CLINICAL AND LABORATORY STANDARDS INSTITUTE®

2nd Edition

VET03

Methods for Antimicrobial Broth Dilution and Disk Diffusion Susceptibility Testing of Bacteria Isolated From Aquatic Animals

This guideline provides the most up-to-date techniques for the determination of minimal inhibitory concentrations and zones of inhibition of aquatic bacteria and criteria for data interpretation and quality control testing.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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Replaces VET03-A and VET04-A2

Methods for Antimicrobial Broth Dilution and Disk Diffusion Susceptibility Testing of Bacteria Isolated From Aquatic Animals

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Abstract

Antimicrobial susceptibility testing (AST) is recommended to determine which antimicrobial agents should be considered for treating a bacterial pathogen. Many bacteria that cause disease in aquatic animals have growth conditions that vary substantially from routine terrestrial bacterial pathogens. It has thus become desirable to develop guidelines for standardizing AST methods for organisms isolated from aquatic animals that prefer or need certain conditions, such as lower temperatures, diluted media, or longer incubation times.

Clinical and Laboratory Standards Institute guideline VET03—*Methods for Antimicrobial Broth Dilution and Disk Diffusion Susceptibility Testing of Bacteria Isolated From Aquatic Animals* describes broth dilution and disk diffusion, and it includes a series of procedures to standardize the way the tests are performed on Groups 1 and 3 aquatic bacteria. Group 1 nonfastidious bacteria grow readily in cation-adjusted Mueller-Hinton broth (CAMHB) and on Mueller-Hinton agar and are readily cultured at temperatures of 22°C ± 2°C and 28°C ± 2°C. Group 3 nonfastidious gliding bacteria grow in diluted CAMHB and are readily cultured at temperatures of 18°C or 28°C, depending on the species.

The supplemental VET04[1] tables used with this guideline represent the most current information for antimicrobial agent selection, interpretation, and QC using the procedures described in VET03. The QC ranges for *Escherichia coli* ATCC® 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC® 33658 when tested at 18°C, 22°C, 28°C, and 35°C ± 2°C (ie, *E. coli* only) are listed in VET04[1] for different antimicrobial agents important to global aquaculture.

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Foreword

The global aquaculture industry is composed of many fish species, which have substantially different bacterial microbiota that grow at different optimal temperatures. Thus, CLSI has standardized antimicrobial susceptibility testing (AST) methods and established QC ranges at 18°C±2°C, 22°C±2°C, and 28°C±2°C. These temperatures were chosen based on temperatures most frequently used for testing; recommendations of the Subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST), the Working Group on Aquaculture AST Methods (VET03 WG), and the Working Group on Aquatic Animals; and to coordinate efforts with researchers from other countries. In the case of zoonotic pathogens from aquatic sources or tropical fish species, clinicians may request AST conducted at 35°C±2°C. In these cases, refer to CLSI documents VET01,[2] VET08,[3] VET06,[4] and M45[5] for the appropriate QC organisms, ranges, and to the extent possible, interpretive categories.

In this revision of VET03, the broth dilution and disk diffusion procedures for testing aquatic bacteria were consolidated into one guideline, with reformatting of the guideline to follow a laboratory's path of workflow, defined as the sequential processes of preexamination, examination, and postexamination. Several chapters and subchapters have been added or expanded, as described in the Overview of Changes. An overview of the AST process is provided in the beginning of this guideline in the new Figure 1 (see Chapter 3) and at the beginning of each method chapter (see Chapters 4 and 5), with various testing methods shown in easy-to-follow step-action tables throughout this guideline. Other improvements have been made in this guideline by incorporating relevant updates derived from CLSI documents VET01,[2] M02,[6] and M07,[7] and the *M02 Disk Diffusion Reading Guide,*[8] and by adding new antimicrobial agents or testing guidelines for aquatic bacterial pathogens.

The current edition of CLSI document VET04[1] (formerly VET03/VET04), a volume of tables that includes clinical breakpoints (susceptible, intermediate, and resistant) and epidemiological cutoff values (ECVs) (ie, wild-type cutoffs) for fish pathogens, is made available with this guideline to ensure users are aware of the latest Subcommittee on VAST performance guidelines related to both the methods and the information presented in the tables. Previously published tables should be replaced with the current editions for interpreting breakpoints and ECVs. Significant changes in VET04[1] since 2014 include additional fish-specific ECVs for the pathogens *Aeromonas salmonicida, Aeromonas hydrophila, Flavobacterium columnare,* and *Flavobacterium psychrophilum.*

This guideline represents the collective efforts of the Subcommittee on VAST, the VET03 WG, and the Working Group on Aquatic Animals to produce a guidance document describing recommended broth dilution and disk diffusion susceptibility testing methods for bacteria isolated from aquatic species. The Subcommittee on VAST relied heavily on the initial efforts of those who organized the 1998 *Workshop on MIC Methodologies in Aquaculture* and the subsequent publication of draft protocol developed at the workshop.[9] These documents outlined the problems encountered when comparing data created by laboratories that were using different methods, because those data usually varied greatly from laboratory to laboratory. The published methods[9] were termed "tentative" by the authors to indicate that there were a number of unresolved issues. Members of the current Subcommittee on VAST resolved some of these issues, such as the development of QC ranges for QC strains, in previous editions of this guideline.

