the colour changes equivalent to the growth control well, and the plates were monitored twice daily until the required colour change was observed. The MIC was recorded as the lowest concentration of antibiotic that inhibited visible colour change of the medium at the time when a colour

change could be observed in the growth control without antibiotic. MICs were obtained after 24 to 48 hours depending on the strains tested. All MICs were determined twice to confirm results, and repeated a third time if the end points for any antibiotic differed by more than one dilution.

4.2.7 AGAR DISK DIFFUSION TEST

Susceptibility testing was performed with the agar disk diffusion method described by Bauer, A. W., Kirby, W. M. M., Sherris, J. C. & Turck, M. (1966) on Columbia blood agar (Difco) supplemented with 6 % horse blood. The antimicrobial drugs tested were enrofloxacin, oxytetracycline and tilmicosin. Due to the unavailability of florfencol disks, it was not included in the test. After incubation for 24 hours, the zones of inhibition was measured using a caliper, and each zone interpreted by reference to the zone diameter interpretive standards in NCCLS document M31-A (1999).

4.3 RESULTS

The minimum inhibitory concentrations of the antibiotics to which the *Mmm*LC field isolates were susceptible are shown in Table 4.3 and Table 4.4. Duplicate tests did not vary by more than one serial two-fold dilution. For enrofloxacin fifty per cent of the isolates showed an MIC value of 0.25 µg $m\ell^{-1}$, and twenty percent of the isolates had an MIC values of 0.5 µg $m\ell^{-1}$. Thirty per cent of the isolates yielded an MIC value of ≤0.125 µg $m\ell^{-1}$. The MIC_{50} and MIC_{90} were 0.025 µg $m\ell^{-1}$ and 0.35 µg $m\ell^{-1}$, respectively (Fig.4.1).

The MIC range for florfenicol was 2.0-4.0 µg $m\ell^{-1}$, and the MIC_{90} was 2.8 µg$m\ell^{-1}$ (Fig. 4.2). Oxyteytracycline showed activity against all isolates of *Mmm*LC, with a range of MIC values between 1.0 µg $m\ell^{-1}$ and 2.0 µg $m\ell^{-1}$, and a mean MIC value of 1.1 µg $m\ell^{-1.}$ Ninety per cent of the strains yielded an MIC≤1.0 µg $m\ell^{-1}$ (Fig. 4.3). The MIC_{90} of spiramycin for the isolated strains was 6.0. µg $m\ell^{-1}$ (Fig. 4.4).

Table 4.3 Selected *Mmm*LC field strains and their minimum inhibitory concentrations for four antibiotics

Antibiotic	MIC (µg mℓ$^{-1}$)	No of isolates
Enrofloxacin	≤0.125	3
	0.25	5
	0.5	2
Florfenicol	2.0	8
	4.0	2
Oxytetracycline	≤1.0	9
	2.0	1
Spiramycin	≤4.0	7
	8.0	3

Table 4.4 MIC_{50} and MIC_{90} of field isolates of *MmmLC* and the type strain

Antimicrobial drug	MIC (µg mℓ$^{-1}$) for field strains (n=10)		MIC (µg mℓ$^{-1}$) for type strain (Y-Goat)
	50 %	90 %	
Enrofloxacin	0.025	0.35	0.125
Florfenicol	ND*	2.8	2.0
Oxytetracycline	ND	1.0	1.0
Spiramycin	ND	6.0	4.0

