In Vitro Susceptibility of Equine-Obtained Isolates of Corynebacterium pseudotuberculosis to Gallium Maltolate and 20 Other Antimicrobial Agents

Running title: Equine C. pseudotuberculosis susceptibilities

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Abstract: This study’s objective was to determine the in vitro antimicrobial activity of gallium maltolate (GaM) and 20 other antimicrobial agents against equine clinical isolates of Corynebacterium pseudotuberculosis. Growth of cultured isolates was not inhibited by any concentration of GaM. Minimum inhibitory concentration data revealed susceptibility to commonly used antimicrobials.

Keywords: Equine, Corynebacterium pseudotuberculosis, gallium, antimicrobial therapy

This study’s objective was to evaluate the in vitro susceptibility of C. pseudotuberculosis to gallium and 20 conventional antimicrobial agents. We hypothesized that equine isolates of C. pseudotuberculosis would be susceptible to commonly used antimicrobials and GaM.

One hundred equine isolates of C. pseudotuberculosis were obtained from the Texas A&M University Veterinary Medical Teaching Hospital (VMTH) Clinical Microbiology Laboratory repository and from the Diagnostic Bacteriology/Mycology Section at the Texas Veterinary Medical Diagnostic Laboratory (TVMDL). Isolates were confirmed as C. pseudotuberculosis using rapid identification test kits (VMTH – RapID CB Plus system, TVMDL – API Coryne). Isolates were stored at -80°C in trypticase soy broth supplemented with 10% glycerol.

The minimum inhibitory concentration (MIC) of GaM against isolates of C. pseudotuberculosis was evaluated by macrodilution. Isolates were transferred from frozen stock onto brain-heart infusion (BHI) agar plates and allowed to grow for 48 hours at 37°C. One or 2 isolated colonies were then selected from each plate and inoculated into 5 mL of BHI broth (supplemented with 0.1% Tween 80 to prevent clumping of the C. pseudotuberculosis colonies, and 20 µg/mL 2,2’-dipyridyl to chelate free iron) and incubated at 37°C for 24 hrs.
Following incubation, each isolate was re-suspended in the broth to spectrophotometrically achieve an optical density of 0.08-0.10 at a wavelength of 600 nm (optically comparable to that of a 0.5 McFarland standard). Each suspension contained approximately 3x10^6 colony forming units (CFU)/mL of *C. pseudotuberculosis*. The suspensions were immediately diluted in the broth to achieve a working concentration of 1x10^6 CFU/mL, which was combined with each GaM dilution to achieve a final concentration of 5x10^5 CFU/mL.

Two-fold serial dilutions of GaM in BHI broth were made to achieve 6 concentrations ranging from 1,024 µM to 32 µM. The diluted inoculum for each isolate was added 1:1 to each concentration of GaM in 12 mm x 75 mm round-bottom tubes for final concentrations of GaM of 512, 256, 128, 64, 32, and 16 µM. Positive (inoculum only) and negative (without inoculum) controls were prepared for each isolate or GaM dilution. Additionally, 5 isolates were tested by the same methods, but replaced GaM with gentamicin. Tubes were incubated for 24 hours at 37°C on a shaker and read by visual inspection. Growth or no growth was recorded. Measurements were considered invalid if no growth was noted in the positive control. For each isolate, MIC was defined as the lowest concentration of GaM inhibiting visible growth in the tube.

