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Antimicrobial activity of gallium against virulent *Rhodococcus equi in vitro* and *in vivo*

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Rhodococcus equi, a facultative intracellular bacterium, causes severe pneumonia in foals. Evidence suggests that most foals become infected very early in life, when they have immature or ineffective innate immune responses. This study evaluated the antimicrobial activity of gallium against *R. equi*, as a potential chemoprophylactic and therapeutic agent. *Rhodococcus equi* was grown in media with various concentrations of gallium nitrate (GN), with and without excess iron. GN significantly inhibited growth and killed *R. equi*, and these effects were abolished with excess iron. Antimicrobial effects of Ga appear to be related to its interference with iron metabolism. Mice were treated orally with gallium maltolate (GaM), 10 or 50 mg/kg BW, or distilled H_2O prior to and after experimental infection with *R. equi*. Six days post-infection, organs were harvested and *R. equi* concentrations assessed, and serum gallium concentrations determined. GaM was absorbed in a dose-dependent manner, and *R. equi* tissue burdens were greater in control mice than in all GaM-treated mice. GaM may aid in the control of disease by preventing development of overwhelming *R. equi* tissue burdens prior to the establishment of requisite innate and adaptive immune responses.

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INTRODUCTION

Infectious diseases are a major concern to the equine industry on a worldwide basis, and respiratory disease is a primary cause of disease and death in foals (Cohen, 1994). *Rhodococcus equi*, a gram-positive, soil-saprophytic, facultative-intracellular pathogen, is the causative agent of the most severe form of pneumonia in foals (Prescott, 1991; Hondalus, 1997). *Rhodococcus equi* is also an emerging opportunistic pathogen of immunosuppressed people, particularly AIDS patients (Takai *et al.*, 1995). The bacterium, which shares many characteristics with *Mycobacterium tuberculosis*, is able to replicate within macrophages, eventually destroying them and producing granulomatous lesions (Giguere & Prescott, 1997).

Several characteristics of the *R. equi*–host interaction indicate its uniqueness among equine infectious diseases, and have marked effects on the prevention and control of the disease process. *Rhodococcus equi* foal pneumonia is endemic on some farms, but young foals residing on those farms are only sporadically affected even though exposure to the organism is widespread (Cohen *et al.*, 2000). Passive transfer of maternal immunity to *R. equi* does not appear to be protective (Martens *et al.*, 1991). Young foals are commonly affected by *R. equi*, whereas juvenile and adult horses rarely develop *R. equi*-induced disease (Prescott, 1991). Most foals with spontaneous disease appear to become infected very early in life (Horowitz *et al.*, 2001), even though clinical signs of disease may not become apparent for several weeks or months (Prescott, 1991). A subpopulation of neonatal foals appears to have transiently ineffective or inefficient immune responses, which may render them more susceptible to infection with *R. equi* (Martens *et al.*, 1988; Chaffin *et al.*, 2004), and may help explain the age-related susceptibility to infection and sporadic distribution of clinical disease.

On the basis that some foals may not possess the requisite immunologic armamentarium early in life to control *R. equi*, and that there may not be sufficient time for foals to mount an adequate adaptive immune response before becoming infected, it may be most appropriate to develop prophylactic strategies (e.g. chemoprophylaxis or passive immunization) that provide immediate protection during the perinatal period. It is anticipated that such strategies may prevent infectious organisms from growing

to overwhelming numbers before innate immune responses sufficiently mature or specific adaptive immunity can be established.

Ferric iron (Fe^{3+}) is crucial for the survival and replication of most pathogens, thereby providing a potential target for prophylactic and therapeutic strategies. A variety of antimicrobial strategies that act by interfering with microbial acquisition and utilization of iron have been developed (Byrd & Horwitz, 1991; Weinberg, 1994; Bernstein, 1998). Gallium (Ga), a trivalent semi-metal that is chemically similar to Fe^{3+}, is taken up by some bacteria (Bernstein, 1998). The ability of gallium to inhibit growth of intracellular bacteria by interfering with bacterial iron metabolism was established *in vitro* by incorporating gallium nitrate ($Ga(NO_3)_3$; GN) in culture media with *Mycobacterium* spp. (Olakanmi *et al.*, 2000). Those investigators further demonstrated that the antimicrobial effect was due to the Ga moiety of the GN, not the nitrate. A new formulation of gallium, gallium maltolate ($Ga(C_6H_5O_3)$; GaM), which attains high bioavailability when administered orally to a variety of animal species, was recently developed by one of the authors (L.B.) (Bernstein *et al.*, 2000). The purpose of this study was to investigate the potential use of gallium as a chemoprophylactic and chemotherapeutic agent for the control of *R. equi* infections.

