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VET04-A

Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Approved Guideline

This document provides the most up-to-date techniques for the determination of minimal inhibitory concentrations (MICs) of aquatic bacteria by broth micro- and macrodilution, 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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Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Approved Guideline

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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 susceptibility testing standards for organisms that prefer or require conditions, such as lower temperatures, semisolid media, or supplemented media (e.g., NaCl, serum).

Clinical and Laboratory Standards Institute (CLSI) document VET04-A—*Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Approved Guideline* describes a standardized broth dilution method and quality control criteria for testing Group 1 aquatic bacteria. These organisms grow readily in cation-adjusted Mueller-Hinton broth (CAMHB), 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 ten different antimicrobial agents (ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, ormetoprim-sulfadimethoxine, oxolinic acid, oxytetracycline, and trimethoprim-sulfamethoxazole).

Future editions of this document will incorporate additional data, as they become available. Still needed are methods for testing other groups of aquatic bacterial 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 describing a standardized broth dilution susceptibility testing 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 publication of Alderman and Smith's draft protocols developed at the workshop.[1] These documents outlined the problems encountered when comparing data created by laboratories that were 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 broth dilution susceptibility testing of Group 1 aquatic organisms. 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 CLSI document M42—*Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated From Aquatic Animals* for agar disk diffusion 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 drugs used in other medical disciplines against bacteria isolated from the aquatic environment. 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 in the testing medium. It is proposed that both of these organisms be used as quality control organisms for broth dilution susceptibility testing. With respect to *Aeromonas salmonicida* subsp. *salmonicida*, there is a ban on importation of this organism in several nations and thus, *E. coli* may be used in its place.

We have optimized testing conditions primarily for the Group 1 (see Table 1) organisms and hope that this guideline will engender future studies with other groups of bacteria. Such organisms include the obligate halophiles, gliding bacteria, and gram-positive cocci.

The global aquaculture industry is comprised of many fish species, which have substantially different bacterial flora and grow at different temperature optimums. Thus, quality control ranges have been established at three different temperatures, 22 ± 2 °C, 28 ± 2 °C, and 35 ± 2 °C (*E. coli* only) (see Tables 6 through 9). These temperatures were chosen based on temperatures most frequently used for testing, recommendations of the VAST-AWG, and also to coordinate our 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 9 or CLSI/NCCLS document M31—*Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for*

[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)

Bacteria Isolated From Animals, for the appropriate QC organisms, ranges, and interpretive criteria.

Since this is a collective effort, recognition must go to the collaborating laboratories who conducted the broth microdilution standardization study. In addition, we appreciate the assistance of the Subcommittee on Veterinary Antimicrobial Susceptibility Testing, especially Dr. Thomas Shryock for his help with the CLSI consensus process, and to Dr. Robert Walker for his guidance on developing control strains and review of the document. We also thank 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 from the Oak Ridge Associated Universities, 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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It is important for users of VET04-A to recognize that commercial susceptibility testing devices are not addressed in this guideline. The methods described herein are generic reference procedures that can be used for routine susceptibility testing by clinical laboratories, or that can be used by clinical laboratories to evaluate commercial devices for possible routine use. Some laboratories could find that a commercial dilution, antibiotic gradient, colorimetric, turbidimetric, fluorometric, or other method is suitable for selective or routine use.

Key Words

Antimicrobial susceptibility, aquaculture, aquatic, broth microdilution, macrodilution, minimal inhibitory concentration (MIC)

Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Approved Guideline

1 Scope

This guideline provides veterinary and aquatic animal disease diagnostic laboratories with currently recommended antimicrobial broth dilution susceptibility testing methods for bacteria isolated from aquatic animals, primarily Group 1 organisms (see Table 1), including criteria for quality control testing with two quality control strains.

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

We have not addressed interpretive criteria 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 M42—*Methods for Antimicrobial Disk 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) 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] There has been quite an "evolution" of legislation and drug approvals for aquaculture in many nations during the last 50 years. Using the United States as an example, 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 are approved by the FDA, three of which are available for use in aquaculture fish species: florfenicol to control enteric septicemia in catfish; ormetoprim-sulfadimethoxine to control furunculosis in salmonids and enteric septicemia in catfish; and oxytetracycline monoalkyl trimethyl ammonium for selected indications in salmonids, catfish, and lobsters (see U.S. 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. Antimicrobial agents mentioned in this document are not endorsed for use in fish-farmed species. Clinicians must consult the regulations in their countries and also those of countries to which the fish may be exported.

There are over 70 species of bacteria capable of causing disease in aquatic animals.[16-21] Table 3 includes a short list of the most commonly isolated aquatic pathogens. In addition to the organisms well-characterized as pathogens of aquatic animals, there are numerous instances where isolated organisms are either partially identified or unidentified. In such cases, as for identified pathogens, antimicrobial susceptibility testing of the isolates is indicated prior to initiating therapy. Susceptibility tests are also