CLINICAL AND LABORATORY STANDARDS INSTITUTE®

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# VET03-A

## Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Approved Guideline

This document provides the most up-to-date techniques for disk diffusion susceptibility testing of aquatic species isolates, and criteria for quality control testing.

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

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## Abstract

Antimicrobial susceptibility testing is recommended to determine which antimicrobial agents should be considered for treating a bacterial pathogen. Many bacteria that cause disease in aquatic animals require growth conditions that vary substantially from routine terrestrial bacterial pathogens. It has thus become desirable to develop antimicrobial testing standards for organisms that prefer or require conditions such as lower temperatures, semisolid media, or supplemented media (e.g., NaCl, serum).

This guideline describes the standard agar disk diffusion method and quality control criteria for testing Group 1 aquatic bacteria. These organisms can grow readily on standard Mueller-Hinton agar, and are readily cultured at temperatures of 22 ± 2 °C and 28 ± 2 °C. Quality control ranges for *Escherichia coli* ATCC® 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC® 33658 when tested at 22 °C, 28 °C, and 35 ± 2 °C (*E. coli* only) are listed for different antimicrobial agents used to varying degrees in global aquaculture.

Future editions of this guideline will incorporate additional data, as they become available. Still needed are methods for testing other groups of aquatic pathogens, such as the gliding bacteria, obligate halophiles, and gram-positive cocci. In addition, interpretive criteria will also need to be developed, which requires a correlation between pharmacokinetic/pharmacodynamic properties of the drug, *in vitro* susceptibility data, and clinical outcomes.

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## Foreword

This CLSI guideline represents the collective efforts of the Subcommittee on Veterinary Antimicrobial Susceptibility Testing Aquaculture Working Group (VAST-AWG) to produce a guidance document for standardizing an antimicrobial disk susceptibility test method for bacteria isolated from aquatic species. The working group has relied heavily on the initial efforts of those who organized the *Workshop on MIC Methodologies in Aquaculture, Weymouth, 1998* and the subsequent Alderman and Smith publication of the draft protocols developed at the workshop.[1] These documents outlined the problems encountered when comparing data created by laboratories using different methods, since those data usually varied greatly from laboratory to laboratory. The methods published by Alderman and Smith were termed "tentative" by the authors to indicate there were a number of "unresolved issues."

Members of the current VAST-AWG have expanded the work of the European group by targeting some of these unresolved issues, such as the development of quality control ranges for quality control strains. We have limited this guideline to the disk diffusion susceptibility testing of Group 1 aquatic organisms (Table 3). This guideline contains the current best thinking of scientists in the field and their recommendations for conducting a particular test. We have not addressed the issues of interpretive criteria. It is hoped that this guideline and the CLSI document M49—*Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated From Aquatic Animals* for broth dilution testing will evolve and include additional standardized susceptibility testing methods and interpretive criteria for antimicrobial agents used to treat bacterial infections in aquatic species.

Since we are attempting to harmonize standards as an international effort, we have chosen to include agents that are used in some nations, but may not be used in other nations. In addition, concerns have been raised about changes in susceptibility of bacteria exposed to antimicrobials in the environment, from either human or veterinary use. It is therefore important to have standardized methods for testing those bacteria isolated from aquatic organisms to drugs used by other medical disciplines. Finally, if more antimicrobials are approved for use in aquaculture—especially those already in use in other areas of agriculture—standards will already be in place.

We have chosen to characterize two quality control strains based on their susceptibility profiles and global availability. *Aeromonas salmonicida* subsp. *salmonicida* (ATCC®[a] 33658; NCIMB[b] 1102) and *Escherichia coli* (ATCC® 25922; NCIMB 12210) are both susceptible to a wide range of antimicrobials, grow well at low temperatures, and have proven to be stable after numerous passes on the testing medium. It is proposed that both of these organisms be used as quality control organisms for disk diffusion susceptibility testing. There is a ban on importation of *A. salmonicida* in several nations; E. *coli* may be used in its place.

We have optimized testing conditions primarily for Group 1 (Table 3) organisms and hope that this guideline will engender future studies with other groups of bacteria. Such organisms include the obligate halophilic bacteria and the gliding bacteria, which require specialized media.

The global aquaculture industry is comprised of many fish species, which have substantially different bacterial flora and grow at different temperature optimums. Thus, we have established the quality control ranges at both 22 $\pm$ 2 °C and 28 $\pm$ 2 °C (Tables 6 through 8). These temperatures were chosen based on temperatures most frequently used for testing, recommendations of the VAST-AWG, 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 susceptibility data conducted at 35 ± 2 °C. In those cases, refer to Table 5 or CLSI/NCCLS document M31—*Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals,* for the appropriate QC organisms, ranges, and interpretive criteria.

