Antimicrobial Activity of Gallium Nitrate against *Mycobacterium avium* subsp. *paratuberculosis* in Neonatal Calves


**Background:** *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the agent of Johne’s disease in cattle, is a facultative intracellular bacterium that is dependent on ferric iron for its survival and replication. Gallium (Ga), a trivalent semimetal that shares many similarities with ferric iron and functions as an iron mimic has been shown to have in vitro antimicrobial activity against several microorganisms, including MAP.

**Objectives:** (1) To investigate the antimicrobial activity of Ga in calves experimentally infected with MAP; and (2) to monitor for potential adverse effects of Ga on calf health.

**Animals:** Twelve Holstein calves.

**Methods:** Randomized blind controlled experiment. Beginning at 10 days of age (study day 1), the experimental calves (n = 6) were treated with 20 mg/kg gallium nitrate daily for 45 days. On study days 4 and 5, all calves were challenged with a PO dose of a live field strain MAP. Treated calves were monitored daily for adverse effects. Calves were euthanized on study day 100, and 29 tissue samples and 1 fecal sample were collected from each calf. Samples were cultured for MAP by MGIT liquid culture system, Herrold’s Egg Yolk Medium culture, or both.

**Results:** No adverse effects were observed in the treated calves. Treatment was associated with a significant reduction in MAP tissue burden when compared with control calves (P = .017).

**Conclusions and Clinical Relevance:** Chemoprophylactic treatment of calves with Ga before and during the period of high susceptibility decreased MAP tissue colonization in experimentally infected neonatal calves.

**Key words:** Cattle; Johne’s disease; Paratuberculosis; Prophylaxis.

Johne’s disease (JD) is a chronic, granulomatous infection of the intestinal tract of cattle and other ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Most calves probably become infected with MAP very early in life. For this reason, control programs are based primarily on preventing direct or indirect transmission of MAP organisms from adult cattle to young replacement stock on the farm. No current drugs are approved for the prevention or treatment of JD in cattle. Vaccination has been shown to decrease MAP fecal shedding in infected animals and, in conjunction with husbandry changes, can decrease the incidence of infection in herds. However, vaccination does not fully prevent infection and is available only on a limited basis in the United States. Prophylactic administration of an antimicrobial agent to neonatal calves during the period of high susceptibility and exposure may represent an additional approach to the prevention of infection. Monensin sodium, a monovalent polyether antibiotic the properties of which include anticycclidal activity and improved feed utilization in cattle, has shown some efficacy against MAP in both in vitro and in vivo situations. However, in a clinical trial, reduction in fecal shedding in cattle receiving monensin was found to be marginal and the biological effect of this reduction remains unknown. In addition, the generalized use of antibiotics in food-producing animals is now more scrutinized by the general public, and may represent an added impediment to this approach.

MAP is a facultative intracellular bacterium that can survive and reproduce within monocytes and macrophages. Ferric iron is crucial for its survival and replication, thereby providing a potential target for prophylactic and therapeutic strategies. Ferric iron sequestration is used by hosts, mediated primarily by transferrin, but also by lactoferrin and ferritin, as an innate defense mechanism. Mycobacteria, however, can produce and use siderophores, thereby circumventing this defense mechanism. Gallium (Ga) is a trivalent semimetal that shares many similarities with ferric iron and functions as an iron mimic. Ga is preferentially taken up by phagocytes at sites of inflammation, and its biologic effect appears to relate to its ability to substitute for ferric iron in many cellular metabolic pathways and disrupt them. In vitro antimicrobial activity of Ga has been shown against various microorganisms, including *Rhodococcus equi, Pseudomonas aeruginosa, Mycobacterium tuberculosis*, and MAP. The antimicrobial activity of Ga in calves experimentally infected with MAP is of interest for further study.
activity of Ga against R. equi also was demonstrated in vivo in mice treated PO with Ga maltolate and experimentally infected with R. equi.14

The objectives of this study were (1) to investigate the ability of the metallic compound Ga to inhibit MAP infection in neonatal calves; and (2) to monitor and evaluate potential adverse effects of Ga on calf health. We hypothesized that the use of gallium nitrate (GaN) in neonatal calves is safe, and that treatment of calves with GaN before and during exposure to MAP organisms could prevent or decrease MAP infections in calves.

