



Sub-MIC Effects of Aureomycin,[®] Aureo S 700,[®] and Temperature on Growth Kinetics and Outer Membrane Protein Expression in *Mannheimia haemolytica* and *Haemophilus somnus*

Abstract

A study was conducted to compare the effect of full, $1/4$, $1/2$, $1/8$, and $1/16$ MIC of Aureomycin[®] and Aureo S 700[®] (AS700) at incubation temperatures of 37°C and 41°C on growth and outer membrane protein expression of *Mannheimia haemolytica* and *Haemophilus somnus*. Two clinical and one non-virulent isolates of both *M. haemolytica* and *H. somnus* were utilized.

The MIC for the two clinical *M. haemolytica* isolates were the same at 37°C and 41°C, and growth kinetics indicated that they were actually better adapted for growth at the febrile temperature than at normal body temperature.

With one exception, growth of the clinical isolates of *M. haemolytica* was severely inhibited in the presence of Aureomycin and AS700 at $1/2$ and $1/4$ MIC. In most cases, growth was also notably reduced for the 2 clinical isolates at $1/16$ MIC. The nonvirulent isolate exhibited only moderate to slightly reduced growth at $1/2$ and $1/4$ MIC, and in most cases there was not much difference between growth at $1/8$ or $1/16$ MIC, and no antibiotic.

Temperature effects on *H. somnus* were not apparent, with some exceptions. The exceptions were those strains having much lower MIC for Aureomycin and AS700 at 41°C compared to 37°C. All 3 *H. somnus* strains exhibited limited growth at $1/2$ and $1/4$ MIC, with the exception of 1 reference strain at 41°C, which had a very low MIC (<0.25 µg/mL). Some strains which had high initial MIC also experienced limited growth at both $1/8$ and $1/16$ MIC.

Based on the methods used in these studies, it could not reliably be concluded that sub-MIC antibiotic levels or

temperature had an effect on expression of *M. haemolytica* or *H. somnus* outer membrane proteins.

The results of these studies suggest that Aureomycin and AS700 are more effective at 37°C than 41°C against *M. haemolytica*, thereby indicating that antibiotic administration prior to development of febrile response is more likely to be effective in treatment or prevention of BRD.

Introduction

Gram-negative bacteria *Mannheimia haemolytica* and *Haemophilus somnus* are important pathogens involved in bovine respiratory disease (BRD). As with other bacteria, the anticipated effectiveness of antibiotics against these two pathogens traditionally has been determined through minimum inhibitory concentration (MIC) determinations, defined as the minimum amount of an antibiotic required to kill or inhibit growth of an organism *in vitro*. There is, however, little correlation between MIC values and *in vivo* treatment success, and MIC determinations may at best be a rough guideline for antibiotic selection.

In vitro sub-MIC concentrations (less than MIC) of an antibiotic can negatively impact bacteria in a number of ways, some of which include affecting either ultra-structure and antigenicity, adherence to epithelial cells, synthesis/secretion of pathogenic enzymes,¹ and/or the efficiency of phagocytosis.² It is known that sub-MIC concentrations of Aureomycin (chlortetracycline) and AS700 (chlortetracycline/sulfamethazine) can reduce lung lesions caused by *H. somnus*.³

Successful treatment regimes for bacterial infections are not totally reliant on the action of the antibiotic. Ultimately,

the animal's immune system must combat the infection and restore some degree of normality. Antibiotics do not kill all target bacteria, and any reduction in pathogen numbers, or adverse effect on the bacteria itself or its virulence factors, will enable the animal to more effectively combat infection.

Study Objective

A study was conducted to determine the effects of sub-MIC concentrations of chlortetracycline (hereafter referred to as Aureomycin) and the combination of chlortetracycline and sulfamethazine (hereafter referred to as AS700) on *Mannheimia haemolytica* and *Haemophilus somnus*.

Antibiotic concentrations studied were full, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$ MIC, using incubation temperatures of 37°C and 41°C. Parameters measured and compared to these MIC levels included growth kinetics and outer membrane protein expression of *M. haemolytica* and *H. somnus*.

Rationale for Parameters Measured

Growth Kinetics

Studies have indicated that sub-MIC levels of antibiotics reduce the growth rates of bacterial strains.⁴ One study showed that some antibiotics altered log-phase and diminish growth rates.⁵ The mode of action of Aureomycin is the inhibition of protein synthesis; sulfonamides inhibit folate synthesis, thus inhibiting purine and pyrimidine synthesis.⁶ Inhibition of protein synthesis and purine/pyrimidine synthesis would both be expected to reduce growth rate.

Outer Membrane Proteins

The outer membrane of Gram-negative bacteria is composed primarily of phospholipids, lipopolysaccharide, and a group of outer membrane proteins (OMP), which comprise approximately 50% of the outer membrane mass.⁷ OMP help maintain cell structure, assist in transportation of nutrients and substances involved in bacterial cell defense, and are involved in adhesion to other cells.⁸ Some OMP expressed by Gram-negative bacteria are involved in scavenging iron from the host (a prerequisite for bacterial growth) and, therefore, are important determinants of virulence. Additionally, because of their makeup, some may modulate the immune response during infection. OMP are also involved in resistance mechanisms of some intracellular bacteria to the effects of phagocytosis.⁹

While the exact function of many *M. haemolytica* and *H. somnus* OMP have not yet been determined, and the

exact role these proteins play in virulence is speculative, a decrease in expression of OMP may cause a decrease in the acquisition of growth-promoting nutrients. This could negatively affect strain virulence in the host. It is also possible that the adhesion properties of these strains may be impacted by the reduced expression of some OMP, which would reduce the effectiveness of *M. haemolytica* or *H. somnus* in causing infection. Alterations in OMP expression would provide evidence of structural and functional alterations of the bacterial cell wall. These changes may result in altered adhesion, nutrient acquisition, or permeability, thus altering virulence.

