**In vitro antimicrobial susceptibility of *Mycoplasma mycoides mycoides* large colony and *Arcanobacterium pyogenes* isolated from clinical cases of ulcerative balanitis and vulvitis in Dorper sheep in South Africa**

A Kidanemariam*a, J Gouwsb, M van Vuurenc, and B Gummowd

**ABSTRACT**

The *in vitro* activities of enrofloxacin, florfenicol, oxytetracycline and spiramycin were determined against field isolates of *Mycoplasma mycoides mycoides* large colony (MmMLC) by means of the broth microdilution technique. The minimum inhibitory concentrations (MICs) of these antimicrobial drugs were determined for a representative number of 10 isolates and 1 type strain. The susceptibility of *Arcanobacterium pyogenes* to enrofloxacin, oxytetracycline and tilmicosin was determined by means of an 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/ml, respectively. This study has shown that resistance of MmMLC against enrofloxacin, florfenicol, oxytetracycline and spiramycin was negligible. All the field strains of *A. pyogenes* that were tested were susceptible to enrofloxacin, oxytetracycline and tilmicosin with mean inhibition zones of 30.6, 42.3 and 35.8 mm, respectively. Although there is lack of data on *in vivo* efficacy and *in vitro* MIC or inhibition zone diameter breakpoints of these antimicrobial drugs for MmMLC, 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.

**Key words**: *Arcanobacterium pyogenes*, Dorper sheep, minimum inhibitory concentrations, *Mycoplasma mycoides mycoides*, ulcerative balanoposthitis and vulvovaginitis.

**INTRODUCTION**

Ulcerative balanoposthitis and vulvovaginitis of sheep is a venereal disease characterised by erosion and ulceration of the glans penis and vulval labia. It has been described in several countries

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by application of MmmLC intravaginally into healthy ewes. In another study Kidanemariam found that MmmLC and *Arcanobacterium pyogenes* were the 2 most common bacterial species isolated from the lesions of clinically affected cases, the former being isolated from 61.5% and the latter from 44.2%. He suggested a possible synergistic role between these 2 organisms in the disease process and found that the odds ratio analysis was that when the 2 organisms were present together they were 53.5 times more likely to occur in clinically affected sheep than in unaffected sheep.

Ulcerative balanitis and vulvitis is at present controlled by application of antimicrobial drugs. The objective of this study was to determine the *in vitro* sensitivity of MmMLC and *A. pyogenes* isolated from field cases of UBV to a variety of these drugs in order to assist in the selection of those most appropriate for the treatment of clinical cases.

At present there are no internationally accepted protocols for testing the susceptibility of mycoplasmas for antimicrobials which is ideally done by determination of their minimum inhibitory concentrations (MICs). Different methods and media are used in different laboratories. However, Hannan in his review article described the general principles and guidelines. In mycoplasmology the MIC is defined as the lowest concentration of an antimicrobial drug that will inhibit visible growth of the mycoplasma under review as judged by the colour change of the medium due to metabolism of the substrate. This method was followed in this study.

The antimicrobial drug susceptibility of *A. pyogenes* was determined using an agar disc diffusion method.

**MATERIALS AND METHODS**

**Cultures of MmMLC**

Ten mycoplasma field isolates obtained from affected sheep during an investigation of UBV in Dorper sheep and the type strain, *MmmLC* (Y-Goat) (NCTC 11706), were selected for the study. Purifi-
cation of the cultures was based on the descriptions of several authors. 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. After incubation for 3 days at 37 °C 10-fold dilutions were made and loopfuls of each were streaked onto plates containing Hayflick’s agar. These were incubated and single-colony picks were made from those plates on which the colonies showed 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 MmmLC using the indirect immunofluorescent antibody test (IFAT). The strains were stored at −80 °C until used for determination of their susceptibility to antimicrobial drugs.

**Culture of A. pyogenes**

The *A. pyogenes* strains used in this study were field isolates obtained from genital swabs of affected sheep. Nine representative isolates were selected for the test and were grown on horse blood agar at 37 °C for 24 hours.

**Media for broth microdilution tests**

For the microdilution test, the purified strains of MmmLC were grown at 37 °C aerobically in an atmosphere containing 5% CO₂. The medium used was 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.

**Standardisation of inocula**

The 10 isolates and type strain of MmmLC were removed from cryostorage and allowed to thaw at room temperature. A panel of 9 tubes each containing 3.6 mℓ of inoculum and 50 mℓ of the antimicrobials when 50 mℓ of inoculum was added. The concentration ranges of the antimicrobial agents, after the addition of 50 µℓ of inoculum, are listed in Table 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.

