Short Communication

Comparison of the antimicrobial activities of gallium nitrate and gallium maltolate against *Mycobacterium avium* subsp. *paratuberculosis* in vitro

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A B S T R A C T

Johne's disease (JD) is an enteric infection of cattle and other ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). This study compared the antimicrobial activities of gallium nitrate (GaN) and gallium maltolate (GaM) against two field MAP isolates by use of broth culture. The concentrations that resulted in 99% growth inhibition of isolates 1 and 2 were, respectively, 636 µM and 183 µM for GaN, and 251 µM and 142 µM for GaM. For both isolates, time to detection was significantly higher for GaM than GaN. These results suggest that GaM is more efficient than GaN in inhibiting MAP growth in vitro.

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Johne's disease (JD) is a chronic intestinal infection of cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Most new infections occur in young animals during the first few days of life. Control programs aim at preventing transmission of MAP to young stock but on farms with many infected cattle, exposure of susceptible calves is almost inevitable. Currently no drugs are approved for prevention of JD in cattle in the United States, and vaccination is not fully protective (Sweeney et al., 2009).

MAP, a facultative intracellular bacterium, survives and reproduces within monocytes and macrophages. Iron is crucial for its survival and replication (Olakamni et al., 2000), thereby providing a potential control target. Gallium (Ga), a trivalent semimetal with many similarities to ferric iron, functions as an iron mimic. At inflammation sites Ga is taken up by phagocytes, interferes with iron-dependent cellular pathways, and causes bacterial death (Olakamni et al., 2000). In vitro antimicrobial activity of Ga has been demonstrated against numerous microorganisms (Bernstein, 2013; Bonchi et al., 2014), including intracellular bacteria such as MAP (Fecteau et al., 2011a). Activity of Ga against MAP was also demonstrated in experimental infection where prophylactic treatments of calves with Ga nitrate (GaN) challenged with a live MAP field strain resulted in decreased tissue colonization (Fecteau et al., 2011b). However, despite reducing MAP bioburden in treated calves, none were completely protected from infection.

Ga maltolate (GaM), a novel preparation of Ga, has higher oral Ga bioavailability than GaN and is more lipid-soluble (Bernstein et al., 2000; Martens et al., 2007). This study was designed to compare in vitro antimicrobial activities of GaN and GaM against two field isolates of MAP. We hypothesized that, because of its lipid solubility, GaM would have better penetration of MAP, and show superior antimicrobial activity compared to GaN.
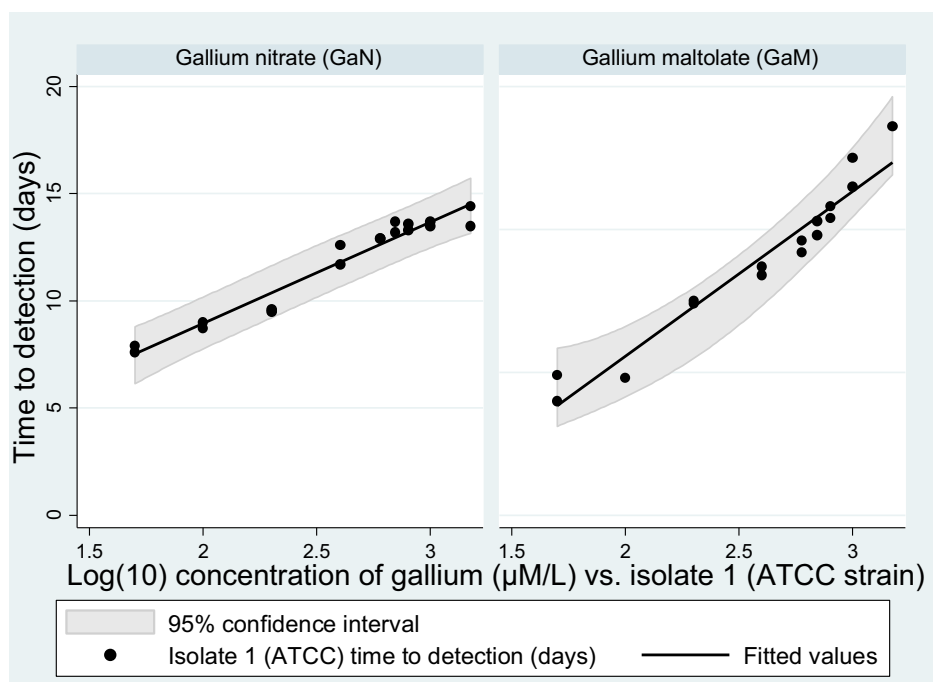
Two MAP strains isolated from naturally infected cows ('isolate 1' – ATCC 700535; 'isolate 2' – undesignated) were chosen for their different Ga sensitivities (Fecteau et al., 2011a). A stock solution of each isolate was prepared using a single-colony swab specimen from a Herrold egg yolk culture suspended in sterile saline (0.9% NaCl) (Fecteau et al., 2011a) to form a suspension with an approximate concentration of 10^6 MAP CFU/mL (determined by serial dilution on solid media).