MATERIALS AND METHODS

Culture study

Bacteria and growth conditions

Virulent, *vapA*-positive *R. equi* (ATCC 33701) were grown in brain heart infusion broth (Beckton-Dickinson, Cockeysville, MD, USA) (BHIB) with 10% newborn calf serum (Sigma Chemical Co, St Louis, MO, USA) for 48 h at 35 °C with rotation (Laboratory rotator, model 099A; Glas-Col, Terre Haute, IN, USA) (10 r.p.m.). Bacterial cells were pelleted by centrifugation at 1600 ***g*** for 10 min and washed three times with sterile phosphate-buffered saline (PBS; Gibco BRL, Frederick, MD, USA). The concentration of bacteria was determined spectrophotometrically (Smartspec 3000; Bio-Rad Laboratories, Hercules, CA, USA) at an optical density of 600 nm (OD_{600}), and approximately 5×10^6 colony-forming units (CFU)/mL were inoculated into *R. equi* minimal media (MM) (Kelly *et al.*, 2002). MM was used as the control medium, and MM without added iron (MM-Fe) was used to assess the effects of gallium on growth of *R. equi*. The iron concentrations of MM (2.9 μM Fe) and MM-Fe (0.5 μM Fe) were determined by inductively coupled plasma optical emission spectrometry, as previously described (Jordan *et al.*, 2003). All media were prepared in polypropylene beakers (VWR, Aurora, CO, USA) with molecular grade water (Milli-Q plus, 18Ω, pH 7.0; Millipore, Molsheirn, France), and sterilized through a 0.2-μM cellulose acetate filter into polystyrene containers (Nalgene polystyrene filter units, PES membrane, VWR). In all experiments, concentrations of bacteria were determined by 10-fold serial dilutions cultured in duplicate on trypticase soy agar with 5% sheep red blood cells (Becton-Microbiology Systems, Cockeysville, MD, USA). Bacterial concentrations were determined at 0, 8, 24, and 48 h and reported as CFU/mL. All growth studies were conducted in triplicate.

Addition of gallium nitrate

Citrate-buffered GN, in a 0.1 M sterile solution, was kindly provided by Genta Inc. (Ganite; Genta Inc., Berkeley Heights, NJ, USA). The concentrations of *R. equi* grown in MM-Fe containing GN at 50, 100, 150, and 200 μM (3.5, 7, 10.5, and 14 μg/mL) were determined at 0, 8, 24, and 48 h and compared with the concentrations of *R. equi* grown in MM.

Addition of iron

Excess iron, as $FeCl_3$, was added to MM-Fe containing GN to ascertain its effect on GN-induced growth suppression of *R. equi*. Concentrations of *R. equi* grown in: (i) MM; (ii) MM-Fe + 150 μM GN; and (iii) MM-Fe + 150 μM GN + 25 μM $FeCl_3$ were compared at 0, 8, 24, and 48 h incubation.

Mouse study

Bacteria and growth conditions

Virulent *R. equi* (ATCC 33701) were grown at 37 °C, with rotation (10 rpm), in BHIB, with 10% newborn calf serum. After 24 h incubation, 1.25 mL of the culture (containing approximately 1×10^8 *R. equi*/mL) were transferred to 25 mL fresh broth and incubated for 48 h under the same conditions. The bacteria were centrifuged at 1600 ***g*** for 10 min. The concentration of bacteria was determined spectrophotometrically at OD_{600}, adjusted to a concentration of approximately 1×10^7 CFU/mL with PBS, and stored in aliquots at −80 °C.

Mice

Eighteen, 5- to 6-week-old, female, BALB/c mice, weighing approximately 20 g, were obtained from a commercial source (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA). GaM was kindly provided by Titan Pharmaceuticals, Inc. (South San Francisco, CA, USA). The mice were obtained in groups of six, for each of three independent studies. In each study, the six mice were separated into three treatment groups as follows: (i) distilled water (control); (ii) low-dose GaM (10 mg/kg); and (iii) high-dose GaM (50 mg/kg). Prior to initiation of the study, mice were acclimated for 10 days and fed a basal rodent diet containing 60 p.p.m. iron (Basal Test Diet #5755; Purina Mills, St Louis, MO, USA). Mice were administered 150 μL of distilled water (controls), or distilled water containing either 1.33 mg/mL GaM or 6.65 mg/mL GaM via oral gavage (Animal feeding needles, VWR) daily for 10 days. On the fourth treatment day, frozen aliquots of *R. equi* were thawed at 37 °C in a water bath and gently mixed. The mice were lightly anesthetized with methoxyfluorane (Pitman-Moore, Mundelein, IL, USA) in a closed chamber, and 200 μL of the bacterial suspension, containing approximately 2×10^6 CFU of *R. equi*, was injected into the peritoneal space with a 27 G × 5/8″ needle (Monoject, VWR). The concentration of viable *R. equi* at the time of infection was

Table 1. Gallium nitrate-induced growth suppression and death of virulent *Rhodococcus equi* (ATCC 33701)

Growth conditions	Mean cell density (CFU/mL)*,† after growth times (h) of 0	8	24	48
MM	3.62×10^5	6.37×10^{6a}	3.55×10^{8a}	5.21×10^{8a}
MM-Fe + 50 μM GN	3.62×10^5	3.57×10^{6a}	1.81×10^{6b}	7.30×10^{6b}
MM-Fe + 100 μM GN	3.62×10^5	1.26×10^{6a}	1.58×10^{6b}	7.28×10^{5c}
MM-Fe + 150 μM GN	3.62×10^5	1.58×10^{6a}	1.70×10^{6b}	6.89×10^{5c}
MM-Fe + 200 μM GN	3.62×10^5	1.15×10^{6a}	1.62×10^{6b}	4.16×10^{5c}

MM, minimal media (2.9 μM Fe); MM-Fe, minimal media without added iron (0.5 μM Fe); GN, gallium nitrate; ATCC, American Type Culture Collection.

