Antimicrobial Activity of Tulathromycin and 14 Other Antimicrobials Against Virulent *Rhodococcus equi* In Vitro*

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**CLINICAL RELEVANCE**

This study determined the antimicrobial activity of tulathromycin against *Rhodococcus equi* in vitro. Ninety-eight virulent isolates of *R. equi* from equine clinical cases were examined, of which 20 isolates were macrolide resistant. A custom 96-well antimicrobial susceptibility testing plate was used, allowing 14 additional antimicrobials to be tested against *R. equi*. Isolates were cultured with various concentrations of antimicrobials, and minimal inhibitory concentration (MIC) values were determined. Tulathromycin was found to have poor activity in vitro against *R. equi* isolates susceptible or resistant to macrolides, with MIC<sub>50</sub> and MIC<sub>90</sub> values >64 μg/mL for all isolates. MIC values for other macrolides tested were similar to previously published data.

*The costs associated with this study were covered by funds from the Link Equine Research Endowment.
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Rhodococcus equi bronchopneumonia is a well-recognized cause of morbidity and mortality in foals 3 weeks to 6 months of age. Successful antimicrobial therapy is required for foal survival. Therapy with orally administered macrolide antimicrobials has greatly improved survival rates for foals with R. equi pneumonia. However, azithromycin, clarithromycin, and erythromycin, the macrolides most commonly used for treatment, require oral dosing more than once daily, which can be labor-intensive and have a negative effect on compliance. Thus, a treatment with a longer dosing interval would be highly desirable, particularly for farms with large numbers of affected foals.

In addition, evidence of R. equi isolates resistant to the commonly used macrolides (azithromycin, clarithromycin, and erythromycin) as well as rifampin is emerging. The overall prevalence of R. equi isolates resistant to macrolides or rifampin has been reported as 4%. Foals infected with macrolide-resistant R. equi isolates have a significantly lower survival rate (25% survival) than foals infected with macrolide-susceptible isolates of R. equi (70% survival). As a result, evaluation of other treatment options for macrolide-resistant isolates of R. equi is needed. In addition, it is important to evaluate the extent to which resistant isolates are also resistant to newer classes of macrolides.

Tulathromycin is a semisynthetic veterinary macrolide approved for the treatment of bovine and porcine respiratory disease. It is an injectable antimicrobial with a long elimination half-life (7.6 days in the lung tissue of cattle and 6 days in the lung tissue of swine); thus, a single dose provides approximately 1 week of coverage in these food animal species. Tulathromycin is specifically labeled for the treatment of respiratory disease associated with Mannheimia haemolytica, Pasturella multocida, Histophilus somni, Actinobacillus pleuropneumoniae, Bordatella bronchiseptica, and Haemophilus parasuis, all of which are gram-negative organisms. Tulathromycin has been shown to be rapidly absorbed, widely distributed, and slowly eliminated when administered intramuscularly in foals. In addition, tulathromycin accumulates in bronchoalveolar cells of foals. Tulathromycin has been used for the treatment of pulmonary abscesses in foals and appeared to be well tolerated. However, the lack of in vitro susceptibility data against equine respiratory pathogens prevents the rational use of tulathromycin for the treatment of R. equi pneumonia in foals. The objective of this study was to determine the in vitro antimicrobial activity of tulathromycin against multiple strains of R. equi, including strains resistant to macrolides. A secondary objective was to determine the in vitro activity of 14 other antimicrobials against R. equi.

### MATERIALS AND METHODS

A total of 98 virulent isolates of R. equi collected from clinically affected foals between January 1, 1998, and December 31, 2008, was selected for the study, with 20 isolates identified as resistant to the macrolides azithromycin, clarithromycin, or erythromycin (hereafter termed macrolide-resistant organisms). All isolates had previously been identified as virulent R. equi by using a multiplex polymerase chain reaction assay that simultaneously detects R. equi and the vapA gene. Before susceptibility testing, all isolates were tested for purity and identified by morphologic characteristics. Seventy-eight isolates were obtained from a repository maintained in the Equine Infectious Disease Laboratory at Texas A&M University, and 20 isolates were contributed by Dr. Giguère, including 19 macrolide-resistant isolates described elsewhere.

