

Determination of the Antibacterial Activity of Gamithromycin Against Pathogens of Bovine Respiratory Disease

Ronald K. Tessman¹

Donald J. Bade²

¹*Merial, Fulton, Missouri*

²*Microbial Research Incorporated, Fort Collins, Colorado*

Corresponding author:

Ronald K. Tessman

3239 Satellite Blvd., Duluth, GA 30096-4640

Phone: 573-642-5977

E-mail: ron.tessman@merial.com

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ABSTRACT

A study was conducted to determine the time and concentration of gamithromycin at which a 3-log kill (99.9%) of select *H somni*, *M haemolytica*, and *P multocida* isolates is achieved. Initially, 12 isolates each of *H somni* and *M haemolytica* and five isolates of *P multocida* were triplicate tested. Each isolate was previously identified and determined to have a gamithromycin MIC equivalent to the MIC₉₀ of the respective species. Isolates were triplicate susceptibility tested to gamithromycin. Kill curves at concentrations of 0×, 1×, and 4× the MIC₉₀ of the species were tested at 0, 4, 8, 10, and 24 hours. By 8 hours and at all subsequent evaluations, the cell count for *M haemolytica* at gamithromycin 1 µg/mL fulfilled the 3-log decrease, and this dose was considered bactericidal. A mean 3-log reduction was achieved for *P multocida* between 10 and 24 hours, and gamithromycin 1 µg/mL was considered bactericidal.

INTRODUCTION

Gamithromycin is an azalide 15-membered semi-synthetic macrolide antibiotic that has been developed for treatment and prevention of bovine respiratory disease (BRD).¹⁻³ Studies of the pharmacokinetic and pharmacodynamic properties of gamithromycin showed that a single subcutaneous dose of gamithromycin at 6 mg/kg provides rapid therapeutic and persistent activity in the control and prevention of BRD pathogens *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*.^{1,3} In feedlot studies and studies with cattle assembled at sales for shipment to feedlots, gamithromycin administered at 6 mg/kg once by subcutaneous injection to cattle has provided significantly ($P \leq 0.05$) lower mean depression scores, respiratory character scores, rectal temperatures than cattle treated with saline.⁴⁻⁶ Mortality rates also were reduced in the cattle treated with gamithromycin compared with those treated with placebo.⁴ The current study was one of a series of in vitro studies to provide data on the antimicrobial activity of gamithromycin. Gamithromycin MIC₉₀

values have been determined to be 1,¹ and 0.5 µg/mL for *M haemolytica*, *P multocida*, and *H somni*, respectively, in North American isolates. The purpose of the study was to determine the time and concentration of gamithromycin at which a 3-log kill (99.9%) of select *H somni*, *M haemolytica*, and *P multocida* isolates is achieved.

MATERIALS AND METHODS

Test material

Gamithromycin was manufactured in accordance with pharmaceutical Good Manufacturing Practice as provided by the sponsor (Merial Ltd.).

Test bacteria

Selected isolates of each species were obtained from two diagnostic laboratories (Kansas State Veterinary Diagnostic Laboratory, Manhattan, Kansas and Newport Laboratories, Worthington, Minnesota). Initially, 12 isolates each of *H somni* and *M haemolytica* and five isolates of *P multocida* were triplicate MIC tested. Each isolate was previously identified and determined to have a gamithromycin MIC equivalent to the MIC90 of the respective species. The target MIC90 values 0.5 µg/mL for *H somni* and 1.0 µg/mL for *M haemolytica* and *P multocida* are the values that appear on the US product label. Five isolates each for *H somni* and *M haemolytica* were randomly selected from those available for testing. The five isolates originally provided for *P multocida* were those included for testing.

Each isolate was verified as being pure and having typical colony morphology prior to testing in the study. Viability and morphology of the isolates tested by streaking the frozen sample, plating to 5% sheep blood agar (BA), and incubating overnight at 36°±2°C. *Histophilus somni* were incubated in 5%±2% CO₂.

