CLINICAL AND LABORATORY STANDARDS INSTITUTE®

3rd Edition

VET04

Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated From Aquatic Animals

This document includes updated tables for the Clinical and Laboratory Standards Institute veterinary antimicrobial susceptibility testing guideline VET03.

A CLSI supplement for global application.

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Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated From Aquatic Animals

Ron A. Miller, MS, PhD
Charles M. Gieseker, MS, PhD
Ruben Avendaño-Herrera, PhD
Sandrine Baron, PhD
Nicky Buller, PhD, BSc
Claire R. Burbick, DVM, PhD, DACVM
Rungtip Chuanchuen, DVM, MS, PhD
Inger Dalsgaard, DVM, PhD
Annelies M. Declercq, PhD, MSc
Patricia S. Gaunt, DVM, PhD, DABVT
John P. Hawke, PhD
Hui-Min Hsu, DVM, MS, PhD
Renate Reimschuessel, VMD, PhD
Esteban Soto, Med Vet, MSc, PhD, DACVM
Peter R. Smith, BA, PhD
David Verner-Jeffreys, PhD, MSc, BSc
Ching Ching Wu, DVM

Abstract

The data in the tables are valid only if the methodologies in CLSI document VET03[1] are followed. This guideline contains information about disk and broth dilution susceptibility test procedures for bacteria isolated from aquatic animals. The clinical importance of antimicrobial susceptibility test results demands that these tests be performed under optimal conditions and that laboratories have the capability to interpret results based on the most current clinical breakpoint or epidemiological cutoff value interpretive categories.

The tables presented in VET04 represent the most current information for drug selection, interpretation quality control using the procedures standardized in VET03[1]. Users should replace previously published tables with these new tables. Changes in the tables since the previous editions appear in boldface type. Users should consider the interpretive categories presented in these tables most useful to isolates of *Aeromonas salmonicida, Aeromonas hydrophila, Flavobacterium columnare,* and *Flavobacterium psychrophilum.* Careful extrapolations may be possible to other bacterial species and with other similar antimicrobial agents, but only after consulting CLSI document VET09.[2] Fish disease diagnostic laboratories that typically conduct susceptibility testing less often than once per week should consult this document for revised guidance for frequency of QC.

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Foreword

It is important for users of CLSI document VET03[1] and VET04 to recognize that the standard methods described in CLSI documents are reference methods. These methods may be used for routine antimicrobial susceptibility testing of bacteria isolated from aquatic animals. The Working Group on Aquatic Animals envisions adding more aquaculture pathogens and antimicrobial agents to these (clinical) breakpoint and epidemiological cutoff value (ECV) tables as the data become available. Data needed to develop more clinical breakpoints could include, for example, a clinical effectiveness report that may be correlated with minimal inhibitory concentrations and/or zone diameters for a suspected pathogen obtained using standard methods. **If such data are available, individuals are strongly encouraged to contact any member of the Working Group on Aquatic Animals.**

Breakpoints and ECVs (defined in Sections II and III) established by CLSI may differ from those approved by various authorities for many reasons, including the use of different susceptibility databases, differences in data interpretation, and different public health policies. Differences also exist because CLSI proactively evaluates the need for changing clinical breakpoints. The reasons why veterinary breakpoints may change and the manner in which CLSI evaluates data and determines veterinary breakpoints are outlined in CLSI document VET02.[3]

Following a decision by CLSI to change an existing breakpoint, regulatory authorities may also review data to determine how changing a breakpoint may affect the safety and effectiveness of the antimicrobial agent for the approved indications. If the regulatory authority changes a breakpoint, commercial device manufacturers may have to conduct a clinical laboratory trial, submit the data to the regulatory authority, and await review and approval. For these reasons, a delay of more than the suggested CLSI "tentative" period of one year may be needed if a breakpoint change is to be implemented by a device manufacturer.

Instructions for Use of Tables

These instructions apply to:

