Wisconsin Veterinary Diagnostic Laboratory
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Antimicrobial Susceptibility Testing Diagnostic Aid

WVDL primarily uses the broth microdilution method for antimicrobial susceptibility testing, although the Kirby Bauer, disk diffusion, method is still employed when needed. In this document, we attempt to answer many of the frequently asked questions regarding antimicrobial susceptibility testing. In particular, we will describe how the WVDL reports resistant, intermediate and susceptible and which antimicrobials should be reported or used based on the Antimicrobial Group as defined by the Clinical Laboratory Standards Institute (CLSI). Additionally, we will discuss the importance of efficacy ratios. It is our hope that this document will be useful to our clients, the practicing veterinarians.

- **What is the broth microdilution method?** The broth microdilution method is a liquid culture method whereby a standard amount of bacteria are inoculated into the wells of a 96-well micro-titer plate that contain different dilutions of antimicrobial drugs. For example in the standard bovine/porcine panel (see image below of BOPO7F layout; ThermoFisher Trek Diagnostic Systems), four wells contain the antibiotic ceftiofur (XNL) with dilutions of 8, 4, 2, and 1 µg/ml and four wells contain spectinomycin (SPE) with dilutions of 64, 32, 16 and 8 µg/ml, respectively. After 18-24 hours, the plates are examined visually for evidence of bacterial growth. Results are recorded as minimum inhibitory concentrations (MIC), the lowest concentration without growth, and reference tables are used to determine if the bacteria are Sensitive (S), Intermediate (I) or Resistant (R) to the antimicrobial drugs as determined by the CLSI guidelines[1,2] (CLSI.org).

thermoscientific

SENSITITRE™ BOVINE/PORCINE PLATE FORMAT

Plate Code: **BOPO7F** Plate Type: **MIC**

	1	2	3	4	5	6	7	8	9	10	11	12
A	PEN 0.12	PEN 0.25	PEN 0.5	PEN 1	PEN 2	PEN 4	PEN 8	TET 0.5	TET 1	TET 2	TET 4	TET 8
B	AMP 0.25	AMP 0.5	AMP 1	AMP 2	AMP 4	AMP 8	AMP 16	GEN 1	GEN 2	GEN 4	GEN 8	GEN 16
C	TIA 0.5	TIA 1	TIA 2	TIA 4	TIA 8	TIA 16	TIA 32	TIP 1	TIP 2	TIP 4	TIP 8	TIP 16
D	TYLT 0.5	TYLT 1	TYLT 2	TYLT 4	TYLT 8	TYLT 16	TYLT 32	TIL 2	TIL 4	TIL 8	TIL 16	NEO 4
E	NEO 8	NEO 16	NEO 32	TUL 8	TUL 16	TUL 32	TUL 64	ENRO 0.12	ENRO 0.25	ENRO 0.5	ENRO 1	ENRO 2
F	CLI 0.25	CLI 0.5	CLI 1	CLI 2	CLI 4	CLI 8	CLI 16	DANO 0.12	DANO 0.25	DANO 0.5	DANO 1	POS
G	XNL 0.25	XNL 0.5	XNL 1	XNL 2	XNL 4	XNL 8	GAM 1	GAM 2	GAM 4	GAM 8	SDM 256	POS
H	FFN 0.25	FFN 0.5	FFN 1	FFN 2	FFN 4	FFN 8	SPE 8	SPE 16	SPE 32	SPE 64	SXT 2/38	POS

ANTIMICROBICS

AMP	Ampicillin
CLI	Clindamycin
DANO	Danofloxacin
ENRO	Enrofloxacin
FFN	Florfenicol
GAM	Gamithromycin
GEN	Gentamicin
NEO	Neomycin
PEN	Penicillin
POS	Positive Control
SDM	Sulphadimethoxine
SPE	Spectinomycin
SXT	Trimethoprim / sulfamethoxazole
TET	Tetracycline
TIA	Tiamulin
TIL	Tilmicosin
TIP	Tildipirosin
TUL	Tulathromycin
TYLT	Tylosin tartrate
XNL	Ceftiofur

