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Initial Determinations of Susceptibility Testing Interpretive Criteria and Quality Control Guidelines for Tiamulin, A Pleuromutilin Derivative Used in Veterinary Practice

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ABSTRACT

Background: Tiamulin is a widely used antimicrobial uniquely applied to the therapy and prevention of animal respiratory and GI infections, usually in swine. Interpretive susceptibility testing criteria and QC ranges have not been determined for routine laboratory procedures using NCCLS methods.

Methods: A total of 393 non-fastidious and 129 fastidious pathogens (human and animal) were tested by recommended NCCLS M31 (1999) methods and on specified media. These included: staphylococci (250), enterococci (71), Enterobacteriaceae (24), *A. pleuropneumoniae* (APP; 52), *A. suis* (21), *E. rhusiopathiae* (11), *H. parasuis* (18), *S. suis* (27) and other species (11). NCCLS (M37) recommended QC strains were used for all QC experiments (MIC and 30-µg disks) in 8 laboratories.

Results: Tiamulin was active against *S. aureus* (MIC_{50}, 1 µg/ml), CoNS (MIC_{50}, ≤0.5 µg/ml), and *P. multocida* (PM; MIC_{50}, 16 µg/ml) strains among the rapidly growing aerobes. At a susceptible MIC breakpoint of ≤4 or ≤8 µg/ml, correlate zones would be approximately ≥15 mm, categorizing staphylococci as susceptible and PM as intermediately susceptible. Fastidious pathogenic animal species also exhibited two distinct susceptible populations: APP and *A. suis* at 8-16 µg/ml and other species at ≤4 µg/ml. Zone breakpoint criteria for these strains tested on VFM agar varied (15 to 25 mm) by species. QC studies established ranges for APP ATCC 27090 (8-32 µg/ml; 12-18 mm), *S. aureus* ATCC 29213 (0.5-2 µg/ml; 25-32 mm), *S. pneumoniae* ATCC 49619 (0.5-4 µg/ml).

Conclusions: Tiamulin in vitro susceptibility testing by NCCLS veterinary methods were established including interpretive breakpoint criteria and QC guidelines. Small zone diameter breakpoints for APP may require a modified disk concentration if diagnostic problems emerge.

INTRODUCTION

Quality control (QC) limits for monitoring the MIC and disk diffusion test susceptibility of veterinary antimicrobial agents must be established to ensure the precision and accuracy of the test procedures. Recently developed guidelines for susceptibility testing veterinary pathogens have followed those established previously by the National Committee for Clinical Laboratory Standards (NCCLS) for testing of fastidious and non-fastidious human pathogens. The multicenter QC study described here reports QC ranges of the MICs and disk diffusion zone diameters for tiamulin using NCCLS-recommended reference strains.

An additional study was conducted to document the sustained activity of tiamulin against selected rapidly growing and fastidious animal pathogens to establish choices of diagnostic reagents, and to determine susceptibility testing interpretive criteria. Tiamulin is a pleuromutilin derivative antimicrobial used in the control and treatment of veterinary Gram-positive and -negative respiratory and enteric pathogens with a particular emphasis toward infections in swine.

MATERIALS AND METHODS

QC study. Tiamulin hydrogen fumarate was obtained from Boehringer Ingleheim Vetmedica, Inc. (St. Joseph, MO). Two disk (30-µg) lots were obtained from Becton Dickinson Microbiology Systems (Cockeysville, MD). The MIC and disk diffusion test QC protocol designs were based on NCCLS recommendations. Eight separate laboratories contributed testing results in each trial. Broth microdilution and disk diffusion susceptibility tests were performed according to NCCLS standards. The QC strain *A. pleuropneumoniae* ATCC 27090 was tested in Veterinary Fastidious Medium (VFM), Haemophilus Test Medium (HTM), and Mueller-Hinton (MH) broth supplemented with lysed horse blood (LHB). *S. aureus* ATCC 25923 disk tests were performed on MH agar (MHA) and *A. pleuropneumoniae* ATCC 27090 disk tests were performed on chocolate MH agar (CMHA). Three different lots of MH broth were obtained from three manufacturers and served as the basis for the VFM, HTM and LHB media used in the broth microdilution trays. Three different CMHA base lots and three MHA base lots were utilized for disk diffusion testing of *A. pleuropneumoniae* ATCC 27090 and *S. aureus* ATCC 25923, respectively.

