Evaluation of the in vitro activity of gallium nitrate against *Mycobacterium avium* subsp *paratuberculosis*

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Objective—To evaluate the in vitro susceptibility of various field isolates of *Mycobacterium avium* subsp *paratuberculosis* (MAP) to gallium nitrate.

Sample—10 isolates of MAP, including 4 isolated from cattle, 2 isolated from bison, 1 isolated from an alpaca, and 3 isolated from humans.

Procedures—The in vitro susceptibility to gallium nitrate was tested by use of broth culture with detection of MAP growth by means of a nonradiometric automated detection method. For each MAP isolate, a series of 7 dilutions of gallium nitrate (concentrations ranging from 200 to 1,000 µM) were tested. Gallium nitrate was considered to have caused 90% and 99% inhibition of the MAP growth when the time to detection for culture of the MAP stock solution and a specific concentration of gallium nitrate was delayed and was similar to that obtained for culture of the MAP stock solution (without the addition of gallium nitrate) diluted 1:10 and 1:100, respectively.

Results—Gallium nitrate inhibited MAP growth in all 10 isolates. The susceptibility to gallium nitrate was variable among isolates, and all isolates of MAP were inhibited in a dose-dependent manner. Overall, the concentration that resulted in 90% inhibition ranged from < 200 µM for the most susceptible isolates to 743 µM for the least susceptible isolates.

Conclusions and Clinical Relevance—Gallium nitrate had activity against all 10 isolates of MAP tested in vitro and could potentially be used as a prophylactic agent to aid in the control of MAP infections during the neonatal period. (Am J Vet Res 2011;72:1243–1246)

**Abbreviations**

<table>
<thead>
<tr>
<th>ATCC</th>
<th>American Type Culture Collection</th>
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<tr>
<td>MAP</td>
<td><em>Mycobacterium avium</em> subsp <em>paratuberculosis</em></td>
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<td>TTD</td>
<td>Time to detection</td>
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Paratuberculosis (Johnne’s disease) is an important, costly enteric infection of cattle and other ruminants and is caused by MAP. It is generally accepted that most calves become infected with MAP soon after birth. Therefore, control programs are based on preventing transmission from adult cattle that shed MAP organisms in feces to young replacement stock on the farm. Control programs specifically focus on management of the birthing area (calving pen), feeding management for colostrum and milk, and rearing of young stock. No drugs are currently approved for the prevention or treatment of Johnne’s disease in cattle. Monensin sodium, a monovalent polyether antimicrobial whose properties include anticoccidial activity and improvement in feed use in cattle, has some efficacy against MAP in vitro and in vivo. Vaccination, available on a limited basis in the United States, may reduce the prevalence of clinical disease and has been associated with reduced colonization of intestinal tissues in experimental studies, but vaccinated cattle are not fully protected against infection and can still shed MAP in their feces.

*Mycobacterium avium* subsp *paratuberculosis* is a facultative intracellular bacterium that is able to survive and reproduce within monocytes and macrophages. Ferric iron is crucial for survival and replication of MAP, thereby providing a potential target for prophylactic and therapeutic strategies. Sequestration of ferric iron (primarily through the production of transferrin, but also via the production of lactoferrin and ferritin) is used by hosts as an innate defense mechanism. However, mycobacteria can produce and use siderophores, thereby circumventing this defense mechanism. Gallium, a trivalent semimetal that shares many similarities with ferric iron and functions as an iron mimic, has been found to have activity against various microorganisms, including *Rhodococcus equi*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*. The ability of gallium to inhibit growth of intracellular bacteria

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by interfering with bacterial iron metabolism has been established in vitro by incorporating gallium nitrate in culture media for *M. tuberculosis* and *Mycobacterium avium*.3,4 However, to the author’s knowledge, the susceptibility of MAP to gallium has not been established. Protocols for antimicrobial susceptibility testing of MAP have not yet been standardized, but typically, variations of the broth macrodilution method used for slow-growing nontuberculous mycobacterium, such as *M. avium*, have been used.15–17 In the study reported here, susceptibility testing was conducted in broth culture by use of a mycobacterial detection system.