- **Table 1:** MIC and zone diameter breakpoints to be used for isolates of *A. salmonicida*
- **Tables 2A through 2D:** MIC and zone diameter ECVs to be used for isolates of *A. salmonicida* and *A. hydrophila,* and MIC ECVs to be used for isolates of *F. columnare* and *F. psychrophilum*
- **Table 3:** bacterial QC strains used for aquaculture antimicrobial susceptibility tests
- **Tables 4A through 4F:** MIC QC ranges for *E. coli* ATCC® 25922 and *A. salmonicida* subsp. *salmonicida* ATCC® 33658 to be used for all tests incubated under the following conditions:
  - **Table 4A:** 22°C±2°C for 24–28 hours in cation-adjusted Mueller-Hinton broth (CAMHB)
  - **Table 4B:** 22°C±2°C for 44–48 hours in CAMHB
  - **Table 4C:** 28°C±2°C for 24–28 hours in CAMHB
  - **Table 4D:** 35°C±2°C with QC ranges included for additional organisms
  - **Table 4E:** 18°C±2°C for 92–96 hours in diluted CAMHB
  - **Table 4F:** 28°C±2°C for 44–48 hours in diluted CAMHB
- **Tables 5A through 5D:** disk diffusion QC ranges for *E. coli* ATCC® 25922 and *A. salmonicida* subsp. *salmonicida* ATCC® 33658 to be used for all tests incubated under the following conditions:
  - **Table 5A:** 22°C±2°C for 24–28 hours
  - **Table 5B:** 22°C±2°C for 44–48 hours
  - **Table 5C:** 28°C±2°C for 24–28 hours
  - **Table 5D:** 35°C±2°C with QC ranges included for additional organisms
- **Table 6:** table of solvents and diluents for preparing stock solutions of antimicrobial agents
- **Table 7:** example of how to prepare dilutions for broth dilution susceptibility tests

I. Selecting Antimicrobial Agents for Testing and Reporting

A. **Testing:** Bacterial pathogens frequently isolated from fish and shellfish and the diseases known to be caused by these organisms are listed in Appendix A. Selecting the most appropriate antimicrobial agents to test and report is a decision best made by each laboratory in consultation with veterinarians, infectious diseases practitioners, clinical pharmacologists, and antimicrobial stewardship teams, if available. The recommendations for each organism group include antimicrobial agents that show acceptable *in vitro* test performance. Considerations in the assignment of antimicrobial agents to specific test/report groups include clinical efficacy, prevalence of resistance, minimizing emergence of resistance, cost, regulatory agency–approved clinical indications for use, and current consensus recommendations for first-choice and alternative agents. Tests of selected agents may be useful for infection control and/or monitoring purposes.

B. **Reporting:** Each laboratory should decide which antimicrobial agents to routinely test and report. In many countries, veterinary oversight is a regulatory requirement for the use of antimicrobial agents in food animals, including fish.

Although regulatory requirements for drug use are beyond the scope of this document, veterinarians are responsible for understanding the legal limitations of prescribing drugs for animals.

When unexpected resistance is confirmed, it should be reported to the veterinarian. For additional information and guidelines for routine reporting, see VET03,[1] Subchapter 2.3. Guidelines for reporting pathogens with intrinsic resistance to antimicrobial agents (see CLSI document VET08,[5] Appendix B) are discussed in VET03,[1] Subchapters 4.9.3 and 5.8.4 and in CLSI document VET01,[4] Subchapter 2.4.4.

II. Breakpoints and Interpretive Category Definitions

A. **Breakpoint** – minimal inhibitory concentration (MIC) or zone diameter value used to categorize an organism as susceptible, intermediate, resistant, or nonsusceptible; **NOTE 1:** MIC or zone diameter values generated by a susceptibility test can be interpreted based on established breakpoints; **NOTE 2:** See **interpretive category (for breakpoints); NOTE 3:** Also known as "clinical breakpoint."

B. **Interpretive category (for breakpoints)** – category derived from microbiological characteristics, pharmacokinetic-pharmacodynamic parameters, and/or clinical outcome data; **NOTE 1:** Minimal inhibitory concentration (MIC) or zone diameter values generated by a susceptibility test can be interpreted based on established breakpoints; **NOTE 2:** Categories used for breakpoints include susceptible, intermediate, resistant, and nonsusceptible.

EXAMPLE:

Interpretive Category	Breakpoints	
	MIC, µg/mL	Zone Diameter, mm
Susceptible	≤4	≥20
Intermediate	8–16	15–19
Resistant	≥32	≤14
Nonsusceptible	>1	<17

MIC or zone diameter value breakpoints or interpretive categories are established per CLSI document VET02[3] (or CLSI document M23[11] for human medical breakpoints) for categories of susceptible, intermediate, and resistant (and nonsusceptible, when appropriate).

- **susceptible (S)** – a category defined by a breakpoint that implies that isolates with an MIC at or below or a zone diameter at or above the susceptible breakpoint are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used, resulting in likely clinical efficacy.