For MIC testing, each of the eight participating laboratories performed 10 replicate tests with each of three lots of VFM and HTM, and the *A. pleuropneumoniae* ATCC 27090 reference strain (total of 240 tests for each of the two media). Enrofloxacin was tested as a control on one lot each of VFM and HTM. For disk diffusion testing, each of the eight laboratories performed 10 replicate tests over three days with each of the two disk lots on three different lots of CMHA or MHA with the two reference strains. Enrofloxacin (5-µg) disk was tested concurrently as a control. This study design produced 60 replicate zone diameters for each of the reference strains in each participating laboratory, or 480 zones per reference strain overall, excluding the enrofloxacin control values. However, the results from one lot of CMHA with the *A. pleuropneumoniae* ATCC 27090 reference strain were observed to have significant variation compared to the results with the remaining two lots in all participating laboratories.

Interpretive Criteria Study. The organisms tested included both human and veterinary pathogens totaling 518 strains. Included were 392 non-fastidious bacteria: *Aerococcus* spp. (3), *Enterococcus* spp. (71), *Staphylococcus aureus* (150), *coagulase-negative staphylococci* (99), *Streptococcus* spp. (4), *P. multocida* (31) Enterobacteriaceae (24 strains; 8 species), *Acinetobacter baumannii* (2), *Pseudomonas aeruginosa* (6) and *Stenotrophomonas maltophilia* (2). Also tested were 126 fastidious veterinary pathogens: *Actinobacillus pleuropneumoniae* (52), *A. suis* (21), *Erysipelothrix rhusiopathiae* (11), *Haemophilus parasuis* (18), and *Streptococcus suis* (24).

Broth microdilution testing was performed using four media formulations: non-fastidious bacteria were tested in cation-adjusted Mueller-Hinton broth (CAMH) whereas fastidious veterinary pathogens were tested in Veterinary Fastidious Medium (VFM), Haemophilus Test Medium (HTM), and Mueller-Hinton broth supplemented with 5% lysed horse blood (LHB). The technical details of the veterinary NCCLS documents were followed.

The 30-µg disks were produced by Becton Dickinson Microbiology Systems (Cockeysville, MD) and used following NCCLS methods M2-A7 and M31-A. The non-fastidious isolates were tested on Mueller-Hinton agar (MHA) and the fastidious isolates were tested on chocolate MHA (CMHA). The fastidious organisms were tested using incubation conditions of 35ºC in 5-7% CO_2 for 20-24 hours and the non-fastidious organisms were tested in ambient air and 35ºC for 16-18 hours. The results were compared by scattergram, regression statistics and error-rate bounding analysis. Breakpoints for susceptibility were proposed based on available tiamulin pharmacodynamic data and NCCLS guidelines.

RESULTS

QC Study

- Results with control agent, enrofloxacin, for the broth microdilution (0.015 - 0.06 µg/ml) and the disk diffusion tests (range for *A. pleuropneumoniae* [APP] ATCC 27090, 31 - 38 mm and for *S. aureus* ATCC 25923, 27 - 31 mm) were all within NCCLS recommended QC ranges.
- The MIC results for APP ATCC 27090 obtained using VFM established a clear tiamulin modal value (16 µg/ml) and the resulting proposed QC range of 8 - 32 µg/ml encompassed all of the reported MICs. Tiamulin tested in HTM broth, if used, was assigned a four-dilution QC range (4 - 32 µg/ml; Table 1).
- Tiamulin MIC QC range for the *S. aureus* ATCC 29213 strain have previously been determined by our group (0.5 - 2 µg/ml) and have been published in NCCLS tables. In the same earlier experiment, the range for *S. pneumoniae* ATCC 49619 was determined by NCCLS methods at 0.5 - 4 µg/ml.
- Disk diffusion QC results for tiamulin tested against *S. aureus* ATCC 25923 showed nearly identical median and mean zones of inhibition (29.0 versus 28.7 mm), and the proposed QC range of 25 to 32 mm encompassed 95.8% of reported zone results.
- Disk diffusion QC results for tiamulin and APP ATCC 27090 with two media lots were highly consistent both within and among the participating laboratories. The QC range using the method of Gavan et al is 12 to 18 mm (97.0% of results) and equal to the median zone ± 3 mm (Table 2).