Susceptibility testing

Isolates that were identified as typical for each of the species were triplicate susceptibility tested to gamithromycin. Each MIC test originated from a separate colony. The assay purity of gamithromycin was 99.0%

(990 µg/mL). A stock solution of gamithromycin at 5120 µg/mL was prepared by dissolving 0.0517 g of the powder in 10 mL of 0.1 M, pH 6.0 phosphate buffer with the aid of sonification. The test material was divided into four aliquots, three of which were stored at or below -65°C until used. Each aliquot was identified, including the expiration date of the sample.

Preparation of the pH 6.0, 0.1 M phosphate buffer was accomplished as follows:

Solution X: 1.58 g of anhydrous sodium phosphate dibasic was dissolved in distilled water and made up to a final volume of 100 mL. Solution Y: 1.2 g of anhydrous monosodium phosphate was made up to a final volume of 100 mL. The complete buffer (50 mL) included 6.15 mL of Solution X mixed with 43.85 mL of solution Y, with a pH of 5.92.

Dilutions of gamithromycin ranging from 0.06 µg/mL to 32 µg/mL were prepared. Each well of the 96-well MIC plate was filled with 100 µL of the appropriate dilution. Growth control wells (positive and negative) were filled with 100 µL of the appropriate broth.

Cation-adjusted Mueller Hinton Broth (MHB) was used for testing *P multocida* and *M haemolytica*. Veterinary fastidious medium (VFM) was used for *H. somni*. MIC plates inoculated with *P. multocida* and *M. haemolytica* and their respective quality control organisms (*Staphylococcus aureus*, *Enterococcus faecalis*, and *H somni*) were incubated at 36°±2°C for 20.0 hours. MIC plates inoculated with *H. somni* were incubated at 36°±2°C with 5%±2% CO₂ for 22.0 hours.

Additional quality control tests included positive growth control wells for each isolate; sterility checks of plates at each testing date; and inoculum counts from positive control wells of one reference strain and up to four study isolates.

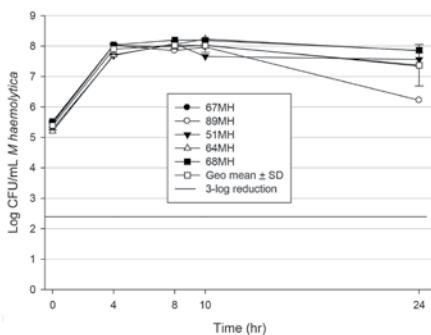
MIC results were interpreted as the lowest concentration of gamithromycin that completely inhibited growth of the organism as detected by the unaided eye.

Time kill curves

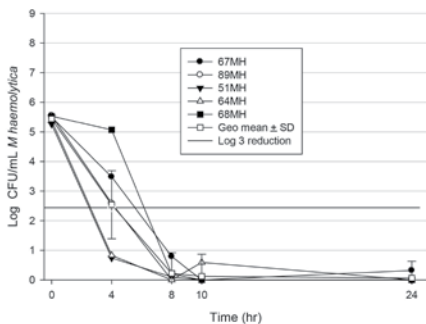
Time kill procedures were performed in triplicate. Each organism was grown overnight on a BA plate at $36^{\circ}\pm 2^{\circ}\text{C}$ (aerobically for *P multocida* and *M haemolytica* and *H somni* in $5\%\pm 2\%$ CO_2). Growth from the BA plate was suspended in 5 mL MHB until turbidity equivalent to 0.5 McFarland was reached. To reach the desired theoretical concentration of 5×10^5 colony-forming units (cfu), 350 μL of the suspension was diluted into 5 mL

Figure 1. Numbers of *Mannheimia haemolytica* at sampling times for gamithromycin doses 0 $\mu\text{g/mL}$ (0 \times) (A); 1 $\mu\text{g/mL}$ (1 \times) (B); and 4 $\mu\text{g/mL}$ (4 \times) (C).