*Cell densities determined by enumerating CFU in serial dilutions plated on trypticase soya agar with sheep RBC.

†Average of triplicate-independent experiments.

[a,b,c]Within a column, values with different superscripts are significantly ($P < 0.05$) different.

determined by duplicate cultures of 10-fold serial dilutions on trypticase soy agar with 5% sheep red blood cells. On the tenth day of treatment (6 days post-infection), mice were gavaged with their respective doses of distilled water or GaM 2 h prior to termination of the study, anesthetized with methoxyfluorane and killed by cardiac snip.

Sample collection and analyses

Spleens, lungs, and livers were aseptically harvested and weighed. Each organ was homogenized with a tissue grinder (Wheaton, Millville, NJ, USA) in 2 mL of sterile PBS and the volume of homogenate was recorded. The *R. equi* concentration (CFU/mL) of tissue homogenates was determined by duplicate quantitative culture of 10-fold serial dilutions. The numbers of bacteria per gram of tissue were determined by dividing the product of bacterial concentration (CFU/mL) and homogenate volume by the weight of the organ.

Following cardiac snip, whole blood was aspirated from the thoracic cavity and serum was harvested and stored at −80 °C for determination of gallium concentration. Samples were thawed at 37 °C and diluted with 1% ultra-pure HNO_3 (Seastar Baseline; Seastar Chemicals Inc., Sidney, BC, Canada) in deionized water in preparation for gallium analysis by inductively coupled plasma-mass spectroscopy (Model DRC 2; Perkin Elmer, Foster City, CA, USA) (ICP/MS) using the ^{71}Ga isotope and ^{103}Rh as an internal standard. Weighted linear calibration was performed with a blank and four external standards (0.2, 2.0, 20, and 200 μg/L). Data were acquired in peak hopping mode, using the autolens feature and three replicate reads per determination. Calibration and baseline determinations were performed before and after the analytical run.

Statistical analysis

Data for bacterial numbers were described by use of mean ± SD of experiments conducted in triplicate. Data were examined both as measured and because of skewness in the distribution of some of the data and their variances, following natural logarithmic transformation. For the *in vitro* culture experiments, the effects of fixed covariates of time and media on bacterial concentrations were analyzed using analysis of variance with the method of Sidak for *post-hoc* testing (Sidak, 1967). Data obtained from experimentally challenged mice were also analyzed using analysis of variance. A *P*-value ≤ 0.05 was considered statistically significant.

RESULTS

Gallium nitrate inhibits growth of R. equi in culture

The concentrations of *R. equi* at 0, 8, 24, and 48 h were compared when cultivated in MM-Fe supplemented with various concentrations of GN and in MM without GN (Table 1). Significant effects of GN were not observed at 8 h. At 24 and 48 h incubation, there were significant ($P \leq 0.01$) decreases in concentrations of *R. equi* cultivated in media containing 50, 100, 150, or 200 μM GN, compared with those grown in MM. At 48 h, 50 μM GN produced a nearly 2-log order decrease in *R. equi* concentration relative to control levels, and 100, 150, or 200 μM GN produced a further significant decrease (Table 1).

Excess iron inhibits GN-induced growth suppression

To determine whether the inhibitory effect of GN on growth of *R. equi* was caused by interference with, or disruption of, iron metabolism, iron-override experiments were conducted. Concentrations of *R. equi* grown in MM and in MM-Fe + 150 μM GN + 25 μM $FeCl_3$ were significantly ($P \leq 0.001$) greater than concentrations of *R. equi* grown in MM-Fe + 150 μM GN at 24 h and 48 h (Table 2).

Status of mice

One mouse in the low-dose treatment group died on the fourth day of the study, shortly after receiving its fourth dose of GaM and being infected with *R. equi*. Gross lesions were not apparent in this mouse and postmortem autolysis precluded accurate

Table 2. Reversal of gallium nitrate-induced growth suppression and death of virulent *Rhodococcus equi* (ATCC 33701) with excess iron

Growth conditions	Mean cell density (CFU/mL)*,† after growth times (h) of 0	8	24	48
MM	3.62×10^5	6.37×10^{6a}	3.55×10^{8a}	5.21×10^{8a}
MM-Fe + 150 μM GN	3.62×10^5	1.58×10^{6a}	1.70×10^{6b}	6.89×10^{5b}
MM-Fe + 150 μM GN + 25 μM $FeCl_3$	3.62×10^5	1.05×10^{7a}	3.90×10^{8a}	5.84×10^{8a}

See Table 1 for key.

microscopic evaluation. None of the other mice exhibited clinical signs of disease or gross lesions.

