Sensitivity of *Brucella* spp to tetracycline and its analogues

I. D. FARRELL, P. M. HINCHLIFFE, AND L. ROBERTSON

*From the Public Health Laboratory, Royal Infirmary, Meadow Street, Preston PR1 6PS*

**SYNOPSIS** The sensitivity to eight tetracyclines of 100 strains of brucellae, comprising strains of *Brucella abortus*, *Br. melitensis*, and *Br. suis*, was determined. Demethylchlortetracycline was the most effective against all the groups of brucellae, whereas oxytetracycline and chlortetracycline were the least effective. The mean MIC value for demethylchlortetracycline, doxycycline, lymecycline, and tetracycline was \( \leq 1 \mu g/ml \). Strains of *Br. abortus* biotype 2 and *Br. suis* were the most sensitive strains examined.

Tetracyclines are the most effective of the chemotherapeutic agents in the treatment of brucellosis (Spink, 1964; Rizzo-Naudi *et al.*, 1967). Robertson *et al.* (1973) have shown that strains of *Brucella abortus* are almost uniformly sensitive to tetracycline, and the minimum inhibitory concentration (MIC) values are between four and eight times less than the peak plasma levels. Steigbigel *et al.* (1968), in an extensive study, found significant differences in the *in vitro* antimicrobial activity of tetracycline and its various chemical analogues.

In this investigation the sensitivity of strains of *Br. abortus*, *Br. melitensis*, and *Br. suis* were determined to tetracycline and seven of its analogues. The ditch plate method, described by Robertson *et al.* (1973) as a suitable method for determining the MIC of various antibiotics to brucellae, was used after further preliminary investigation.

**Material and methods**

**BRUCELLA STRAINS**

The 100 strains of brucellae used in this study were isolated from human and animal sources. They were classified according to the criteria of Alton and Jones (1967) into 67 strains of *Br. abortus*, 23 strains of *Br. melitensis*, and 10 strains of *Br. suis*, and divided into their biotypes. The strains of *Br. abortus* were either isolated from cow's milk or cultured from the blood of patients with brucellosis in north-west Lancashire, the strains of *Br. melitensis* were isolated from patients infected abroad, and the strains of *Br. suis* were isolated from animal sources abroad.

The strains were grown on serum-dextrose (SD) agar, prepared from blood agar base No. 2 (Oxoid) incorporating 5% horse serum and 1% dextrose. All plates were incubated in air containing 10% CO\(_2\) at 37°C for three days.

**TETRACYCLINE SOLUTION**

Stock solutions of the following eight tetracyclines were prepared in 10% dimethylformamide (DMF): chlortetracycline, demethylchlortetracycline, doxycycline, lymecycline, methacycline, minocycline, oxytetracycline, and tetracycline. They were obtained as powders of known potency from Mast Laboratories, Liverpool.

The solutions were prepared as required and sterilized by membrane filtration.

**MINIMUM INHIBITORY CONCENTRATION OF THE CONTROL STRAIN**

The WHO prototype *Br. abortus* biotype 2 was used as the control strain and the MIC of each tetracycline was determined as follows:

A series of two-fold dilutions of each of the tetracyclines was prepared in SD agar plates ranging from 4 \( \mu g/ml \) to 0.1 \( \mu g/ml \). Control plates were prepared containing the highest proportion of DMF used in the antibiotic plates, that is, 1 in 200. A primary suspension of the control strain was prepared in 0.1% peptone water from a three-day SD agar plate to give approximately \( 5 \times 10^8 \) colony-forming units per ml.

The MIC was taken as the lowest concentration of antibiotic which completely inhibited growth or which allowed only a small number of discrete colonies to grow when the surface of the antibiotic
plate was inoculated with approximately 10^7
brucellae.

DITCH PLATE METHOD

Calibration of regression lines
SD agar was poured into 90 mm flat-bottomed
plastic Petri dishes, and, when set, a 15 mm wide
strip of agar was removed along the diameter of each
plate. The appropriate concentration of antibiotic
was added to molten SD agar at a temperature of
48° to 50° in a ratio of one part antibiotic to 19
parts of medium, and this SD-antibiotic agar was
used to fill the ditches.

The suspension of the control strain was inoculated
onto a series of ditch plates containing two-fold
dilutions in the range 160 μg/ml to 10 μg/ml of
oxytetracycline and 20 μg/ml to 0.6 μg/ml of
the other tetracyclines.