- **What is the MIC?** The MIC is the highest dilution (lowest concentration) of antimicrobial drug that completely inhibits bacterial growth. If available, interpretation guidelines (S, R, I) that have been established by CLSI are reported with MIC values. Occasionally, other interpretation guidelines are reported. For example, when no interpretive guidelines have been established for the specific bacteria/drug/animal species combination being tested, the MIC result may be reported with 'No Interpretation' (typically abbreviated as NI, or NA or NM). Resistant (R) may be reported without MIC as the interpretation may be determined by a different antimicrobial in the same drug class. CLSI guidelines change periodically in response to growing research, and this may result in new interpretations. The WVDL works diligently to stay current with those changes.

- **What are break-points?** Breakpoints are the MIC values used as cutoffs for each interpretation category established by CLSI. These breakpoints, and their associated interpretations, are specific to each organism-drug-animal species combination. For example, in a case of bovine pneumonia involving *Pasteurella multocida,* the break-points for ceftiofur are ≤ 2 (S), 4 (I) and ≥ 8 (R) µg/ml.

- **What is the Kirby Bauer disk diffusion test?** The Kirby Bauer test is a qualitative assay whereby disks of paper are impregnated with a single concentration of different antibiotics. The disks are placed on the surface of an agar plate that has been inoculated with test bacteria. During incubation, the antibiotics diffuse outward from the disks creating a concentration gradient. After 18-24 hours, the zone diameter (zone of inhibition) is measured and reference tables are used to determine if the bacteria are Sensitive (S), Intermediate (I) or Resistant (R) to the antimicrobial drugs. The WVDL uses the Kirby Bauer method to determine methicillin/oxacillin resistance.

CLSI Antimicrobial Groups

The WVDL follows the Clinical Laboratory Standards Institute (CLSI) guidelines, which provide specific parameters for determining when a bacterium, isolated from a particular host species, is resistant, intermediate or sensitive to a particular antimicrobial. As an example, there are specific breakpoints for particular antimicrobial agents that have been established for bovine respiratory disease pathogens such as *Pasteurella multocida*, *Mannheimia haemolytica* and *Histophilus somni*. These breakpoints do not apply to these bacteria isolated from non-respiratory tissues from cattle. Additionally, these breakpoints do not apply to non-bovid species such that a *P. multocida* isolated from a cat would not get these same breakpoints. Therefore, the CLSI guidelines utilize a grouping system for interpretations of antimicrobial agents and their uses for veterinary pathogens.

- **Group A:** includes antimicrobial **agents with VETERINARY-SPECIFIC breakpoints and interpretive categories that are considered appropriate for routine, primary testing for food and companion animals. These antimicrobial agents are considered first to report and use**, and are preferred over using those with human medical breakpoints. These Group A compounds have demonstrated an acceptable level of correlation between *in vitro* susceptibility test results and clinical outcome.
- **Group B:** includes antimicrobial agents with veterinary-specific breakpoints and interpretive categories but are considered antimicrobials that should only be tested and reported as '**drugs of last resort'**. The Subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST) considers these antimicrobials to be 'drugs of last resort' and concern exists that their use could select for resistance, which could be transferred from animals to humans. The veterinary laboratory can report these at their discretion but are mostly used for antimicrobial resistance monitoring.
- **Group C:** includes antimicrobial agents that **use HUMAN medical breakpoints** and interpretive categories. These agents may perform adequately, but outcomes for many veterinary applications have not been demonstrated. The veterinary laboratory can report these at their discretion.