* ND= Not determined

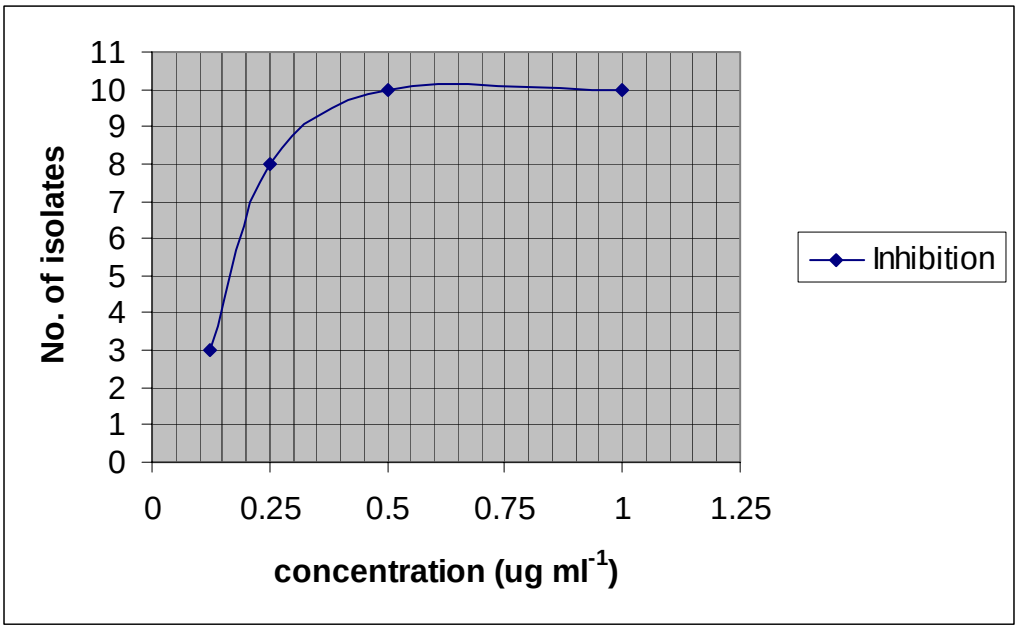

Fig. 4.1 Probit graph to determine the MIC_{50} and MIC_{90} of enrofloxacin

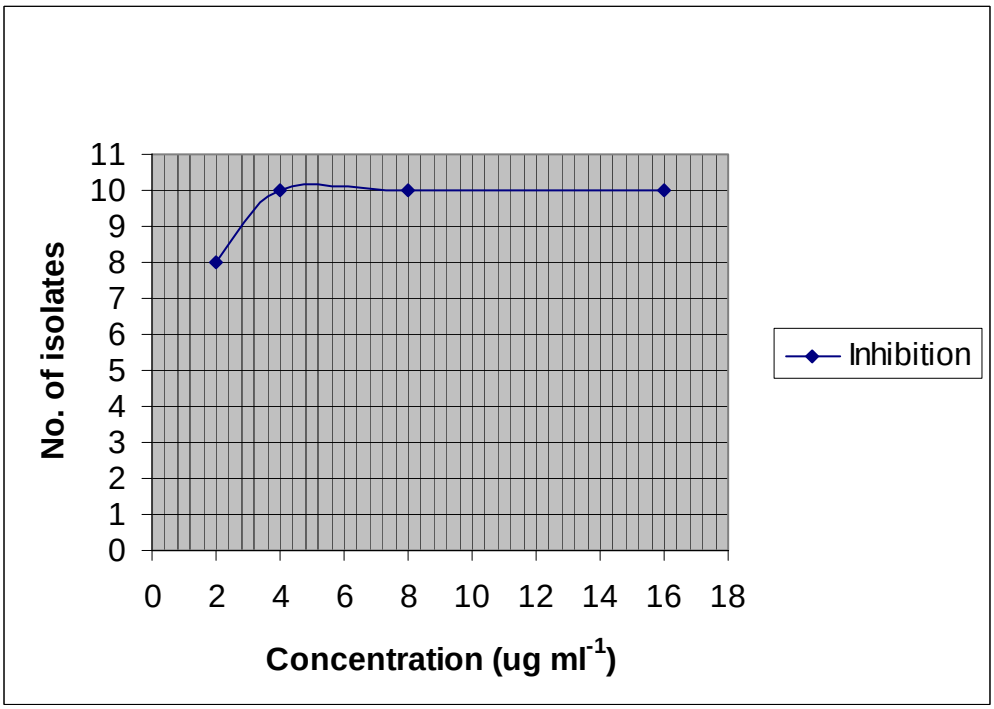

Fig.4.2 Probit graph to determine the MIC_{90} of florfenicol

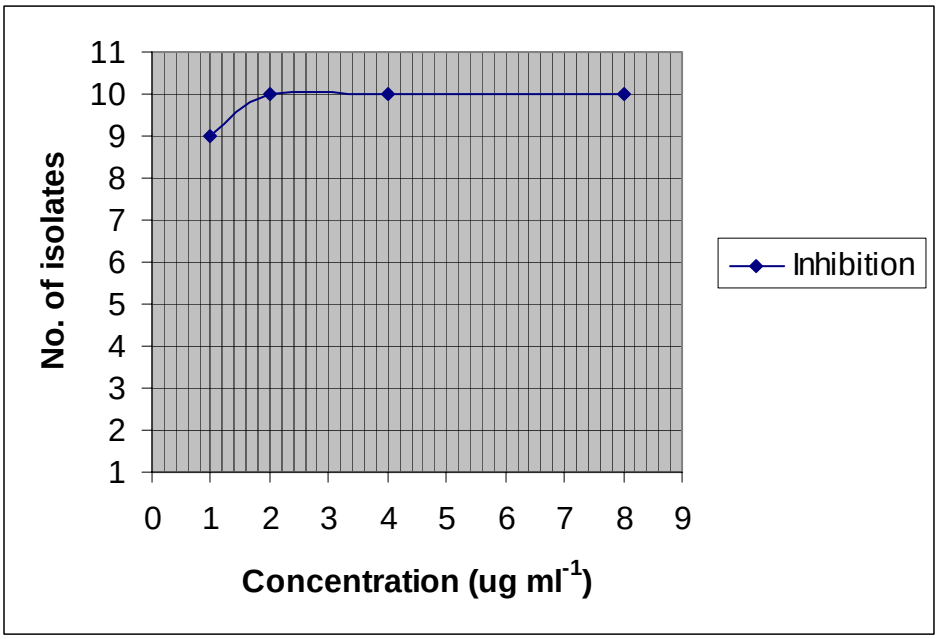

Fig. 4.3 Probit graph to determine MIC_{90} of oxytetracycline

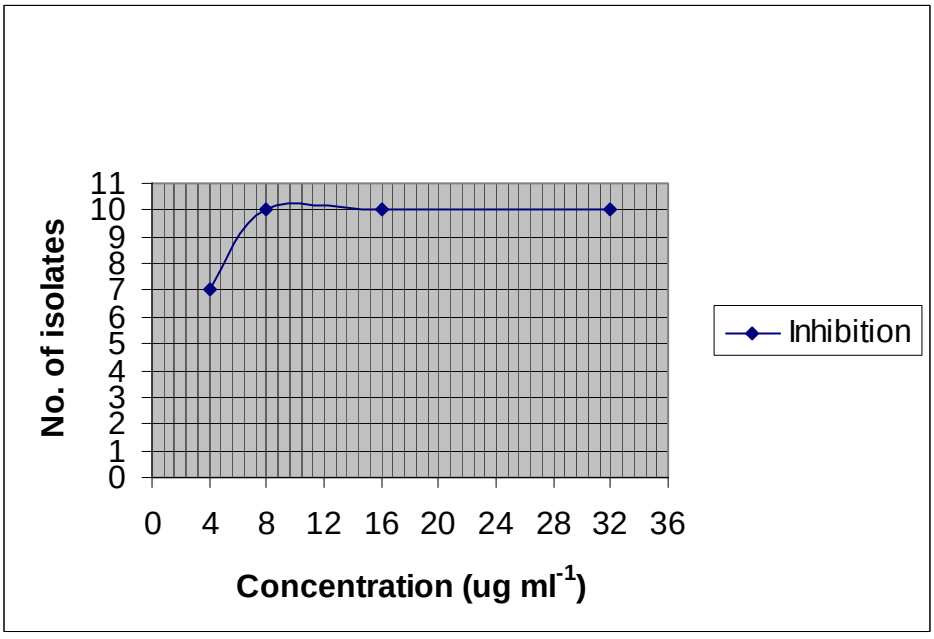

Fig. 4.4 Probit graph to determine MIC_{90} of spiramycin

Table 4.5 The mean MIC values for the tested drugs against *Mmm*LC field isolates

Antimicrobial drug	MIC values for field strains (n=10)		
	Mean (µg mℓ$^{-1}$)	sem‡	Range (µg mℓ$^{-1}$)
Enrofloxacin	0.24	0.044	0.125-0.50
Florfenicol	2.4	0.155	2.0-4.0
Oxytetracycline	1.1	0.014	1.0-2.0
Spiramycin	5.0	0.440	4.0-8.0

‡ Standard error of the mean

The nine field starins of *A. pyogenes* were susceptible to all antimicrobial drugs tested. The inhibition zone diameters of the tested drugs are presented in Table 4.6.

Table 4.6 Zones of inhibition of the three drugs on blood agar (n=9)

Antimicrobial drug	Mean (mm)	sem‡	Range	Zone diameter interpretive standard (NCCLS, 1999)	
				Susceptible	Resistant
Enrofloxacin	30.6	1.8	26.8-32.4	≥20	≤16
Oxytetracycline	42.3	1.4	36.8-47.3	≥23	≤18
Tilmicosin	35.8	4.12	31.7-39.0	≥14	≤10

‡ Standard error of the mean