Materials and Methods

Study Design

Randomized blind controlled experiment.

Animals

The study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Twelve healthy newborn Holstein male calves were obtained from a nearby JD-negative commercial dairy. Calves were separated from their dams within 1 hour after birth and fed 4 L of colostrum that was collected from their own dams. Within 72 hours after birth, the calves were transported to the New Bolton Center Research Isolation Barn where they were housed in individual pens and fed milk replacer and calf starter grain according to normal husbandry methods. Calves were randomly assigned (in blocks of 4) to treatment or control group (n = 6 per group).

Experimental Design

Beginning at 10 days of age (study day 1), the experimental group calves (n = 6) were treated daily with 20 mg/kg of GaN powdera mixed with the morning milk replacer meal. This dose had been extrapolated from existing pharmacokinetic data on oral Ga maltolate administration in neonatal foals,17 and from a pilot study of oral GaN administration in neonatal calves (data not shown). The treated calves were fed GaN every day for 45 days, at which time treatment was discontinued and normal feeding resumed. On 2 occasions (study days 4 and 5), all 12 calves were challenged with an oral dose of 5 × 107 colony-forming units (CFU) of a live field strain MAP (American Type Culture Collection 700,535; prepared as described previously).18 Briefly, first-passage MAP seed stock that was originally cultured from a bovine fecal sample was frozen at −70°C. Before the start of the experiment, an aliquot of seed stock was thawed, propagated in mycobacterial growth medium, and stored at 4°C until used in the mycobacterial challenge. The MAP was suspended in milk replacer, and the calves voluntarily suckled the suspension from a syringe. Calves were euthanized on study day 100 by IV of barbiturates.

Clinical Observations and Sample Collection

Upon arrival at the facilities, all calves were weighed and blood was obtained for determination of plasma total protein concentration by refractometer. For calves assigned to the treatment group, blood was obtained before treatment for CBC, serum biochemistry profile, and determination of plasma fibrinogen concentration. These samples again were obtained on the treated calves before necropsy (study day 100) to monitor for adverse effects of Ga on calf health. Throughout the treatment period, calves were monitored twice daily for any possible adverse effects, including diarrhea, decreased appetite, dullness, hematuria, lameness, and oral ulcerations. In addition, blood was collected from each calf before MAP challenge (study day 4) and before necropsy (study day 100) to test for MAP antibody with a commercial ELISA kit according to the manufacturer’s recommendations. All calves were weighed again before necropsy (study day 100). At necropsy, 29 tissue samples and 1 fecal sample were collected from each calf for MAP culture. Tissue samples included duodenum, 10 sites of jejunum and adjacent lymph nodes, 3 sites of ileum, 2 ileocecal lymph nodes, ileocecal valve, cecum, and spiral colon.

Table 1. Median (range) time to detection (TTD) of MAP in liquid culture for selected tissues from gallium-treated and untreated controls.a

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TTD (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
</tr>
<tr>
<td>Jejunum</td>
<td>60 (46.8–60)</td>
</tr>
<tr>
<td>Jejunal Ln</td>
<td>42.4 (12.0–60)b</td>
</tr>
<tr>
<td>Ileum</td>
<td>20.2 (14.4–22.0)</td>
</tr>
<tr>
<td>Ileocecal Ln</td>
<td>14.2 (12.0–21.1)</td>
</tr>
<tr>
<td>Cecum</td>
<td>49.7 (35.7–60)b</td>
</tr>
<tr>
<td>All tissues</td>
<td>21.5 (6.3–60)b</td>
</tr>
</tbody>
</table>

MAP, Mycobacterium avium subsp. paratuberculosis; Ln, lymph node.

a A time of 60 days was assigned to those samples with no growth after 60 days of incubation. Sites displayed are from 5 representative sites (total of 29 sites sampled from each calf, 6 calves per group).
b Results significantly different from control, P < .05.