**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 were made. To each well of the microwell plate, 50 µℓ of diluted culture containing 10⁴ ccu/ml was added. The plates were sealed with transparent self-adhesive tape to prevent evaporation, and then incubated at 37 °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 1 dilution.

**Agar disk diffusion test**

Susceptibility testing of *A. pyogenes* was performed using an agar disk diffusion method on Columbia blood agar (Difco) supplemented with 6% horse blood. The antimicrobial drugs tested were enrofloxacin, oxytetracycline and tilmicosin. Owing to the unavailability of florfenicol disks, the drug was not included in the test. After incubation for 24 hours, the diameters of the zones of inhibition were measured using a calliper. Each zone diameter was interpreted by reference to the zone diameter interpretative standards in NCCLS document M31-A³⁸.

**RESULTS**

The minimum inhibitory concentrations of the antibiotics to which the MmmLC field isolates were susceptible are shown in Tables 3 and 4. Duplicate tests did not vary by more than 1 serial 2-fold dilution. For enrofloxacin 50% of the isolates showed an MIC value of 0.25 µg/ml; 20% of the isolates had an MIC value of 0.5 µg/ml. Thirty per cent of the isolates yielded a MIC value of ≤0.125 µg/ml. The MIC₅₀ and MIC₉₀ were 0.025 µg/ml and 0.35 µg/ml, respectively (Fig. 1). The MIC range for florfenicol was 2.0-4.0 µg/ml and the MIC₉₀ was 2.8 µg/ml.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrofloxacin</td>
<td>≤0.125</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>2.0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>2</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>≤1.0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>≤4.0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>3</td>
</tr>
</tbody>
</table>
Oxytetracycline showed activity against all isolates of *Mmm*LC, with a range of MIC values between 1.0 µg/ml and 2.0 µg/ml, and a mean MIC value of 1.1 µg/ml (Table 5). Ninety per cent of the strains yielded an MIC ≤ 1.0 µg/ml (Fig. 3). The MIC<sub>90</sub> of spiramycin for the isolated strains was 6.0 µg/ml (Fig. 4).

The 9 field strains of *A. pyogenes* were susceptible to all 3 antimicrobial drugs tested. The inhibition zone diameters of the tested drugs are presented in Table 6.

**DISCUSSION**

One of the aims of quantitative studies of antimicrobial sensitivity is to assist in choosing an effective antimicrobial to control an infection. *In vitro* antimicrobial activity, however, 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 responsible organism<sup>24</sup>.

Many methods have been used to obtain MIC data for veterinary *Mycoplasma* species, which make it difficult to compare the results reported from different laboratories. This lack of standardisation has been caused partly by the wide variation in nutritional requirements and culture conditions needed for different *Mycoplasma* spp. and partly 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<sup>23</sup>, and MIC values for reference strains are recommended to be within ±1 dilution of the expected value.

The microdilution method used in this study was that recommended by Hannan<sup>18</sup>. However, apart from the colour change in the medium, the end point could also be determined using the extent of the mycoplasma growth (or lack of it), which was visible at the bottom of the plate as ‘buttons’. This was made possible by the high growth rate of the mycoplasma isolates in the test, which made it easy to interpret the results.

The MICs for the antimicrobial drugs tested for mycoplasma isolates were generally in agreement with the MIC breakpoints of the same antibiotics against bacterial pathogens<sup>25</sup>. 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.<sup>3,9,16</sup>

While no MIC breakpoints are available for mycoplasmas in general and for *Mmm*LC strain Y-Goat in particular, the

<table>
<thead>
<tr>
<th>Antimicrobial Drug</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (&lt;µg/ml&gt;)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (&lt;µg/ml&gt;)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (&lt;µg/ml&gt;)&lt;sub&gt;Y-Goat&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrofloxacin</td>
<td>0.025</td>
<td>0.35</td>
<td>0.125</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>ND*</td>
<td>2.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>ND</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>ND</td>
<td>6.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

ND = not determined.
shows that enrofloxacin will
for tilmicosin
will be

*Standard error of the mean.

<table>
<thead>
<tr>
<th>Antimicrobial drug</th>
<th>Mean MIC values for field strains (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (µg/mL)</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.24</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>2.4</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>1.1</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*Standard error of the mean.

results of the test were determined in accordance with the NCCLS guidelines25, and were similar to data for veterinary mycoplasmas. Unfortunately, an insufficient number of studies have been performed in small ruminants to determine the efficacy of antibiotics against mycoplasmas associated with genital diseases such as UBV.