4.4 DISCUSSION

One of the aims of quantitative studies of antimicrobial sensitivity is to assist the veterinarian in choosing an effective antimicrobial as a remedy for infections. *In vitro* antimicrobial activity does not always correlate with the *in vivo* efficacy, although a drug showing little or no activity *in vitro* is unlikely to be effective in aiding the body's defences to eliminate the organism (Nosanchuk, J. D., Cleare, W., Franzot, S. P. & Casadevall, A., 1999).

In vitro activity, although important, is only a guide as to whether an antibiotic is likely to be effective in an infection. The minimal drug concentration achieved at the infected site should be approximately equal to the MIC for the infecting organism. However, there is evidence to suggest that even subinhibitory concentrations of antibiotics may enhance phagocytosis and may be effective (Nosanchuk *et al*, 1999). Fluoroquinolones, for example, have been shown to be highly effective at extremely low concentrations, and also possess a prolonged post antibiotic effect (Spoo & Riviere, 1995).

Many methods have been used to obtain MIC data for veterinary *Mycoplasma* species, making it difficult to compare the results from different laboratories. This lack of standardization has been caused partly by the wide variation in nutritional requirements and culture conditions needed for the different mycoplasmas, and also by the lack of internationally agreed standards of performance and interpretation. The broth microdilution susceptibility testing system has been validated for use with human and animal bacterial pathogens (NCCLS, 1999), and MIC values for reference strains are recommended to be within ± one dilution of the expected value.

The microdilution method used in this study considered the recommendations of Hannan (2000). However, apart from the colour change in the medium, the end point could also be determined using the mycoplasma growth, which was visible at the bottom of the plate as buttons. This was made possible by the

high growth rate of the mycoplasma in the test, which made it easy to interpret the results.

The MICs for the antimicrobial drugs tested for mycoplasma were generally in agreement with the MIC breakpoints of the same antibiotics against bacterial pathogens (NCCLS, 1999). Furthermore, the MIC values of enrofloxacin, oxytetracycline and spiramycin obtained for the *Mmm*LC field isolates and type strain were lower than those reported by other investigators for different mycoplasma spp.(Hannan *et al*, 1989; Cooper *et al*, 1993; Ayling *et al*, 2000).