Incubation Temperatures

As a response to disease challenge, and as one of the bodies' natural defense mechanisms, most cattle exhibit an initial increase in body temperature. Most MIC determinations are conducted at incubator temperatures of 37°C in conditions most conducive to bacterial growth and survival. It has been shown that MIC determinations can be incubation-temperature sensitive, with some bacteria exposed to antibiotics in the presence of elevated temperatures exhibiting a 4-fold lowering of MIC.¹⁰ A temperature of 41°C (105.8°F) is not unlike the rectal temperature of cattle exhibiting clinical signs of BRD. Thus, the elevated temperatures sick cattle experience might have a positive effect on the effectiveness of antibiotics.

Materials and Methods

Antibiotic Preparation

A desired stock concentration of 256 µg/mL of Aureomycin was achieved based on a Aureomycin powder purity of 82%. The antibiotic stock solution of AS700 was prepared in the same manner as Aureomycin with the addition of sulfamethazine in a 1:1 ratio.

Bacteria and Source

Three strains of *M. haemolytica* and *H. somnus* were used. *Mannheimia haemolytica* ATCC 55518 was included in the study as a nonvirulent control. Strain 55518 is an attenuated vaccine strain (*aroA* mutant) derived from a virulent pneumonic pasteurellosis isolate from cow lung.¹¹ *Mannheimia haemolytica* clinical isolates D80 and D152 were originally isolated from pneumonic bovine lungs at the Iowa State University Veterinary Diagnostic Laboratory. *Haemophilus somnus* ATCC 700025 is a bovine isolate used for antimicrobial sensitivity testing.¹² *Haemophilus somnus* clinical isolates Hs-91 and 2336 were isolated

TABLE 1. MIC ($\mu\text{g/mL}$) of Aureomycin and AS700 on *M. haemolytica* strains.

Strain	37°C		41°C	
	Aureo.	AS700	Aureo.	AS700
55518	1	1	0.25	0.5
D80	16	32	16	16
D152	16	32	16	16

from pneumonic bovine lung; Hs-91 was isolated from calf lung during an experimental challenge at Iowa State University, and 2336 is a virulent isolate from pneumonic calf lung.¹³

Determination of MIC: *Mannheimia haemolytica*

A serial 2-fold macro-broth dilution method was performed at both 37°C and 41°C to determine the MIC of Aureomycin and AS700 for *M. haemolytica*.¹⁴ To be considered valid, MIC determinations for each of the 3 replicates had to be within plus or minus one dilution of each other. If necessary, additional replicates were run until 3 replicates were obtained within these limits. The MIC was defined as the lowest concentration of antibiotic at which bacterial growth was not detected, and Table 1 reflects those values.

Determination of MIC: *Haemophilus somnus*

The MIC for *H. somnus* were determined with the same standards used for *M. haemolytica* strains and are listed in Table 2.

Determination of Growth Kinetics

The growth kinetics of all strains were determined in the presence of each sub-MIC antibiotic concentration at both 37°C and 41°C. Cultures were incubated until the control cultures (without antibiotics) reached stationary phase, and bacterial densities were estimated turbidimetrically at 30-minute intervals by measuring the optical density at a wavelength of 620 (OD_{620}). Units of measurement indicate the amount of light absorbed at this wavelength, with increased absorption indicating increased bacterial density. Growth kinetic assays for each *M. haemolytica* and *H. somnus* strain were performed in triplicate from at least 2 separate stationary-phase starter cultures and antibiotic stock solutions.

TABLE 2. MIC ($\mu\text{g/mL}$) of Aureomycin and AS700 on *H. somnus* strains.

Strain	37°C		41°C	
	Aureo.	AS700	Aureo.	AS700
700025	1	2	<0.25	<0.25
91	1	1	1	0.25
2336	4	4	4	2

Determination of Outer Membrane Proteins

The impact of Aureomycin and AS700 ($1/4$, $1/2$, $1/8$, and $1/16$ MIC) on OMP for all strains of *M. haemolytica* and *H. somnus* was determined at 37°C and 41°C using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

OMP were isolated from broth cultures of the 3 *M. haemolytica* and the 3 *H. somnus* strains under each antibiotic and temperature condition in duplicate using a protocol that selectively enriches for OMP.¹⁵ Protein concentrations were determined using the bicinchoninic acid (BCA) assay by determining the OD_{562} in the OMP extracts and comparing to known concentrations of bovine serum albumin. SDS-PAGE was performed for each of the duplicate OMP preparations from each *M. haemolytica* and *H. somnus* strain at each antibiotic and temperature combination. The approximate molecular weights of major and minor bands were determined by plotting the log molecular weights of the protein standards vs the distance each band migrated, and then performing a linear regression on each of the standard curves.