Isolates of R. equi were tested for their suscep-
The objective of this study was to determine the in vitro antimicrobial activity of tulathromycin against multiple strains of Rhodococcus equi.

suspended in sterile water to turbidity equal to a 0.5 McFarland standard. Ten microliters of the suspension were transferred into 10 mL of cation-adjusted Mueller-Hinton broth (CAMHB; TREK Diagnostic Systems, Cleveland, OH) for a final bacterial concentration of $1 \times 10^5$ colony-forming units (cfu)/mL. Each well of the plate was then inoculated with 100 μL of CAMHB. Positive (antimicrobial-free) and negative (sterile CAMHB) control wells were included on each plate. The plates were sealed with adhesive covers and incubated in room air at 35°C. The plates were read manually following 24 and 48 hours of incubation, with each well recorded as visible growth or no growth. Although 24 hours is the standard incubation time for most MIC protocols, the plates were also read after 48 hours due to the relatively slow growth of the R. equi organism. Plates were considered valid only if there was sufficient growth in the positive control well.

Control strains used to validate the assay at weekly intervals included Staphylococcus aureus ATCC 29213 and R. equi ATCC 33701. S. aureus ATCC 29213 was selected as a control strain because it is the standard gram-positive indicator organism for the veterinary susceptibility plates used for this study. Reference MICs were available for the S. aureus ATCC 29213 control isolate from the Clinical and Laboratory Standards Institute (CLSI). For the R. equi ATCC 33701 isolate, the MICs of azithromycin, ceftiofur, clarithromycin, doxycycline, enrofloxacin, erythromycin, gentamicin, rifampin, and vancomycin were also determined using a macrodilution broth dilution technique as previously published and in accordance with the guidelines established by the CLSI. This served as an additional step for the validation of the custom-ordered veterinary susceptibility plates. For the broth dilution, a standard inoculum of $5 \times 10^5$ cfu/mL was used for each isolate. For each antimicrobial agent, concentrations tested represented twofold dilutions between 256 and 0.03 μg/mL. All MIC determinations were performed in triplicate, and the median value was used. MIC was determined as the first dilution with no bacterial growth after 24 hours of incubation at 37°C. Control strains used to validate the broth macrodilution assay were S. aureus ATCC 29213, Escherichia coli ATCC 25922, and Enterococcus faecalis ATCC 29212.

The MIC for each isolate was defined as the lowest concentration of an antimicrobial to
completely inhibit visible growth in the well. The MIC required to inhibit growth of 50% of isolates (MIC<sub>50</sub>) and the MIC required to inhibit growth of 90% of isolates (MIC<sub>90</sub>) were determined for both macrolide-susceptible and macrolide-resistant isolates. MIC<sub>50</sub> values were compared between macrolide-resistant and macrolide-sensitive isolates at each time point (i.e., 24 and 48 hours’ incubation) using the Wilcoxon rank sum test. Because of the multiplicity of comparisons, an overall type I error rate (P value) of 0.05 was applied, using the method of Bonferroni. Comparisons were made for 15 antimicrobials; therefore, the Bonferroni-adjusted threshold of significance for an individual comparison became P &lt;(0.05/15) = P &lt;0.0033.

**RESULTS**

Of the 98 *R. equi* isolates tested, results for two isolates (one macrolide susceptible and one macrolide resistant) were excluded because bacterial growth was identified in the negative control well for each isolate after 48 hours of incubation. As a result, 19 macrolide-resistant and 77 macrolide-susceptible isolates were used to determine MIC values. In addition, *S. aureus* 29213 and *R. equi* 33701 were tested as
control strains. The MICs obtained for *S. aureus* 29213 were within the reference range proposed by the CLSI.\textsuperscript{13} The MICs obtained for *R. equi* 33701 were similar to MICs determined using a macrodilution broth dilution technique as previously published.\textsuperscript{12} Table 1 indicates the concentrations of antimicrobials that were evaluated and the MIC results for the control strains.

Tulathromycin was found to have poor in vitro activity against *R. equi*. The MIC\textsubscript{50} and MIC\textsubscript{90} were >64 μg/mL for both macrolide-susceptible and macrolide-resistant isolates of *R. equi* (Tables 2 and 3). The MIC values of tulathromycin for the 77 macrolide-susceptible isolates were as follows: after 24 hours of growth, two isolates had an MIC of 32 μg/mL, 27 isolates had an MIC of 64 μg/mL, and 48 isolates had an MIC >64 μg/mL. All macrolide-susceptible isolates had an MIC >64 μg/mL after 48 hours of growth. All macrolide-resistant isolates had an MIC >64 μg/mL regardless of growth time. Although the MIC\textsubscript{50} and MIC\textsubscript{90} values for tulathromycin were identical after 24 and 48 hours of growth, the MIC values for tulathromycin were significantly (*P* = .0015; Bonferroni-adjusted significance, *P* < .0033) lower for macrolide-suscep-
tible isolates than for macrolide-resistant isolates after 24 hours of growth. However, the MIC of all isolates was >64 μg/mL after 48 hours of growth, such that there was no significant difference between the MICs for tulathromycin between macrolide-resistant and macrolide-susceptible isolates after 48 hours.