Sample Processing

Laboratory technicians processing the tissue and fecal samples were blinded to the calves’ experimental group assignment. Tissue and fecal samples were processed for MAP culture using hexadecylpyridinium chloride decontamination.19,20 Fecal samples were cultured by 2 systems: the MGIT16 liquid broth method, and solid Herrold’s Egg Yolk Medium (HEYM). Tissue samples were cultured by the MGIT liquid broth method only.

For the liquid broth method, MGIT tubes were first fortified with egg yolk and growth supplement. Tubes then were inoculated with each tissue or fecal sample (1 tube/sample) and incubated at 37°C in the BACTEC 960 instrument,16 which automatically detects mycobacterial growth by measure of bacterial oxygen consumption. The time to detection (TTD) for each tube was recorded in days. Any tube that did not signal by end of protocol (56 days) was considered negative for mycobacterial growth. For purposes of analysis, a TTD of 60 days was assigned to cultures that yielded negative results. Confirmation that detected growth was because of MAP was made with a commercial RT-PCR kit according to the manufacturer’s recommendation.

For the HEYM culture method, fecal samples (4 tubes/sample) were cultured for MAP by a method described previously.19 Cultures were read every 2 weeks for a period of 16 weeks, at which time the total number of CFU/tube was recorded.

Statistical Analysis

Mean body weight (arrival and necropsy) and total plasma protein concentrations of treated and nontreated calves were compared with Student’s t-test. For this experiment, the outcome variable was TTD in liquid culture for each tissue sample. Cox survival regression analysis was chosen to test the difference in TTD between treated calves and controls. More specifically, this was performed by...
including the TTD for each tissue from each calf in the model. Additionally, survival analysis was performed separately for each tissue site. Analysis was performed by the statistical software Stata 11.0.\(^4\) Values of \( P \leq .05 \) were considered statistically significant. For fecal cultures, the 2 outcome variables were (1) total number of CFUs/calf (sum of all 4 tubes/fecal sample), and (2) the TTD of each fecal sample. Descriptive statistics were used to describe fecal culture results.

**Results**

**Pretreatment Clinical Comparisons**

Mean weight at presentation was 103 lb (range, 93–120 lb) for the treated calves and 104 lb (range, 83–131 lb) for the nontreated calves. Mean plasma total protein concentration at presentation was 6.5 g/dL (range, 6.0–7.4 g/dL) for the treated calves and 6.4 g/dL (range, 5.5–7.2 g/dL) for the nontreated calves. There were no significant differences in plasma total protein and body weight between treated and nontreated groups, indicating that group randomization was effective.

**Impact on Calf Health**

No adverse effects were recorded on the treated calves throughout the treatment period. Both pre- and post-treatment routine blood test results were within the reference range for all treated calves. Mean weight at necropsy was 329 lb (range, 299–359 lb) for the treated calves and 332 lb (range, 306–369 lb) for the nontreated calves. The difference was not significant.

**Immune Response**

Calves were seronegative for antibodies against MAP at both time points.

**Tissue Colonization**

A significant difference in TTD was found between treated and control calves \( (P = .017) \) (Table 1). Treatment with GaN was associated with a significant delay in TTD (33% prolonged) of the tissue samples from the treated calves when compared with control calves (hazard ratio, 0.67; 95% confidence interval, 0.477–0.931).

**Fecal Shedding**

When evaluated using culture with HEYM, 3 calves from the treated group had positive cultures with 1, 1, and 2 MAP colonies, respectively. In the control group, 4 calves had positive cultures with 1, 1, 7, and 20 MAP colonies, respectively. When evaluated by MGIT liquid culture, 4 calves from the treated group had positive cultures with TTDs of 24.1, 24.2, 36, and 38.4 days. In the control group, 2 calves were positive with TTDs of 19.5 and 23.9 days. Overall, 4 calves in each group had low levels of MAP detected in feces by MGIT liquid culture, HEYM techniques, or both at the time of necropsy.

