DOCUMENT PREPARED FOR RMRDSA PROJECT 9.2A.5 (Balanoposthitis in small ruminants)

LITERATURE STUDY

1. The South African sheep industry

Almost 72 million hectares of land in South Africa is located in semi-arid and arid climatic zones. The arid regions are most suitable for livestock production, and currently 70% of these areas are used exclusively for this purpose, representing it as the largest and most important agricultural practice within the country (Schoeman *et al.,* 2010; Spies, 2011). The ability of sheep to thrive under adverse conditions and the fast return on capital investment have resulted in sheep being the most abundant livestock species in the country, with an estimated population of 24.3 million (DAFF, 2014). The distribution of sheep flocks throughout South Africa and the percentage distribution within the different provinces can be seen in Figures 1 and 2, respectively.

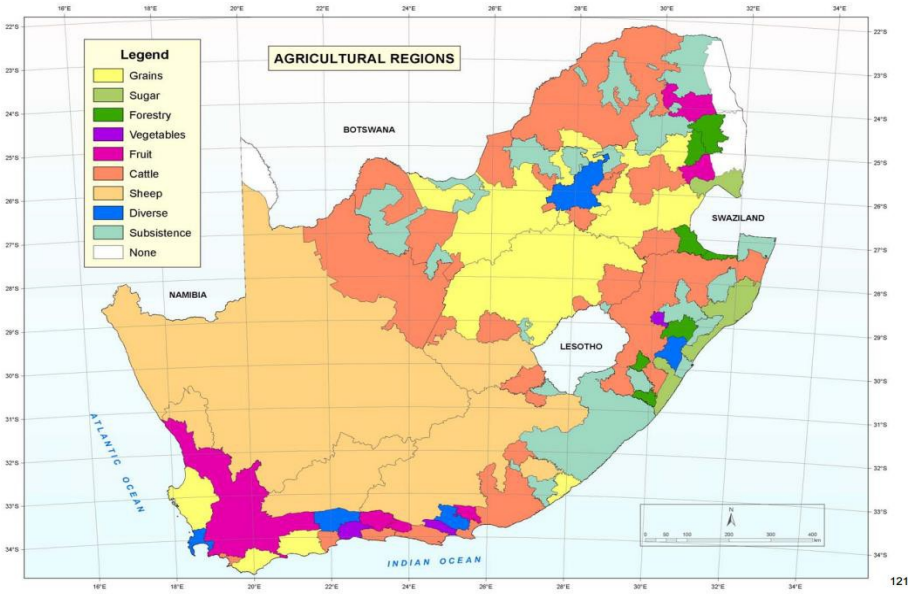

Figure 1. Regional distribution of sheep within South Africa (source: Agricultural Geo-Referenced Information System, 2007).

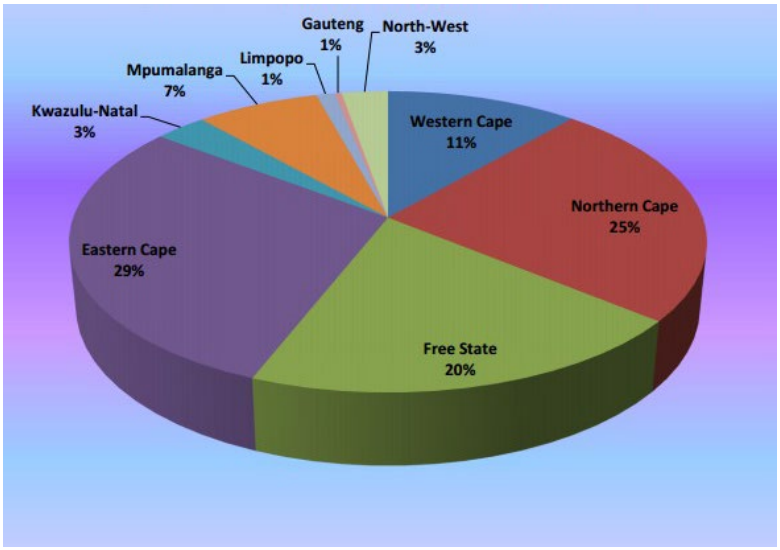

Figure 2. The percentage distribution of sheep per province according to 2013 statistics (source: DAFF, 2014).

In South Africa, there are currently twenty breeds that are commercially produced for wool and/or mutton. The mutton industry in South Africa plays an important part in food security, assisting in supplying the growing demand for animal protein. The demand is however usually higher than the supply, and currently South Africa is considered a net importer of mutton (DAFF, 2014). When breeds are considered, the Dorper breed represents the largest non-wool mutton sheep population and is recognized as the top mutton breed in South Africa. This breed however is currently affected by a venereal disease of unknown aetiology termed ulcerative balanoposthitis that affects both rams and ewes (termed vulvovaginitis). In rams, ulcerative balanoposthitis (UB) is a combination of balanitis and posthitis. Balanitis is where inflammation of the glans penis is observed, and posthitis is characterized by inflammation on the prepuce.

Gummow and Staley (2000) suggested that UB is a disease that primarily affecting Dorper populations in South Africa, with fewer cases observed in other sheep and goat breeds. Ulcerative balanoposthitis was first reported in Dorper flocks in 1976 in the Calvinia district of the Northern Cape Province. The disease has since, presumably due to the distribution of Dorper flocks, been reported in the Free State, KwaZulu-Natal, and Eastern and Western Cape provinces, with the highest incidence of the disease observed in the Free State Dorper populations (Trichard *et al.,* 1993; Gummow & Staley, 2000).

Dorper flocks affected by UB experienced depressed lambing percentages as much as a 50% decline due to the refusal of rams and/or ewes to mate because of the inflammation and resulting discomfort and/or pain associated with UB infection (Bath & De Wet, 2000). The incidence of UB also prevent Dorper producers to participate in breeder and stud auctions, which in turn restrict the national trade of sheep stock. These consequences of UB along with forced culling of diseased sheep, as a means to control the disease, makes UB a disease of economic importance in SA.

Almost 40 years after the discovery of the disease, the causative agent of UB has not yet been conclusively identified and with few studies related to the disease in SA, researchers have attributed the aetiology of the disease to different infectious organisms. Initially, studies of UB in South Africa were through conventional microbiological and molecular methods using morphological appearance; biochemical, serological (Trichard *et al.,* 1993; Kidanemariam, 2003; Ali, 2012) and DNA based-tests (Ali, 2012) to identify and classify cultured bacterial species recovered from affected and unaffected individuals. the respective studies on the incidence and cause of UB in SA have illustrated inconsistent isolation of different bacterial species from clinically infected specimens and have suggested that the aetiology of the disease may be multi-microbial in nature.

The existing studies on UB in sheep have highlighted the need to identify the causative agent(-s) of UB, and to formulate guidelines that can limit the transmission of the infection, or prevent the occurrence of the infection in commercial Dorper sheep flocks. Studies have been conducted on the microbial population present in the reproductive tract of the ewe (El-Arabi *et al*., 2014; Swartz *et al.,* 2014), but information for rams is scant.

2. Reproductive diseases and their role in sustainable livestock production

Reproductive diseases affecting the production efficiency of livestock production systems have been identified across the world in a number of livestock species including bulls (Hancock *et al.,* 2015), pigs (Teankum, 2006), goats (Gouletsou & Fthenakis, 2015) and sheep (Kidanemariam, 2003). Both non-infectious and/or infectious agents can contribute to the incidence of diseases, with a myriad of microorganisms involved in infections, and in the case of the reproductive system, functioning can be impaired or inhibited.

In some cases, microbial pathogens contributing to the incidence of a disease may not yet have been established or can be complicated and hard to determine due to multiple organisms contributing to the disease (Yoo, 2010).

Infectious reproductive diseases adversely affect the performance and the welfare of animals, resulting in most cases in significant economic losses in flocks affected. Economic losses occur as a consequence of the lower reproductive output in flocks, high culling rates of infected individuals, bans on international trade and restricted participation of rams in national sales and auctions (Picard-Hagen *et al.,* 2015). The degree to which the production efficiency of flocks are affected will depend on the aetiological agent and the manner in which it originally gained access to the animal population, and it is transmitted between animals in the same or a different population.

The effects of infectious reproductive diseases range from structural or functional defects of the reproductive system that can cause partial or complete reproductive failure, to tampering with the libido and mating ability of males. A decline in semen quality and fertility has also been associated with reproductive diseases (Toe *et al.,* 1994).

2.1 Common reproductive diseases of livestock

The most common reproductive diseases affecting livestock include epididymitis, orchitis, contagious ecthyma, ulcerative dermatosis and ovine posthitis. These diseases are known to affect different parts of the reproductive system and the aetiological agent has been identified in most cases.

Epididymitis is a disease affecting the epididymides that forms part of duct system and is characterized by lesions which cause pathological changes that negatively affects semen quality and fertility. Epididymitis occurs as a direct result of invasion into the epididymis from specific and non-specific bacterial agents. In sheep, the specific bacterial aetiology is thought to differ between mature and pre- pubertal rams, suggesting two forms of the disease, with *Brucella ovis* mainly associated with mature rams, and *Actinobacillus seminis* and other Gram-positive pleomorphic bacteria with pre-pubertal animals (Bagley *et al.,* 1985).