The purpose of the study reported here was to test the in vitro susceptibility of various isolates of MAP to gallium nitrate. We hypothesized that gallium nitrate would have efficacy against MAP and thus could be used as an aid in the treatment or control of paratuberculosis in cattle.

**Materials and Methods**

**Sample**—Ten isolates of MAP were used in the study. The isolates were chosen to include a variety of species of origin and geographic areas in the United States. These included 4 isolates from cattle (California, Pennsylvania, and Vermont), 2 isolates from bison (Midwestern United States), and 1 isolate from an alpaca (Mid-Atlantic State), all of which were obtained from clinically affected animals, and 3 isolates from humans with Crohn’s disease, which were available from the ATCC. A parent stock solution of each isolate was prepared by obtaining a swab specimen of 1 colony grown for 4 to 6 weeks on Herrold egg yolk medium and suspending it in 5 mL of sterile saline (0.9% NaCl) solution that contained 10 sterile 2-mm glass beads. The suspension was homogenized via vigorous vortexing and then was allowed to sit undisturbed for 30 minutes to allow large clumps to settle. Two milliliters of the homogenized suspension was transferred to a sterile glass tube and adjusted by the addition of 20% glycerol in sterile saline solution to achieve a 0.5 McFarland standard, as determined by use of a spectrophotometer.4 Each parent stock suspension was diluted 1:100 in 15% glycerol-saline solution to form a working stock suspension with an approximate concentration of 10^6 MAP CFUs/mL (determined by serial dilutions on solid media). Identity of the isolates as MAP was confirmed on the basis of colony morphology, results of acid-fast staining, mycobactin dependence, and detection of the hspX gene by use of a real-time PCR assay.6

**Preparation of gallium nitrate solution**—A working stock solution of gallium nitrate (4,000 µM) was prepared by diluting 1.303 g of gallium nitrate in 15 mL of sterile water. Then, the pH was adjusted to 6.8 by the addition of saturated aqueous NaCO3, and the mixture was sterilized by passage through a 0.22-µm filter unit. The filtered working stock solution of gallium nitrate was used to prepare test solutions with various concentrations (200, 400, 500, 600, 700, 800, and 1,000 µM) of gallium nitrate.

**Broth cultures and susceptibility testing**—Susceptibility testing was conducted in broth culture by use of a mycobacterial detection system.4 First, tubes of the mycobacterial detection system were fortified with egg yolk and growth supplement and inoculated in triplicate with 100 µL of the working stock suspension of each MAP isolate. Then, serial dilutions of gallium nitrate were added to each tube. In this system, mycobacterial growth was detected automatically by incubation of the tubes in the mycobacterial detection system instrument, which fluorometrically measured bacterial oxygen consumption in the mycobacterial detection system tube. When oxygen consumption reached a threshold, the instrument signaled that the tube had a positive result. Any tube that had not reached the threshold for oxygen consumption by the end of the study (56 days) was considered negative for mycobacterial growth.

The TTD is influenced by the number of mycobacteria in the inoculum and the rate of growth. The strategy for measuring the minimum inhibitory concentration is to determine the concentration of an agent that inhibits MAP growth by 90% and 99%. Thus, 90% and 99% growth inhibition corresponded to a delay in TTD equal to that obtained by diluting the MAP stock solution (without gallium nitrate) to 1:10 and 1:100, respectively. Thus, for each MAP isolate, the undiluted parent stock suspension and 1:10 and 1:100 dilutions of the parent stock suspension were inoculated in triplicate into tubes that did not contain gallium nitrate (growth control tubes; 30 tubes/isolate).

All tubes were incubated at 37°C in the mycobacterial detection system.4 The TTD for each tube was recorded.

