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

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This study's objective was to determine the *in vitro* antimicrobial activities of gallium maltolate (GaM) and 20 other antimicrobial agents against clinical equine isolates of *Corynebacterium pseudotuberculosis*. The growth of cultured isolates was not inhibited by any concentration of GaM. MIC data revealed susceptibility to commonly used antimicrobials.

This study's objective was to evaluate the *in vitro* susceptibility of *Corynebacterium 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 (at VMTH, RapID CB Plus system [Remel, Lenexa, KS]; at TVMDL, API Coryne [bioMérieux, Durham, NC]). Isolates were stored at −80°C in Trypticase soy broth (BD, Franklin Lakes, NJ) supplemented with 10% glycerol.

The MIC of GaM against isolates of *C. pseudotuberculosis* was evaluated by macrodilution. Isolates were transferred from frozen stock onto brain heart infusion (BHI) (BD, Franklin Lakes, NJ) agar plates and allowed to grow for 48 h at 37°C. One or two isolated colonies were then selected from each plate and inoculated into 5 ml of BHI broth (supplemented with 0.1% Tween 80 [Sigma-Aldrich, St. Louis, MO] to prevent clumping of the *C. pseudotuberculosis* colonies and 20 μg/ml 2,2′-dipyridyl [Sigma-Aldrich, St. Louis, MO] to chelate free iron) and incubated at 37°C for 24 h.

Following incubation, each isolate was resuspended in the BHI broth to spectrophotometrically achieve an optical density of 0.08 to 0.10 at a wavelength of 600 nm (optically comparable to that of a 0.5 McFarland standard). Each suspension contained approximately 3×10^6 CFU/ml of *C. pseudotuberculosis*. The suspensions were immediately diluted in the BHI broth to achieve a working concentration of 1×10^6 CFU/ml, which was combined with each GaM dilution to achieve a final concentration of 5×10^5 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 by 75-mm round-bottom tubes (BD, Franklin Lakes, NJ) 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 GaM was replaced with gentamicin (Gibco Invitrogen, Invitrogen Corp., Carlsbad, CA). Tubes were incubated for 24 h 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, the 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 h of incubation, the growth of *C. pseudotuberculosis* was observed in all isolates at every concentration. The MIC of gentamicin was uniformly 2 mg/liter (0.42 μM). The sample size was limited due to the consistency of results.

Standard antimicrobial MIC_{50}s and MIC_{90}s were determined using commercially available veterinary susceptibility plates (TREK Diagnostic Systems, Cleveland, OH). Plate preparation employed a broth microdilution technique, which correlates well with the standard broth dilution method (1).

Isolates were prepared as described in the Clinical and Laboratory Standards Institute (CLSI) (2) approved standard for antimicrobial susceptibility testing. Ten microliters of suspension was transferred into 10 ml of cation-adjusted Mueller-Hinton broth (CAMHB; TREK Diagnostic Systems, Cleveland, OH) for a final bacterial concentration of 1×10^5 CFU/ml. Each well was inoculated with 100 μl of CAMHB. Each plate included positive-control (no-antimicrobial) wells. The plates were sealed and incubated in room air at 35°C. Plates were read by visual inspection at 24 and 48 h postincubation because of the slow growth of the organism (2, 3). 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

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TABLE 1 Summary of MICs of 20 antimicrobial agents for equine isolates of *Corynebacterium pseudotuberculosis*

Antimicrobial	24 h			48 h		
	Range (μM)	MIC_{50} (μM)	MIC_{90} (μM)	Range (μM)	MIC_{50} (μM)	MIC_{90} (μM)
Amikacin	All ≤4	≤4	≤4	≤4 to 16	≤4	≤4
Ampicillin	All ≤0.25	≤0.25	≤0.25	All ≤0.25	≤0.25	≤0.25
Azithromycin	All ≤0.25	≤0.25	≤0.25	All ≤0.25	≤0.25	≤0.25
Cefazolin	All ≤4	≤4	≤4	All ≤4	≤4	≤4
Ceftazidime	≤1 to 32	16	16	≤1 to 32	16	32
Ceftiofur	≤0.25 to 0.5	0.5	0.5	≤0.25 to 0.5	0.5	0.5
Chloramphenicol	All ≤4	≤4	≤4	All ≤4	≤4	≤4
Clarithromycin	All ≤1	≤1	≤1	All ≤1	≤1	≤1
Doxycycline	All ≤2	≤2	≤2	All ≤2	≤2	≤2
Enrofloxacin	All ≤0.25	≤0.25	≤0.25	All ≤0.25	≤0.25	≤0.25
Erythromycin	All ≤0.25	≤0.25	≤0.25	All ≤0.25	≤0.25	≤0.25
Gentamicin	≤1 to 2	≤1	≤1	≤1 to 2	≤1	≤1
Imipenem	All ≤1	≤1	≤1	All ≤1	≤1	≤1
Oxacillin	≤0.25 to >4	2	2	≤0.25 to >4	2	4
Penicillin	≤0.06 to 0.25	≤0.06	≤0.06	≤0.06 to 0.25	0.12	0.12
Rifampin	All ≤1	≤1	≤1	All ≤1	≤1	≤1
Tetracycline	All ≤2	≤2	≤2	All ≤2	≤2	≤2
Ticarcillin	≤8 to 16	≤8	≤8	≤8 to 16	≤8	≤8
Ticarcillin-clavulanate	All ≤8	≤8	≤8	All ≤8	≤8	≤8
Trimethoprim-sulfamethoxazole	All ≤0.5	≤0.5	≤0.5	≤0.5 to 1	≤0.5	≤0.5

to inhibit any visible growth. The MICs required to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of isolates were determined at 24 and 48 h.

Ninety-four isolates were available for antimicrobial susceptibility testing. The MICs obtained for *S. aureus* 29213 and *E. coli* ATCC 25922 were within the reference interval proposed by the CLSI (2). The MIC_{50} and MIC_{90} results at 24 and 48 h are summarized in Table 1.

Gallium maltolate was investigated because *C. pseudotuberculosis* possesses genes for iron uptake that are activated in iron-poor environments (4). *Rhodococcus equi*, a related organism, is susceptible to GaM (5, 6). 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 metallocofactor when iron is not available. A closely related species (*Corynebacterium 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 intramacrophage 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 h of growth showed good agreement. For penicillin and oxacillin, the MIC_{90} values at 48 h were 1 concentration higher than at 24 h. For amikacin and trimethoprim-sulfamethoxazole, the MIC range was greater at 48 h than at 24 h.

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