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 for the particular organism so that there is a good chance of successful treatment. It is, for example, known that the tissue concentrations of enrofloxacin are considerably higher than 1 µg/mL25, which is higher than the breakpoint for most bacterial pathogens. The fact that all 10 MmmLC strains tested had MICs ≤0.5 µg/mL shows that enrofloxacin will likely be an effective drug for the treatment of UBV in Dorper sheep.

In one study27, danofloxacin, a fluoroquinolone, with an MIC of 0.25 µg/ml for Mycoplasma mycoides mycoides small colony (MmmSC) was determined to be as effective for the treatment of pleuropneumonia in cattle. By analogy, the enrofloxacin MIC values of ≤0.25 µg/ml for MmmLC indicate that it should be effective in the treatment of UBV in Dorper sheep.

The MIC90 of enrofloxacin against mycoplasma species has been shown to be 0.01–1.0 µg/ml28. The MIC90 for enrofloxacin in the present study was 0.025 µg/ml, which is within the range obtained by Spoo & Riviere28. Enrofloxacin was found to be 100% effective at 1.25 mg per kg per day per os in pigs with experimentally induced Mycoplasma hyopneumoniae respiratory tract infections. It has also been shown that a mean plasma concentration of 0.6 µg/ml will be attained for enrofloxacin administered to pigs at a dose rate of 2.5 mg/kg body weight28. The same study showed that the mean tissue concentration of enrofloxacin after intramuscular administration will reach between 1.9 and 2.1 µg/ml. These results further support the use of enrofloxacin for the treatment of UBV 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 pathogens25, and it seems, therefore, justifiable to claim that these drugs will be effective against MmmLC infections.

Spiramycin has good tissue penetration ability, reaching concentrations of 25–60 times more than that of serum25. It has also been used successfully to treat contagious bovine pleuropneumonia caused by MmmSC at a dose rate of 25 mg/kg2. It has also been reported that spiramycin has similar applications and effects as those of tylosin, and a much higher in vivo efficacy than that of erythromycin in small ruminants25. 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 a value of MIC90 of 4.0 µg/ml for tilmicosin has been reported29, it is slightly lower than the values for spiramycin. 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 bioavailability20. The potential of this compound in the treatment of microbial infections in food animals intended for human consumption has been demonstrated25. It was initially used for the treatment of bovine respiratory disease caused by Mannheimia haemolytica.

The volume of distribution of oxytetracycline varies markedly (0.32–18.5 l/kg) between animal species and in the different age groups within species2. Owing to their solubility in lipids, tetracyclines are capable of penetrating tissues and becoming widely distributed throughout the body. They have been shown to penetrate well into pulmonary and renal tissues, as well as into bronchial fluids. Concentrations within extracellular tissue fluids are expected to be similar or higher than those in the blood27. 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 MIC90 of tetracycline for the 10 selected isolates of MmmLC tested in this study was

<table>
<thead>
<tr>
<th>Antimicrobial drug</th>
<th>Mean (mm)</th>
<th>SEM*</th>
<th>Range</th>
<th>Zone diameter interpretive standard (NCCLS 1999)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>30.6</td>
<td>1.8</td>
<td>26.8–32.4</td>
<td>≥20</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>42.3</td>
<td>1.4</td>
<td>36.8–47.3</td>
<td>≥23</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>35.8</td>
<td>4.12</td>
<td>31.7–39.0</td>
<td>≥14</td>
</tr>
</tbody>
</table>

*Standard error of the mean.
This value is similar to the observations obtained in other studies13,17, in which a MIC\textsubscript{90} of 1.0 μg/ml was reported. The extensive distribution of oxytetracycline and its in vitro effect against Mmm LC also makes this agent a suitable candidate for the treatment of ulcerative balanitis and vulvitis.

Several investigators have evaluated the susceptibility of \textit{A. pyogenes} to different antimicrobial drugs17. The present study has shown that all the isolates were susceptible to oxytetracycline, enrofloxacin and tilmicosin. A study in Kenya revealed that \textit{A. pyogenes} was susceptible to oxytetracycline20. Tyllosin, erythromycin and enrofloxacin were also found to be effective against strains of \textit{A. pyogenes} isolated from bovines15.

Although data on the in vitro efficacy and in vitro breakpoints for mycoplasmas are incomplete, the MIC results of this study suggest that the 4 antimicrobial drugs will be effective in the treatment of Mmm LC infections of the genital tract of sheep. It should be borne in mind that although only 1 of the 4 antimicrobial drugs, namely oxytetracycline, is registered for use in small stock in South Africa, the other 3 have authorisation for use in other animal species such as cattle and pigs. Their use in small stock would therefore constitute extra-label use, which implies use of a drug in a manner or dosage different from the instructions on the manufacturer’s label. These drugs should therefore be used in small stock only by, or under the supervision of, a veterinarian.

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