While no MIC breakpoints are available for mycoplasma in general and for *Mmm*LC strain Y-Goat in particular, the results of the test was determined in accordance with the NCCLS (1999) guidelines, and were similar to data for veterinary mycoplasmas. Unfortunately, an insufficient number of studies have been performed in small ruminants to determine efficacy of antibiotics against mycoplasmas associated with genital diseases such as ulcerative balanitis and vulvitis.

The pharmacokinetic characteristics of an antimicrobial drug determine the concentrations of that drug that can be achieved in the blood and tissues. These can then be compared with the MICs of the various drugs against a particular pathogen. The effective concentration or breakpoint can be compared with the concentration in the target tissue. This should ideally be higher than the MIC_{90} for the particular organism so that there is a good chance of successful treatment. It is for example known that the tissue concentrations for enrofloxacin are considerably higher than 1 µg $m\ell^{-1}$ (Wiuff, C., Lykkesfeldt, J., Aarestrup, F. M. & Svendsen, O., 2002), which is higher than the breakpoint for most bacterial pathogens. The fact that all ten *Mmm*LC strains tested had MICs ≤0.5 µg $m\ell^{-1}$ shows that enrofloxacin will likely be an effective drug for the treatment of ulcerative balanitis and vulvitis in Dorper sheep.

In one study (Ayling *et al*, 2000), danofloxacin, a fluoroquinolone, with an MIC of 0.25 µg mℓ$^{-1}$ for *Mmm* small colony (SC) was suggested as effective for the treatment of pleuropneumonia in cattle. By analogy, the enrofloxacin MIC values of ≤0.25 µg mℓ$^{-1}$ for *Mmm*LC again indicate that it should be effective in the treatment of ovine ulcerative balanitis and vulvitis in Dorper sheep.

The MIC_{50} of enrofloxacin against mycoplasma species has been shown to be 0.01-1.0 µg mℓ$^{-1}$ from data compiled by Spoo and Riviere, (1995). The MIC_{50} for enrofloxacin in this study was 0.025 µg mℓ$^{-1}$, which is within the range described by Spoo and Riviere, (1995). Enrofloxacin was found to be 100 % effective at 1.25 mg per Kg per day *per os* in pigs experimentally infected with *Mycoplasma hyopneumoniae* in the respiratory tract. It has also been shown that a mean plasma concentration of 0.6 µg mℓ$^{-1}$ will be attained for enrofloxacin administered to pigs at a dose rate of 2.5 mg/kg bwt (Wiuff *et al*, 2002). The same study showed that the mean tissue concentration of enrofloxacin after i.m. administration will reach between 1.9-2.1 µg mℓ$^{-1}$. These results further support the use of enrofloxacin for the treatment of ulcerative balanitis and vulvitis where the MIC values were lower than the expected tissue concentrations.

The MIC values obtained for spiramycin and florfenicol were lower than the MIC breakpoints described for bacterial pathogens (NCCLS, 1999), and it seems, therefore, justifiable to claim that these drugs will be effective against *Mmm*LC infections.

Spiramycin has good tissue penetration ability reaching concentrations of 25-60 times more than that of serum (Prescott, 2000[a]). It has also been used successfully to treat contagious bovine pleuropneumonia caused by *Mmm*SC at a dose rate of 25 mg/kg (Provost, 1974, cited by Prescott, 2000[a]). Prescott (2000[a]) also reported that spiramycin has similar applications and effects to tylosin, and much higher *in vivo* efficacy than erythromycin in small ruminants. Due to the fact that macrolide antibiotics are highly lipid soluble and widely

distributed in body fluids and tissues, spiramycin could effectively be used in combating mycoplasma induced ulcerative genital infections. Although, Kobayashi, H., Morozumi, T., Munthall, G., Mitani, k., Ito, N. & Yamamoto, K., (1996) and Ching Ching Wu, Shryock, T. R., Tsang Long Lin, Mary Faderan & Veenhuizen, M. F., (2000) reported MIC_{90} of 4.0 µg $m\ell^{-1}$ for tilmicosin which is slightly lower than the values for spiramycn, the MIC values for spiramycin in this study are comparable to the breakpoints for bacteria, and would be attainable in the blood and body tissues where the concentration markedly exceeds that of the MICs.