This guideline provides recommended broth dilution susceptibility testing methods for Groups 1 and 3 aquatic bacteria and recommended disk diffusion methods for Group 1 aquatic bacteria. This guideline also contains the current best thinking of scientists in the field and their recommendations for conducting AST on other fastidious aquatic bacteria. It is anticipated that this guideline and its supplement VET04[1] will be kept up-to-date to include additional recommended AST methods, clinical breakpoints, and ECVs for antimicrobial agents used to inform treatment of bacterial infections in aquatic species and detect emerging resistance issues.

Methods for Antimicrobial Broth Dilution and Disk Diffusion Susceptibility Testing of Bacteria Isolated From Aquatic Animals

Chapter 1: Introduction

This chapter includes:

- Guideline's scope and applicable exclusions
- Background information pertinent to the guideline's content
- Standard precautions information
- Terminology information, including:
  - Terms and definitions used in the guideline
  - Abbreviations and acronyms used in the guideline

1.1 Scope

This guideline provides veterinary and aquatic animal disease diagnostic laboratories with currently recommended antimicrobial broth dilution and disk diffusion susceptibility testing methods for nonfastidious bacteria isolated from aquatic animals, including criteria for QC testing with two QC strains. It also provides suggestions as to the testing conditions for other fastidious aquatic animal bacterial pathogens. To avoid confusion, organisms relevant to aquaculture have been grouped (ie, Groups 1 through 5), and the organisms in each group and their corresponding numbers have not changed from previous editions of this guideline. This guideline also provides appendixes and tables describing media and disk preparation, methods for preparing stock solutions and dilutions of antimicrobial agents, and antimicrobial agents used in global aquaculture.

Clinical breakpoints and epidemiological cutoff values (ECVs) are included in VET04.[1] Clinical breakpoints must be established using pharmacokinetic (PK) and pharmacodynamic (PD) data, *in vitro* antimicrobial susceptibility testing (AST) data, and clinical efficacy data. ECVs can be established from susceptibility data distributions alone. For information on how to develop these interpretive categories, consult CLSI document VET02.[10] As more aquatic animal–specific information becomes available, this guideline and VET04[1] will be updated accordingly.

This guideline and its supplement (VET04[1]) are not intended to guide the use of antimicrobial agents that are used for disease prevention or production uses.

1.2 Background

To positively affect clinical outcomes, help maintain antimicrobial effectiveness, assist veterinarians and aquatic animal health care professionals in using antimicrobial agents safely, and minimize the selection of resistant pathogens, laboratories must use a standardized, well-defined method for performing AST. This guideline presents AST methods that provide accurate, reproducible results for bacterial pathogens of aquatic animals. Aquatic animal–specific clinical and epidemiological interpretive categories were established following guidelines presented in CLSI documents VET02[10] and VET05.[11] The need for globally harmonized test methods is essential if interlaboratory minimal inhibitory concentrations (MICs)

or zone-size data are to be compared, eg, in journals, Web postings, and antimicrobial resistance (AMR) monitoring program reports.

Clinical breakpoints and ECVs presented in VET04[1] apply only to isolates of the given bacterial species and if the laboratory has conducted AST according to the specific methods described in this guideline. For antimicrobial agents not approved for use in indicated aquatic animal hosts, the laboratory client or veterinarian assumes all responsibility for efficacy, safety, and violative residue avoidance with the extralabel use of these agents.

1.2.1 Broth Dilution Testing

Broth dilution methods may be used to quantitatively measure the *in vitro* activity of an antimicrobial agent against a given bacterial isolate. To perform the tests, a series of tubes are prepared using a broth medium to which serial concentrations of the antimicrobial agents are added. The tubes are then inoculated with a standardized suspension of the test organism. After incubating at the appropriate temperature for the appropriate time interval, the tests are read, the MIC is determined, and the results are interpreted using approved clinical breakpoints and/or ECVs. The final result is significantly influenced by methodology, which must be carefully controlled if reproducible results (ie, intra- and interlaboratory) are to be achieved.

This guideline describes reference broth dilution (ie, macrodilution and microdilution) methods. The basic components of these methods are largely derived from information contained in published recommendations.[12] Although these methods are standard reference methods, some are sufficiently practical for routine use in aquatic animal, veterinary, and research laboratories.