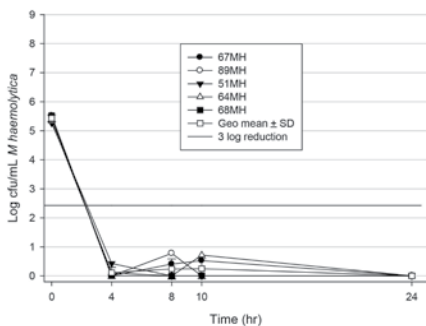
A



B



C



MHB for *M haemolytica* isolates; 625 μL was diluted into 5 mL MHB for *P multocida*; and 600 μL was diluted into 5 mL MHB for *H somni*. To the appropriate tubes, 150 μL of the adjusted suspension was added to fresh, prewarmed ($36^{\circ}\pm 2^{\circ}\text{C}$) 29.75 mL MHB for *M haemolytica* and *P multocida* or to 29.75 mL VFM for *H somni*. The inoculated tubes were incubated aerobically at $36^{\circ}\pm 2^{\circ}\text{C}$ without stirring or shaking for 2 (*M haemolytica* and *P multocida*) or 3 hours (*H somni*).

A stock solution of 5120 $\mu\text{g/mL}$ of gamithromycin was prepared (see Susceptibility testing section above). The stock solution was diluted in MHB or VFM. From the gamithromycin dilutions, final concentrations of 4, 1, and 0 $\mu\text{g/mL}$ were required to achieve concentrations equivalent to 4 \times , 1 \times , and 0 \times the MIC₉₀ of *M haemolytica* and *P multocida*. Final concentrations of 2, 0.5, and 0 $\mu\text{g/mL}$ were required for 4 \times , 1 \times , and 0 \times the MIC₉₀ of *H. somni*. A blank tube for each gamithromycin concentration tested containing no bacteria was exposed to the procedure.

Enumeration of incubated tubes

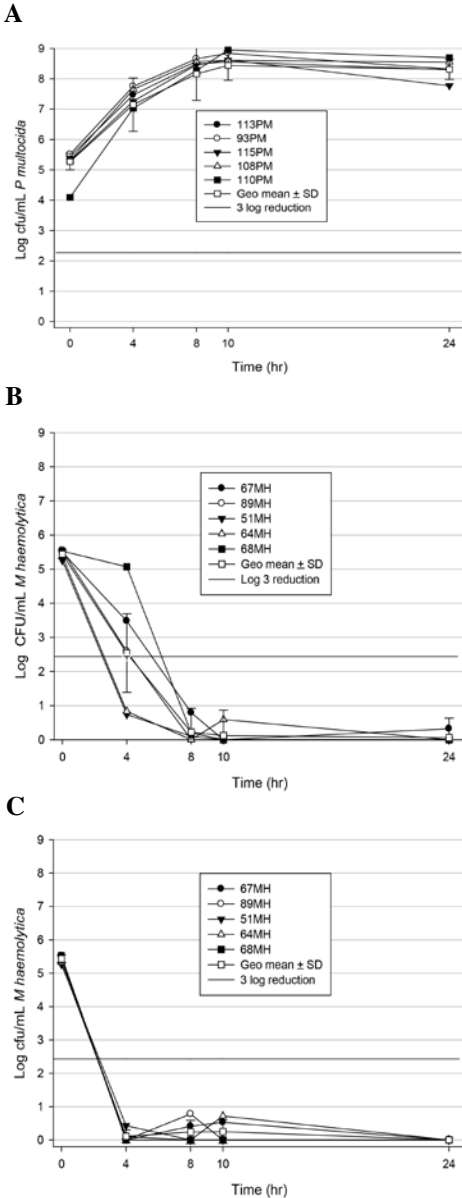
Each culture was enumerated at time 0, followed by incubation aerobically at $36^{\circ}\pm 2^{\circ}\text{C}$ while mixing on a rotating drum. Subsequent enumerations were performed at 4, 8, 10, and 24 hours (± 2 –5 minutes) of incubation.