Gallium maltolate absorption in mice

Serum samples were collected 2 h after the final treatment from all 17 mice that completed the study. However, a laboratory accident resulted in the loss of two serum samples from each experimental group. Consequently, data on serum gallium concentrations are only available for four control, three low-dose GaM, and four high-dose GaM-treated mice. GaM was absorbed in a dose-dependent manner (Fig. 1). After logarithmically transforming the data, the mean concentrations of gallium in sera analyzed by ICP-MS were significantly ($P \leq 0.05$) greater in low-dose GaM-treated mice (mean, 110.5 μg/mL; SE, 0.0316) than in control mice (mean, 0.870 μg/mL; SE, 0.000178), and were significantly ($P \leq 0.05$) greater in high-dose GaM-treated mice (mean, 559.3 μg/mL; SE, 0.136) than in either low-dose or control mice.

Gallium maltolate suppresses growth of R. equi in mice

Median tissue concentrations of *R. equi* were greater in control mice than in GaM-treated mice, and similar between mice treated with low-dose GaM (10 mg/kg BW) and high-dose GaM (50 mg/kg BW) (Table 3). Although not statistically significant, there was a clear trend toward an inverse relationship between serum gallium concentrations and *R. equi* tissue concentrations (Fig. 2).

DISCUSSION

Rhodococcus equi, an intracellular pathogen of macrophages, causes one of the most severe and devastating forms of foal pneumonia. Epidemiologic evidence suggests that most foals with spontaneous *R. equi* pneumonia become infected at a very

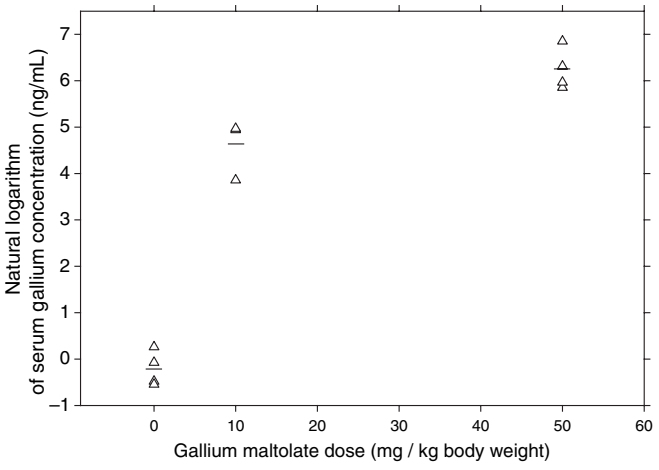

Fig. 1. Scatter plot depicting the natural logarithm of serum gallium concentrations of mice treated by oral gavage with the following: distilled water ($n = 4$), 10 mg/kg GaM ($n = 3$), or 50 mg/kg GaM ($n = 4$). Mean values for each treatment group are indicated by a horizontal line.

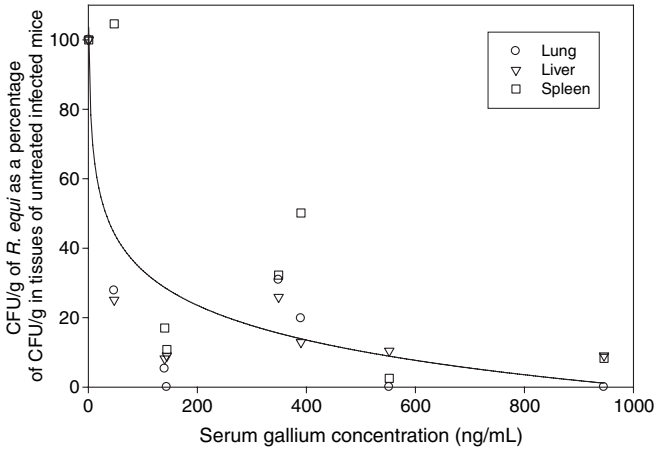

Fig. 2. Colony-forming units (CFU) of *Rhodococcus equi* per gram of mouse tissue (spleen, lung, or liver) expressed as a percentage of the mean CFU/g value for each type of tissue in infected control (untreated) mice (CFU%), as a function of serum gallium concentration ([Ga]) when the animals were killed (2 h following final treatment). The curve corresponds to CFU% = 100 − 14.426 × ln [Ga].