Interpretive Criteria Study

- Two principal populations of susceptible rapidly growing organisms were identified based on relative tiamulin potency: 1) staphylococci at MIC_{90}s of 1 or 2 µg/ml and zone diameters of > 20 mm; and 2) *P. multocida* strains with MICs of 8 - 32 µg/ml and zone diameters of 10 - 21 mm.
- All other organisms such as enterococci, and the enteric and non-fermentative Gram-negative bacilli were resistant to tiamulin. To accurately categorize these two groups of tiamulin-inhibited organisms, the staphylococci were declared susceptible (MIC breakpoint, ≤ 4 µg/ml) and the *P. multocida* as moderately-susceptible or intermediate (MIC range, 8 - 16 µg/ml). The correlate zone breakpoints were susceptible at ≥ 19 mm and resistant at ≤ 11 mm.
- These proposed criteria produce an inter-method accuracy rate of 96.2% and errors were: very major (false-susceptible) = 0.0%, major (false-resistant) = 0.0% and minor errors = 3.8%. The correlation coefficient (r) was 0.98 (Figure 1).
- Like the rapidly growing non-fastidious species tested against tiamulin, the five tested fastidious species groups segregate themselves into two distinct levels of susceptibility to tiamulin. The susceptible species by MIC results in VFM or HTM were: *E. rhusiopathiae*, *H. parasuis* (HTM only; data not shown), and the vast majority *S. suis* isolates (MIC_{50} results, 1 - 2 µg/ml). The moderately-susceptible (intermediate) species groups were: *A. pleuropneumoniae*, *A. suis* and a very small number of *S. suis* (MIC_{90} results, 16 µg/ml).
- The same MIC breakpoints (≤ 4 µg/ml for susceptible; ≥ 32 µg/ml for resistant) were applied to these fastidious species. Interpretive criteria established for the disk diffusion method were: susceptible at ≥ 16 mm and resistant at ≤ 8 mm (Figure 2).
- Although only 108 strains were tested in VFM, the error rates for these species remained acceptable at: very major = 0.0%, major = 0.0%, and minor errors = 10.2% or 89.8% absolute agreement between the reference MIC and disk diffusion test results.
- Applying these criteria *Actinobacillus* spp. isolates, like *P. multocida*, would be categorized as moderately-susceptible (intermediate) to tiamulin requiring appropriate dosing (180 ppm in drinking water) or intramusclar injections where indicated. Other species with tiamulin MIC values at ≤ 4 µg/ml could receive more modest dosing schedules.

Table 1. Distribution statistics from the tiamulin MIC quality control (QC) studies performed in eight laboratories using three lots each of Veterinary Fastidious Medium (VFM) and Haemophilus Test Medium (HTM) for *Actinobacillus pleuropneumoniae* ATCC 27090.

		No. of occurrences at the following MIC (µg/ml)			
Test medium	Laboratory	4	8	16	32
VFMa	A	0	9	18	3
	B	0	11	19	0
	C	0	16	14	0
	D	0	7	23	0
	E	0	3	27	0
	F	0	18	12	0
	G	0	2	26	2
	H	0	5	25	0
	TOTAL	0	[71	164	5][b]
HTMa	A	0	10	20	0
	B	0	18	12	0
	C	0	10	20	0
	D	0	11	19	0
	E	3	12	15	0
	F	0	19	11	0
	G	0	6	24	0
	H	0	19	11	0
	TOTAL	[3	105	132	0][b]

a. Total of 240 tests with each medium.
b. Brackets indicate the proposed QC ranges of MICs.