Orchitis is a reproductive disease of livestock characterized by inflammation of the testes and can be divided into an acute and chronic phase that depends on the aetiological agent and severity of infection. Most causes of orchitis have been associated to preceding infections by epididymitis, with the disease less frequently occurring as a separate entity (Gouletsou & Fthenakis, 2006). The influence of orchitis is evident with reduced fertility with ensued economic losses due to the reduction in the number of offspring lambed and early culling of breeding rams. Bacterial organisms are the most commonly recognised cause of the disease (Gouletsou & Fthenakis, 2006). Viral aetiological agents have also been isolated from cases of orchitis in rams (Gouletsou & Fthenakis, 2015). Specific pyogenic organisms, *Brucella* species and numerous non-specific bacteria are involved in the clinical manifestation of orchitis (Ribeiro *at el.,* 2015) such as *Arcanobacterium pyogenes* (Gouletsou *et al.,* 2004; Gouletsou *et al.,* 2006), *Brucella ovis* and *Brucella melitensis,* which are both important pathogens of small ruminants (Burgess, 1982; Bulgin, 1990), *Actinobacillus seminis* (Heath *et al.,* 1991; Al-Katib & Dennis, 2007), *Corynebacterium pseudotuberculosis* (Van Vuuren & Trichard, 2004c), *Mannheimia haemolytica*, *Bibersteinia trehalosi* or *Pasteurella multocida* (Garcia-Pastor *et al.*, 2009)

Contagious ecthyma is a contagious disease charcaterized by lesions on the preputial orifice, scrotum and penis which come about in a progressive pattern (Billinis *et al.,* 2012). Initially local erythema occurs at the site of infection, followed by the formation of papules, vesicles, pustules and proliferative ulcers that gradually

develop into scabs. The cause of contagious echtyma has been described as a *Parapox virus* that affects both genders of domesticated ruminants, primarily sheep and goats (Hosamani *et al.,* 2009). Secondary infections of contagious ecthyma by bacterial, fungal and maggot origins have been suggested to be common adding to complications of the disease (Hosamani *et al.,* 2009). Contagious ecthyma is transmitted venereally as descrined as a well as by direct or indirect contact (Nandi & Chowdhury, 2011).

Ulcerative dermatosis has been characterized as a disease of sheep. The appearance of the disease is seen in both younger and mature animals, most commonly affecting the latter (Van Vuuren & Trichard, 2004d). The disease manifests in two ways, i.e. crusted ulcerations can occur on either the skin of the lips, legs and feet or it can take on a venereal form, which is characterized with pustulous lesions that develop into ulcers enclosed by scabs on the vulva, the preputial orifice and less commonly the glans penis (Aiello & Moses, 2003). Diagnosis of the disease is often confused with other diseases such as blue tongue, sheep pox, ulcerative balanoposthitis, foot rot and contagious ecthyma. The source of the disease has yet to be classified but it has been suggested to take on a viral form closely related to that of the contagious ecthyma virus.

Ovine posthitis is also known as sheath rot/pizzle rot, *Pisgoed*, urine scald, balanoposthitis, balanitis or non-contagious posthitis. The disease is clearly defined has occurring in two progressive stages (Southcott, 1970). The initial infection occurs on the external prepuce, with necrosis of the external tissue occurring near or around the preputial orifice. Necrosis of the tissue develops into ulcers that become covered by dark scabs. This initial stage is not usually of economic importance, but scabs may join to cover and occlude the orifice with subsequent accumulation of urine in the prepuce. Secondary infection can develop and spread to the internal mucosa of the prepuce forming internal ulcerations. Accumulation of pus, necrotic material and urine around the sheath (Southcott, 1965) as well as swelling of the prepuce (Pemberton, 1959) are common features of the disease. Sometimes severe cases can erupt spreading ulcerations to the glans penis (Van Vuuren & Trichard, 2004d). Rams affected by the disease are less likely to mate with ewes due to the painful nature of the symptoms, and also due to the inability of males to extrude the penis due to swelling and scabbing of the prepuce. The aetiology of the disease has thus been characterized into three important factors: a legume (protein) rich diet that increases the presence of urea in urine and therefore ammonia production; the increased proportion of *Corynebacterium renale* as a consequence of elevated urea levels (Greig, 2007); and thirdly, low testosterone levels which applies only to whethers.

2.1.1 A overview of ulcerative balanoposthitis in livestock

Ulcerative balanoposthitis (UB) and the analogous female condition, ulcerative vulvovaginitis (UV) is a reproductive disease that has been associated with numerous aetiological agents in a number of livestock species including horses (Allen & Umphenour, 2004), cattle (Van Vuuren & Trichard, 2004d; Pritchard *et al.,* 1997), goats (Tarigan *et al.,* 1990; Grewal & Wells, 1986) and sheep (Kidanemariam, 2003; El-Arabi *et al.,* 2014). In the literature the name of the disease may vary and the syndrome has been described in relation to the location of symptoms on the genitalia, with further classification of the female condition according to the clinical appearance of the symptoms (e.g. ulcerative, granulous).

In sheep, UB has been investigated less frequently than UV, with a small number of reports in the UK (Deas, 1983; Rutten, 2012; El-Arabi *et al.,* 2014), Australia (Webb & Chick, 1976), Argentina (Robles *et al.,* 2016) and South Africa (Trichard *et al.,* 1993; Kidanemariam, 2003; Ali, 2012). Furthermore, when the diagnostic method is based solely on clinical evaluation, it is possible that UB may be confused with other reproductive disorders of the penis and prepuce (e.g. contagious echtyma, ulcerative dermatosis and ovine posthitis) due to the similarity of the lesions on the external genitalia; possibly influencing the number of reports on the disease in

the literature. For example, Bath and de Wet (2000)described the aetiology and clinical signs of a disease that are characteristic to UB, but named it pizzle rot, which refers to the reproductive disease caused by a nitrogen-rich diet and the microbial organism *Corynebacterium renale*. El-Arabi *et al.* (2014) also classified UB and pizzle rot as the same disease, incriminating both *Mycoplasma mycoides* species and a *Corynebacterium* species as causative agents involved in the disease.

Other studies such as Bush *et al.* (2006), reported on the occurrence of lesions on the prepuce and penis, but the lack of a comprehensive description of the clinical manifestation and microbial organisms involved cannot differentiate the lesions from that of other infectious diseases of the reproductive system, and the disease involved in producing the lesions remains unclear. Although other reproductive disorders of the external genitalia are not generally associated with mating (Van Vuuren & Trichard, 2004d), thorough investigation of the infectious nature of the lesions and/or the description of the pathogen involved will be required to differentiate them from UB.

2.1.1.1 Epidemiology

Natural transmission of the infectious agent between ram and ewe during sexual activity seems to be the most important way in which the disease is spread throughout and between flocks (Deas, 1983; Trichard *et al.,* 1993), with non-venereal transfer also suggested (Van Vuuren & Trichard, 2004d; Robles *et al.,* 2016). The clinical manifestation has occasionally been reported singly in ewes, with no simultaneous incidence in rams (Cottew *et al.,* 1974; Doig & Ruhnke, 1977; Ball & McCaughey, 1987).

In South Africa, UB is thought to be more prominent in rams, especially young rams (Gummow & Staley, 2000; Kidanemariam, 2003). Higher prevalence in rams may be explained by the higher sexual activity during the breeding season leading to a higher chance of ram-to-ram infection via an infected ewe or an increased likelihood of trauma to the genitals as a result of multiple servicing (Gummow & Staley, 2000). Gummow and colleagues (2010) further implied that these findings may be biased in that rams are examined for breeding soundness prior to the mating season thus are more readily identified with UB than ewes who may be affected but are not readily examined for the disease.

The incidence of UB has been observed before and during the mating season where the outbreak of the acute stage of UB is generally observed a few days after introduction of infected individuals into mating flocks (Trichard *et al.,* 1993; Gummmow & Staley, 2000; Robles *et al.,* 2016). An incubation period of 4-6 days and 18-20 days has been described by Van Vuuren and Trichard (2004) and Deas (1983), respectively. Infected rams are able to copulate and ewes experience normal pregnancy upon fertilization. Depending on the severity of the disease rams may then become reluctant or completely refuse to undergo sexual intercourse due to the painful nature of the lesions involved. Furthermore, in more advanced stages the animal may become depressed and sometimes stand aside and assume an arched back stance (Trichard *et al.,* 1993; Van Vuuren & Trichard, 2004d).

In South Africa, the outbreak of the disease is predominant in Dorper sheep, and is thought to spread to 100% of sheep on primary outbreak with lower incidence of about 50% thereafter, with 2-4% of national flocks affected at any one time (Trichard *et al.,* 1993; Van Vuuren & Trichard, 2004d). These results were concurrent with that of Webb and Chick (1976) that claimed that out of 240 Australian Merino ewes approximately 50% of them had developed a degree of vulvovaginitis. In the UK, Deas (1983) described a lower rate on infection between 20 and 30% of flocks but explained that this may vary according to the severity of the disease.