Commercial systems based primarily or in part on some of these methods are available and may provide results essentially equivalent to the CLSI methods described in this guideline. CLSI does not approve or endorse commercial products or devices. If a laboratory is using a commercial susceptibility test system, the manufacturer's instructions should be followed when performing the commercial test and QC. The laboratory is responsible for ensuring that the performance of a commercial test system has been validated against the reference method.

The broth dilution methods described in this guideline are intended primarily for testing aerobic or facultative bacteria that grow well after incubation in undiluted and diluted cation-adjusted Mueller-Hinton broth (CAMHB). However, alternative media and methods for some fastidious or uncommon organisms are also described. Methods for testing and interpreting data for certain mammalian pathogens that may be pathogenic to aquatic animals (eg, *Vibrio* spp., *Aerococcus* spp.) and are considered infrequently isolated or fastidious bacteria, are included in CLSI documents VET06[4] and M45.[5] Although CLSI document M45[5] provides methods and human medical breakpoints for the mesophilic aeromonads (eg, *Aeromonas hydrophila*), applicability of the human MIC breakpoints available for *A. hydrophila* to aquatic animal medicine is unknown.

This guideline describes methods and QC procedures currently recommended for broth dilution susceptibility tests. When new problems are recognized or improvements in these methods are developed, changes will be incorporated into future editions of this guideline and its supplement, VET04.[1]

1.2.2 Disk Diffusion Testing

Various laboratory methods can be used to measure the *in vitro* susceptibility of bacteria to antimicrobial agents. In many veterinary and medical laboratories, agar disk diffusion is used routinely for testing common, rapidly growing, and certain fastidious bacterial pathogens. This guideline describes the performance, applications, and limitations of the recommended disk diffusion test method.

Chapter 2: Indications for Performing Antimicrobial Susceptibility Tests

This chapter includes:

- Indications for when AST is necessary
- Selecting appropriate antimicrobial agents for routine testing
- Descriptions of the various antimicrobial agent classes
- Guidelines for routine reporting

AST may be performed to guide recommendations concerning the appropriate therapy of specific disease outbreaks or for purposes of monitoring and surveillance of patterns of AMR on a national or regional scale. Published guidance for the prudent use of antimicrobial agents in aquaculture[22] require that susceptibility tests be performed in association with all antimicrobial treatments in aquaculture. When possible, these tests should be performed before starting treatment. When clinical conditions necessitate the rapid initiation of therapy, susceptibility tests should be performed as soon as possible after the start of therapy to confirm the choice of agent being administered.

Unfortunately, not all aquatic pathogens have standardized AST methods or established interpretive categories. When dealing with these isolates, laboratories are strongly encouraged to use the recommended test modifications outlined in VET04[1] Appendixes B and C.

Bacteria with differing antimicrobial susceptibility can be isolated from a single disease outbreak.[23] Therefore, when investigating the susceptibility of bacteria associated with such disease outbreaks, more than one isolate should be examined.

The following shortcut methods of AST are discouraged, because they provide misleading outcomes and can result in poor treatment decisions:

- Mixtures of different types of microorganisms should not be tested in the same susceptibility test (ie, only a pure culture should be tested).

- The practice of conducting susceptibility tests directly with clinical material (eg, normally sterile body fluids and water samples) should be avoided.

- The practice of inoculating susceptibility test plates with colonies directly from selective media should be avoided to prevent carryover of agents that may affect susceptibility test results or carryover of contaminant bacteria growing poorly on the selective medium.

- When the nature of the infection is not clear and the specimen contains mixed organisms or organisms bearing little relationship to the infectious process, susceptibility tests are often unnecessary, may be misleading, and may result in inappropriate use of antimicrobial agents.

2.1 Selecting Antimicrobial Agents for Routine Testing

Selecting the most appropriate antimicrobial agents to test and report is a decision best made by each laboratory in consultation with aquatic animal practitioners. Appendixes B and C list many of the antimicrobial agents used at varying frequencies in global aquaculture, including the status of QC ranges