**Discussion**

In this study, liquid culture was used for MAP detection in the tissue and fecal samples of the experimental calves. Some of the advantages of this culture system are shorter duration of incubation when compared with solid culture media, and automated MAP detection. Quantification of MAP growth also is possible. Liquid culture results previously were shown to correlate inversely with HEYM culture results, where the higher the number of MAP CFUs per sample, the shorter the TTD for that sample.\(^4\) Therefore, prolonged TTD is associated with decreased MAP burden for that sample. In the previously cited study, in which liquid culture TTD and HEYM culture were compared, a 50% prolongation in TTD in the treated group was associated with a 7-fold reduction in CFUs/g of tissue detected by HEYM when compared with the control group. Thus, the authors concluded that although Ga did not prevent infection, a 33% prolongation in TTD may represent a biologically relevant reduction in tissue burden of MAP.

In the study reported here, oral treatment of calves with GaN before and after experimental challenge with MAP resulted in less extensive intestinal and lymph node tissue colonization in treated calves, compared with findings in control calves. GaN treatment was associated with a significant prolongation of TTD in the treated calves, which can translate to a decrease in MAP tissue burden. However, although GaN treatment decreased tissue colonization compared with control calves, infection was not prevented in any treated calves. Because calves were euthanized 13 weeks after challenge, it is not possible to know how many of them eventually would have developed clinical disease, and if treatment with GaN would have delayed the onset of clinical disease. Additional studies will be necessary to determine whether a reduction in MAP tissue load early in infection would lead to improved long-term outcome. In addition, fecal shedding at necropsy was detected in an equal number of calves per group. This underlines the importance of management practices directed at decreasing exposure of young stock to MAP, and that the addition of chemoprophylactic treatment of neonatal calves with GaN could represent an additional tool in decreasing MAP tissue burden in infected calves.

In the study described here, GaN was chosen over gallium maltolate (GaM). The main reason for this choice was the substantial cost difference between GaN and GaM, making the use of GaM cost prohibitive in food-producing animals. Previous studies have shown Ga salts (Ga chloride, GaN) to be less well absorbed PO than other formulations of Ga such as GaM.\(^21\) From a previous experiment evaluating the in vitro efficacy of GaN against 10 different MAP strains, the concentrations of GaN that resulted in 90 and 99% MAP growth inhibition for the challenge strain used here were 440 and 1,000 \(\mu\)M of GaN, respectively.\(^16\) In a pilot study measuring serum and fecal Ga concentrations in 2 neonatal calves receiving 20 mg/kg of GaN daily PO for 5 days, the average serum and fecal Ga concentration after 5 days of treatment were 6 and 5,027 \(\mu\)M, respectively (data not shown). Based on the results of the study reported here,
the antimicrobial effect of Ga may be intraluminal and high serum Ga concentrations may not be necessary, but this remains unclear. Additional pharmacokinetic and dose-response studies with GaN may be needed.

In humans, adverse effects associated with the IV administration of GaN include gastrointestinal irritation (nausea, vomiting, and diarrhea), bone pain, and renal toxicity.\(^2\)

Neonatal calves treated PO with 4 mg/kg of gallium chloride twice daily developed severe intestinal lesions, alterations in enteric microflora, and diarrhea.\(^2,21\) GaM has been reported to be better tolerated when administered PO than is GaN.\(^2,25\) In the study reported here, no adverse effects were detected in the calves throughout the treatment period, which lasted 45 days. Mean body weight at necropsy was equal in both groups, showing that treatment with GaN did not have a negative effect on calf growth. However, studies evaluating the toxicity of GaN in neonatal calves may be needed to further ensure its safety. Additional concerns regarding the use of GaN in cattle including drug residues, cost of prophylactic treatment, and environmental impact also would have to be considered.

## Footnotes

\(^a\) Sigma-Aldrich Co, Milwaukee, WI

\(^b\) Middlebrook 7H9 broth, BD Diagnostic Systems, Sparks, MD

\(^c\) Paracheck, Biorcor Animal Health, Omaha, NE

\(^d\) BACTEC MGIT, BD Diagnostic Systems

\(^e\) Tetracore Vet Alert, Tetracore Inc, Rockville, MD

\(^f\) StataCorp LP, College Station, TX

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## References