Results

Growth Kinetics: *M. haemolytica*

The cultural growth kinetics of the 6 strains cultivated under different temperature and antibiotic conditions can be seen in Figures 1-3. With one exception, growth of the clinical isolates D80 and D152 was severely inhibited in the presence of both antibiotics at $1/2$ and $1/4$ MIC (never achieving an OD_{620} of 0.1). Growth of strain D152 was severely inhibited at concentrations as low as $1/8$ MIC (Figures 2 and 3). In fact, the growth curves demonstrate no detectable growth at some sub-MIC within the 6 hours the growth curves were conducted, thereby indicating that

FIGURE 1: Growth kinetics of *M. haemolytica* strain 55518 in the presence of sub-MIC of Aureomycin and AS700 at 2 temperatures. Each value represents the mean OD₆₂₀ readings from 6 cultures originating from 2 independent starter cultures.

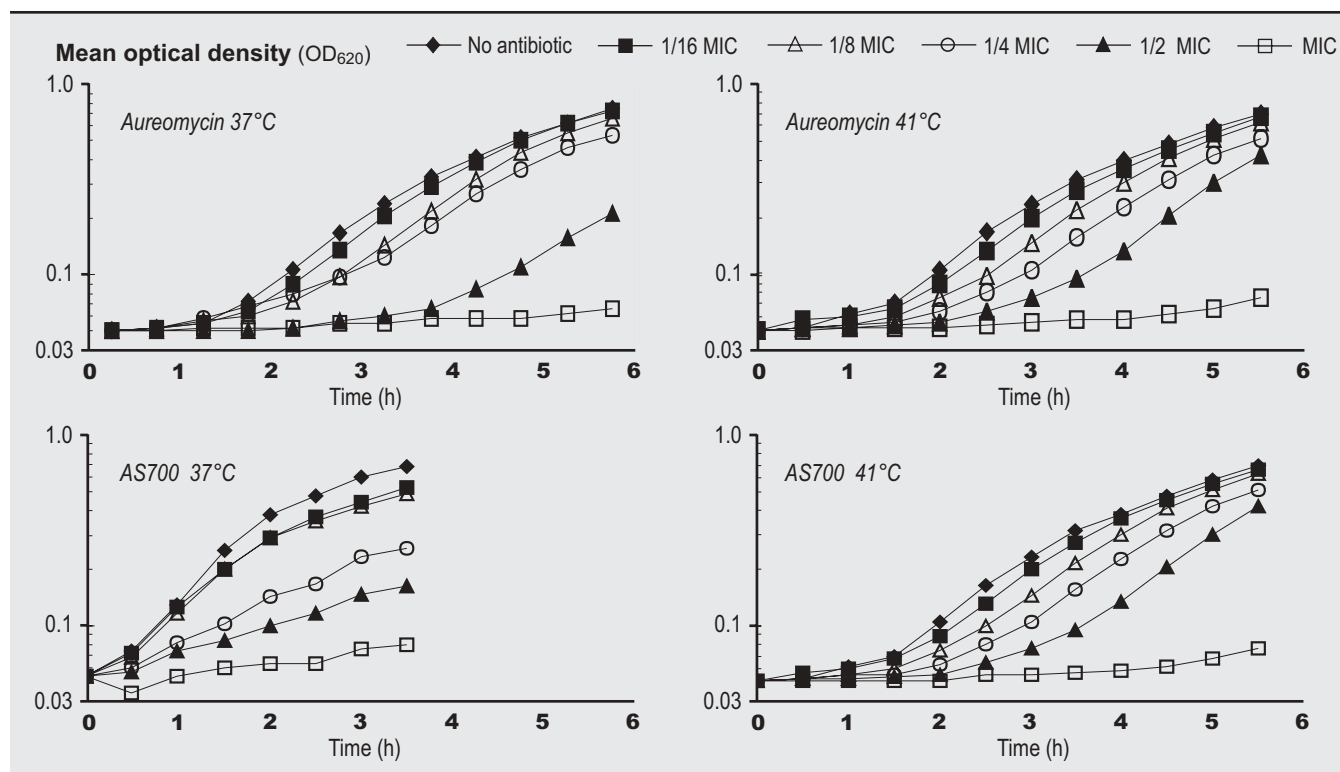


FIGURE 2: Growth kinetics of *M. haemolytica* strain D80 in the presence of sub-MIC of Aureomycin and AS700 at 2 temperatures. Each value represents the mean OD₆₂₀ readings from 6 cultures originating from 2 independent starter cultures.