Due to the implications of the disease on the sexual activity of rams and its high infection rates within and across flocks, the disease has been identified as economically important to the South African mutton industry (Trichard *et al.,* 1993). Bath and de Wet (2000) estimated that as a consequence of the repressed reproductive performance of rams lambing percentages may be reduced to 50% comparing to usual standards of 100% or more. Results from a survey conducted by Gummow and Staley (2000) on ulcerative balanoposthitis in South Africa were in agreement with these results stating that the 56.4% of ewes infected with the disease observed a lambing percentage less than a 100%. Furthermore, economic losses have also been described as a result of increased culling of diseases animals and inability of stock and stud owners to participate in national sales and auctions (Kidanemariam, 2003).

2.1.1.2 Clinical manifestation

The clinical symptoms of the male condition are more consistent in the literature than in ewes, and differ only in their location on the male genitalia. In rams the disease can affect the glans penis or the glans penis and the prepuce with ulcerative balanitis (Webb & Chick, 1976; Ball *et al.,* 1991; Kidanemariam, 2003) and ulcerative balanoposthitis (El- Trichard *et al.,* 1993; Gummow & Staley, 2000; Arabi *et al.,* 2014; Robles *et al.,* 2016) describing the disease, respectively.

The onset of UB can generally be defined by hyperaemia and inflammation of the mucosal membrane of the penis (Van Vuuren & Trichard, 2004d). As the infection progresses small scattered papulo-vesicular lesions that develop into erosions or ulcers are observed on the glans penis (Webb & Chick, 1976; Deas, 1983; Trichard *et al.,* 1993; Kidanemariam, 2003; El-Arabi *et al.,* 2014; Robles *et al.,* 2016). The penis may also be covered with fibrinous or mucopurulent exudate (Ball *et al.,* 1991; Van Vuuren & Trichard, 2004d; El-Arabi *et al.,* 2014; Robles *et al.,* 2016). The acute ulcers, sometimes filled with blood clots, are sensitive and easily tear, and during servicing attempts blood may ooze from the lesions staining the wool surrounding the vulva and prepuce in ewes and rams, respectively (Deas, 1983; Bath & de Wet, 2000; Kidanemariam, 2003; Robles *et al.,* 2016). Blood markings surrounding the genitals are usually regarded as the first sign that the infection has been established in a flock (Pritchard *et al.,* 2008).

In more progressive stages erosion can be extensive and ulcers may cover most of the glans penis and advance to the preputial orifice (Deas, 1983; Trichard *et al.,* 1993; El-Arabi, 2014; Robles *et al.,* 2016). Furthermore, scabs can form over the ulcers leaving exposed raw, bleeding surfaces if removed. In less frequent but more complicated cases, phimosis and para-phimosis of the prepuce can occur and the penis cannot be retracted into the sheath (Trichard *et al.,* 1993; Kidanemariam, 2003). Sometimes necrotic material can occlude the preputial opening and the prepuce may be swollen and oedematous making it difficult and in some cases impossible to extrude the penis, even manually (Deas, 1983; Webb & Chick, 1976). The variations in the gross clinical appearance described by several authors have been suggested to depend on the invasion from secondary bacteria and the stage and severity of infection (Vuuren & Trichard, 2004d).

2.1.1.3 Aetiology

Ulcerative lesions of the penis and prepuce have been described in the literature since the early 1900's. It is only now towards the later part of the 20th century that ovine ulcerative balanoposthitis has become more prominent in the literature, especially in South Africa and the UK. Greig (2007) suggests that ulcerative balanitis (balanoposthitis) that cannot be explained by a *Parapox virus* (ORF) or a urease-producing diphteroid such as *Corynebacterium renale*, is one that is classified as having an unknown aetiology. In several

countries, many assumptions of infectious agents associated with the disease have been deduced, however this has been achieved with little confidence and inconsistent experimental isolation of specific organisms to back it up (Webb & Chick, 1976; Deas, 1983; Linklater & Smith, 1993; Kidanemariam, 2003; Van Vuuren & Trichard, 2004d; Pritchard *et al.,* 2008; Ali, 2012; Robles *et al.,* 2016).

Although it was previously thought that the disease was not associated with Chlamydophila or a virus (Webb & Chick, 1976; Jones *et al.,* 1983; Deas, 1983; Kidanemariam, 2003; Trichard *et al.,* 1993); more recent studies conducted on flocks in Switzerland and the UK have isolated ovine *Herpes virus* type 2 from vulvar and penile specimens, suggesting its possible role in UB/UV (Pritchard *et al.,* 2008; Rutten, 2012). Pritchard *et al.* (2008) observed the possible association between shedding ovine *Herpers virus* type 2 and the early stages of the condition.

Growing evidence suggests that the microbial cause of UB/UV could be a mollicute belonging to the genera *Mycoplasma*, *Ureasplasma, Achoeloplasma* or a combination thereof (Van Vuuren & Trichard, 2004d). Although *Ureaplasma* species have been isolated in the normal genital tract of ewes, a number of serotypes and unidentified species have been associated with UB/UV (Cottew *et al.,* 1974; Livingstone & Gauer, 1983; Trichard *et al.,* 1993; Kidanemariam, 2003; Ali, 2012). *Acholeplasma axanthum* and *Acholeplasma laidlawi* have been isolated from cases of UV and UV/UB, respectively; their role in the disease is still unknown (Jones *et al.,* 1983; Kidanemariam, 2003; Ali, 2012). *Mycoplasma* species from vulvar lesions (Jones *et al.,* 1983) and cases of vulvovaginitis (Cottew *et al.,* 1974), vulvitis (Ball & McCaughey, 1987), ulcerative vulvovaginitis (Trichard *et al.,* 1993) ulcerative vulvitis (Kidanemariam, 2003; Ali, 2012) and ulcerative balanoposthitis (Trichard *et al.,* 1993) and ulcerative balanitis and rams have been reported (Kidanemariam, 2003; Ali, 2012). Furthermore, unpublished data that granular-vesicular vaginitis and balanitis could be reproduced in sheep following the application of a combination of mollicutes to the mucous membrane of the genitals is proof that either one or more species could thus be involved in the disease (Van Vuuren & Trichard, 2004d).

The first *Mycoplasma* isolate to be associated with the disease in sheep was *Mycoplasma* species 2D (Cottew *et al.,* 1974; Linvingstone & Gauer, 1983), however its presence as a natural inhabitant of the reproductive tract (Linvingstone & Gauer, 1983) with no subsequent research to verify its role as an aetiological agent warranted further investigation of the disease by other authors.

In South Africa, the disease was first identified in 1976 in Dorper flocks in the Calvinia district of the Northern Cape. It has since been presumed to have spread throughout the country where sheep are reared and Dorper flocks prevail (Gummow & Staley, 2000). The first bacteriological study on the incidence of UB in Dorper flocks was conducted by Trichard *et al.* (1993) who proposed another *Mycoplasma* species, *Mycoplasma mycoides mycoides* large colony (MMMLC), as the primary causative agent of UB. Kidanemariam (2003) supported these findings and reported consistent isolation of MMMLC in diseased Dorper sheep around the country compared to healthy ones, with an isolation rate of 61.5% and 6%, respectively. The MMMLC has not been clearly defined as being associated with reproductive diseases in sheep, and is generally considered a *Mycoplasma* occurring in goats, isolated in cases of polyarthritis, conjunctivitis, keratitis, pneumonia and cervical abscesses (Rosendal, 1994). Kidanemariam (2003) also went on to suggest a synergistic relationship with *Arcanobacterium pyogenes* in that it had concurrently high isolation rates (31.7%) with MMMLC and its pyogenic nature could be involved in the more progressive clinical stages of the disease, with 74% of strains isolated from severe clinical cases of UB/UV.

Ali (2012) went on to molecularly characterize the *Mycoplasma* species isolated from cases of UB, from both new samples and samples previously collected by Kidanemariam (2003). Ali (2012) found that although the

number of isolates used in this study was small, comparing to Kidanemariam (2003) and Trichard *et al.* (1993) MMMLC had a poor isolation rate (~6%), with *Mycoplasma arginini* being the most frequently isolated *Mycoplasma* species. Although *Mycoplasma arginini* has been isolated from animals suffering from mastitis, pneumonia, arthritis and reproductive diseases (Ali, 2012) its role in UB is undetermined and its natural occurrence in the genital tract (Kidanemariam, 2003) as well as presumed low pathogenicity in animals would propose otherwise (Ali, 2012)-rephrase sentence-too long. The findings from these studies suggest that the aetiological agent involved in ulcerative lesions of the external genitalia in South Africa is still unresolved.