At each time point, 3 mL was removed from each tube and plated onto BA using a combination of standard and/or spiral plating and the 50- μL deposition setting. At time 0, the removed aliquot from each tube plus an additional 1:10,000 dilution was spiral plated. At the 4- to 24-hour times, a 1:10,000 dilution was prepared and plated, in addition to the 1:100 dilution for the tubes containing 0 μL gamithromycin/mL. At these sampling times, all tubes containing gamithromycin at 4, 2, 1, or 0.5 $\mu\text{g/mL}$ had 1 mL of the aliquot plated to BA, and the aliquot plus a 1:100 dilution was spiral plated. If the turbidity of the culture increased, additional dilutions were plated at the discretion of the technician.

The BA plates were incubated aerobically at $36^{\circ}\pm 2^{\circ}\text{C}$ for *P multocida* (17.0–21.0 hours) and *M haemolytica* (17.0–23.0 hours) or in 5%±2% for *H somni* 20.0–23.75 hours).

Counts (cfu/mL) in triplicate at each

Figure 2. Numbers of *Pasteurella multocida* at sampling times for gamithromycin doses 0 µg/mL (0x) (A); 0.5 µg/mL (1x) (B); and 4 µg/mL (4x) (C).



time point for each isolate and concentration were calculated for plotting of the mean and standard deviation. Gamithromycin was considered bactericidal if there was a decrease of at least 103 (3 log kill), which was consistent over time, with no return of the bacterial count at later time points within the 103 cfu/mL kill area.

RESULTS

Each time kill curve for *M haemolytica* and *P multocida* isolates started at nearly the same concentration (mean ranged from 5.27 log to 5.44 log) at time 0, regardless of gamithromycin dose level. The time kill curves for the five *H somni* isolates at time 0 ranged from 4.16 log to 4.24 log.

Mannheimia haemolytica

The mean decrease in the concentration of *M haemolytica* from the 1 µg/mL gamithromycin concentration was 2.9 log by 4 hours (Figure 1). By 8 hours and at all subsequent evaluations, the cell count for each individual isolate and the mean viable cell count at 1 µg/mL gamithromycin fulfilled the 3-log decrease, and this dose was considered bactericidal. All isolates tested at 4 µg/mL gamithromycin resulted in a 3-log decrease in *M haemolytica* cells at all sampling times (Figure 1).

Pasteurella multocida

Rate of kill for gamithromycin at 1 µg/mL against *P multocida* was slower than that for *M haemolytica*. A mean 3-log reduction was achieved between 10 and 24 hours (Figure 2). All five isolates demonstrated a >3-log reduction at 24 hours in tests of gamithromycin 1 µg/mL. Kill tests for gamithromycin at 4 µg/mL showed a >3-log decrease in *P multocida* at 4 hours for the mean and for three isolates. One additional isolate reached a 3-log decrease by 8 hours, and all isolates were killed by >3 log by 24 hours. One isolate was slow to be killed at both 1 µg/mL and 4 µg/mL gamithromycin. Gamithromycin is considered bactericidal at 1 µg/mL and at 4 µg/mL.

Histophilus somni

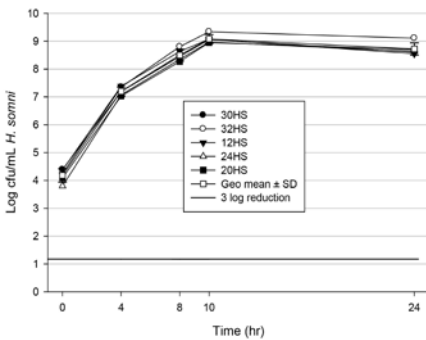
None of the five *H somni* isolates had a

3-log decrease in tests with gamithromycin 0.5 µg/mL (Figure 3). Four of five isolates tested for gamithromycin 2 µg/mL reached a 3-log decrease at 4 hours. The single isolate that did not reach goal at 4 hours did not achieve a 3-log decrease throughout the test period; however, at 10 hours, this isolate approached the 3-log decrease (2.96-log).