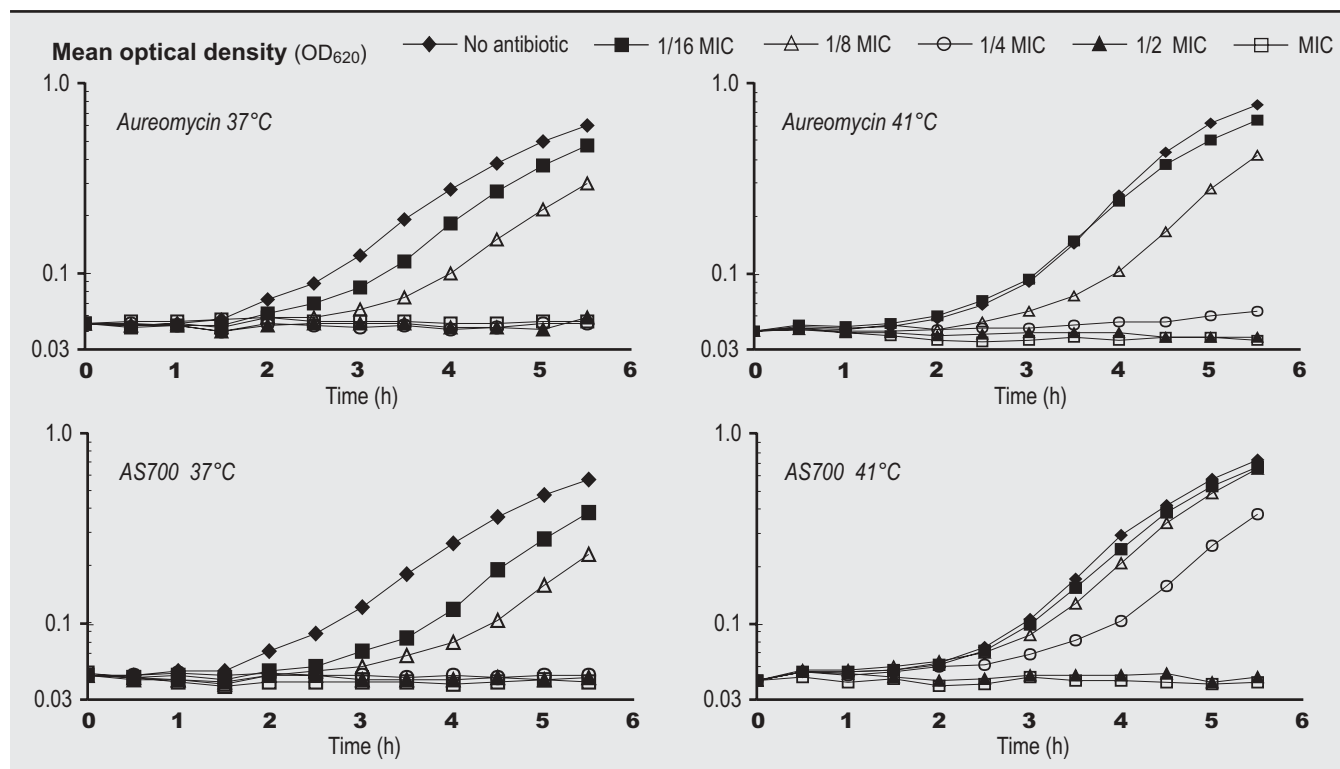
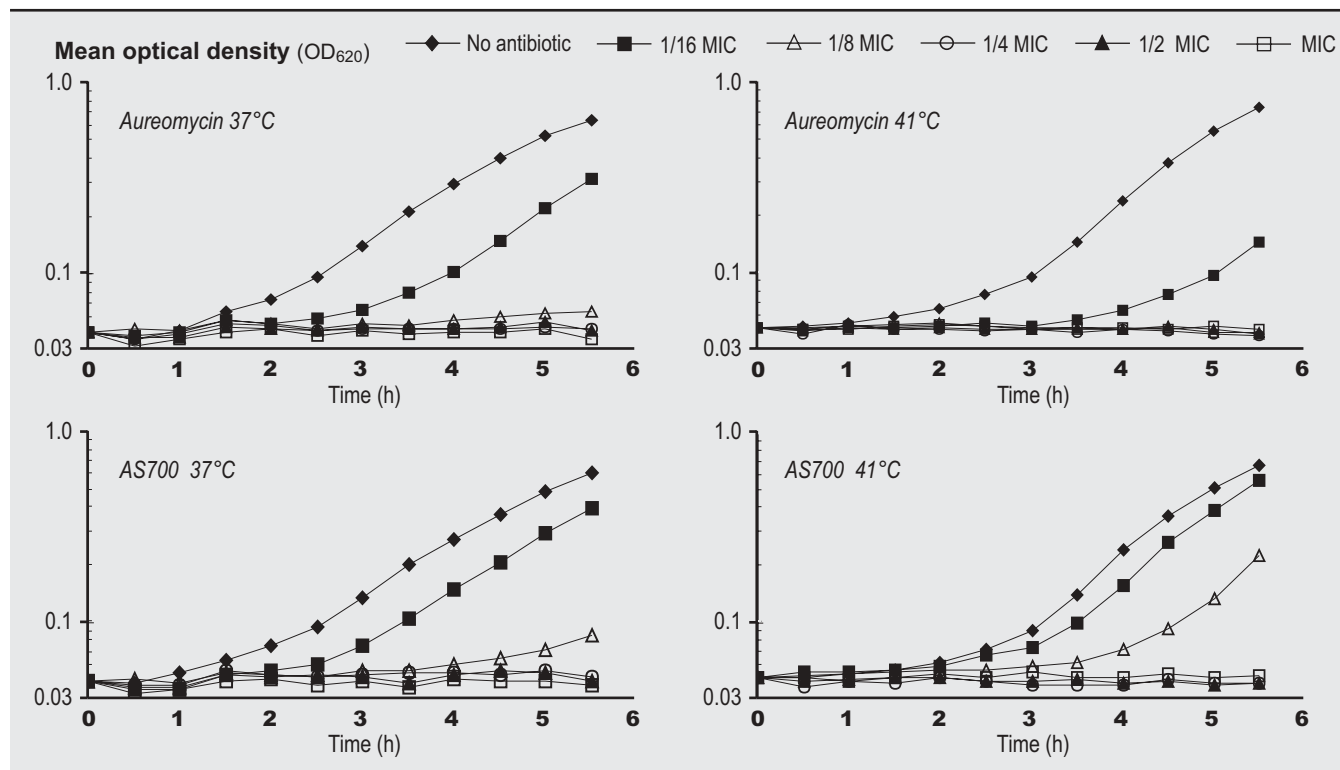