Table 3. Median (range) of *Rhodococcus equi* tissue concentrations in mice experimentally infected with virulent *R. equi* (ATCC 33701) and treated with water or gallium maltolate (GaM) (CFU/g tissue)

Treatment	Liver	Lung	Spleen
Control (water)	3575 (396–609 000)	2233 (0–76 100)	32 950 (9500–2 040 000)
GaM (10 mg/kg)	1989 (268–6090)	303 (0–2940)	14 500 (1470–140 000)
GaM (50 mg/kg)	2830 (430–6290)	906 (0–2410)	28 650 (1110–82 600)

young age (i.e. within the first few days of life) (Horowitz *et al.*, 2001), before they have time to develop an effective adaptive immune response. During primary infection with many intracellular bacteria the host must rely on innate immune defenses to prevent organisms from growing to overwhelming numbers before specific adaptive immunity can be generated and expressed (Pedrosa *et al.*, 2000; Bennouna *et al.*, 2003). Thus, innate immunity is likely important for controlling early *R. equi* infections. Evidence suggests that immaturity of innate immune responses and individual variability in those responses may contribute to increased susceptibility to infection by *R. equi* (Boyd *et al.*, 2003; Kohler *et al.*, 2003; Chaffin *et al.*, 2004). On these bases, strategies designed to work in conjunction with innate immune responses may prevent or control early infections, thereby providing the host additional time for immune system maturation and for development of an adequate adaptive immune response.

Despite an abundance of iron in host tissues and fluids, the amount of free iron available for use by invading organisms is minute (Ratledge & Dover, 2000). Iron withholding is an essential antimicrobial component of the innate immune complex. Iron-binding proteins (e.g. transferrin, lactoferrin) bind virtually all free iron, thereby rendering it unavailable to most microorganisms. Some pathogens, however, can acquire iron from these iron-binding proteins or via siderophores (Weinberg, 1995, 1998; Boelaert, 1996; Ratledge & Dover, 2000). Recent evidence has demonstrated the importance of iron for growth and survival of *R. equi*, and the ability of *R. equi* to acquire iron from transferrin and lactoferrin (Jordan *et al.*, 2003). In foals, virulence of *R. equi* is strongly associated with the presence of an 80–90 kb plasmid that encodes and mediates the expression of vapA, a 15–17 kDa virulence-associated protein (Takai *et al.*, 1993; Martens *et al.*, 2000). Although the exact function of vapA is unknown, there is compelling evidence of its potential role in iron acquisition and utilization by *R. equi* (Jordan *et al.*, 2003; Rahman *et al.*, 2003; Ren & Prescott, 2003).

On the basis that gallium interferes with iron-dependent metabolic pathways in *Mycobacterium* spp. and suppresses growth (Olakanmi *et al.*, 2000), we hypothesized that gallium would suppress the growth of *R. equi* in a similar manner. In this study we: (i) demonstrated the ability of GN to suppress growth of *R. equi in vitro*; (ii) determined that these growth-suppressing effects were abrogated by the addition of excess iron, and thus were most likely due to interference with iron acquisition and utilization; (iii) documented that mice readily absorb orally administered GaM in a dose-dependent manner; and (iv) showed that mice that received oral GaM before, and for a short time after, experimental infection with *R. equi* had lower concentrations of bacteria in vital organs than untreated control mice.

A variety of preventative and therapeutic strategies for bacterial infections have been developed that act by interfering with microbial iron acquisition and utilization (Byrd & Horwitz, 1991; Weinberg, 1994; Bernstein, 1998). Gallium is a semimetal that accumulates in rapidly dividing tumor cells, areas of inflammation, macrophages, neutrophils, and some bacteria (Tsan, 1986; Bernstein, 1998). The antimicrobial effects of gallium are related to its ability to compete with iron for the binding sites of host iron-binding proteins (e.g. transferrin and lactoferrin) and siderophores, which are sources of iron for many intracellular pathogens (Emery, 1986; Tsan, 1986; Bernstein, 1998). Bacteria acquire gallium, instead of iron, from the host iron-binding proteins and incorporate it in metabolic pathways and enzymes that require Fe^{3+}. Many of these enzymes, such as ribonucleotide reductase, are crucial for cell cycle regulation and DNA synthesis. Because gallium, unlike iron, is unable to undergo redox cycling (i.e. reduction from trivalent to divalent state), these enzymes are inactivated and the net result is inhibition of DNA synthesis and bacterial replication, and ultimately cell death (Bernstein, 1998).

Gallium utilizes both transferrin-dependent and -independent pathways to enter mammalian cells. When plasma concentrations of gallium exceed approximately 50 μM, transferrin is saturated, and the majority of the unbound gallium exists as gallate, $Ga(OH)_4^-$, which is rapidly excreted in the urine and can be nephrotoxic (Bernstein, 1998). This most commonly occurs when gallium enters the bloodstream (via intravenous administration) at a rate that exceeds the binding capacity of transferrin. The risk of adverse effects can be minimized by oral or subcutaneous administration of gallium, as it will enter the bloodstream more slowly and follow the endogenous pathway of iron uptake.