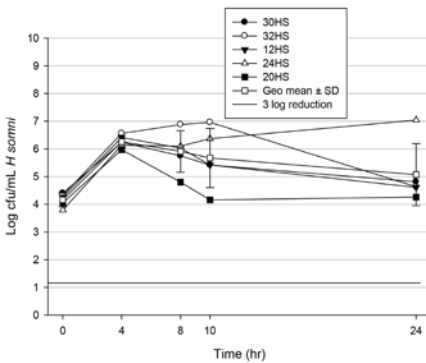
Additional MIC testing was per-

Figure 3. Numbers of *Histophilus somni* at sampling times for gamithromycin doses 0 µg/mL (0×) (A); 0.5 µg/mL (0.5×) (B); and 2 µg/mL (4×) (C).

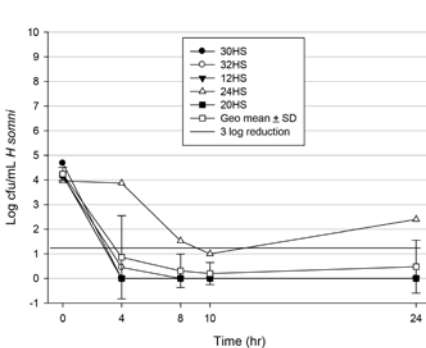
A



B



C



formed on samples taken from multiple sampling times for the isolate that failed to reach goal at all gamithromycin concentrations. The MIC of the isolate was consistently 0.5 µg/mL, except for one replicate, which was 4 µg/mL at 24 hours.

Overall results

A summary of the results for each species and each dose of gamithromycin is shown in Figure 4.

DISCUSSION

At the approved dosage (6 mg/kg), gamithromycin administered by subcutaneous injection is rapidly absorbed from the injection site, reaching peak plasma levels within 1 hr.1 Distribution from plasma into tissues is equally rapid, reaching maximal concentrations (18.5 µg/g) in the lungs within 24 hr.1 In vitro data (unpublished) demonstrate that gamithromycin is both bactericidal and bacteriostatic when higher concentrations are present in lung tissue. MIC90 values for *H somni*, *M haemolytica*, and *P multocida* in Europe have been reported to be between 0.5 and 1 µg/mL, and earlier studies indicated that concentrations of gamithromycin levels in the lungs exceeded those values for up to 15 days.1

Based on results from earlier studies, gamithromycin has demonstrated excellent bioavailability and absorption, dose proportionality of area under the plasma concentration × time curve, with extensive distribution to lung tissue and low level of plasma protein binding.3 In field studies, gamithromycin administered at 6 mg/kg once by subcutaneous injection to cattle has provided significantly ($P \leq 0.05$) lower mean depression scores, respiratory character scores, and rectal temperatures over the first 10 days after arrival than cattle treated with saline.4 Gamithromycin field studies also demonstrated lower mortality rates for feedlot cattle treated with gamithromycin (0.9%) than for cattle treated with saline (8.5%).6

CONCLUSIONS

Gamithromycin is considered bactericidal against *M haemolytica* and *P multocida* at

the MIC90 concentration of 1 µg/mL as well as at a concentration equivalent to four times the MIC90 (4 µg/mL). Gamithromycin is considered to be bacteriostatic against *H somni* at the MIC90 concentration of 0.5 µg/mL and is considered either bacteriostatic or bactericidal on average at a concentration

four times the MIC90 (i.e., 2 µg/mL); however, the actual bactericidal concentration of gamithromycin for all tested isolates of *H somni* was not determined in this current study.

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Figure 4. Summary of the gamithromycin kill curves against *Mannheimia haemolytica* (A), *Pasteurella multocida* (B), and *Histophilus somni* (C)