FIGURE 3: Growth kinetics of *M. haemolytica* strain D152 in the presence of sub-MIC of Aureomycin and AS700 at 2 temperatures. Each value represents the mean OD₆₂₀ readings from 6 cultures originating from 2 independent starter cultures.



up to 24 hours (the length of incubation for the MIC determinations) may be required for detectable growth at these concentrations. In most cases, growth was also notably reduced for the 2 clinical isolates at $1/16$ MIC. By contrast, nonvirulent isolate 55518 exhibited only moderate to slightly reduced growth at $1/2$ and $1/4$ MIC (Figure 1), and in most cases there was not much difference between growth at $1/8$ MIC, $1/16$ MIC, and no antibiotic.

Temperature also affected the growth kinetics of the 3 *M. haemolytica* strains, particularly the clinical isolates. Even though the MIC for these 2 isolates were the same at 37°C and 41°C, the growth kinetics indicated that they were actually better adapted for growth at the febrile temperature than at normal body temperature.

Growth Kinetics: *H. somnus*

All 3 *H. somnus* strains exhibited limited growth at $1/2$ and $1/4$ MIC, with the exception of reference strain 700025 at 41°C which had a very low MIC (<0.25 µg/mL) (Figures 4-6). Growth of strain 2336 (which had the highest MIC for both Aureomycin and AS700) was markedly inhibited at $1/8$

MIC and slightly inhibited at $1/16$ MIC. In most cases, growth of 700025 and Hs-91 at $1/8$ and $1/16$ MIC was markedly inhibited. The exceptions occurred at 41°C for 700025 with both Aureomycin and AS700, and Hs-91 with AS700, where the strains that had very low MIC and growth at $1/8$ and $1/16$ MIC were only slightly affected.

Temperature effects on *H. somnus* were not apparent, with some exceptions. There was apparent improved growth for 700025 at sub-MIC for Aureomycin and AS700 at 41°C compared to 37°C. There was also apparent growth of Hs-91 at sub-MIC for AS700 at 41°C compared to 37°C. However, as described above, these strains had much lower MIC for these antibiotics at 41°C compared to 37°C. Therefore, the antibiotic concentrations used for the growth kinetics study at 37°C were higher than those used at 41°C, making it difficult to draw conclusions on temperature effects.

***M. haemolytica* Outer Membrane Protein**

SDS-PAGE revealed 4 major OMP in *M. haemolytica*, based on band intensity. All 4 bands were consistently

FIGURE 4: Growth kinetics of *H. somnus* strain 700025 in the presence of sub-MIC of Aureomycin and AS700 at 2 temperatures. Each value represents the mean OD₆₂₀ readings of 3 independent determinations.