Sixty-four isolates were used for gallium susceptibility testing. Following 24 hours of incubation, growth of *C. pseudotuberculosis* was observed in all isolates at every concentration. The MIC of gentamicin was uniformly 2 mg/L (0.42 µM). The sample size was limited due to the consistency of results.
Standard antimicrobial 50% and 90% MICs were determined using commercially available veterinary susceptibility plates. Plate preparation employed a microbroth dilution technique, which correlates well with the standard broth dilution method. Isolates were prepared as described in the Clinical and Laboratory Standards Institute (CLSI) approved standard for antimicrobial susceptibility testing. Ten microliters of suspension were transferred into 10 ml of cation-adjusted Mueller-Hinton broth (CAMHB) for a final bacterial concentration of $1 \times 10^5$ cfu/ml. Each well was inoculated with 100 µl of CAMHB. Each plate included positive (no antimicrobial) control wells. The plates were sealed and incubated in room air at 35°C. Plates were read by visual inspection at 24 and 48 hours post-incubation because of the slow growth of the organism. Results (growth/no growth) were recorded. Plates were considered invalid if there was insufficient growth in the positive control. The assay was validated weekly using *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922. The MIC for each isolate was defined as the lowest concentration of an antimicrobial to inhibit any visible growth. The MIC required to inhibit 50% (MIC$_{50}$) and 90% (MIC$_{90}$) of isolates were determined at 24 and 48 hours.

Ninety four isolates were available for antimicrobial susceptibility testing. The MIC values obtained for *S. aureus* 29213 and *E. coli* ATCC 25922 were within the reference interval proposed by the CLSI. The MIC$_{50}$ and MIC$_{90}$ results at 24 and 48 hours are summarized in Table 1.

Gallium maltolate was investigated because *C. pseudotuberculosis* possesses genes for iron uptake that are activated in iron-poor environments. *Rhodococcus equi*, a related organism, is susceptible to GaM. In the present study, GaM failed to inhibit the growth of *C. pseudotuberculosis*.
Possible explanations for GaM’s lack of effect on the growth of *C. pseudotuberculosis* include the potential to utilize other metals as a metallo-cofactor when iron is not available. A closely related species (*C. ammoniagenes*) has been shown to utilize manganese in this way.\(^7\) Another possibility is the potential for the organism to use siderophores to obtain iron. Related bacteria use siderophores to obtain iron from intra-macrophage ferritin.

*Corynebacterium pseudotuberculosis* also contains a cluster of iron-acquisition genes\(^8\) that may have enabled the use of chelated iron.

Because *C. pseudotuberculosis* failed to grow in minimal media, BHI supplemented with 20 µg/ml of 2,2’-dipyridyl and 0.1% Tween 80 was selected based on the results of Billington *et al.*\(^9\) The chelating effects of 2,2’-dipyridyl on gallium are unknown; it is possible that the GaM was chelated, mitigating any possible antibacterial effects.

MIC values were similar to those reported for mixed-source isolates.\(^10\)–\(^12\) Because of phenotypic differences between the equine and ovine strains and our interest in equine medicine, only equine isolates were evaluated.\(^10\) The antimicrobials tested were selected because they were included on the standard commercially available antimicrobial susceptibility plate. Although most antimicrobials performed well against *C. pseudotuberculosis* *in vitro*, *in vivo* activity is likely to be limited to drugs with good lipid solubility and intracellular activity.

The MIC values after 24 and 48 hours of growth showed good agreement. For penicillin and oxacillin, the MIC\(_{90}\) values at 48 hours were 1 concentration higher than at 24 hours. For amikacin and trimethoprim-sulfa the MIC range was greater at 48 hours than at 24 hours.

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116  **References:**
118     antimicrobial drug susceptibility testing: a collaborative study. Antimicro. Agents
119     Chemo. **17:**464-469.
120  2. **Clinical and Laboratory Standards Institute (CLSI).** 2008. Performance standards for
121     antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals;
122     approved standard – 3rd edition. Clinical Laboratory Standards Institute, Wayne, PA.
124     *Corynebacterium pseudotuberculosis*: microbiology, biochemical properties,
125     pathogenesis and molecular studies of virulence. Vet Res. **37:**201-218.


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**Table 1**: Summary of Mean Inhibitory Concentrations (MIC) (µM) for 20 antimicrobial agents to equine isolates of *Corynebacterium pseudotuberculosis*. 

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159 Table 1: Summary of Mean Inhibitory Concentrations (MIC) (µM) for 20 antimicrobial agents to equine isolates of *Corynebacterium pseudotuberculosis*.