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Short communication

In vitro antimicrobial activity of gallium maltolate against virulent *Rhodococcus equi*

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ABSTRACT

The objective of this study was to determine the *in vitro* antimicrobial activity of gallium maltolate (GaM) against *Rhodococcus equi*. A total of 98 virulent bacterial isolates from equine clinical cases were examined, of which 19 isolates were known to be resistant to macrolides and rifampin. Isolates were cultured with various concentrations of GaM and minimal inhibitory concentration (MIC) values were determined after 24 and 48 h. Both the MIC₅₀ and the MIC₉₀ after 24 h of growth were 558 ng/mL (8 μM) and after 48 h of growth were 2230 ng/mL (32 μM). There were no apparent differences between MICs of macrolide-resistant and macrolide-susceptible isolates.

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1. Introduction

Rhodococcus equi, a soil-saprophytic, Gram-positive, facultative-intracellular bacterium, replicates in and destroys macrophages. It primarily causes severe pneumonia in foals as a result of pyogranulomatous lesions in the lungs (Prescott, 1987; Ainsworth et al., 1998; Cohen et al., 2000). Pulmonary disease is most common; however, extrapulmonary infection and immune-mediated inflammatory disorders also occur (Reuss et al., 2009). Disease caused by *R. equi* is economically important to the equine breeding industry with high morbidity and mortality rates (Ainsworth et al., 1998).

Treatment of *R. equi* pneumonia is frequently prolonged, costly, and not always successful (Ainsworth et al., 1998). The combination of rifampin and macrolides, including erythromycin, clarithromycin, and azithromycin, has become the preferred treatment for treating *R. equi* infections in foals (Giguère, 2001). Macrolide resistance with a prevalence of 4% has been documented for isolates of *R. equi* obtained from foals (Giguère et al., 2008). Foals infected with these resistant isolates have a significantly greater case-fatality rate compared to foals infected with susceptible isolates (Giguère et al., 2008).

Because of the threat of macrolide and rifampin resistance, there is a need to explore alternative antimicrobial agents for treatment of foals infected with *R. equi*. Gallium, a trivalent semimetal with characteristics similar to Fe³⁺, has been used to exploit the iron dependency and inhibit bacterial growth of various microorganisms, including *R. equi*. Gallium binds to plasma transferrin, from which it can be taken up by macrophages, the target cell of the *R. equi* bacterium (Bernstein et al., 2000;

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Olakanmi et al., 2000; Harrington et al., 2006). Gallium competes with Fe^{3+} for uptake by intracellular bacteria. The trivalent gallium is incorporated into and consequently inactivates iron-dependent metabolic and reproductive enzyme pathways. When administered orally to mice, GaM is readily absorbed and reduces tissue concentrations of *R. equi* (Martens et al., 2007b). With the ability to decrease intercellular concentrations *in vitro* (Martens et al., 2007a) and safety after oral administration in foals (Martens et al., 2007b), the use of GaM as a treatment for *R. equi* infection has shown some promise.

Minimum inhibitory concentrations (MICs) of gallium against *R. equi* have not yet been determined. Thus, the primary objective of this study was to determine minimum inhibitory concentrations of GaM against both macrolide-resistant and macrolide-susceptible isolates of *R. equi*, and to determine if there was a difference between the susceptible and resistant isolates.

2. Materials and methods

The MICs of GaM were evaluated for 98 randomly selected virulent isolates of *R. equi* after 24 and 48 h of incubation. All isolates of *R. equi* were obtained from clinical specimens of infected foals collected from 1998 to 2008. A majority of the isolates were selected from a repository maintained in the Equine Infectious Disease Laboratory at Texas A&M University and the macrolide and rifampin-resistant isolates were previously described (Giguère et al., 2008). Virulence was defined as positive results of multiplex PCR with primers specific for *R. equi* and for the gene encoding the virulence-associated protein A (*vap A*) (Halbert et al., 2005). All isolates were tested for purity and identified by morphologic characteristics. Of the 98 isolates, 19 were identified as resistant to the macrolides azithromycin, clarithromycin, and erythromycin as well as rifampin (hereafter termed macrolide and rifampin-resistant organisms) and 79 were defined as macrolide and rifampin-susceptible, on the basis of MIC test results and guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2009). Comparisons of the MICs between macrolide and rifampin-resistant and macrolide and rifampin-susceptible isolates were made using the Wilcoxon rank-sum test; a significance level of $p < 0.05$ was used.

Mean inhibitory concentrations were determined by the macrodilution method. Fresh isolates were grown in minimal media without iron (Robinson, 1982) for 24 h at 37 °C. *R. equi* colonies were suspended in sterile water to spectrophotometrically achieve an OD (625 nm) of 0.08–0.10, an optically comparable turbidity to that of a 0.5 McFarland standard. The suspension contained approximately 1.28×10^7 colony forming units (CFU)/mL of *R.*

equi. Immediately after preparation, the inoculum suspension was diluted using minimal media without iron to achieve 5×10^5 CFU/mL in each tube to be used with each GaM concentration. GaM was dissolved in iron-free minimal media using 2-fold serial dilutions to obtain an initial concentration of 8922 ng/mL (128 μM) and a final concentration of 279 ng/mL (4 μM). The diluted inoculum was then added to each of the GaM suspensions. A positive control tube of iron-free minimal media without GaM was made for each organism tested and a negative control tube without inoculum was made for each dilution. The inoculated tubes were briefly vortexed and incubated at 37 °C on a shaker. The tubes were read manually following 24 h of incubation and recorded as growth or no growth.