- **Group D:** include antimicrobial agents that are regulatory agency-approved for use in the specific animal species. Although quality control data is available, these antimicrobial agents **DO** NOT have CLSI-approved veterinary-specific or human medical breakpoints or interpretive categories. These agents may be approved for use in other animal species and have veterinary-specific breakpoints in those animals. However, **it is not recommended to use breakpoints set for a particular animal species to be applied to a different animal species.** This is because there are differences in dosages and pharmacokinetics between animals, people and between animal species. Thus, these agents should be reported selectively before extra-label use agents (Group D), but after agents in Group B.
- **Group E:** includes antimicrobial agents that are **NOT APPROVED** but may be used in an extra-label manner per the Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA) guidelines in the US. These agents may be selectively tested and reported and are often used for antimicrobial resistance monitoring. Group E may also include certain antimicrobial agents that are used only for a specific infection site (such as nitrofurantoin for treating urinary tract infections) in non-food-producing animals.[1]

Table 1: Antimicrobial Agents that could be Considered for Routine Testing by Veterinary Microbiology Laboratories.

	Animal Species						
Test/Report Group	Swine	Cattle	Bovine Mastitis	Poultry	Horses	Dogs	Cats
Group A- Vet-specific breakpoints					Amikacin	Amikacin	
						Amoxicillin-clavulanate	Amoxicillin-clavulanate
	Ampicillin	Ampicillin			Ampicillin	Ampicillin	Ampicillin
					Cefazolin	Cefazolin	
						Cefovecin	Cefovecin
			Cefoperazone				
	Ceftiofur	Ceftiofur	Ceftiofur		Ceftiofur		
						Cephalexin	
						Cephalothin	
						Clindamycin	
		Danofloxacin					
						Difloxacin	
					Doxycycline	Doxycycline	
	Enrofloxacin	Enrofloxacin			Enrofloxacin	Enrofloxacin	Enrofloxacin
	Florfenicol	Florfenicol					
		Gamithromycin					
					Gentamicin	Gentamicin	
			Kanamycin-Cephalexin				
						Marbofloxacin	Marbofloxacin
					Minocycline	Minocycline	
						Orbifloxacin	Orbifloxacin
	Penicillin G	Penicillin G			Penicillin G		
			Penicillin-novobiocin				
			Pirlimycin				
	Pradofloxacin	Pradofloxacin				Pradofloxacin	Pradofloxacin
		Spectinomycin					
	Tetracycline	Tetracycline				Tetracycline	
	Tiamulin						
	Tildipirosin	Tildipirosin					
	Tilmicosin	Tilmicosin					
	Tulathromycin	Tulathromycin					
Group B- vet-specific breakpoints; drugs of last resort						Ceftazidime	
				Enrofloxacin		Chloramphenicol	
						Levofloxacin	
						Piperacillin-tazobactam	
Group C- human breakpoints							Amikacin
			Ampicillin				
					Azithromycin	Azithromycin	
							Cefazolin

					Chloramphenicol		Chloramphenicol
	Clindamycin						Clindamycin
						Colistin	
							Doxycycline
	Erythromycin	Erythromycin	Erythromycin	Erythromycin	Erythromycin	Erythromycin	Erythromycin
				Gentamicin			Gentamicin
							Minocycline
						Nitrofurantoin	
	Oxacillin	Oxacillin	Oxacillin	Oxacillin	Oxacillin	Oxacillin	Oxacillin
	Penicillin	Penicillin	Penicillin	Penicillin	Penicillin	Penicillin	Penicillin
					Rifampin	Rifampin	Rifampin
	Sulfonamides	Sulfonamides		Sulfonamides		Sulfonamides	Sulfonamides
			Tetracycline	Tetracycline	Tetracycline		
						Tobramycin	Tobramycin
				TMS	TMS	TMS	TMS
Group D- Only QC ranges available (breakpoints not established)	Apramycin						
				Ceftiofur		Ceftiofur	
	Cefquinome	Cefquinome	Cefquinome		Cefquinome		
				Clindamycin			
	Gamithromycin						
	Gentamicin						
	Spectinomycin			Spectinomycin			
	Tylosin	Tylosin					
Group E- drugs that may be tested and selectively reported if isolate is resistant to Group A, B or C agents	Amikacin	Amikacin					
		Gentamicin					
					Imipenem	Imipenem	Imipenem
						Linezolid	Linezolid
					Meropenem	Meropenem	Meropenem
	TMS	TMS					
	Tylvalosin			Tylvalosin			
					Vancomycin	Vancomycin	Vancomycin