- **intermediate (I)** – a category defined by a breakpoint that includes isolates with MICs or zone diameters within the intermediate range that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates; **NOTE:** The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher-than-normal dosage of a drug can be used. This category also

Table 1. (Continued)

Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm			Interpretive Categories and MIC Breakpoints, µg/mL			Comments
		S	I	R	S	I	R	
Tetracyclines								
Oxytetracycline	30 µg	≥28	22–27	≤21	≤1	2–4	≥8	**(6)** Class representative for tetracyclines **(7)** Established based on: • Visual inspection of zone diameter and MIC distributions of 323 *A. salmonicida* isolates[3,4] • Clinical correlations – Atlantic salmon held at 13°C in freshwater commercial conditions and dosed *ad libitum* 75 mg/kg body weight for 10 consecutive days[5] – Atlantic salmon held at 14°C in freshwater laboratory conditions and dosed *ad libitum* 75 mg/kg body weight for 10 days[6] • PK data – Various nonsalmonid species held at 15 to 30°C in freshwater and/or saltwater laboratory conditions and dosed *ad libitum* 82.8 mg/kg body weight for 10 days[7] – Rainbow trout held at 12°C in freshwater laboratory conditions and dosed by gavage 74 mg/kg body weight for 10 days[8]
Quinolones								
Oxolinic acid	2 µg	≥30	25–29	≤24	≤0.12	0.25–0.5	≥1	**(8)** Established based on: • Visual inspection of zone diameter and MIC distributions of 323 *A. salmonicida* isolates[3,4] • Clinical correlations – Atlantic salmon held at 10°C in freshwater commercial conditions and dosed *ad libitum* 10 mg/kg body weight for 10 days[9] – Atlantic salmon held at 14 to 16°C in saltwater commercial conditions and dosed *ad libitum* 20 mg/kg body weight for 2 days then 10 mg/kg body weight for 4 days[10] – Atlantic salmon held at 10°C in freshwater experimental conditions, experimentally challenged, and dosed *ad libitum* 10 mg/kg body weight for 10 days[11] • PK data – Cod held at 8°C in saltwater laboratory conditions and dosed intravenously once with 12.5 mg/kg body weight[12] – Rainbow trout held at 16°C in freshwater laboratory conditions and dosed intravenously once with 10 mg/kg body weight[13]

Abbreviations: ATCC®, American Type Culture Collection; CAMHB, cation-adjusted Mueller-Hinton broth; I, intermediate; MHA, Mueller-Hinton agar; MIC, minimal inhibitory concentration; PK, pharmacokinetic; QC, quality control; R, resistant; S, susceptible.

Table 1
MIC and Zone Diameter Breakpoints for
Aeromonas salmonicida

Table 2B. MIC and Zone Diameter Epidemiological Cutoff Values for *Aeromonas hydrophila*

Testing Conditions	
Medium:	**Broth dilution: CAMHB** **Disk diffusion: MHA**
Inoculum:	**Growth method or direct colony suspension, equivalent to a 0.5 McFarland standard**
Incubation:	**28°C±2°C; ambient air; 24–28 hours**

Routine QC Recommendations (see Tables 4C and 5C for acceptable QC ranges)

***Escherichia coli* ATCC®[a] 25922**
***Aeromonas salmonicida* ATCC® 33658**

General Comments

(1) These ECVs are applicable only to isolates of *Aeromonas hydrophila* tested under quality controlled conditions, as described in VET03.[1] Before results for test strains are interpreted, QC test results should be ensured to be within the ranges specified in Table 4C.

(2) ECVs presented here were established solely based on a statistical analysis of MIC and zone diameter data obtained from the ECOFFinder[2] and the normalized resistance interpretation method.[3] A geographically diverse set of 104 test isolates originated from 13 different countries. ECVs can be used as a measure of the emergence of strains with reduced susceptibility to a given agent. They are not breakpoints, and, thus, proven clinical relevance has not yet been identified or approved by CLSI or any regulatory agency.

(3) The isolates used to establish these ECVs were not from fish that were part of a clinical field trial. These ECVs should be used in the establishment of interpretive categories, as described in CLSI document VET02.[4]

(4) See the Instructions for Use of Tables, Section H for definitions of ECVs and interpretive categories for ECVs.

NOTE: Information in boldface type is new or modified since the previous edition.

Table 6. Solvents and Diluents for Preparing Stock Solutions of Antimicrobial Agents