The experimental samples were considered valid only if sufficient growth was noted in the control sample. The MIC for each isolate was defined as the lowest concentration of GaM to completely inhibit visible growth. The MIC required to inhibit growth of 50% of the isolates (MIC₅₀) and 90% of the isolates (MIC₉₀) was determined for macrolide and rifampin-susceptible and macrolide and rifampin-resistant isolates.

3. Results

A total of 98 *R. equi* isolates were evaluated including 79 macrolide and rifampin-susceptible and 19 macrolide and rifampin-resistant isolates. Both the MIC₅₀ and the MIC₉₀ for GaM against the 98 isolates at 24 h were 558 ng/mL (8 μM); the range was from 279 to 1115 ng/mL (4–16 μM) with a standard deviation of 125.5. MICs at 48 h of incubation were uniformly higher (data not shown).

There was no significant difference between macrolide and rifampin-resistant and macrolide and rifampin-susceptible isolates in MIC values of GaM at 24 h ($p = 0.172$). After 24 h, Both the MIC₅₀ and the MIC₉₀ for GaM against the 19 macrolide and rifampin-resistant isolates and the 79 macrolide and rifampin-susceptible isolates were 558 ng/mL (8 μM); the MIC after 24 h was 558 ng/mL (8 μM) for each resistant isolate, and the range of the MICs after 24 h for the 79 susceptible isolates was from 279 to 2230 ng/mL (4–32 μM) with a standard deviation of 139.5. (Table 1). There was no significant difference in MIC values of GaM between macrolide and rifampin-resistant and macrolide and rifampin-susceptible isolates at 48 h (data not shown).

4. Discussion

Strategies to control or prevent *R. equi* pneumonia in foals on endemic farms are limited. As several other

Table 1
MIC data for gallium maltolate against *R. equi*.

Isolates	MIC ₅₀ (ng/mL)	MIC ₉₀ (ng/mL)	Range (ng/mL)	Standard deviation
All isolates (98 total)	558	558	279–1115	125.5
Macrolide and rifampin-resistant isolates (19 total)	558	558		0
Macrolide and rifampin-susceptible isolates (79 total)	558	558	279–1115	139.5

bacterial diseases have been inhibited by exploiting the iron dependency of the pathogenic organism (Byrd and Horwitz, 1991; Bernstein et al., 2000), the use of GaM for the treatment or prevention of *R. equi* has been proposed. The primary objective of this study was to provide *in vitro* susceptibility data of GaM against *R. equi*. In the study, both the MIC₅₀ and the MIC₉₀ after 24 h of growth were 558 ng/mL (8 μM). There were no apparent differences between macrolide and rifampin-resistant and macrolide and rifampin-susceptible isolates.

Recent studies have proposed a serum concentration of gallium of 700 ng/mL (10 μM) to be used as a target for therapeutic use against pneumonia caused by *R. equi* in foals (Martens et al., 2007a). In one study, a serum gallium concentration of 700 ng/mL reduced *R. equi* tissue burdens by approximately 90% in mice treated prophylactically with GaM and experimentally infected with virulent *R. equi* (Harrington et al., 2006). In an evaluation of the pharmacokinetics of GaM in foals, a serum gallium concentration of 700 ng/mL was achievable following intragastric administration (Martens et al., 2007a). In the current study, both the MIC₅₀ and the MIC₉₀ after 24 h were 558 ng/mL (8 μM), which is in range of concentrations achieved in plasma following intragastric administration of GaM. However, *in vitro* susceptibility does not necessarily equate to clinical efficacy. The therapeutic effectiveness of gallium against *R. equi* is largely dependent upon the achieved concentration in infected tissues, primarily macrophages. While the MIC data reported here do not tell us about intracellular concentrations, it is expected that gallium would concentrate in inflammatory cells such as macrophages (Olanmi et al., 2000).

The emergence of *R. equi* isolates that are resistant to macrolides and rifampin is a growing concern (Giguère et al., 2008). Most likely, many factors may have contributed to the development of these resistant strains. The use of macrolides for the treatment of *R. equi* has been long-standing and widespread. Additionally, many breeding farms affected by *R. equi* foal pneumonia have implemented screening tests such as serial monitoring of results of white blood cell concentrations (Giguère et al., 2003) or thoracic ultrasound examinations (Slovic et al., 2005) in an effort to detect and initiate early therapy of foals with subclinical disease. Because foals with positive screening tests may be treated with macrolides, the practice of screening likely increases the number of subclinical foals that are treated. It is plausible that increased use of macrolides will increase pressures for development of resistance of *R. equi* to macrolides, increasing the need for alternative therapies. In this study, there was no significant difference in MICs of GaM for macrolide and rifampin-resistant and macrolide and rifampin-susceptible isolates. Recently, GaM failed to be effective for chemoprophylaxis against *R. equi* pneumonia when administered once daily (~30 mg/kg; PO) for the first 2 weeks of life (Chaffin et al., 2009). Nevertheless, further evaluation of GaM as a treatment against clinical or subclinical disease by *R. equi* is warranted.

This study is not without limitations. The study only evaluated the activity of GaM against *R. equi*; however, many other bacterial organisms have been shown to cause pneumonia in foals. GaM might be effective for the treatment of other respiratory pathogens of foals. Importantly, this study evaluated the activity of GaM *in vitro*, which may not correlate with efficacy *in vivo*. The sample size of 98 *R. equi* isolates is modest. None of these limitations, however, vitiates the clinical importance of the results.

To the researchers' knowledge, this study is the first providing data regarding the antimicrobial activity of GaM against virulent *R. equi*. Further evaluation of GaM as a therapeutic agent, either as monotherapy or in conjunction with other antimicrobial agents, is warranted.

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