Florfenicol, an amphenicol, has a broad range of activity because of wide tissue distribution and high bioavailability (Prescott, 2000[b]). Spoo & Riviere (1995) showed the potential of this compound in the treatment of microbial infections in food animals intended for human consumption. It was initially used as a treatment for bovine respiratory disease caused by *Mannheimia haemolytica*.

The volume of distribution of oxytetracycline varies markedly (0.32-18.5 ℓ/kg) between animals and in the different age groups within species (Riviere & Spoo, 1995). Due to the solubility in lipids, teracyclines are capable of penetrating tissues and becoming widely distributed throughout the body. It has been shown to penetrate well into pulmonary and renal tissues, as well as into bronchial fluids. Concentrations within the extracellular tissue fluid are expected to be similar or higher than in blood (Riviere & Spoo, 1995). Higher concentrations of tetracyclines in tissues as such could dictate their increased usage in the treatment of infections caused by a wide variety of microorganisms. The MIC_{90} of tetracycline for the ten selected isolates of *Mmm*LC tested during this study was 1.0 µg $m\ell^{-1}$. This value is similar to the observations of Hannan, P. C. T., Windsor, H. M. & Ripley, P. H. (1997), Aarestrup, F. M., Friis, N. F. & Szancer, J. (1998) and Ching Ching *et al* (2000), who reported an MIC_{90} of 1.0 µg $m\ell^{-1}$. The extensive distribution of oxytetracycline and its *in vitro* effect against *Mmm*LC make this agent also a suitable candidate for the treatment of ulcerative balanitis and vulvitis.

Several investigators have evaluated the susceptibility of *A. pyogenes* to different antimicrobial drugs (Yoshimura, H., Kojima, A. & Ishimaru, M., 2000). The present study has shown that all the isolates were susceptible to oxytetracycline, enrofloxacin and tilmicosin. A study in Kenya revealed that *A. pyogenes* was susceptible to oxytetracycline (Mulei & Gitau, 1993). Tylosin, erythromycin and enrofloxacin were also found to be effective against strains of *A. pyogenes* isolated from bovines (Yoshimura *et al*, 2000).

Although data on the *in vivo* efficacy and *in vitro* MIC breakpoints for mycoplasma are incomplete, MIC results of this study suggest that all 4 antimicrobial drugs will be effective in the treatment of mycoplasmal infections, particularly of *Mmm*LC of the genital tract of sheep. It should be borne in mind that although only one of the 4 antimicrobial drugs, namely oxytetracycline, is registered for use in small stock in South Africa, the others have marketing authorization for use in other species such as bovines and porcines. Their use in small stock would therefore constitute the use of an extra-label (unapproved) drug. The latter is a drug that is used in a manner or dosage different from the instructions on the manufacture's label. These unapproved drugs should therefore be used in small stock only by or under the supervision of a veterinarian.

CHAPTER V

5 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

It is acknowledged that certain shortcomings were present in the study design in terms of the selection of control groups that came from the same population where the cases were. However, there will not be a significant variation in the final statistical results and conclusions of the findings due to the fact that the case and control groups were selected by examining the genitalia for presence or absence of lesions of an ulcerative nature. The interpretaion was, therefore, made in association with the sources of the specimens and the identified microorganisms.

Although, the sensitivity and specificity of the microbiological techniques influence the detection of microorganisms, the procedures employed in the current study were standardised and acceptable.