Stock 20 mM solutions of GaN and GaM were prepared by dissolving each complex in liquid growth medium (Middlebrook 7H9, BD Diagnostic Systems). The GaN solution pH was adjusted to 6.8 by addition of saturated $NaHCO_3$; GaM required no adjustment. Each stock was sterilized by passage through a 0.22-µm filter, and diluted with liquid growth medium to give Ga concentrations of 50, 100, 200, 400, 600, 700, 800, 1000, and 1500 M in the incubation tubes.

Susceptibility testing was conducted using a mycobacterial detection system (BACTEC MGIT, BD Diagnostic Systems). Incubation tubes were first fortified with 500 µL egg yolk and 800 µL growth supplement (MGIT Growth Supplement, BD Diagnostic Systems), then inoculated in duplicate with 100 µL of the MAP isolate stock, and 700 µL of serial dilutions of GaN or GaM. In addition, for each

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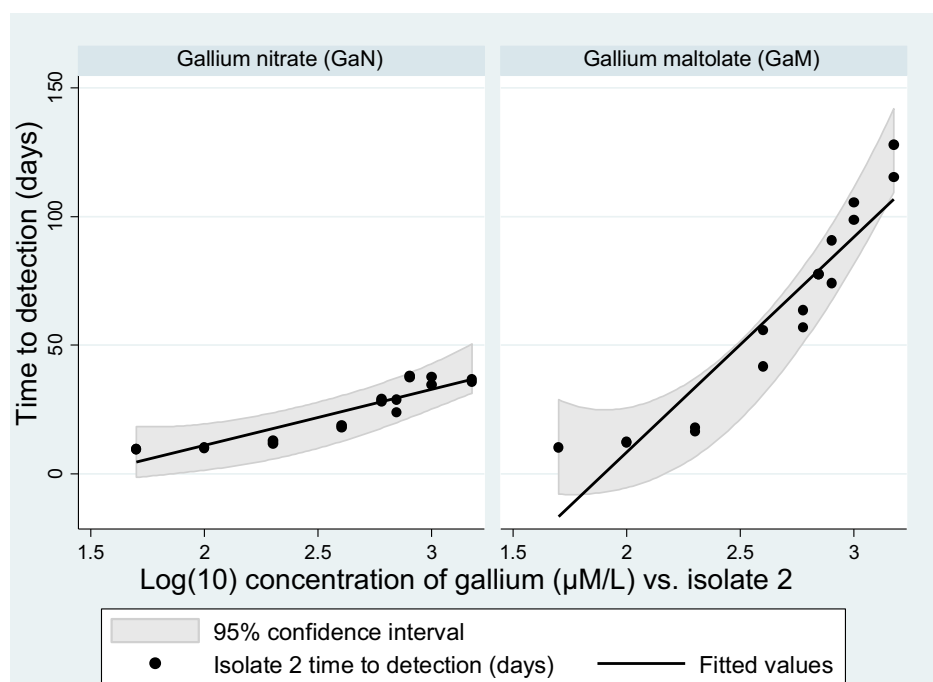*E-mail address:* mfecteau@vet.upenn.edu (M.E. Fecteau).

Fig. 1. Dose–response relationship with 95% CI for time to detection in days of MAP isolate 1 (ATTC strain) in the presence of increasing (50–1500 μM) concentrations of gallium nitrate (GaN; range 7.6–13.5 days) or gallium maltolate (GaM; range 9–18.6 days).