Apart from MMMLC, a mixed microflora of other mollicutes and pathogenic and non-pathogenic bacteria have also been isolated less frequently from the mucosal membranes of the penis and prepuce in affected sheep in South Africa. Mollicutes isolated included *Mycoplasma agalactiae* (Kidanemariam, 2003), *Mycoplasma bovigenatalium*, *Mycoplasma mycoides capri*, *Mycoplasma* species group 7, unidentified *Mycoplasma* and Ureaplasma species (Trichard *et al.,* 1993; Kidanemariam, 2003), *Mycoplasma arginini* (Trichard *et al.,* 1993; Kidanemariam, 2003; Ali, 2012), *Acholeplasma laidlawii* (Kidanemariam, 2003; Ali, 2012), *Mycoplasma ovine/caprine serogroup II*, and *Mycoplasma canadense* (Ali, 2012). A number of these mollicutes are known to be pathogenic and have been involved in other livestock diseases (Ali, 2012).

The opportunistic bacteria *Arcanobacterium pyogenes* has been most consistently isolated from field cases of UB/UV in South Africa (Van Vuuren & Trichard, 2004d) and is presumed to be a secondary bacterial infection to *Mycoplasma* causing the progressive purulent clinical manifestations observed (Kidanemariam, 2003). *Arcanobacterium pyogenes* has also been significantly isolated in a number of other cases of UV/UB around the world (Pritchard *et al.,* 2008; Robles *et al.,* 2016). Some other known pathogenic bacteria included *Erysipelothrix rhusiopathiae, Pasteurella multocida, Enterococcus faecalis, Haemophilus somnus* also known as *Histophilus somni, Eschericia coli* and Staphylococci and Streptococci species. These were isolated less frequently and most had concurrently similar isolation rates in healthy animals (Kidanemariam, 2003).

Other studies on UB/UV where *Mycoplasma* species were not identified in diseased specimens were also reported in the literature. In Argentina, *Arcanobacterium pyogenes* and *Pasteurella multocida* were the most prevalent bacterial species isolated in Patagonian Merinos that experienced UB/UV (Robles *et al.,* 2016). Bacteriological findings in Scottish sheep, showed that the main predominant bacterial species isolated in rams and ewes infected with the disease differed. *Mannheimia haemolytica* and Streptococcus species formed the largest percentage of isolates in rams with concurrently higher isolation rates of Corynebacteria and Staphylococci species in ewes (El-Arabi *et al.,* 2014). Intravaginal inoculation of a mixture of bacterial strains of the *Haemophilus/Histophilus* group isolated from field cases of UV in Northern Ireland was able to reproduce a disease identical to that of the field condition in both ewes and rams, following mating (Ball *et al.,* 1991). *Mycoplasma*, *Ureaplasma* and viruses were not isolated prior to or during the experiment. *Haemophilus/Histophilus* strains were the only bacteria consistently isolated from the mucosa of the vulva and glans penis of experimental cases.

3. History of analytical methods used in the identification of bacteria in South African cases of UB

In order to make a valid clinical diagnosis and develop an effective treatment that can aid in the management and control of an infectious disease, identification and classification ofmicrobes involved in the incidence of the diseases, is essential. Identification of bacterial species can be described as assigning an unknown microbial organism to a particular class in an existing classification (Priest, 2003). In clinical microbiology the aim is usually directed at characterizing bacteria to the most appropriate classification level that is

informative enough to associate an organism(-s) to a disease. In some cases a bacterial taxon (not necessarily at the species level) may stand out from the expected microbiome, and therefore species level identification is not required. In others, species level identification is essential to differentiate organisms from that of the normal microflora where no differences can be seen at the higher classification levels. Some diseases may be linked with a change in the microbial diversity, which then result in a shift in community composition between the healthy and the diseased state. In other scenarios, an increase in the relative abundance of bacteria which normally occur in the animal, to higher than normal levels, may also result in the incidence of a disease.

Previous research on ulcerative balanoposthitis in South Africa has been aimed at identifying a causative agent from swabs and scrapings taken from the mucosal lining of the penis and prepuce of rams. These studies investigated suspected pathogens for their isolation rate in healthy and diseased sheep, with significant differences further suggesting the association of a pathogen(-s) to the disease. The methods used to identify bacteria in clinical samples included broad categorization of bacteria by means of culturing, followed by more specific definitive tests that allowed researchers to characterize the bacteria present.

The above-mentioned UB-related studies have focussed primarily on identifying bacteria from the class mollicutes, specifically *Mycoplasma*, which have previously been associated with other reproductive diseases in livestock species (Basemen & Tuly, 1997; Nicholas *et al.,* 2008) and are currently considered potential aetiological agents of UB, although the cause is still unresolved. Attempts to identify other pathogenic bacteria have also also been condcted. No attempts at characterizing and comparing the entire bacterial microbiome surrounding the penis in healthy and diseased Dorper rams were carried out. A description of the diagnostic methods previously used as well as their advantages and disadvantages are described in the sections below.

3.1 Culture identification

Culturing of bacteria is a technique that has been practiced for over 50 years and is described as the golden standard in diagnostic microbiology (Padmanabhan *et al.,* 2013). This technique involves the isolating and proliferation of bacteria from clinical specimens on various growth media under different environmental conditions, allowing researchers to make initial presumptions of bacterial identity according to their growth requirements and characteristics. Studies on cases of UB in South Africa have been carried out with the aim to culture and investigate the bacterial composition of clinical samples; to determine bacterial colonies that could be involved in the infection; and obtain sufficient growth of pure organisms for further identification and classification using various types of tests (Didelot *et al*., 2012).

The initial step involved in the clinical diagnosis of an aetiological agent(-s) in UB in South Africa has been based on the cultivation of bacteria (Trichard *et al.,* 1993; Kidanemariam, 2003) and *Mycoplasma* (Trichard *et al.,* 1993; Kidanemariam, 2003; Ali, 2012) on a battery of standard media known to support the growth and routine isolation of most pathogenic bacteria (Quinn *et al.,* 2011) and *Mycoplasma* species (Nicholas *et al.,* 2008Along with specialised media, the environmental conditions under which these cultures were grown differed across studies but remained within the scope of what is suggested optimal growth conditions for culturing most pathogens (Razin, 1994; Quinn *et al.,* 2011).

In order for bacteria to be cultivated, similar growth conditions found *in-vivo* need to be established *in- vitro*, and the use of specific media and conditions relating but not limited to the nutrient requirements, temperature, pH, moisture, and oxygen and carbon dioxide requirements should allow for such organisms to proliferate under artificial conditions (Quinn *et al.,* 2011). For example, Trichard *et al.* (1993) incubated some

cultures at 10% CO2 in order to cultivate bacteria from the *Haemophilus* and *Actinobacillus* groups that have previously been identified as causing reproductive diseases in sheep (Bagley *et al.,* 1985; Van Vuuren & Trichard, 2004a & b), which can only optimally grow in higher than normal CO2 concentrations.

The need to create a perfect *in-vitro* environment plays an important role in diagnostic microbiology in that it is not possible to produce a culture environment specific to all bacteria and growth conditions will be chosen according to pathogens suspected of being present in clinical samples, thus limiting the number of pathogens tested for. It is well accepted in the literature that not all bacterial species can be cultured and it is believed that about 10% of infectious bacterial pathogens are unculturable or difficult to grow *in-vitro* (Didelot *et al.,* 2012). For example, the diagnosis of *Mycoplasma* from infectious diseases using conventional culture techniques can be complicated in that some *Mycoplasma* species are unculturable and the minority of *Mycoplasma* existing in different habitats that have been cultivated grow slowly (several days to weeks) and poorly even on the best *Mycoplasma* medium available and are often overgrown by other bacteria in samples (Razin, 1994; Cai *et al*., 2014). In addition, bacteria that grow rapidly in a culture often outgrow species that grow slowly in the same culture, thus removing what could be an "important" organism from further analysis. Bacterial cultivation can be very laborious, time-consuming and in many cases requires skill and equipment (Bowler *et al.,* 2001; de Boer *et al.,* 2010) thus making it near impossible to test for an entire bacterial population within a clinical sample.

3.1.1 Definitive identification

Colonial morphology, physical traits such as colour and motility, and other key growth characteristics observed on primary bacterial isolation media are used to make a presumptive bacterial identification to higher levels of classification, with the choice of more specific identification procedures thereafter based on the need for a definitive identification of an organism down to species level (Snyder & Atlas, 2006). Different types of tests have been described in the literature that can be used as additional or alternative definitive bacterial identification methods to bacterial culture (Quinn *et al*., 2011). Assays previously used in South Africa for definitive bacterial identification were based on previously cultured bacteria and included biochemical, serological and molecular assays, depending on the nature of the study.

3.1.1.1 Biochemical assays

Conventional culture methods are the most widely used methods in diagnostic microbiology and base definitive identification of bacteria on phenotypic analysis that look most commonly at biochemical characteristics with other physiological, chemotypic (particularly fatty acid components) and metabolic characteristics also studied (Quinn *et al.,* 2011).