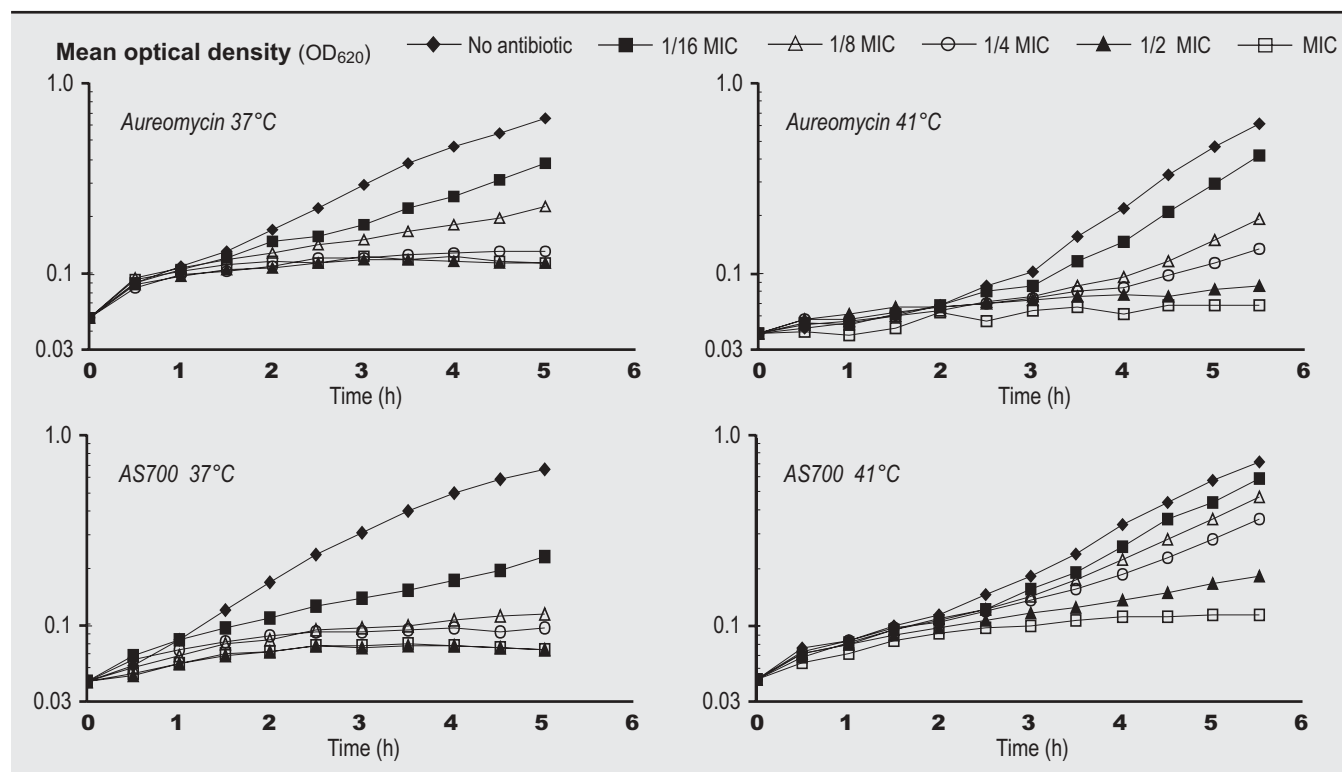


FIGURE 5: Growth kinetics of *H. somnus* strain Hs-91 in the presence of sub-MIC of Aureomycin and AS700 at 2 temperatures. Each value represents the mean OD₆₂₀ readings of 3 independent determinations.

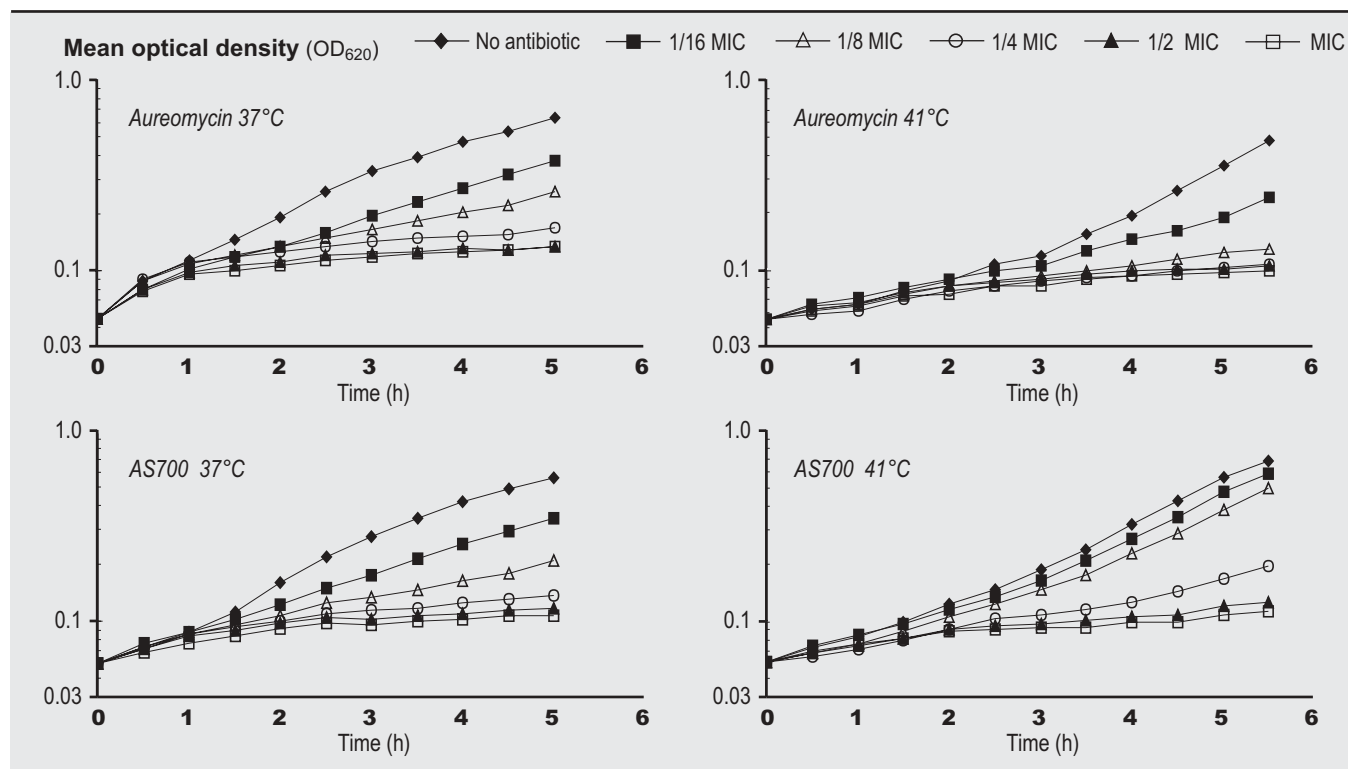
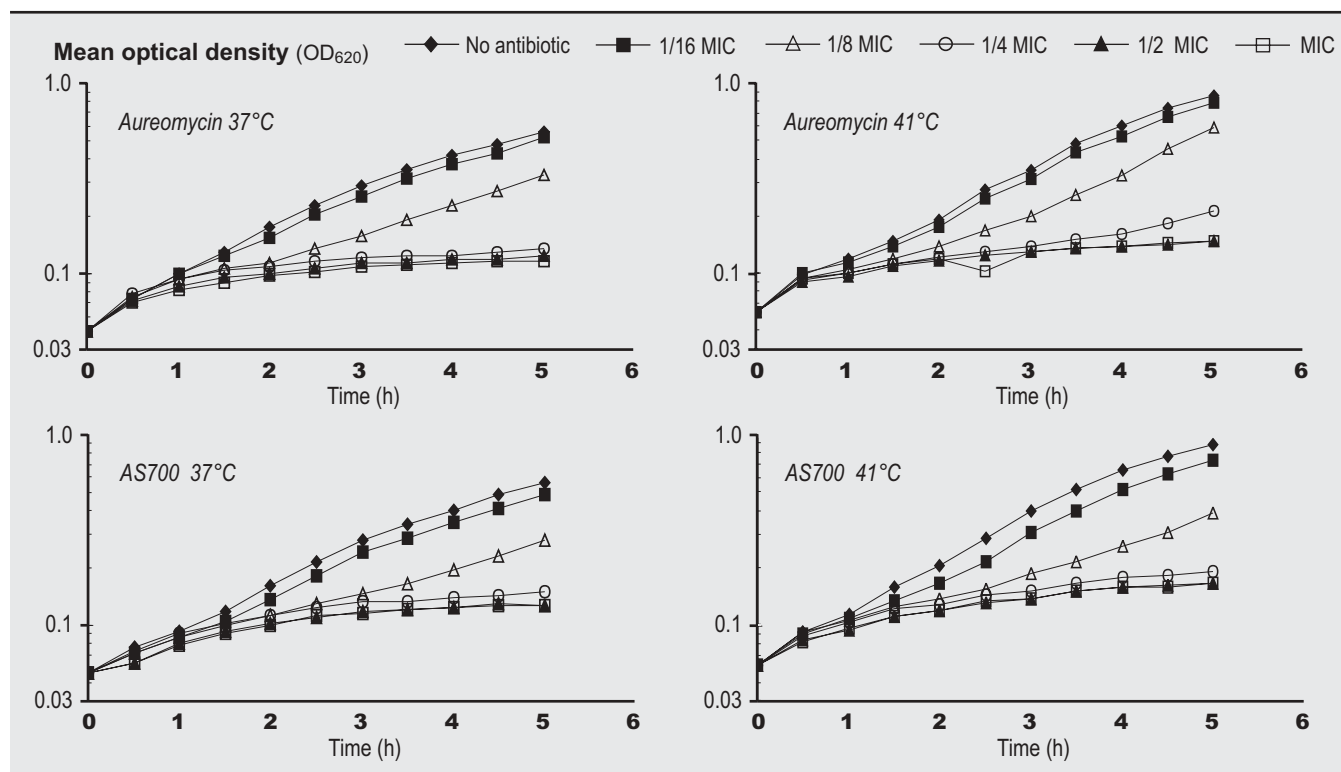