A regression line of the form y = mx + c, which
described the relation between the logarithm of
the concentration of antibiotic (y) and the size of
the inhibition zone in millimetres (x), was calculated
for each tetracycline by the least squares method
(Mosteller et al., 1973).

MIC of the 100 strains under test
All strains were inoculated onto two ditch plates
containing a high and low concentration of anti-
biotic respectively. This ensured that, whatever the
sensitivity of the strain, a measurable zone would
be obtained on at least one of the ditch plates. The
concentrations in the ditches were 20 μg/ml and 10
μg/ml for chlortetracycline, oxytetracycline, and
methacycline and 10 μg/ml and 1.25 μg/ml for the
other tetracyclines.

Five brucella strains were inoculated onto each half
of the ditch plate by streaking a loopful of inoculum
at right-angles to the ditch from the plate periphery
to the nearside edge of the ditch. Thus each ditch
plate would accommodate 10 strains without mutual
interference between adjacent strains growing on the
ditch plate.

The MIC for the test strains was calculated using a
sensitivity factor, as previously described by
Robertson et al. (1973), as follows:

\[ \text{MIC test strain} = \text{MIC control strain} \times \]
\[ \text{sensitivity factor.} \]

The sensitivity factor was obtained by subtracting
the zone of inhibition of the test strain from that of
the control strain, multiplying by the slope (m) of
the regression equation, and taking the anti-
logarithm.

The MIC values obtained by the conventional
agar dilution procedure were compared with those
obtained by the ditch plate method for 25 strains
of brucellae. Both methods gave comparable results
and confirmed that the ditch plate method was
appropriate for the present investigation.

Results

The extremes and mean of the MIC values of each
tetracycline are shown in the table for the brucella
strains classed according to their species and bio-
type. These figures indicate that strains of Br.
abortus biotype 2 and Br. suis are the most sensitive
to all eight antibiotics, while for the remaining bio-
types the mean MIC values are similar for any one of
the antibiotics. The efficacy of the tetracyclines
varied significantly in vitro; oxytetracycline and
chlortetracycline were the least effective of the tet-
rcyclines, even for the more susceptible strains.
Demethylchlortetracycline was the most effective
agent in that the mean MIC was the lowest for each

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biotyp</th>
<th>No. of</th>
<th>Range and mean^1 of MIC values (μg/ml) of:</th>
<th>Chlor</th>
<th>Demeth</th>
<th>Doxy</th>
<th>Lytme</th>
<th>Metha</th>
<th>Mino</th>
<th>Oxytet</th>
<th>Tetra</th>
<th>HSD^4</th>
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<td>0-2-05</td>
<td>0-2-20</td>
<td>0-3-12</td>
<td>0-4-30</td>
<td>0-5-25</td>
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<td>2</td>
<td>17</td>
<td>0-8-1-6</td>
<td>0-04-02</td>
<td>0-09-09</td>
<td>0-2-04</td>
<td>0-1-04</td>
<td>0-1-08</td>
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<td>0-12</td>
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Table  Sensitivity of 100 brucella strains to eight tetracyclines

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^1The mean is shown in parentheses.

^4HSD = Honestly significant difference; this allows a pair-wise comparison of the mean MIC values for each of the tetracyclines (Haber and Runyon, 1973).
Sensitivity of Brucella spp. to tetracycline and its analogues

The cumulative total of brucella strains sensitive to increasing concentrations of the eight antibiotics is depicted in the figure. It can be seen that the tetracyclines form two groups: the first group includes demethylchlortetracycline, lymecycline, doxycycline, tetracycline, tetracycline, and minocycline, and the second includes chlortetracycline and oxytetracycline, methacycline occupying an intermediate position. In the first group, the concentration of antibiotic to which 50% of the strains were sensitive ranges from 0.2 μg/ml (demethylchlortetracycline) to 0.65 μg/ml (tetracycline), whereas in the second group the range was 2.9 μg/ml (chlortetracycline) to 3.7 μg/ml (oxytetracycline).