**Statistical analysis**—Results were assessed by use of a mixed-effect regression analysis whereby triplicate tubes were clustered, variation in TTD of the triplicate tubes was treated as a random effect, and isolates and concentrations were treated as fixed effects. A Tukey ladder analysis revealed that transformation of the data by use of the mathematical reciprocal of TTD (1/TTD) would best achieve normality. The interaction between each tested gallium nitrate concentration for all isolates combined was tested by use of post hoc comparisons. Values of P ≤ 0.05 were considered significant. For 2 of the tested MAP isolates (bovine isolates 1 and 2), results for the 500 µM gallium nitrate concentration were not available. Therefore, the 500 µM gallium nitrate concentration was excluded from the analysis. Finally, for each MAP isolate, the 90% and 99% inhibition concentrations were calculated on the basis of the concentration of gallium nitrate that resulted in prolongation of the undiluted working suspension inoculum median TTD equal to that of the growth control tubes (containing no gallium nitrate) diluted 1:10 and 1:100, respectively. When the critical concentration was between 2 experimental points, the concentration was calculated via linear interpolation by use of the results for only the concentrations above and below the critical points.

**Results**

Growth of all 10 MAP isolates was inhibited by gallium nitrate in a dose-dependent manner (Figure 1). There was significant variation in TTD on the basis of
the concentration of gallium nitrate ($P = 0.01$) and the MAP isolate ($P \leq 0.05$). A significant interaction was detected between MAP isolate and the gallium nitrate concentration. Post hoc comparisons revealed that for all MAP isolates, TTD for all gallium nitrate concentrations > 200µM was significantly different from the TTD for the growth control tubes (MAP without gallium nitrate). In addition, for all pairwise comparisons, the TTD at each sequential concentration differed significantly from the TTD for the next higher concentration, except for 600 and 800µM.

Overall, the concentration of gallium nitrate that resulted in 90% growth inhibition ranged from < 200µM for the most susceptible isolates to 743µM for the least susceptible isolates (Table 1). For 4 of the tested isolates, 99% inhibition was not achieved at the highest gallium nitrate concentration (1,000µM).

**Table 1**—Concentration of gallium nitrate that resulted in 90% and 99% growth inhibition for 10 MAP isolates in vitro.

<table>
<thead>
<tr>
<th>MAP isolate</th>
<th>Gallium nitrate concentration (µM)</th>
<th>90% inhibition</th>
<th>99% inhibition</th>
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<tbody>
<tr>
<td>Bovine 1</td>
<td>286</td>
<td>467</td>
<td>NA</td>
</tr>
<tr>
<td>Bovine 2 (ATCC 700535)</td>
<td>440</td>
<td>1,000</td>
<td>NA</td>
</tr>
<tr>
<td>Bovine 3</td>
<td>286</td>
<td>630</td>
<td>NA</td>
</tr>
<tr>
<td>Bovine 4</td>
<td>611</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Bison 1</td>
<td>&lt; 200</td>
<td>&lt; 200</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Bison 2</td>
<td>440</td>
<td>933</td>
<td>NA</td>
</tr>
<tr>
<td>Alpaca</td>
<td>&lt; 200</td>
<td>&lt; 200</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Human 1 (ATCC 43544)</td>
<td>743</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Human 2 (ATCC 43015)</td>
<td>566</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Human 3 (ATCC 43546)</td>
<td>730</td>
<td>NA</td>
<td>NA</td>
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</table>

The 90% and 99% growth inhibition corresponded to a delay in TTD equal to that obtained for culture of the MAP stock solution (without gallium nitrate) diluted 1:10 and 1:100, respectively. NA = Not achieved at the highest gallium nitrate concentration of 1,000µM.