In spite of the reported widespread occurrence and economic importance of ulcerative balanitis and vulvitis in South Africa there have thus far been few reports on the clinical signs, pathology and aetiology of this disease. During this investigation, the clinical signs of the disease were clearly described and it was concluded that posthitis and vaginitis are not typical for the disease as seen in Dorper sheep in South Africa. It is suggested that the terms ulcerative balanitis and vulvitis, which describe the lesion more appropriately, are used.

The present study threw some light on the prevalence of mycoplasmas in the genital tract of apparently healthy sheep and at the same time identified the mycoplasma pathogen associated with ulcerative balanitis and vulvitis. The findings of this investigation suggested the involvement of mycoplasma, particularly *Mycoplasma mycoides mycoides* large colony variant, in the clinical

disease in Dorper sheep in South Africa. Because *Mmm*LC was isolated from 90.1 % of sheep with clinical signs and supported by earlier investigations, it was concluded that this microorganism could be the primary pathogen of balanitis and vulvitis in sheep. The study furthermore demonstrated the synergism between *A. pyogenes* and *Mmm*LC. Affected sheep were 53.45 times more likely to have both *Mmm*LC and *A. pyogenes* than healthy sheep, which emphasizes the multifactorial nature of the disease.

Additional findings of the study were the apparent association between age and the likelihood of acquiring the disease, and the severe form of the lesions of ulcerative balanitis and vulvitis. Young sheep were 2.5 times more prone to develop lesions than adult sheep. In other words adult animals are less likely to be affected, or even if affected the lesions would present as mild or inapparent. Therefore, it is likely that the source of outbreaks could be persistently (latent) infected carrier animals.

The major consequences of balanitis and vulvitis in sheep occurring in the Republic of South Africa directly relate to reduction in conception rate and eventual reduction in lambing percentage, disease control costs, control efforts and low value of infected sheep, particularly rams.

There is a need for reliable information for the treatment of ulcerative genital infections in sheep in South Africa. Accordingly, in this study, the *in vitro* antimirobial efficacy of fluoroquinolone (enrofloxacin), macrolide (spiramycin, & tilmicosin), tetracycline (oxytetracycline) and amphenicol (florfenicol) was determined.

The *in vitro* activities of the 4 antibiotics used against 10 *Mmm*LC field isolates and the susceptibility of *A. pyogenes* to the same classes of drugs point to the fact that they are likely to be effective in the control of ulcerative balanitis and vulvitis. These antimicrobial drugs proved to possess good antimycoplasma and antibacterial activity against all the tested strains, and therefore have potential

advantages provided that similar results are obtained *in vivo* under field conditions. Their pharmacokinetc features also made them suitable for the treatment of ulcerative genital infections.

5.2 RECOMMENDATIONS

Based on the findings of this study and clinical observations, it is recommended that:

- Pre-mating clinical examination of rams and ewes should be instituted to detect animals with visible lesions of ulcerative balanitis and vulvitis to allow for treatment until recovery is achieved. The affected animals should not be used for breeding purposes until no clinical signs are visible, although this is no guarantee that recovered animals will not transmit the disease.

- A strict procedure to avoid contact between healthy and infected flocks should be instituted at every farm where the disease is known to exist.

- Newly introduced sheep from any source should not be allowed to mix with the flock without prior clinical examination.

- Enrofloxacin, florfenicol, oxytetracycline, spiramycin and tilmicosin have proved to be effective against all tested *Mmm*LC and *A. pyogenes* field isolates, and are therefore the antibiotics of choice in the treatment of ulcerative balanitis and vulvitis in sheep in South Africa.

- It is recommended that susceptibility testing be continued and that more field strains be isolated during outbreaks to monitor the efficacy of the antimicrobial drugs used against *Mmm*LC.

- The clinical efficacy of these drugs (dose, dosage interval & duration) should be confirmed in infected sheep.

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