Observations from a previous study suggested that the iron requirement of *R. equi* is very low (Jordan *et al.*, 2003). In the present study, *R. equi* MM was used as the control media, and MM-Fe as the principal media in all GN studies, on the following bases: (i) growth characteristics of *R. equi* in MM are well characterized (Boland & Meijer, 2000; Jordan *et al.*, 2003); (ii) MM contains more iron than needed for optimal *R. equi* growth (Jordan *et al.*, 2003); (iii) iron concentration in MM-Fe more nearly reflects that available to bacteria *in vivo* (Jordan *et al.*, 2003); (iv) *R. equi* growth rates are similar in MM and MM-Fe (Carnes, 2002); and (v) excessive iron interferes with antimicrobial effects of gallium (Olakanmi *et al.*, 2000). The addition of various concentrations of GN to culture media inhibited the growth of *R. equi* during the first 24 h of incubation. Higher concentrations of GN resulted in bacterial death by 48 h incubation, as shown by decreased CFU counts. GN thus appears to initially induce bacterial stasis, by inhibiting DNA synthesis and cell replication, which then results in cell death. The same pattern has been observed in mammalian cell cultures, where cells unable to replicate due to gallium exposure soon undergo apoptosis (Bernstein, 1998). Olakanmi *et al.* (2000) similarly demonstrated a delayed bactericidal effect of GN on intracellular *M. tuberculosis*.

The antimicrobial effects of gallium were completely abolished when media were supplemented with excess iron, providing evidence that GN interferes with iron-dependent mechanisms that are crucial to the growth and survival of *R. equi*. Our findings were similar to those obtained in a previous study in which the *in vitro* antimicrobial activity of GN for *M. tuberculosis* and *M. avium* was due to Ga interference with bacterial iron metabolism (Olakanmi *et al.*, 2000). Our *in vitro* studies

indicated that GN causes significant inhibition of growth and results in death of *R. equi*, but because of the concerns associated with toxicity, as well as the need for continuous monitoring and infusions (Bernstein, 1998), the parenteral administration of GN to horses would not be practical. Rather, oral administration would be far superior in terms of safety, cost, and ease of administration.

On the basis of the *R. equi* antimicrobial effects demonstrated in the *in vitro* aspect of this study, we investigated the ability of orally administered GaM to control *R. equi* in experimentally infected mice. GaM has high oral bioavailability and safety in humans and a variety of animal species (Bernstein *et al.*, 2000), and is not associated with the gastrointestinal irritation caused by oral GN and gallium chloride (Fettman *et al.*, 1987), or the renal toxicity caused by intravenous GN (Bernstein *et al.*, 2000). GaM is available as a moderately water-soluble powder that is stable at room temperature, has a pleasant odor and little flavor, and is relatively inexpensive to produce.

Although pneumonia is the primary disease caused by *R. equi* in horses and people (Prescott, 1991), a laboratory animal model for pneumonia is lacking. The virulence status of *R. equi* isolates has been established by infecting mice either i.v. or i.p. and quantifying the concentrations of bacteria in their spleens, lungs, and livers (Takai *et al.*, 1992; Giguere *et al.*, 1999). The i.v. route of infection is more commonly reported; however, we elected to use the i.p. route in this study because of the relative ease and accuracy of i.p. inoculation and the report of induction of less severe disease (Takai *et al.*, 1992). The finding in this study of lower concentrations of *R. equi* in the lungs than in spleens and livers is consistent with previous experimental systemic infections of mice with *R. equi*. As the lung is not the primary target organ in mice systemically infected with *R. equi*, information obtained from such mice may not be directly applicable to horses or people having lung infections, but our results do demonstrate the likely efficacy of oral GaM against *R. equi* infections in a mammalian host.

The mouse in the low-dose GaM treatment group that died appeared bright and alert, but progressively lost body weight following the initial GaM treatments. On the basis that no other mice in the study showed evidence of illness, the death of this mouse was most likely precipitated by the gavage administration of GaM. Gavage is the traditional method of delivering small volumes of substances to the stomach of mice. The procedure, however, is not risk-free. It can cause mechanical damage to the oropharyngeal and esophageal tissues, thereby interfering with food consumption, and administered substances can overflow and transport contaminants into the lung causing irritation or infection (Craig & Elliott, 1999). Alternatively, i.p. inoculation of the *R. equi* could have resulted in trauma to abdominal organs. Mice were lightly anesthetized, positioned in dorsal recumbency and the abdominal wall elevated during inoculations in an effort to circumvent such complications. There was no evidence of major organ trauma at the time of necropsy.

Gallium, in the form of GaM, was absorbed in a dose-dependent manner following oral administration. Although the loss of two serum samples from each experimental group markedly reduced the statistical power of the resulting gallium concentration data, there was still a significant difference between all three treatment groups. These data on oral bioavailability are in agreement with previous observations in a variety of animal species (Bernstein *et al.*, 2000). Serum concentrations of gallium are useful for assessing drug absorption and relative availability, but do not take into account gallium that has left the serum and entered other tissues, where gallium is known to concentrate (Bernstein, 1998). The capacity of gallium to accumulate in areas of inflammation and infection, macrophages, neutrophils, and some bacteria provides an even greater source with which to combat infectious agents (Tsan, 1986; Bernstein, 1998).