**Discussion**

Numerous antimicrobials have been used for the treatment of individual animals with paratuberculosis, but antimicrobial treatment has not been used on a herd-wide basis to prevent infection in susceptible calves. The use of antimicrobials or other compounds for this purpose may represent a novel preventative approach. In cattle, the use of gallium is particularly attractive because it is not an antimicrobial; thus, concerns about the public health impact of antimicrobial resistance could be avoided.

The in vitro efficacy of tilmincosin phosphate against a single isolate of MAP was determined in a study in 2004. In that same study, the in vitro activity of monensin sodium against 1 isolate of MAP was also determined. Studies conducted to evaluate the efficacy of gallium against $R$ equi, a facultative intracellular bacterium that causes severe bronchopneumonia in foals, revealed promising results. It has been found that gallium is efficacious in inhibiting the growth of $R$ equi in vitro, that tissue burden of $R$ equi is lower in gallium-treated mice, and that gallium maltolate is bioavailable when given orally to neonatal foals. On the basis of these findings, it has been proposed that gallium could suppress the growth of or kill intracellular $R$ equi when prophylactically administered to susceptible neonatal foals. The pattern of infection of foals with $R$ equi is similar to that for MAP infection in calves because it is believed that both infections develop soon after birth.

In the study reported here, gallium nitrate caused at least 90% growth inhibition of all 10 isolates of MAP tested in broth culture. Growth of MAP was inhibited in a dose-dependent and variable manner among MAP isolates. In other words, different isolates responded differently to gallium nitrate, which was reflected by the variability in the inhibitory concentrations (Table 1). In the mixed-effect regression analysis, the 500µM concentration of gallium nitrate was excluded. This omission would not have affected the conclusion that growth inhibition was a dose-dependent phenomenon. In the determination of the 90% and 99% microbial growth inhibition, the missing value for 500µM may have had a small influence on the results of 2 isolates (bovine isolates 1 and 2) because the critical values were approximately 500µM for both isolates. However, in both cases, the critical values would still have been between 400 and 600µM.

Results of the present study are comparable with those of other studies conducted to investigate in vitro activity of gallium against other microorganisms. In 1 study, investigators reported 90% growth inhibition of several isolates of $M$ tuberculosis and $M$ avium for concentrations between 250 and 1,000µM. Fifty micromoles of gallium nitrate inhibited the growth of $R$ equi in media that did not contain additional iron. The biological effect of gallium appears to relate to its ability to substitute for iron in many biomolecular processes.

The isolates used in the present study were chosen from our laboratory bank and ATCC to include a variety of sources of origin (human and animal species) and geographic areas of the United States. Further characterization of the isolates to rule out genetic similari-
ties was not performed prior to the study. Because susceptibility to gallium nitrate was variable among the 10 isolates used in this study, it is possible that the efficacy of prophylactic administration of gallium to susceptible animals on a farm where MAP is endemic may depend on the types of isolates present on the farm.

Evaluation of the in vivo effect of dietary supplementation of iron on the efficacy of gallium was beyond the scope of this study. However, in vitro experiments have revealed that the growth inhibition induced by gallium is prevented in a concentration-dependent manner by excess iron, which implies that gallium acts primarily by interfering with bacterial iron metabolism.\

Most animals become infected with MAP in the neonatal period and may remain infected for life. Disease eradication from a herd is extremely difficult in part because of the long incubation period and the lack of definitive diagnostic tests during the early phases of infection. Therefore, disease control is often primarily directed at preventing new infections within replacement animals. In addition to early separation of a calf from its dam, colostrum management, and decreasing the pressure of infection through culling of high-shedding adult animals, strategies aimed at decreasing the risk of new infections in calves could include the prophylactic administration of gallium during the first few months after birth. Use of this compound, administered as a chemoprophylactic agent to calves during the period of greatest susceptibility, would represent a novel control strategy for the reduction of MAP transmission on infected farms. Further studies are needed to evaluate the bioavailability and safety of gallium nitrate in neonatal calves and to determine the in vivo activity of gallium nitrate against MAP in neonatal calves.

References