FIGURE 6: Growth kinetics of *H. somnus* strain 2336 in the presence of sub-MIC of Aureomycin and AS700 at 2 temperatures. Each value represents the mean OD₆₂₀ readings of 3 independent determinations.



present in all of the *M. haemolytica* OMP preparations regardless of antibiotic or temperature conditions. Minor *M. haemolytica* OMP were also identified that had lower band intensity. There were some variations in the expression of these 4 minor OMP under different antibiotic and temperature conditions, but no clear trend was evident. Although there was some detectable variation in expression of the OMP, it could not reliably be concluded that sub-MIC antibiotic levels or temperature had an effect on expression of *M. haemolytica* OMP.

H. somnus Outer Membrane Protein

Sub-MIC antibiotic levels or temperature had no effect on expression of *H. somnus* OMP.

Conclusions

Studies have indicated that sub-MIC levels of antibiotics reduce the growth rates and increase log-phase of bacterial strains.^{4,5,16,17} This study is consistent with those earlier reports and demonstrates that the *M. haemolytica* and *H. somnus* clinical isolates had especially limited growth at $1/2$, $1/4$, and sometimes $1/8$ MIC for both Aureomycin and AS700.

This is logical, considering that Aureomycin's mode of action is inhibition of protein synthesis, and sulfonamides inhibit purine and pyrimidine synthesis (through interference with folate metabolism). Inhibition of protein synthesis and purine/pyrimidine synthesis would both be expected to reduce growth rates. Assuming that similar growth inhibition occurs at equivalent antibiotic concentrations *in vivo*, these findings suggest a plausible mechanism for the demonstrated clinical efficacy of sub-MIC administration of these antibiotics; namely, inhibition of bacterial growth *in vivo* would slow the progression of infection and presumably allow the host immune system to more readily clear the pathogen. In addition, the MIC results of this study indicate that *H. somnus* is more sensitive to Aureomycin and AS700 than *M. haemolytica*.