Please see Vet01 Supplement for more information.[1]

The WVDL solely uses breakpoints supplied by CLSI and will be reporting per Table 1. Therefore, the WVDL will report mostly Group A and Group C antimicrobial agents based on the pathogen and what species and tissue in that host species the pathogen was isolated from. On occasion, some Group B, D and E antimicrobials may be interpreted with an MIC and interpretive criteria based on CLSI Vet01[1] and Vet09[2] guidelines. An example is applying *M. haemolytica* breakpoints for bovine respiratory disease to other members of the *Pasteurellaceae* family is acceptable[2]. As well, the CLSI Vet09 extrapolates the *Staphylococcus aureus* breakpoints and interpretive criteria for bovine mastitis so that Gram-positive cocci (but not *Enterococcus*) can be interpreted.[2] **Therefore, the WVDL reports fewer antimicrobials with interpreted categories than it has in the past.** Veterinarians can always contact the WVDL for more information regarding AST or if additional antimicrobial agent breakpoints are needed.

Note, for bovine respiratory disease pathogens susceptibility to ampicillin is only interpreted when MICs ≥ 0.25 ug/mL indicating R. The current method cannot determine S (≤ 0.03) or I (0.06-0.12) as the method does not contain those antimicrobial concentrations. Following CLSI Vet 09[2], *M. haemolytica* breakpoints for bovine respiratory disease is now applied to other members of the *Pasteurellaceae* family such as *Biberstienia* and *Gallibacterium* species. As well, the CLSI Vet09 extrapolates the *Staphylococcus aureus* breakpoints and interpretive criteria for bovine mastitis so that Gram-positive cocci (but not *Enterococcus*) can be interpreted.[2] Interpretations for bovine metritis and mastitis caused by *Enterobacteriaceae* have been extrapolated from *E. coli* breakpoints.[2] Interpretations for bovine respiratory disease, metritis and mastitis have been extrapolated for camelid, caprine, cervid and ovine species. Veterinarians can always contact the WVDL for more

information regarding antimicrobial susceptibility testing (AST) or if additional antimicrobial agent breakpoints are needed.

Efficacy Ratios[1]:
Efficacy ratios can be used to calculate which antimicrobial drug has the highest predicted efficacy or activity against a given bacterial isolate.

- **What are efficacy ratios?** Efficacy ratios (ER) are calculated by taking the resistant break-point MIC value and dividing it by the MIC value obtained as a result of susceptibility testing using the broth microdilution method. It is a tool that can be used to evaluate the relative efficacy of different antimicrobial drugs. For example, recently the WVDL obtained the following MIC results from an isolate of *Mannheimia haemolytica* from a bovine sample.

Drug	**Test Result MIC (µg/ml)**	**Interpretation**	**Resistant Break-point MIC (µg/ml)**	**Efficacy Ratio (ER)**
Ceftiofur	0.5	S	≥ 8	16
Florfenicol	0.25	S	≥ 8	32
Spectinomycin	16	S	≥ 128	8
Gamithromycin	2	S	≥ 16	8
Tulathromycin	8	S	≥ 64	8

In this example, the ER for ceftiofur is 16. This was calculated by taking the resistant break-point MIC for ceftiofur (8 µg/ml) and dividing it by the measured MIC of 0.5 µg/ml. Efficacy ratios for all other drugs were calculated in the same way.

Given these results, even though this isolate is “Susceptible” to all the drugs listed above, florfenicol has the greatest predicted efficacy, based on an ER of 32. It is important to remember that several factors influence the decision of which antimicrobial drug should be used for any particular case, and the final decision ultimately lies with the practicing veterinarian.

References:
[1]CLSI Performance Standards for Antimicrobial Disk and Dilution Susceptibility tests for Bacteria Isolate from Animals. CLSI, Vet01, Edition 5.
[2]Understanding Susceptibility test Data as a Component of Antimicrobial Stewardship in Veterinary Setting. CLSI, Vet09, Edition 1.