Antimicrobial Agent	Solvent[a]	Diluent[a]
	Unless otherwise stated, a minimum amount of the listed solvents should be used to solubilize the antimicrobial powder.	**The final stock solution should be diluted as stated below.**
Amikacin	Water	Water
Amoxicillin	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Amoxicillin-clavulanate	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Ampicillin	Phosphate buffer, pH 8.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Apramycin	95% ethanol[b]	Water
Azithromycin	95% ethanol or glacial acetic acid[b,c]	Broth media
Azlocillin	Water	Water
Aztreonam	Saturated solution sodium bicarbonate	Water
Carbenicillin	Water	Water
Cefaclor	Water	Water
Cefadroxil	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cefamandole	Water	Water
Cefazolin	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Cefdinir	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cefditoren	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cefepime	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Cefetamet	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cefixime	Phosphate buffer, pH 7.0, 0.1 mol/L	Phosphate buffer, pH 7.0, 0.1 mol/L
Cefmetazole	Water	Water
Cefonicid	Water	Water
Cefoperazone	Water	Water
Cefotaxime	Water	Water
Cefotetan	DMSO[b,d]	Water
Cefoxitin	Water	Water
Cefpodoxime	0.10% (11.9 mmol/L) aqueous sodium bicarbonate	Water
Cefprozil	Water	Water
Ceftazidime	Sodium carbonate[g]	Water
Ceftibuten	1/10 vol DMSO[b,d]	Water
Ceftiofur	Water or broth	Water or broth
Ceftizoxime	Water	Water
Ceftriaxone	Water	Water
Cefuroxime	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Cephalexin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cephalothin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cephapirin	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Cephradine	Phosphate buffer, pH 6.0, 0.1 mol/L	Water
Chloramphenicol	95% ethanol	Water
Cinoxacin	1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve	Water
Ciprofloxacin	Water	Water
Clarithromycin	Methanol or glacial acetic acid[b,c]	Phosphate buffer, pH 6.5, 0.1 mol/L
Clavulanate	Phosphate buffer, pH 6.0, 0.1 mol/L	Phosphate buffer, pH 6.0, 0.1 mol/L
Clinafloxacin	Water	Water
Clindamycin	Water	Water
Colistin[a]	Water	Water
Dalbavancin	DMSO[b,d]	Water
Danofloxacin	1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve	Water
Difloxacin	1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve	Water
Dirithromycin	Glacial acetic acid[b,c]	Water
Doripenem	0.85% physiological saline	0.85% physiological saline
Doxycycline	Water	Water

Related CLSI Reference Materials*

M02 **Performance Standards for Antimicrobial Disk Susceptibility Tests. 13th ed., 2018.** This standard covers the current recommended methods for disk susceptibility testing and criteria for quality control testing.

M02QG **Disk Diffusion Reading Guide. 1st ed., 2018.** The Disk Diffusion Reading Guide provides photographic examples of the proper method for reading disk diffusion susceptibility testing results.

M07 **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 11th ed., 2018.** This standard covers reference methods for determining minimal inhibitory concentrations of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

M23 **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters. 5th ed., 2018.** This guideline discusses the necessary and recommended data for selecting appropriate breakpoints and quality control ranges for antimicrobial agents.

M24 **Susceptibility Testing of Mycobacteria, *Nocardia* spp., and Other Aerobic Actinomycetes. 3rd ed., 2018.** This standard provides protocols and related quality control parameters for antimicrobial susceptibility testing of mycobacteria, *Nocardia* spp., and other aerobic actinomycetes.

M39 **Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data. 4th ed., 2014.** This document describes methods for recording and analysis of antimicrobial susceptibility test data, consisting of cumulative and ongoing summaries of susceptibility patterns of clinically significant microorganisms.

M100 **Performance Standards for Antimicrobial Susceptibility Testing. 30th ed., 2020.** This document includes updated tables for the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards M02, M07, and M11.

VET01 **Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 5th ed., 2018.** This standard covers the current recommended methods for disk diffusion susceptibility testing and the reference methods for determining minimal inhibitory concentrations of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution for veterinary use.

VET02 **Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents. 3rd ed., 2008.** This document addresses the required and recommended data needed for selection of appropriate interpretive standards and quality control guidance for new veterinary antimicrobial agents.

VET03 **Methods for Antimicrobial Broth Dilution and Disk Diffusion Susceptibility Testing of Bacteria Isolated From Aquatic Animals. 2nd ed., 2020.** This guideline covers the current recommended methods for broth micro- and macrodilution and disk diffusion susceptibility testing of aquatic species isolates and criteria for quality control testing.

VET08 **Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 4th ed., 2018.** This document includes updated tables for the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standard VET01.

VET09 **Understanding Susceptibility Test Data as a Component of Antimicrobial Stewardship in Veterinary Settings. 1st ed., 2019.** This report provides veterinarians with the information needed to successfully acquire and interpret antimicrobial susceptibility test results. It promotes common understanding between the veterinarian and the veterinary microbiology laboratory by providing example culture and susceptibility reports and animal species–specific guidance on applying breakpoints to interpret susceptibility test results.

* CLSI documents are continually reviewed and revised through the CLSI consensus process; therefore, readers should refer to the most current editions.

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950 West Valley Road, Suite 2500, Wayne, PA 19087 USA

P: +1.610.688.0100 **Toll Free (US):** 877.447.1888 **F:** +1.610.688.0700

E: customerservice@clsi.org **www.clsi.org**

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