Bacteria are divided into different groups that are known to have specific biochemical characteristics (Healing, 1993) and the type and number of biochemical tests required to identify a bacterium will vary from one group to another. Some bacterial taxa require fewer tests and are easier to differentiate than others. A number of biochemical assays can be routinely used for many groups of bacteria (e.g. fermentation or utilization of carbohydrates, oxidase, amino acid degrading enzymes, nitrate reduction) whilst others are confined to a single family, genus or species (e.g. coagulase test for Staphylococci, pyrrolidonyl arylamidase test for Gram-positive cocci) (Baron, 1996). Specific growth media, nutrients, chemicals or growth conditions are applied by biochemical tests to elicit observable and measurable responses from microorganisms that can in turn be used to identify and classify them (Gupta, 2011). These tests are abundant throughout the literature, ranging in their specificity, sensitivity and reproducibility, and the type used largely depends on

the pathogen suspected as well as the knowledge, expertise and preferences of the researcher in terms of the protocols followed (Healing, 1993).

Using broader as well as more specific biochemical assays specifically designed to test the catabolic properties of different types of bacteria, Trichard *et al.* (1993) and Kidanemariam (2003) followed standard criteria and interpretation of results from laboratory protocols, literature (Stephens *et al.,* 1983; Razin & Freundt, 1984; Quinn *et al.,* 2011) and purchased commercial analytical systems (API 10S, API Coryn, Microbat 12A and 12B) and kits (staph and strep kits). Using these specific biochemical tests, they were able to isolate and characterize a number of pathogenic bacteria from healthy and diseased rams that fall under characteristics of Gram-negative and –positive bacteria along with other mollicutes (Trichard *et al.,* 1993; Kidanemariam, 2003).

Conventional methods have been perfected over many years and are still the preferred method in diagnostic microbiology. This approach does however, like any other method, have several drawbacks which limit accurate diagnosis. Phenotypic tests can be subjective and interpretation of results can be highly biased according to the technologist and his or her experience (Stager & Davis, 1992). When phenotypic profiles do not follow standard criteria as a result of for example evolution, environmental stress factors, unusual microorganisms which are rare or may result from new strains or poorly updated commercial bacterial identification databases, the accuracy of biochemical tests may be questioned and it is possible that tests are interpreted to fit expectations (Kolbert & Persing, 1999; Petti *et al.,* 2005). Furthermore, some biochemical tests cannot sufficiently differentiate bacteria to species level, for several reasons that may include similarity in phenotypic profiles with other bacteria or phenotypic paucity rendering them indistinguishable (Vandamme *et al.,* 1996). Bosshar *et al.* (2003) demonstrated that out of a 136 known aerobic gram-positive rods isolated from clinical specimens only a minority of isolates were identified using phenotypic methods. Classification was only achieved at the genus level, with 71 of 136 (52.2%) isolates phenotypically characterized at the genus level and the remaining 65 of 136 (47.8%) isolates could not be discriminated at any taxonomic level. In this case additional non- phenotypic tests will be required to further characterize these isolates (Gupta, 2011).

Studies on *Mycoplasma* suggest that characterization of species from cultures can be achieved through the aid of biochemical tests (Gois *et al*., 1974). Although biochemical tests are still widely used, the diagnosis of *Mycoplasma* through these assays are limited, with consequential increase in the dependence of other tests such as serological and molecular assays (Gois *et al.,* 1974). This is especially true for the *Mycoplasma mycoides* cluster, under which the suspected aetiological agent of UB, MMMLC, is grouped, where only a few biochemical and physiological properties can be used to differentiate them (Cottew *et al.,* 1987).

Many authors have described biochemical tests used in the classification of *Mycoplasma* which include but is not limited to glucose fermentation, arginine utilization, urea hydrolosis, sensitivity to digitonin, serum digestion, tetrazolium HCL reduction, phosphatase activity, catalase and oxidase tests and metabolisms of a number of carbohydrates (Kidanemariam, 2003). Only one study in South Africa carried out by Ali (2012), used biochemical assays to characterize *Mycoplasma* species in rams affected with UB. Ali (2012) biochemically characterized *Mycoplasma* present in clinical samples by following standard protocols developed by Erno and Stipkovits (1973) that involved the use of several of the biochemical tests previously mentioned, preceded by a standard staining method (Gram's stain), additional growth on a selective medium and further differentiation according to lactose fermentation. The biochemical tests used by Ali (2012) proved to be limited in their ability to differentiate *Mycoplasma* to species level and additional molecular tests were used to further differentiate these *Mycoplasma*s.

3.1.1.2 Serological identification

Serological tests have been extensively used in differentiating *Mycoplasma* species, and can include amongst others immunoperoxidase, immunofluorescent antibody assays (IFA), Western immunoblotting, complement fixation, and enzyme linked immunoabsorbent assays (ELISA) (Clyde, 1964; Rosendal & Black, 1972; Krogsgaard-Jensen, 1972; Goll, 1994; Thacker & Talkington, 1995; Nicholas *et al.,* 2008), with some of these techniques being more specific and/or sensitive than others. Trichard *et al.* (1993) and Kidanemariam (2003) made use of the direct-IFA (uses only one antibody) and indirect-IFA (uses two antibodies) techniques, respectively, which have proven to be highly species- specific tests for the identification of various *Mycoplasma* species observed in the penile samples of South African Dorper rams, with higher sensitivity suggested with the indirect-IFA technique (Rosendal & Black, 1972). As a result, the indirect-IFA technique is now the most commonly used method of the two (Turgeon, 2015).

The serological techniques rely on fluorophore-conjugated antibodies that fluoresce on attachment to its complementary *Mycoplasma* antigen. These tests although specific, may be subjective in that fluorescent signal intensity used to determine the presence of a specific *Mycoplasma* cannot be quantified and grading of the intensity signal depends on the opinion of the researcher, which could lead to false positives Furthermore, this test is generally suitable to test for a small number of bacteria (Gillespie, 1994). In *Mycoplasma* identification, the antibodies filtered from antisera prepared from experimental animals as a response to a number of reference *Mycoplasma* strains are required which makes this test time-consuming and limits the identification of *Mycoplasma*s to only those reference strains used. Trichard *et al.* (1993) and Kidanemariam (2003) limited *Mycoplasma* identification to 9 *Mycoplasma* strains, although suggesting that several mollicutes could be involved in the disease.

The diagnosis of *Mycoplasma*s is complicated by the fact that members of the genus seem to be closely related phenotypically and genotypically. Serologically, the results achieved for members of the *Mycoplasma mycoides* cluster are often difficult to interpret with serological cross-reactions not uncommon. Although not believed to be observed by Trichard *et al.* (1993) and Kidanemariam (2003), cross-reactions have been observed amongst *Mycoplasma* species such as between *Mycoplasma capricolum* subspecies *capripneumoniae* and *Mycoplasma* species strain PG50 as well as some other strains of *Mycoplasma capricolum* subspecies *capricolum* (Bolske *et al.,* 1988). Confirmation of these *Mycoplasma* species with serological assays often require further tests to characterize them, which include protein profiling (Thiaucourt *et al.,* 2000) and DNA hybridization (Bonnet *et al.,* 1993).

3.1.1.3 16S rRNA gene sequencing

Although conventional phenotypic methods and serological tests are readily used to identify bacteria these tests require live cultures and experience in diagnostic microbiology. The methods are also considered to be subjective resulting in misclassifications; and are sometimes limited in their ability to identify bacteria to species level or differentiate bacteria within the same bacterial taxa, which will then require additional tests to further characterize them.

Some of the limitations of phenotypic and serological bacterial characterization techniques can be overcome by using molecular genotypic assays as a complementing or alternative mode of identification (Fihman *et al.,* 2007; Petti, 2007; Monteserin *et al.,* 2016). Ali (2012) was the first researcher in South Africa to make use of molecular technology as a complementary test to characterize previously cultured *Mycoplasma* isolated from

Dorper rams affected with UB. Genotypic identification methods in general are considered to be quicker, objective, highly sensitive and specific, and reproducible compared to phenotypic methods (Fihman *et al.,* 2007; Srinivasan, 2015).

Genotypic tests are not limited to providing a complementary test to bacterial cultures, and can be conducted from bacterial DNA extracted directly from clinical samples. It can be used to overcome the limitations of culture-based identification of bacteria mainly related to the identification of bacterial species that are unculturable, rare or difficult to grow *in-vitro*, with the added benefit of novel species discovery of previously unculturable organisms (Woo *et al.,* 2003). The most obvious advantages of 16S gene sequencing of bacterial DNA directly from samples is that researchers are not required to be experienced in diagnostic microbiology and that there is no need to suspect infectious agents prior to experimentation, omitting other potential pathogens through limited culture media, growth conditions and phenotypic tests aimed at specific pathogens, as well as poor expertise. All bacteria present have the ability to be identified.

The analytical properties of genetic material, directed at deoxyribonucleic acid and ribonucleic acid (DNA and RNA), have allowed a number of genotypic methods to be developed under headings such as molecular hybridization, molecular fingerprinting, microarrays, PCR and gene sequencing (Quinn*et al.,* 2011).