Step	Action	Comment
3	Optimally within 15 minutes of preparation, dilute the adjusted inoculum suspension in water, saline, or broth.	Each well should contain approximately 5×10^5 CFU/mL (range, 3.3–6.6 × 10^5 CFU/mL). The dilution procedure to obtain this final inoculum varies according to the method for delivering the inoculum to the individual wells and must be calculated for each situation. For microdilution tests, the exact inoculum volume delivered to the wells must be known to make this calculation. For example, if the volume of broth in the well is 0.1 mL and the inoculum volume is 0.01 mL, the 0.5 McFarland suspension (1×10^8 CFU/mL) should be diluted 1:20 to yield 5×10^6 CFU/mL. When 0.01 mL of this suspension is inoculated into the broth, the final test concentration of bacteria is approximately 5×10^5 CFU/mL (or 5×10^4 CFU/well in the microdilution method). Because of the larger-size Group 3 flavobacteria, twice the amount of the 0.5 McFarland suspension is needed. Therefore, in the example above, the suspension should be diluted 1:10. Guidance for 96-well broth microdilution panels typically inoculated with 11 mL of CAMHB is shown for dried and frozen panels: • Dried panels (ie, 0.1 mL inoculum volume) – Group 1: dilute 0.5 McFarland suspension 1:200 – Group 3: dilute 0.5 McFarland suspension 1:100 • Frozen panels (ie, 0.05 mL inoculum volume) – Group 1: dilute 0.5 McFarland suspension 1:100 – Group 3: dilute 0.5 McFarland suspension 1:50

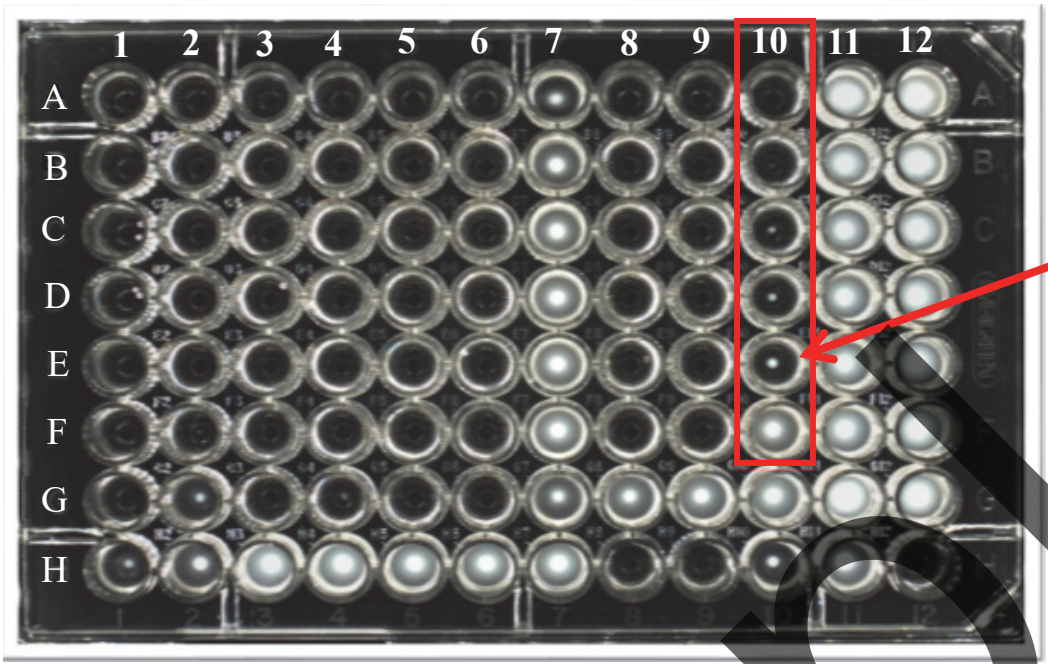

Figure 5. Trimethoprim-Sulfamethoxazole: 80% Inhibition End Point. From top to bottom, wells A10 to F10 are 152/8 to 9.5/0.5 µg/mL. The MIC is well E10, which is designated by the arrow. Well G12 is the growth control with no antimicrobial agent, and well H12 is the negative growth control.

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 **M02 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.

M29 **Protection of Laboratory Workers From Occupationally Acquired Infections. 4th ed., 2014.** Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

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.

M45 **Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. 3rd ed., 2016.** This guideline informs clinical, public health, and research laboratories on susceptibility testing of infrequently isolated or fastidious bacteria that are not included in CLSI documents M02, M07, or M100. Antimicrobial agent selection, test interpretation, and quality control are addressed.

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.

VET04 **Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated From Aquatic Animals. 3rd ed., 2020.** This document includes updated tables for the Clinical and Laboratory Standards Institute veterinary antimicrobial susceptibility testing guideline VET03.

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

Related CLSI Reference Materials (Continued)

VET05 **Generation, Presentation, and Application of Antimicrobial Susceptibility Test Data for Bacteria of Animal Origin. 1st ed., 2011.** This report offers guidance on areas in which harmonization can be achieved in veterinary antimicrobial surveillance programs with the intent of facilitating comparison of data among surveillance programs.

VET06 **Methods for Antimicrobial Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria Isolated From Animals. 1st ed., 2017.** This document provides guidance for antimicrobial agent disk and dilution susceptibility testing, criteria for quality control testing, and breakpoints for fastidious and infrequently tested bacteria for veterinary use.

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 veterinary 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.

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