MAP isolate, the stock suspension and 1:10 and 1:100 dilutions of that suspension were inoculated in triplicate into tubes without any Ga (growth controls). All tubes were incubated at 37 °C. Time to detection (TTD) for each tube was recorded in days. The 90% and 99% growth inhibitory concentrations (IC) were determined by comparing prolongation of TTD in Ga-containing tubes to TTD for 1:10 (90% inhibition) or 1:100 dilutions (99% inhibition) as described elsewhere (Heifets, 1988; Ardito et al., 2001).

Results were analyzed by means of robust multiple linear regression (Stata 13; Stata Corp). Predictor variables were Ga (nitrate or maltolate) and $\log_{10}$ concentration of Ga (50–1500 μM). TTD was the outcome variable. IC 90%, IC 99% and 95% confidence intervals (CI) were determined from the regression based on average TTD for the 1:10 and 1:100 MAP dilutions, respectively.

Regression diagnostics indicated that models were both valid and reliable. Both Ga compounds significantly delayed growth of each

Fig. 2. Dose–response relationship with 95% CI for time to detection in days of MAP isolate 2 in the presence of increasing (50–1500 μM) concentrations of gallium nitrate (GaN; range 9.6–38.1 days) or gallium maltolate (GaM; range 10.3–127.9 days).

MAP isolate. Additionally, increase in TTD was significantly correlated to concentration of GaN or GaM (dose-dependent growth inhibition). Specifically, for isolate 1, when the influence of GaN or GaM was accounted for, each log_{10} unit increase in concentration was predicted to prolong TTD by 5.2 days (95% CI 4.6–5.8) (Fig. 1). For isolate 2, an even greater prolongation of TTD of 48.9 days (95% CI 31.8–65.9) was predicted (Fig. 2).

GaM demonstrated superior activity against both isolates manifested as prolonged TTD compared to GaN. For isolate 1, the regression model predicted that GaM prolonged TTD by 2.2 days (95% CI 1.8–2.7). For isolate 2, the GaM effect was markedly greater (TTD = 35.6 days, 95% CI 23.8–47.4).

For isolate 1 the average and standard deviation of TTD for the 1:10 and 1:100 dilutions were 10.2 ± 0.6 days and 12.8 ± 0.5 days, respectively. Equivalent values for isolate 2 were 12.5 ± 0.1 and 15.5 ± 0.1 days. Concentrations of GaN and GaM that resulted in 90% and 99% growth inhibition of both isolates are enumerated in Table 1.

We determined that both Ga formulations showed dose-dependent antimicrobial efficacy in vitro against each MAP isolate. Moreover, regardless of MAP isolate, GaM was significantly superior to GaN.

When dissolved in aqueous solution, Ga salts such as nitrate dissociate into Ga hydroxide and corresponding acids, which renders them poorly lipid soluble. GaM, however, is a metal–organic coordination complex that remains intact at pH values between about 5 and 8, is soluble in both water and lipids and as such can penetrate cell membranes, including those of bacteria. It is likely that these features are the reason for its superior antimicrobial effect.

Based on these results, GaM is more efficient than GaN in inhibiting MAP growth in vitro. Although the data presented here are promising, additional research is needed to investigate the effect of GaM on a larger collection of MAP isolates from veterinary sources, as well as its efficacy in preventing MAP infection in vivo. Finally, thorough safety and economical analyses would have to be completed before GaM could be used in cattle.

Conflict of interest statement

None of the authors of this paper has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table 1
Concentrations of GaN and GaM that resulted in 90% and 99% growth inhibition for two MAP isolates in vitro.

	IC90 (95% CI) μM		IC99 (95% CI) μM	
	GaN	GaM	GaN	GaM
Isolate 1	190 (164–220)	94 (74–122)	636 (556–728)	251 (214–294)
Isolate 2	140 (102–194)	132 (99–179)	183 (138–243)	142 (105–190)

GaN, gallium nitrate; GaM, gallium maltolate; MAP, *Mycobacterium avium* subsp. *paratuberculosis*, IC, inhibitory concentration; CI, confidence intervals.