The molecular technique gene sequencing is currently the gold standard for defining bacteria and can be described as the sequencing of a specific section of a bacterial genome previously amplified using polymerase chain reaction (PCR). The 16S ribosomal RNA (rRNA) gene is approximately 1550 nucleotide base pairs (bp) long and in certain bacterial taxa multiple copies can exist throughout the genome (Větrovský & Baldrian, 2013). The 16S gene has widely been accepted in the literature as a tool for identifying bacterial isolates (Patel, 2001; Mignard & Flandrois, 2006; Revetta *et al.,* 2010; de Melo Oliveira *et al.,* 2013; Monteserin *et al.,* 2016) and diagnosing microbial diseases (Trotha *et al.,* 2001; Lecouvet, 2004; Lau *et al.,* 2006; Woo *et al.,* 2007; Kuhn *et al.,* 2011; Srinivasan *et al.,* 2015). It is currently the most extensively used bacterial gene in clinical microbiology and can be used in its entirety or in smaller sections to characterize bacteria, depending on the sequencing technology used (which will be described in the next section). Its popularity lies in its degree of conservation/universality across the domain bacteria with few other genes as equally conserved (Clarridge 3rd, 2004).

The 16S gene consists of eight highly conserved regions that flank nine hypervariable regions across the bacterial domain (Armougom & Raoult, 2009). The regions that exhibit variable nucleotide sequences can be used to compare DNA homology between bacteria in turn differentiating them according to species. Thus, the 16S rRNA sequence of a bacterium is a genotypic feature which allows the identification of organisms at the species level. Furthermore, the conserved regions of the 16S gene can be used to identify bacteria covering most taxa or differentiate organisms belonging to a specific group (Baker *et al.,* 2003). In the most recent study on UB in South Africa, Ali (2012) characterizedthe *Mycoplasma* diversity in sheep affected with UB by means of 16S rRNA sequencing. Hecharacterized *Mycoplasma* from sequenced PCR and cloned PCR products previously amplified from a forward primer derived from a section of the 16S gene universal to all bacteria and a reverse primer from a section of the 16S gene conserved across all *Mycoplasma*s; generating a DNA fragment that covered approximately 1078bp of the 16S gene (~1550bp) and that was specific to *Mycoplasma*.

During bacterial identification, 16S rRNA sequences are assigned according to the similarity of the query (amplified) 16S rRNA sequence to 16S rRNA reference sequence located in a database. Thus 16S sequence classification and taxonomic resolution is constrained to reference databases and to organisms that have

previously been identified and sequenced. Like phenotypic techniques, 16Sgene sequencing also uses a cut-off value for acceptable levels of similarity to identify bacterial species. Although the value is not as well-defined as should be and no consensus algorithm has been used to calculate this value, it is suggested by most taxonomists that a percent identity score of ≥97% and ≥99% between a query sequence and a reference sequence is usually sufficient to define the 16S rRNA sequence to a genus and a species, respectively (Petti, 2007). In the literature, disparities have been observed in the total percentage of bacteria identified to species level across all cut-off definitions (even with no definition) (Clarridge 3rd, 2004), suggesting that this parameter plays an important role in definitive pathogen diagnosis. Higher percentage of species are usually classified at no or lower identity cut-off values, this however increases the chance of misclassification.

Several studies compared the ability of 16S gene sequencing with conventional methods in identifying clinically important bacterial groups from cultures (Bosshard *et al.,* 2003; Heikens *et al.,* 2005; Song *et al.,* 2005; Adderson, 2008; Rhoads *et al.,* 2012). From these studies it is deduced that when 16S rRNA sequencing is used to identify rare bacteria and bacteria with unusual biochemical profiles, a more effective degree of identification can be achieved.

Two separate studies investigated the potential of the MicroSeq 500 16S gene-based identification system to identify bacteria and found that this system was able to characterize 81% of clinically significant bacterial isolates with ambiguous phenotypic profiles (Woo *et al.,* 2003) and 89.2% of unusual aerobic Gram-negative bacilli to species level (Tang *et al.,* 1998). When 16S rRNA sequencing was used to resolve the identification of phenotypically unidentified bacterial isolates the method proved to be effective, with more than almost 90% of isolates defined (Drancourt *et al.,* 2000).

When testing the ability of routine identification by the 16S rRNA in the clinical laboratory, 16S rRNA was able to characterize 243 out of 382 clinical isolates of mycobacteria which are known to be clinically important pathogens and are in general slow-growing and/or difficult to identify phenotypically (Clarridge 3rd, 2004). Species-specific identification of *Campylobacter* has been reported as problematic due to the absence of suitable biochemical assays. 16S rRNA sequencing was demonstrated as a reliable tool in differentiating most *Campylobacter* species with exceptions observed with *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* that could not be differentiated due to identical or almost identical 16S rRNA gene profiles (Gorkiewicz *et al.,* 2003). This is one of the limitations of 16S gene sequencing, and usually alternative genes may be used to further differentiate these organisms. One such gene, ropB, has been described as a complementary gene in differentiating between *Bacillus cereus* and *Bacillus anthracis* that have identical 16S gene sequences (Blackwood *et al.,* 2004).

In South Africa, Ali (2012) made use of biochemical assays to detect isolates from the genus *Mycoplasma*, and characterized *Mycoplasma*s to species level using 16S rRNA sequencing (Sanger sequencing technology), in an attempt to verify previous findings on UB suspecting MMMLC as the primary aetiological agent. Ali (2012) isolated species that had previously been identified in other studies (Trichard *et al.,* 1993; Kidanemariam, 2003) as well three new species of *Mycoplasma*, two that had never been identified in cases of UB (*Mycoplasma* Mmm. jvc1 and *Mycoplasma* species USP 120) and one (*Mycoplasma canadense*) never observed in sheep in South Africa. Ali (2012) succeeded in identifying MMMLC, however noted that the rate at which it was isolated (2 out of 34 isolates) was much smaller than expected, when compared to much higher isolation rates suggested in previous studies on the disease (Trichard *et al.,* 1993; Kidanemariam, 2003). Although the number of isolates tested for was small, the low isolation rate of MMMLC may suggest an overestimation in the role of MMMLC as an aetiological agent brought about as a result of serological cross-reactions with other *Mycoplasma* species, which is a common flaw of serological identification methods

(Bolske *et al.,* 1988).

Janda and Abbott (2007) reviewed the use of 16S gene sequencing in clinical diagnosis and found that in most cases (>90%) only genus identification is possible with species identification that ranges from 65-83% according to studies. Bacterial groups including but not limited to Enterobacteriaceae, Mycobacteria, Achromobacter, Stenotrophomonas and Actinomyces have demonstrated resolution problems at these levels. As is suggested from these findings, no technique can be a 100% effective in identifying bacteria and although 16S rRNA sequencing can be useful in classifying bacteria especially those difficult to classify using other techniques, it is not without limitations and species level identification is not always guaranteed.

The lack of resolution to the species level can be attributed to a number of reasons mainly related to the size of the 16S gene DNA fragment sequenced; the chosen hypervariable region if only a section of the 16S gene is used; the sequence reference database used; and the quality of the 16S sequences produced. Although Clarridge, 3rd (2004) has suggested that the initial 500bp of the gene is sufficient to classify most clinical bacterial isolates to species level, sequencing of the whole 16S gene is often required to distinguish bacteria between taxa, where sometimes even the whole 16S gene is not enough (Gorkiewicz *et al.,* 2003). It is not to say that species characterization cannot be achieved using DNA fragments that are less than 500bp long, but that the smaller a DNA fragment is the smaller the resolution and the less confidently the sequence can be attributed to a bacterial taxon at the species level. Tewari *et al.* (2011) compared the use of a DNA fragment of ~500bp and another of ~40bp, and found that the longer 16S gene fragment could identify 87% of isolates to species level compared to 43% using the shorter section. Furthermore, not all short fragments could be identified to the genus, family or order taxonomic levels as compared to 100% identification by the 500bp.

Some short 16S rRNA sequenced gene fragments have proved to have taxonomic resolution closerto that of full length 16S sequences as a result of the variable region they cover. Some hypervariable regions are more variable across bacterial taxa than others, thus have higher potential of discriminating between bacterial taxa. Recovery and coverage levels of bacterial taxa using different 16S regions measured relative to the results achieved using full length sequences, observed that the V6 hypervariable region did poorer than other regions in identifying organisms from the gut microbiota at the genus level (Lui *et al.,* 2008).

The bacterial identification and discriminative ability of the 16S rRNA gene using a reference sequence database relies on four key components, i.e. the deposition of complete and good quality nucleotide sequences into databases; correct taxonomic classification of each sequence deposited; the number of 16S rRNA sequences deposited; and the taxonomic-resolution of sequences available, especially as longer sequences are added to databases. It is no secret that the sequences found in reference databases contain errors especially those that were deposited years back prior to the development of high-fidelity, automated systems (Ashelford *et al.,* 2005). Although these errors are believed to be corrected, they and errors due to newer sequencing technologies are still observed at varying degree throughout the available databases such as Genbank, RDP and Greengenes (DeSantis *et al.,* 2006; Cole *et al.,* 2009; Benson *et al.,* 2013). For example, Genbank accepts any taxonomically named sequence deposited and is not peer reviewed (Clarridge 3rd, 2004). A study by Clayton *et al.* (1995) revealed that for every two 16S rRNA gene sequence coding for the same bacteria, a minimum of 26% had more than 1% sequencing errors, and 50% had more than 2% sequencing errors.