Earlier reports on the effects of sub-MIC of antibiotics on bacterial growth have not taken into account the effect of growth at febrile temperature. The finding that the *M. haemolytica* clinical isolates were better adapted for growth at febrile temperature both in the absence of antibiotics and in the presence of sub-MIC of Aureomycin and AS700 in this study was unexpected. The optimal

temperature for growth of *M. haemolytica* is reported to be 37°C, with growth occurring from 25°C to 40°C.¹⁸

However, this finding is not surprising in light of the fact that febrile response is typical of BRD and is often used as a prognostic indicator. These results indicate that Aureomycin and AS700 are more effective at 37°C than 41°C against *M. haemolytica*, thereby suggesting that antibiotic administration prior to development of febrile response is more likely to be effective in treatment or prevention of BRD. This supports the philosophy that metaphylaxis with either Aureomycin or AS700 is best utilized early in disease onset when calves are not critically ill.

There was no detectable variation in the expression of major OMP from either *M. haemolytica* or *H. somnus* as a result of growth in the presence of sub-MIC concentrations of Aureomycin or AS700 or as a result of growth at 41°C. Some variation was detected in expression of some of the minor *M. haemolytica* OMP, but it could not definitively be concluded that it occurred as a result of varying environmental conditions.

The ability of Aureomycin and AS700 to decrease morbidity due to BRD and improve performance in cattle has been documented.^{19,20,21} This study demonstrated that *in vitro* sub-MIC concentrations of Aureomycin and AS700 affect the growth kinetics of both *M. haemolytica* and *H. somnus*, offering a plausible mechanism for the demonstrated clinical efficacy of sub-MIC of Aureomycin and AS700.

The impact on clinical disease when growth kinetics are altered requires further observation. This study, however, gives insight to a possible mechanism by which sub-MIC levels of Aureomycin and AS700 can positively affect disease outcome, and underscores the misconceptions that may occur when MIC is used as the sole method to evaluate an antibiotic's effect on a particular bacterium.

References

- Lorian V. Medical relevance of low concentrations of antibiotics. *J Antimicrob Chemother* 1993; 31(Suppl D):137-148.
- Gemmell CG. Antibiotics and expression of microbial virulence factors: implications for host defenses. *J Chemother* 1991 3(Suppl 1):105-111.
- Alpharma Inc. Effects of Aureo S 700 and Aureomycin in reducing lung lesions in *Haemophilus somnus*-challenged calves. Alpharma Technical Bulletin CD0329.
- Atkinson BA et al. Sublethal concentrations of antibiotics, effects on bacteria and the immune system. *Crit Rev Microbiol* 1982; p 101-138.
- Shah PM et al. Activity of amikacin at subinhibitory levels. *J Antimicrob Chem* 1976; 2:97-100.
- Sherris JC et al. *Medical Microbiology*. 1994 Appleton & Lange, Norwalk, p 200-204.
- Koebnik R et al. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* 2000; 37:239-253.
- Wexler HM. Outer-membrane pore-forming proteins in gram-negative anaerobic bacteria. *Clin Infect Dis* 2002; 35(Suppl 1): S65.
- Cockayne A. Bacterial Cell Walls. *Encyclopedia of Immunology*, 2nd edition. p 321-22.
- Mackowlak P et al. Effect of temperature on antimicrobial sensitivity of bacteria. *J Inf Dis* 1982; 145(4).
- Tatum FM, Briggs RE, Halling SM. Molecular gene cloning and nucleotide sequencing and construction of an *aroA* mutant of *Pasteurella haemolytica* serotype A1. *Appl Environ Microbiol* 1994; 60:2011-2016.
- McDermott PF et al. Standardization of broth microdilution and disk diffusion susceptibility tests for *Actinobacillus pleuropneumoniae* and *Haemophilus somnus*: quality control standards for ceftiofur, enrofloxacin, florfenicol, gentamicin, penicillin, tetracycline, tilmicosin, and trimethoprim-sulfamethoxazole. *J Clin Microbiol* 39:4283-4287.
- Corbeil LB et al. Cloning and expression of genes encoding *Haemophilus somnus* antigens. *Infect Immun* 1988; 56:2736-2742.
- Watts JL, Chengappa MM, Cole JR et al. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: approved standard. *National Committee on Clinical Laboratory Standards* 1999; Wayne, PA.
- Newton JC et al. Outer membrane protein profiles of *Edwardsiella ictaluri* from fish. *Am J Vet Res* 1990; 51:211-215.
- Greenwood D. Interactions between antibacterial drugs below the minimal inhibitory concentration. *Rev Infect Dis* 1979; 1:807-812.
- Lorian V. Some effects of subinhibitory concentrations of antibiotics on bacteria. *Bull N Y Acad Med* 1975; 51:1046-1055.
- Carter GR. Genus I. *Pasteurella* T revision 1887. In: *Bergey's Manual of Systematic Bacteriology*, JG Holt, ed., 1984; p 552-557. Williams & Wilkins, Baltimore, MD.
- Perry TW et al. Use of chlortetracycline for treatment of new feedlot cattle. *J Anim Sci* 1986; 62:1215-1219.
- Gallo GF, Berg JL. Efficacy of a feed-additive antibacterial combination for improving feedlot cattle performance and health. *Can Vet J* 1995; 36:223-229.
- Kreikemeier K et al. Influence of delayed processing and mass medication with either chlortetracycline (CTC) or tilmicosin phosphate (Micotil) on health and growth of highly stressed calves. *Kansas State University Agricultural Experiment Station Report of Progress* 773:23-27.



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