Table 2. Distribution of zone diameters from the tiamulin disk diffusion quality control (QC) studies performed in eight laboratories using *Actinobacillus pleuropneumoniae* ATCC 27090.

	Tiamulin results by laboratory:								
Zone diameter (mm)	A	B	C	D	E	F	G	H	All results (cum %)
12								2	2 (0.7)[a]
13	1		1		2	1	5	5	15 (5.7)[a]
14	5		10		10	12	11	8	56 (24.6)[a]
15	6	2	14		7	24	23	7	83 (52.5)[a]
16	17	3	14		14	3	1	10	62 (73.4)[a]
17	8	9	1	9	3			2	32 (84.2)[a]
18		6		25	1			6	38 (97.0)[a]
19				6	3				9 (100.0)

a. Proposed QC range at 12 to 18 mm (97.0% of results) and equal to the median ± 3 mm.

FIGURE LEGEND

Figure 1: Scattergram comparing reference tiamulin MIC results to zone diameters around 30-mg tiamulin disks when testing 390 strains of non-fastidious, rapid-growing organisms. Horizontal solid lines show the proposed MIC susceptible (≤ 4 mg/ml) and resistant criteria (≥ 32 mg/ml). Vertical solid lines show the correlate interpretive zone diameters of ≥ 18 and ≤ 11 mm, respectively (96.2% inter-method agreement).

Figure 1.

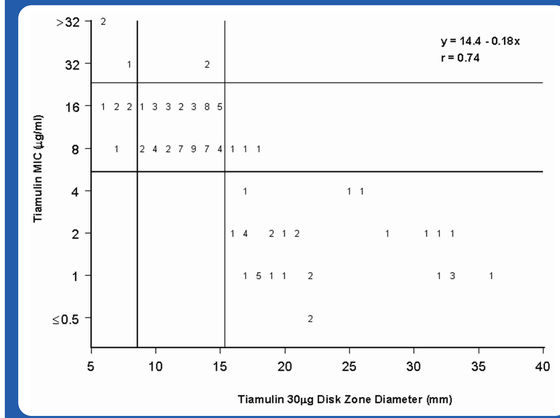
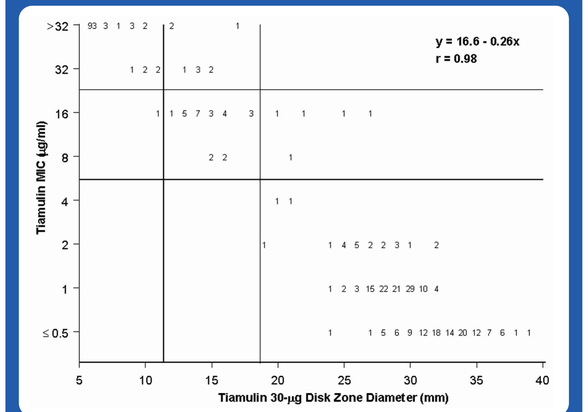


FIGURE LEGEND

Figure 2: Scattergram comparing reference tiamulin MIC results in VFM broth compared to zone diameters around 30-mg tiamulin disks when testing the fastidious veterinary pathogens: A. pleuropneumoniae, A. suis, E. rhusiopathiae, and S. suis (108 total strains). Horizontal solid lines indicate the proposed MIC breakpoints of ≤ 4 mg/ml = susceptible and ≥ 32 mg/ml = resistant. Disk diffusion breakpoints are shown by solid vertical lines (≥ 16 and ≤ 8 mm).

Figure 2.



CONCLUSIONS

- The newly established MIC and zone diameter QC ranges for tiamulin provide important new information required for testing of this agent and extends the number of antimicrobial agents for veterinary use that can be tested by NCCLS methods. The use of these proposed QC ranges will improve the accuracy and validity of quantitative and qualitative susceptibility testing data.
- These suggested interpretive criteria and NCCLS methods will facilitate the reporting of more accurate and valid susceptibility testing data. Care must be exercised to test pathogens on appropriate media and to apply the species-specific quality assurance guidelines.

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