Poor quality reference sequences diverge the identity score between a query sequence and the reference sequence, where ambiguous bases reduce the score preventing an identity match to the desired level of taxonomy (e.g. family instead of species). In addition, these errors may lead to wrong best identity match

between a query and a reference sequence. In other words, it is possible that a query sequence may match, by random chance, to another reference sequence rather than the erroneous reference sequence it is actually meant to match to, thus assigning a wrong taxonomic classification to the query sequence.

Partial 16S rRNA reference sequences in databases also reduce the taxonomic resolution potential of longer query sequences, for the latter will be classified according to the classification of the shorter reference sequence if longer reference sequences are not available. Furthermore, it is also possible that a number of sequences are misclassified in databases. Bacterial 16S sequences are usually assigned to a taxon based on phenotypic tests; and in some cases when phenotypic traits are poorly described or tests are faulty, the wrong taxonomic label may be assigned to a 16S rRNA reference sequence and subsequently to query sequences (Clarridge 3rd, 2004). When many sequences are analysed at once it is hard to determine whether a sequence is difficult to identify or that it is incorrectly identified.

As mentioned by Drancourt *et al.* (2000) errors are also observed in query sequences affecting taxonomic resolution and correct species identification. These errors may arise as a result of inefficient PCR amplification and/or poor sequencing. It is possible that during PCR amplification, suboptimal reaction conditions, such as improper temperature, may cause amplification to be less sensitive or non- specific or may stop earlier than expected (producing 16S rRNA sequences that are shorter than expected). These types of errors cannot always be avoided all together but can be minimized by testing various reaction conditions that provide the most sensitive and specific PCR reaction (Wilson, 1997). PCR amplification with reduced sensitivity (i.e. increased base mismatch) and specificity (i.e. binding primers to the wrong region of the 16S gene) can produce sequences that are classified at lower resolutions, that are wrongly classified, and that are not amplified from the desired variable region. This can cause biases in the bacterial diversity and representation of organisms. In addition, incomplete amplified sequences can act as a primer template which can bind to related DNA fragments, generating a product of two sequence combinations which is otherwise known as a chimera, and as a result the new chimeric sequence sequenced can be mistaken for new bacterial taxa, amplifying the bacterial diversity of a sample (Edgar *et al.,* 2011). The rate of chimerism has been reported by Haas *et al.* (2011) to range between 5% and 45%.

The number of sequence errors produced during sequencing vary according to the sequencing platform used. For example an error rate (i.e. number of errors per total nucleotide bases sequenced) of 1-2% has been reported using the Roche 454 sequencing platform, mainly due to errors related to homopolymers (Margulies *et al.,* 2005). Sequence errors in query sequences have the same effect on bacterial identification than sequencing errors observed in 16S reference sequences, previously mentioned.

The reference databases available to researchers vary in diversity and overall taxonomic structure and do not always agree on species names or taxonomy (Werner *et al*., 2011; Santamaria *et al*., 2012).This will influence the taxonomic classification of 16S sequences, ultimately affecting the perceived diversity. It is possible that a bacterial taxon (phylum to genus level) is under-represented in one database as compared to another, and the representation of this bacterial taxon will thus also be under-represented in the 16S data when assigning an identity to 16S query sequences, as compared to using a database where the taxon is highly represented (Huse *et al.,* 2008). In addition, for some genera too few species have been sequenced and deposited in the databases and the identity score for a particular query sequence never exceeds the genus level (i.e. 97% identity score) (Drancourt *et al.,* 2000). For example, Genbank comprises the largest databank of nucleotide sequences with over 100 000 sequences of the 16SrRNA gene deposited (http://www.ncbi.nlm.nih.gov/genbank/) to match against, as compared to a commercially available MicroSeq database with ~2000 16S rRNA sequences (Petti, 2007). Furthermore, not all databases are

updated regularly, thus new sequences and sequences withhigher taxonomic resolution available cannot be accessed. The Greengenes reference database (DeSantis *et al*., 2006), for example is updated only periodically.

Apart from limitations of 16S gene sequencing to correctly classify sequences and provide sequences of high taxonomic resolution, as determined according to the sequences available in the reference databases, the observed diversity can also be influenced by DNA extraction and primer selection. DNA extraction protocols have been recognised in the literature with special attention to their ability and efficiency in extracting DNA from all bacterial taxa and the effects thereof on bacterial diversity analysis (Feinstein *et al.,* 2009; Wesolowska-Andersen *et al.,* 2014; Hart *et al.,* 2015). The disruption and lysis of bacterial membranes during the first step of DNA extraction can be biased towards specific bacterial taxa due to differences in cell wall integrity and structure. Gram-positive bacteria generally require bead- beating to break down the cell wall and expose the DNA molecule. Drancourt *et al.* (2000) observed that 2% of clinical bacterial isolates could not be identified due to inappropriate DNA extraction methods.

The choice of a primer or primer set used to amplify regions of the 16S rRNA gene and the hypervariable regions sequenced vary across the literature *(*Baker *et al.,* 2003; Edwards *et al.,* 2006; *Sogin et al.,* 2006; Roesch *et al.,* 2007; Andersson *et al., 2008;* Frank *et al.,* 2008*;* Huse *et al.,* 2008; Liu *et al.,* 2008; Wang & Qian, 2009). The selection of a primer or primer set has been described as the most important step in accurate 16S gene sequencing analysis, in that suboptimal primer pairs can lead to under- or over-representation of bacterial taxa, or select against a specific species or group if they match the consensus sequence poorly (Hamady & Knight, 2009; Klindworth *et al.,* 2012). For example, where a primer is not conserved across all bacterial taxa present in a clinical sample, some mismatches between a primer and the complementary 16S DNA fragment may occur, decreasing the efficiency by which it amplifies the DNA fragment, causing that taxa to be under-represented in the sample. This effect can usually be decreased by the use of degenerative primers.

No true consensus on what the best universal (i.e. conserved across all bacterial taxa) primer pair has yet been agreed upon (Schloss *et al.,* 2011) and the coverage of bacterial taxa differ according to the taxa present in microbial communities analysed (Huse *et al.,* 2008; Soergel *et al*., 2012). Klindworth *et al.* (2012) experimented with a number of universal primer pairs and found variations in the coverage percentages, which ranged from 76.5% - 96.7%, with differences in the number of phyla that could be recovered. For example, they reported that through modelling experiments the primer pair S-D-Bact- 0785-b-A-18 and S-D-Bact-0785-a-A-21 is believed to cover 49 out of 59 phyla from the bacterial domain as compared to the primer pair S-D-Bact-0564-a-S-15 and S-D-Bact-0785-b-A-18 that had a slightly lower overall coverage for bacteria but only failed to detect 4 bacterial phyla.

The limitations of 16S rRNA sequencing to some extent affects final bacteria identifications and the observed bacterial diversity. A number of methods have however been developed to overcome or reduce these constrains, such as choosing a curated reference database that is frequently updated and has a low rate of sequence errors; maximizing the length of the DNA fragment sequenced; choosing a hypervariable region that is highly variable and can maximize bacterial differentiation; and making sure the best primer pairs, PCR reaction conditions and DNA extraction methods are chosen. The ability of 16S gene sequencing as a viable method in characterizing bacteria cannot be rejected.

4. High throughput next generation sequencing

With the advent of sequencing technology bacterial identification is no longer limited to cultures and identifying a small number of bacteria per sample. Microbial communities can now be sequenced and identified with limited bias by using universal 16S rRNA primers that during a PCR reaction amplify all microbial nucleic acids present in a sample. The ability to identify all bacteria has several advantages such as the identification of unculturable bacteria and the identification of polymicrobial infections (Cai *et al*., 2014), which is presumed a challenge for culture-based methods (Fenollar *et al.,* 2006; Roger *et al.,* 2009). In addition, characterizing all bacteria removes the need for researchers to make presumptions about the pathogen(-s) that may be involved in a disease of unknown aetiology, allowing all pathogens to be identified rather than testing for a select few. UB is both a disease of unknown aetiology and one suspected of polymicrobial infection.