Discussion

Tetracyclines are still the chemotherapeutic agents of choice in the treatment of brucellosis (Garrod et al., 1973) with the possible exception of co-trimoxazole (Hassan et al., 1971; Giunchi et al., 1971). In this investigation we have found that the efficacy of the tetracyclines varies significantly in vitro. The method of analysis used was that of one-way analysis of variance, whereby comparison is made between each tetracycline for replicate strains from one brucella type. The honestly significant difference (HSD) is shown in the table for each brucella group. Oxytetracycline and chlortetracycline were the least effective of the tetracyclines, even for the more susceptible strains of Br. abortus biotype 2 and Br. suis. Demethylchlortetracycline was the most effective agent in that the mean MIC value was the lowest for each group of brucella examined. The mean MIC values for lymecycline, doxycycline, and tetracycline were always < 1 μg/ml, whereas methacycline and minocycline appear to hold an intermediate position. The comparative performance of each tetracycline within the entire brucella sample examined is shown graphically in the figure.

The interpretation of in-vitro antibiotic susceptibilities for the in-vivo clinical situation is complex. The albumin fraction of plasma certainly binds many antibacterial agents, and similar reversible binding may occur with proteins of various tissues and with non-protein macromolecules. Plasma binding can be measured experimentally but results vary. Kunin (1967) reported that tetracycline and demethylchlortetracycline were 24% and 41% protein bound respectively, whereas other workers have reported corresponding figures of 65% and 90% respectively (Kucers, 1972). Plasma binding is measured in an in-vitro environment which is comparatively static, and often concentrations of antibiotic in excess of expected therapeutic levels are used. However, the in-vivo situation is a complex dynamic system of many competitive activities. The importance of the
reversibility of plasma binding is reflected in the undoubted effectiveness of agents such as cloxacinil which are known to be highly protein bound. We have chosen to examine the in-vitro efficacy of the various tetracyclines on a weight-to-weight basis and have not attempted to interpret our findings in the light of reported values for serum binding of tetracyclines.

Oxytetracycline was the least effective in our experiments, and chlortetracycline, perhaps because of its instability in vitro, gave similar results. Some of the more recent tetracyclines compared favourably with tetracycline itself and could have a place in the therapy of brucella infections. It is difficult to select any one of this group as the best for clinical use: lymecycline has not had extensive clinical trials, and the reported incidence of photosensitization and gastrointestinal disturbance associated with demethylchlortetracycline may be a disadvantage despite its effectiveness against brucellae. On the other hand, doxycycline and minocycline may be of value because of their slow rate of excretion, which results in prolonged plasma levels.

We are most grateful to Mrs H. E. Tillett for advice and the statistical analysis of our results. We are indebted to Miss A. V. Holt for excellent experimental assistance.

Appendix

Sensitivity factor

For any one tetracycline concentration let the inhibition zone of the control strain be $S$ millimetres and the zone of the test strain be $T$ millimetres. Then, substituting in the regression equation, the logarithm ($y$) of this tetracycline concentration is $mS + c$.

The sensitivity factor is

$$\frac{C_t}{C_s}$$

where $C_t$ = the concentration of tetracycline required to give a zone of $T$ mm with the test strain, which is the same as that concentration giving a zone of $S$ mm with the control strain, namely, ANTILOG $y$, where $y = mS + c$;

and $C_s$ = the concentration of tetracycline required to give a zone of $T$ mm with the control strain, ie, ANTILOG $y$, where $y = mT + c$.

Therefore

$$\frac{C_t}{C_s} = \frac{\text{ANTILOG} (mS + c)}{\text{ANTILOG} (mT + c)} = \frac{\text{ANTILOG} [(mS + c) - (mT + c)]}{\text{ANTILOG} m (S - T)}$$

Thus $\text{MIC test strain} = \text{ANTILOG} m(S - T) \times \text{MIC control strain}$.

Therefore $\text{MIC test strain} = \text{ANTILOG} m(S - T)$.

The following equations obtained for each of the tetracyclines with the WHO prototype $\text{Br. abortus}$ biotype 2 were as follows:

- **Chlortetracycline**
  \[ y = 0.1 \times x - 0.9 \]

- **Demethylchlortetracycline**
  \[ y = 0.1 \times x - 1.6 \]

- **Doxycycline**
  \[ y = 0.2 \times x - 3.5 \]

- **Lymecycline**
  \[ y = 0.16 \times x - 1.2 \]

- **Methacycline**
  \[ y = 0.17 \times x - 1.7 \]

- **Minocycline**
  \[ y = 0.2 \times x - 2.4 \]

- **Oxytetracycline**
  \[ y = 1.12 \times x - 0.6 \]

- **Tetracycline**
  \[ y = 0.1 \times x - 0.97 \]

References