The MIC values for all antimicrobials after 24 hours and 48 hours of growth are listed in Tables 2 and 3, respectively. In general, the MIC values after 48 hours of growth were one concentration higher (i.e., one lower dilution) than the MIC values after 24 hours of growth. When comparing MIC data between groups after 24 hours of growth, azithromycin, chloramphenicol, clarithromycin, doxycycline, erythromycin, penicillin, rifampin, tilmicosin, and trimethoprim–sulfamethoxazole had significantly (P < .0033, the Bonferroni threshold) higher MICs for macrolide-resistant isolates than for macrolide-susceptible isolates. Although not significant with a Bonferroni adjustment, MIC values for ceftiofur (P = .0119) and vancomycin (P = .0426) tended to be higher for macrolide-resistant isolates after 24 hours of growth. MIC values for enrofloxacin, gentamicin, and linezolid were not significantly different between macrolide-resistant and

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### TABLE 3. MIC<sub>50</sub> and MIC<sub>90</sub> After 48 h Incubation for All Rhodococcus equi Isolates

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Macrolide Susceptible</th>
<th>Macrolide Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt; (μg/mL)</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt; (μg/mL)</td>
</tr>
<tr>
<td>Tulathromycin</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>&gt;8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Linezolid</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Penicillin</td>
<td>&gt;16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Rifampin</td>
<td>≤0.5</td>
<td>≤0.5</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>TMPS</td>
<td>2/38</td>
<td>4/76</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

*TMPS = trimethoprim–sulfamethoxazole.

<sup>a</sup>The MIC<sub>50</sub> was significantly higher for macrolide-resistant isolates than for macrolide-susceptible isolates. Bonferroni-adjusted significance = P < 0.033.
macrolide-susceptible isolates after 24 hours of growth. The MIC data after 48 hours of growth showed similar results, with azithromycin, chloramphenicol, clarithromycin, doxycycline, erythromycin, rifampin, tilmicosin, and trimethoprim–sulfamethoxazole having significantly ($P < .0033$) higher MICs for the macrolide-resistant isolates compared with the macrolide-susceptible isolates. Although not significant, MICs for ceftiofur ($P = .0092$), penicillin ($P = .0063$), and vancomycin ($P = .0098$) tended to be higher for macrolide-resistant isolates after 48 hours of growth. The MIC values for enrofloxacin, gentamicin, and linezolid were not significantly different between macrolide-resistant and macrolide-susceptible isolates after 48 hours of growth.

Azithromycin, clarithromycin, doxycycline, enrofloxacin, erythromycin, gentamicin, linezolid, rifampin, and vancomycin showed high in vitro activity against at least 90% of macrolide-susceptible \textit{R. equi} isolates. Enrofloxacin, gentamicin, linezolid, and vancomycin were highly active in vitro against at least 90% of macrolide-resistant \textit{R. equi} isolates.

**DISCUSSION**

The rational use of tulathromycin for the treatment of \textit{R. equi} bronchopneumonia in foals has been hindered by the lack of in vitro susceptibility data. In the present study, tulathromycin was found to be inactive against \textit{R. equi} in vitro. While in vitro susceptibility does not necessarily equate to clinical efficacy, the MIC$_{90}$ of tulathromycin determined in this study was more than 100-fold above achievable concentrations of tulathromycin in serum and bronchoalveolar cells following intramuscular administration in foals. Thus, despite results suggesting that tulathromycin may be clinically effective for treating abscessing pneumonia in foals, our results indicate that tulathromycin should not be used for the treatment of \textit{R. equi} bronchopneumonia in foals. Tulathromycin may have appeared successful in the treatment of abscessing foal pneumonia because the treated foals may have been infected with bacteria other than \textit{R. equi}, against which tulathromycin could have been effective. It is also possible that some of the treated foals with sonographic pulmonary abnormalities may have recovered spontaneously without treatment.

Tulathromycin is useful for the treatment of bovine and porcine respiratory disease associated with gram-negative respiratory pathogens, such as \textit{Pasteurella}, \textit{Mannheimia}, \textit{Actinobacillus}, and \textit{Histophilus} spp. \textit{R. equi} is a gram-positive, intracellular organism, so it is not entirely surprising that tulathromycin is less effective in vitro than other macrolides that possess a more gram-positive spectrum (azithromycin, clarithromycin, and erythromycin). Despite the altered spectrum, the macrolide-resistant isolates of \textit{R. equi} were more resistant to tulathromycin after 24 hours of growth, suggesting that the mechanism of resistance to tulathromycin may be similar to that for other classes of macrolides. Although the exact mechanism of macrolide resistance in \textit{R. equi} is unknown, the primary mechanisms for bacterial resistance to macrolides are modification of the ribosomal target site by methylation and active efflux by transporter proteins. Bacterial resistance via these mechanisms is thought to induce resistance to all classes of macrolides.