High throughput next generation sequencing (HT-NGS) is a new era of sequencing technologies, that has only been available for the last decade, with an ever growing number of technologies surpassing the abilities of older systems (Van Dijk *et al.,* 2014), varying in their sequencing chemistries, read lengths and throughput capacities (Loman *et al.,* 2012). High-throughput next generation sequencing technologies can generate thousands to millions of sequences in a single run, generating sequence information data of larger magnitudes in a small period of time (Kircher & Kelso, 2010; Buermans & Den Dunnen, 2014). A number of methods of HT-NGS exists and currently 16S amplicon sequencing is one that is widely used to characterize microbial communities. Its ability to generate thousands to millions of sequences in a single run has described 16S amplicon sequencing as an efficient tool in characterizing bacteria from clinical samples with high microbial diversity, where both dominant and lowly abundant or rare bacterial taxa (of which can be pathogens) can be characterized (Siqueira *et al.,* 2012). This means that 16S amplicon sequencing is able to recover greater species richness and characterize almost complete microbial communities from clinical samples with the theoretical ability to identify all bacterial species present, including those not previously cultured. Along with qualitative characterization, molecular profiling of bacterial communities using 16S amplicon sequencing also provides quantitative characterization through more reliable estimates of the relative abundance of bacterial taxa present in clinical samples (Pallen *et al*., 2010). This permits researchers to compare bacterial taxa within and between communities beyond simple presence or absence diagnostic criteria. Further advantages of using16S amplicon sequencing include lower cost, the ability to sequence many samples at once and avoiding cloning biases.

The use of 16S amplicon sequencing in veterinary clinical laboratories is still limited (Tewari *et al.,* 2011) but its use in the veterinary research sector is fairly well documented in the literature (Gill *et al.,* 2006; Costa *et al.,* 2012; Oikonomou *et al.,* 2012; Steelman *et al.,* 2012). These studies and other studies related to human health (Lamont *et al.,* 2011; Dickson *et al.,* 2013; Wade, 2013) have illustrated the role of 16S amplicon sequencing in understanding diseases and the importance of profiling bacterial microbiomes in healthy and diseased states in order to understand the underlying biological and clinical role of bacteria, especially if the disease state is related to changes in community composition from that of the healthy state (Roger *et al.,* 2009). For example, a study that compared the microbial community within the gut of healthy horses and horses that had coilitis, found that the microbiome differed significantly between the two groups and that a shift in microbiome rather than an enrichment in an individual bacterial pathogen may be associated with the disease (Costa *et al.,* 2012).

The ability to characterize a healthy microbiome in order to understand specific diseases has been an innovation of HT-NGS technology such as 16S amplicon sequencing. It is thought that a core microbiome

exists within body sites and maintain a healthy state. Altered microbiomes from that of the healthy states can help researchers determine if the shift in community composition is merely associated as markers for diseases or can also actively contribute to the pathology of the disease (Goodrich *et al.,* 2014). If a shift in the community composition is observed from one state to the other, it is conceivable to narrow down to individual bacterial taxa that are observed to be involved in driving this shift and determine their biological and clinical roles in disease. Furthermore, defining healthy microbiomes allows researchers to identify biological systems that demonstrate natural inter-individuals variations in diversity, limiting later complications in the identification of microbial components and imbalances that may cause or reflect a disease state in such a system (Lloyd-Price *et al*., 2016). An understanding of the structure of a healthy microbiome in the absence of disease is therefore an essential initial step to identifying bacteriological patterns implicated in disease.

Although 16S amplicon sequencing technology surpass Sanger technology on many levels, it is at a cost of 16S rRNA fragment length and sequence quality. High throughput next generation sequencing technologies are only capable of sequencing smaller 16S rRNA fragments, thus affecting the taxonomic resolution of bacteria identified. Nonetheless, newer 16S amplicon sequencing platforms are being developed at a rapid rate to overcome this with sequencing platforms like Illumina Miseq (http://www.illumina.com) that make use of paired-end sequencing exploiting larger read lengths of up to ~550bp that were not previously possible (Schimer *et al.,* 2015). Inevitably, all sequencing technologies produce sequencing errors, some more than others, which if sufficient can mistake a sequence for a new species, inflating species richness of a sample. Higher sequencing depth can however make these errors less relevant, where many copies of the same sequence (with fewer or no errors) can be retrieved. Several strategies have been proposed to deal with these errors which include removing parts of sequences that are of poor quality or ambiguous, or removing sequences all together so that only good quality sequences remain for downstream analysis (Schloss *et al.,* 2011).

With the development of 16S amplicon sequencing, the number of sequences produced per sequencing run has expanded into the millions producing data in the gigabytes range, which poses a challenge for both data storage and analysis (Belak *et al*., 2013). Normal computers do not often have sufficient memory, storage and processing power to analyse these types of datasets and alternative resources such as online servers are usually required. In addition, 16S amplicon sequencing data analysis requires knowledge of bioinformatics, which is the research field focussing on the study of methodsfor retrieving, analysing and storing biological data (Belak *et al.*, 2013). Researchers also require to select amongst a plethora of tools/software available to analyse and make sense of the data, each with their own algorithms and variable parameters, which is too much to describe for the scope of this study. No tool/software is a “one-size-fits-all” and users are required to have knowledge and understanding of the various analyses steps in a given application (such as characterizing a microbiome) and how different software operates at each step, affecting the end results. The type of data analysis steps and the order in which they occur depends on the researcher and no set protocol exists for how data should be analysed. Often the way data is processed depends on the data, the research question and whether sequence quality or quantity or a balance between the two is most important.

Despite all the limitations of 16S rRNA sequencing along with the complications of data analysis, the potential of this method is growing with the development of newer technologies and it remains evident that this is a valuable tool in investigating microbial communities directed at understanding diseases. Although it cannot be expected that all 16S rRNA sequences will be characterized to species level, the microbial diversity no matter the taxonomic resolution, can contribute insight into “what is there “and “how does it differ within and between groups”, forming the basis from which future studies can be directed in an attempt to identify

a causative agents of UB.

5. Latest findings from a MScAgric study, done against the background provided above

The study by Courchay (2017) followed a molecular approach to conduct a preliminary investigation of the microbial population from the mucosal membrane of the prepuce and the glans penis of healthy and diseased Dorper rams and the comparison thereof. This approach included targeted amplification of DNA extracted from samples collected from the genital mucosa, and diseased tissue using the 16S rRNA gene, universal across all bacterial organisms, which were sequenced using 16S amplicon sequencing technology.

In the study, swab samples of the preputial and penile mucosa were collected from 113 rams, of which 40 Dorper ram samples (20 infected, 20 healthy) were chosen for further analysis in this study. Genomic DNA was extracted and amplified based on the V3V4 hypervariable region of the 16S rRNA bacterial gene. Bioinformatics analysis was performed using UPARSE and the ecological and statistical analyses such as Principal Coordinate Analysis (PCoA) was performed in QIIME and XLSTAT. Additional analyses comparing the predicted bacterial microbiota in healthy and diseased populations was carried out using LEfSe. A total of 789 OTUs from 9 964 842 sequences of high quality were obtained from the healthy and diseased communities indicating a high bacterial diversity in the penile environment, higher than previously reported and isolated using culture-based bacterial identification methods. The genus *Corynebacterium* was the most dominant genus identified (20.9%), irrespective of health status. A high inter-sample variation in microbiota was revealed. There were no significant differences in bacterial diversity or community composition between the healthy and diseased ram groups. The microbiota population was thus similar, with a few OTUs of high biological relevance belonging to genera *Fusobacterium* and *Porphyromonas*, as well as uncharacterized genera within *Aerococcaceae* and *Bacteroidale*s that were enriched in the diseased community. MMMLC and *Trueperella pyogenes* were not associated with UB in this study, which is contradictory to previous reports. However, a new *Mycoplasma* species, *Mycoplasma hyopharyngis*, not previously isolated in sheep and in cases of UB in Dorper rams, was identified and although not significantly different, had a higher abundance in the diseased population. The prevalence and predominance of *Corynebacterium* across all samples suggests this genus forms part of the core microbiome of the penile environment. High inter-sample variation in microbiota may depict true biological representation, however, future studies using homogenous ram populations (i.e. same type, same age) under the same environmental factors (i.e. management, feeding regime, etc.) should be conducted to validate these findings. Ulcerative balanoposthitis is not caused as a result of a change in bacterial diversity or community composition but OTUs enriched in the diseased ram population may be disease-specific/disease-associated and their role in UB warrants further investigation. No definitive aetiological agent was thus identified but the OTUs enriched in diseased rams can help direct future studies towards the identification of an aetiological agent of UB.

The study resulted in the development of a protocol for the culture-independent study of ulcerative balanoposthitis in Dorper rams to characterize the bacterial population present in the penile and preputial mucosa of Dorper rams, and provides baseline information for future studies on the causative agent (-s) of UB in Dorper rams.

6. Future studies proposed to supplement the existing data on UB incidence in South African small ruminants

In the project application, it is proposed that the next phase of the research on UB include the following:

- A national survey to establish the historical and current incidence of UB in small ruminants in South Africa.
- Identification of so-called "hot spots" where UB occur, and sampling of males and females. The samples will then be processed according to the developed protocol, and compared to existing findings. Sampling will also be carried out to determine how the immune system of the ram respond to a recurring UB infection.
- Case control study to determine a potential genetic cause for a higher susceptibility of Dorper sheep to UB.

Findings will contribute to the formulation of protocols and management programs to minimise the spread, and potentially also eliminate the occurrence of UB in small ruminant population in South Africa.

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