Data obtained from the mice indicate that the administration of GaM before, and for a short period after, experimental infection resulted in lower concentrations of *R. equi* in vital organs. The median concentrations of *R. equi* in the spleens, lungs, and livers of untreated control mice were approximately 12-, 16- and 42-fold greater, respectively, than those of mice treated with GaM. Although the bacterial burden reductions in GaM-treated mice are not statistically significant, they may very well be clinically relevant. The small number of animals per experimental group severely limited the study's statistical power, and with more mice it is likely that the differences would have been significant. These findings are similar to those demonstrating the effects of GaM on the concentrations of *M. tuberculosis* in tissues of experimentally infected guinea pigs (L.S. Schlesinger, personal communication).

The studies reported here demonstrate that the processes by which *R. equi* acquire and utilize iron are disrupted by gallium, thereby suppressing growth and causing bacterial death. Gallium may serve as a tool for investigating the mechanisms of iron acquisition by *R. equi* and the role of such mechanisms in the pathogenesis of *R. equi* pneumonia. Furthermore, our results suggest that gallium may have prophylactic and therapeutic attributes that would be useful in the control of disease caused by *R. equi*. Additional studies are needed to evaluate the safety and bioavailability of oral GaM in neonatal foals, and to assess its therapeutic effectiveness against *R. equi* and other pathogens.

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REFERENCES

Bennouna, S., Bliss, S.K., Curiel, T.J. & Denkers, E.Y. (2003) Cross-talk in the innate immune system: neutrophils instruct recruitment and

activation of dendritic cells during microbial infection. *The Journal of Immunology*, **171,** 6052–6058.

Bernstein, L.R. (1998) Mechanisms of therapeutic activity for gallium. *Pharmacological Reviews*, **50,** 665–682.

Bernstein, L.R., Tanner, T., Godfrey, C. *et al.* (2000) Chemistry and pharmacokinetics of gallium maltolate, a compound with high oral gallium availability. *Metal-Based Drugs*, **7,** 33–47.

Boelaert, J.R. (1996) Iron and infection. *Acta Clinica Belgium*, **51,** 213–221.

Boland, C.A. & Meijer, W.G. (2000) The iron dependent regulatory protein IdeR (DtxR) of *Rhodococcus equi*. *FEMS Microbiology Letters*, **191,** 1–5.

Boyd, N.K., Cohen, N.D., Lim, W.-S., Martens, R.J., Chaffin, M.K. & Ball, J.M. (2003) Temporal changes in cytokine expression of foals during the first month of life. *Veterinary Immunology and Immunopathology*, **92,** 75–85.

Byrd, T.F. & Horwitz, M.A. (1991) Chloroquine inhibits the intracellular multiplication of *Legionella pneumophilia* by limiting the available iron. *Journal of Clinical Investigation*, **88,** 351–357.

Carnes, M.L. (2002) Iron acquisition and utilization by *Rhodococcus equi*: potential virulence factors. MS Thesis, Department of Large Animal Medicine and Surgery, Texas A&M University, College Station, TX.

Chaffin, M.K., Cohen, N.D., Martens, R.J., Edwards, R.F., Nevill, M. & Smith, R. (2004) Hematologic and immunophenotypic factors associated with development of *Rhodococcus equi* of foals at equine breeding farms with endemic infection. *Veterinary Immunology and Immunopathology*, **100,** 33–48.

Cohen, N.D. (1994) Causes of and farm management factors associated with disease and death in foals. *Journal of the American Veterinary Medical Association*, **204,** 1644–1651.

Cohen, N.D., Chaffin, M.K. & Martens, R.J. (2000) Control and prevention of pneumonia in foals caused by *Rhodococcus equi*. *Compendium on Continuing Education for the Practicing Veterinarian*, **22,** 1062–1070.

Craig, M.A. & Elliott, J.F. (1999) Mice fed radiolabeled protein by gavage show sporadic passage of large quantities of intact material into the blood, an artifact not associated with voluntary feeding. *Contemporary Topics in Laboratory Animal Science*, **38,** 18–23.

Emery, T. (1986) Exchange of iron by gallium in siderophores. *Biochemistry*, **25,** 4629–4633.

Fettman, M.J., Brooks, P.A., Jones, R.L., Mero, K.N. & Phillips, R.W. (1987) Antimicrobial alternatives for calf diarrhea: enteric responses to *Escherichia coli*, deferoxamine, or gallium in neonatal calves. *American Journal of Veterinary Research*, **48,** 569–577.

Giguere, S. & Prescott, J.F. (1997) Clinical manifestations, diagnosis, treatment, and prevention of *Rhodococcus equi* infections in foals. *Veterinary Microbiology*, **56,** 313–334.

Giguere, S., Hondalus, M., Yager, J., Darrah, P., Mosser, D.M. & Prescott, J.F. (1999) Role of the 85-kilobase plasmid and plasmid-encoded virulence-associated protein A in intracellular survival and virulence of *Rhodococcus equi*. *Infection and Immunity*, **67,** 3548–3557.

Hondalus, M.K. (1997) Pathogenesis and virulence of *Rhodococcus equi*. *Veterinary Microbiology*, **56,** 257–268.