[a] ATCC is a registered trademark of the American Type Culture Collection.
[b] National Collection of Industrial and Marine Bacteria (NCIMB, www.ukncc.co.uk)

## Foreword (Continued)

Since this is a collective effort, recognition must go to the Subcommittee on Veterinary Antimicrobial Susceptibility Testing, especially Thomas Shryock for his help with the CLSI consensus process, and to Robert Walker for his guidance on developing control strains and review of the document. We thank also the present and former members of the Aquaculture Working Group, and the organizers and participants of the *Workshop on MIC Methodologies in Aquaculture, Weymouth, 1998,* who began this process. Special thanks must be given to Ron Miller, whose work has provided the data for the long-awaited criteria for quality control testing. Finally, we must acknowledge the U.S. FDA and Oak Ridge Associated Universities for providing support for this effort.

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### *A Note on Terminology*

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in CLSI, International Organization for Standardization (ISO), and European Committee for Standardization (CEN) documents; and that legally required use of terms, regional usage, and different consensus timelines are all challenges to harmonization. In light of this, CLSI recognizes that harmonization of terms facilitates the global application of standards and is an area of immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

Of particular note in VET03-A are two terms whereby CLSI intends to eliminate confusion, over time, through its commitment to harmonization. For the most part, in this guideline, the term *accuracy* is used correctly in its metrological sense, to refer to the closeness of the agreement between the result of a (single) measurement and a true value of a measurand, thus comprising both random and systematic effects. But there are several instances in this document, where accuracy is defined the way ISO defines *trueness*, i.e., the closeness of the agreement between the average value from a large series of measurements and to a true value of a measurand. To facilitate understanding, when used this way, *trueness* has been inserted parenthetically. Also, the terms are defined in the guideline's Definitions section along with explanatory notes. During the next scheduled revision of this document, they will be reviewed for consistency with international use, and revised appropriately.

This document will most likely be used by aquatic disease diagnosticians who may not be familiar with the CLSI terminology; thus we have included an extensive glossary in this guideline, as well as, in the M49 guideline.

**Key Words**

Antimicrobial agent, antimicrobial susceptibility testing, aquatic, disk diffusion, veterinary

## CLSI Working Group on Aquaculture Mission Statement

To develop and to promote performance standards and interpretive criteria for *in vitro* antimicrobial susceptibility testing of bacteria isolated from aquatic organisms.

# Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Approved Guideline

## 1 Scope

This CLSI guideline provides veterinary and aquatic animal disease diagnostic laboratories with currently recommended antimicrobial disk diffusion susceptibility testing methods for bacteria isolated from aquatic animals–primarily Group 1 organisms–including criteria for quality control testing with two quality control strains.

The document also provides appendixes and tables outlining recommended disk concentrations, antimicrobial agents used in global aquaculture, methods for preparing stock solutions and dilutions of antimicrobial agents, and a list of bacteria pathogenic to fish.

Interpretive criteria are not addressed in this guideline. Such criteria must be established using pharmacokinetic and pharmacodynamic data, *in vitro* susceptibility testing data, and clinical efficacy data. Developing interpretive criteria was beyond the scope of this document. As more aquatic animal-specific information becomes available, this document, and CLSI document M49—*Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated From Aquatic Animals*, will be revised to incorporate those data.

## 2 Introduction

One of the great challenges of fish farming (aquaculture) in the United States is the control of disease outbreaks. Throughout history, various compounds have been used to treat fish maladies, including salt, asphalt, and brandy.[2] During the last century, major advances were made in isolation and identification of microorganisms causing disease in aquatic animals.[3-7] Concurrent with advances in diagnostic techniques came advances in production of antimicrobial substances.[8] The first citations on the use of antimicrobials in fish in the U.S. described the use of sulfa drugs to treat furunculosis in trout.[9,10] The early 1950s, with the inclusion of a veterinary medical branch in the FDA, began the era of governmental regulation of veterinary drugs. To date, only four antimicrobial agents have been approved by the FDA, three of which are available for use in aquaculture fish species: florfenicol to control enteric septicemia in catfish, sulfadimethoxine-ormetoprim to control furunculosis in salmonids and enteric septicemia in catfish; and oxytetracycline monoalkyl trimethyl ammonium for selected indications in salmonids, catfish, and lobsters (see FDA Center for Veterinary Medicine website).[11] Federal regulations, however, permit veterinarians to prescribe extra-label uses of certain approved animal drugs and approved human drugs for minor species. A number of publications describe the use of pharmaceuticals in aquaculture, both for food and ornamental species.[12-14] Extra-label drug use is a practice that occurs in many countries, and governmental agencies worldwide are currently grappling with ways to provide proper veterinary care to minor species.[15] Because of potential extra-label drug use in aquaculture, any standardized methods for determining the susceptibility of microbes isolated from aquatic species must include more drugs than those currently approved for use in aquaculture in any given country. The mention of antimicrobial agents in this document is not an endorsement for their use in fish-farmed species. The use of these highly potent drugs remains subjected to the current regulations in force in their respective countries. For export purposes, the regulations in force in the receiving (importing) country may be consulted.

Aquatic animal diseases, for which antimicrobials may be needed, are too numerous to examine here, but several publications are available that can provide the reader with background.[16-20] Plumb cites our current knowledge of over 70 species of bacteria capable of causing disease in aquaculture.[21] Table 1 includes a short list of the most commonly isolated pathogens. In addition to the organisms well characterized as pathogens of aquatic animals, there are numerous instances where isolated organisms are