Most other antimicrobials tested in the study had MIC values similar to previously reported values. The MIC data for other macrolides (azithromycin, clarithromycin, erythromycin, and tilmicosin) were similar to previously published values, as were the MIC data for linezolid. The MIC values for chloramphenicol, doxycycline, enrofloxacin, gentamicin, rifampin, trimethoprim–sulfamethoxazole were also similar to previously published values.
methoxazole, and vancomycin were also similar to previously published values.\textsuperscript{17,19} The MIC\textsubscript{50} and MIC\textsubscript{90} values for the β-lactam antimicrobials (ceftiofur and penicillin) in this study were considerably higher than previously reported data.\textsuperscript{17,19} The reason for this discrepancy is not known. This may have been the result of using a different testing procedure to determine in vitro susceptibility, although the control strains and other antimicrobials had results similar to previously published values.

The macrolide-resistant isolates were found to be resistant to all classes of macrolides tested, and many were resistant to rifampin as well. The only antimicrobials found to be effective in vitro against macrolide-resistant \textit{R. equi} were enrofloxacin, gentamicin, linezolid, and vancomycin. The use of linezolid and vancomycin in veterinary species is not encouraged due to the concern of emerging microbial resistance to these agents in humans. Enrofloxacin administration is not without risk because its use can cause articular cartilage damage in foals.\textsuperscript{20} Gentamicin has been found to be ineffective for the treatment of \textit{R. equi} in vivo, most likely due to poor intracellular concentrations as well as decreased effectiveness in the presence of cellular debris or an acidic environment, such as a pulmonary abscess.\textsuperscript{21}

Although 24 hours is the standard incubation time for most MIC protocols, MIC values were also determined after 48 hours because of the relatively slow growth of the \textit{R. equi} organism.\textsuperscript{11} In general, the MIC values after 48 hours of growth were one concentration higher (i.e., one lower dilution) than the MIC values after 24 hours of growth. This is not surprising, given that additional bacterial growth would be expected with the longer incubation time. When comparing significant differences in MIC results between macrolide-resistant and macrolide-susceptible strains, the MICs for all antimicrobials except tulathromycin remained significantly different after both 24 and 48 hours of growth. The statistically significant difference in the MIC values for tulathromycin between macrolide-resistant and macrolide-susceptible isolates after 24 hours of growth was no longer apparent after 48 hours. This was likely attributable to the high MIC values of tulathromycin against \textit{R. equi} and the fact that after 48 hours of growth all isolates in both groups were found to have an MIC exceeding the highest dilution (i.e., >64 μg/mL). Thus, the absence of statistical significance may have resulted from the range of concentrations studied rather than lack of an actual difference between MIC values for the macrolide-resistant and macrolide-susceptible isolates.

This study has a number of limitations. The range of concentrations selected for tulathromycin was based in large part on previously reported serum and bronchoalveolar lavage fluid cell concentrations.\textsuperscript{6} Using higher concentrations of tulathromycin would have allowed for better characterization of the MIC\textsubscript{50} and MIC\textsubscript{90} of this drug for \textit{R. equi}. However, such extreme concentrations would not be clinically relevant. This study did not evaluate the efficacy of tulathromycin against other equine respiratory pathogens because of budgetary constraints. Tulathromycin may be effective for the treatment of other equine respiratory pathogens, but further study would be required before recommending tulathromycin as an empirical treatment for equine respiratory disease. In addition, the sample size of 98 \textit{R. equi} isolates is modest. The sample size was determined by the cost of custom susceptibility plates and funds available for this project. Nevertheless, to our knowledge, this is the first study to evaluate the susceptibility of \textit{R. equi} to tulathromycin. While the study is not without shortcomings, none of these limitations vitiates the clinical importance of the results.
CONCLUSION

Based on the results of this study, tulathromycin has poor efficacy against \textit{R. equi} in vitro. The MIC\textsubscript{90} of tulathromycin for both macrolide-susceptible and macrolide-resistant isolates of \textit{R. equi} was more than 100-fold above achievable concentrations of tulathromycin in serum and bronchoalveolar cells following intramuscular administration in foals.\textsuperscript{6} Thus, our in vitro findings do not support the use of tulathromycin for the treatment of \textit{R. equi} bronchopneumonia in foals.

REFERENCES