Horowitz, M.L., Cohen, N.D., Takai, S., Becu, T., Chaffin, M.K., Chu, K.K., Magdesian, K.G. & Martens, R.J. (2001) Application of Sartwell's model (log normal distribution of incubation periods) to age at onset and age at death of foals with *Rhodococcus equi* pneumonia as evidence of perinatal infection. *Journal of Veterinary Internal Medicine*, **15,** 171–175.

Jordan, M.C., Harrington, J.R., Cohen, N.D., Tsolis, R.M., Dangott, L.J., Weinberg, E.D. & Martens, R.J. (2003) Effects of iron modulation on growth and viability of *Rhodococcus equi* and expression of virulence-associated protein A. *American Journal of Veterinary Research*, **64,** 1337–1346.

Kelly, B.K., Wall, D.M., Boland, C.A. & Meijer, W.G. (2002) Isocitrate lyase of the facultative intracellular pathogen *Rhodococcus equi*. *Microbiology*, **148,** 793–798.

Kohler, A.K., Stone, D.M., Hines, M.T., Byrne, B.A., Alperin, D.C., Norton, L.K. & Hines, S.A. (2003) *Rhodococcus equi* secreted antigens are immunogenic and stimulate a type 1 recall response in the lungs of horses immune to *R. equi* infection. *Infection and Immunity*, **71,** 6329–6337.

Martens, J.G., Martens, R.J. & Renshaw, H.W. (1988) *Rhodococcus (Corynebacterium) equi*: bactericidal capacity of neutrophils from neonatal and adult horses. *American Journal of Veterinary Research*, **49,** 295–299.

Martens, R.J., Martens, J.G. & Fiske, R.A. (1991) Failure of passive immunization by colostrum from immunized mares to protect foals against *Rhodococcus equi* pneumonia. *Equine Veterinary Journal*, **12** (Suppl.), 19–22.

Martens, R.J., Takai, S., Cohen, N.D., Chaffin, M.K., Liu, H., Sakurai, K., Sugimoto, H. & Lingsweiler, S.W. (2000) Association of disease with isolation and virulence of *Rhodococcus equi* from farm soil and foals with pneumonia. *Journal of the American Veterinary Medical Association*, **217,** 220–225.

Olakanmi, O., Britigan, B.E. & Schlesinger, L.S. (2000) Gallium disrupts iron metabolism of mycobacteria residing within human macrophages. *Infection and Immunity*, **68,** 5619–5627.

Pedrosa, J., Saunders, B.M., Appelberg, R., Orme, I.M., Silva, M.T. & Cooper, A.M. (2000) Neutrophils play a protective nonphagocytic role in systemic *Mycobacterium tuberculosis* infection in mice. *Infection and Immunity*, **68,** 577–583.

Prescott, J.F. (1991) *Rhodococcus equi*: an animal and human pathogen. *Clinical Microbiology Reviews*, **4,** 20–34.

Rahman, M.T., Herron, L.L., Kapur, V., Meijer, W.G., Byrne, B.A., Ren, J., Nicholson, V.M. & Prescott, J.F. (2003) Partial genome sequencing of *Rhodococcus equi* ATCC 33701. *Veterinary Microbiology*, **94,** 143–158.

Ratledge, C. & Dover, L.G. (2000) Iron metabolism in pathogenic bacteria. *Annual Review of Microbiology*, **54,** 881–941.

Ren, J. & Prescott, J.F. (2003) Analysis of virulence plasmid gene expression of intra-macrophage and *in vitro* grown *Rhodococcus equi* ATCC 33701. *Veterinary Microbiology*, **94,** 167–182.

Sidak, A. (1967) Rectangular confidence regions for the means of multivariate normal distributions. *Journal of the American Statistical Association*, **62,** 626–633.

Takai, S., Sasaki, Y. & Tsubaki, S. (1992) Influence of inoculation route on virulence of *Rhodococcus equi* in mice. *Microbiology and Immunology*, **36,** 895–898.

Takai, S., Watanabe, Y., Ikeda, T., Ozawa, T., Matsukura, S., Tamada, Y., Tsubaki, S. & Sekizaki, T. (1993) Virulence-associated plasmids in *Rhodococcus equi*. *Journal of Clinical Microbiology*, **31,** 1726–1729.

Takai, S., Imai, Y., Fukunaga, N., Uchida, Y., Kamisawa, K., Sasaki, Y., Tsubaki, S. & Sekizaki, T. (1995) Identification of virulence-associated antigens and plasmids in *Rhodococcus equi* from patients with AIDS. *Journal of Infectious Diseases*, **172,** 1306–1311.

Tsan, M.F. (1986) Mechanism of gallium-67 accumulation in inflammatory lesions. *Journal of Nuclear Medicine*, **26,** 88–92.

Weinberg, G.A. (1994) Iron chelators as therapeutic agents against *Pneumocystis carinii*. *Antimicrobial Agents and Chemotherapeutics*, **38,** 997–1003.

Weinberg, E.D. (1995) The role of iron in infection. *Current Opinion in Infectious Disease*, **8,** 164–169.

Weinberg, E.D. (1998) Patho-ecological implications of microbial acquisition of host iron. *Reviews in Medical Microbiology*, **9,** 171–178.