Determining Antimicrobial MICs Against Aquaculture Pathogens Using Sensititre® Plates

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INTRODUCTION

Bacterial pathogens are a leading cause of mortality in aquacultured fish.[1] However, only three antimicrobials – florfenicol (AQUAFLOR® (florfenicol) type A medicated article), oxytetracycline (Terramycin® 200 for fish) and sulfadimethoxine/ormetoprim (Romet 30®) – are commercially available for use in the US food-fish industry. Consequently, it is important to monitor the susceptibility of bacterial pathogens to these antimicrobials.

Clinical breakpoints (interpretive criteria) define whether bacteria are susceptible, intermediate or resistant to an antibiotic, and their values are derived from extensive *in vitro*, pharmacokinetic and field trial studies.[2,3] While clinical breakpoints are in place for many bacterial pathogens of terrestrial animals, the only aquaculture pathogen with established clinical breakpoints is *Aeromonas salmonicida*.[4,5] Data to generate clinical breakpoints for fish pathogens should be established in a standardized fashion so they can be consistently reproduced in any laboratory.

Bacteria isolated from fish require different laboratory testing conditions compared to those for terrestrial animals because there are physiological differences, such as the temperature variation between "cold-blooded" fish (poikilotherms) and "warm-blooded" terrestrial animals (homeotherms).[4] Diagnostic laboratories that routinely test bacteria isolated from aquatic species have established their own laboratory-specific clinical breakpoints based on two parameters: specific *in vitro* assays and the clinical response to antimicrobial use.

SUMMARY

- **In US aquaculture, where bacterial infections are a leading cause of mortality, monitoring bacterial pathogen susceptibility to antibiotics is important because there are only three antimicrobials approved for use in foodfish.**
- **When conducted according to standardized methods, the minimal inhibitory concentration (MIC) assay can reliably assess the *in vitro* susceptibility of bacteria to an antibiotic.**

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In an effort to establish formal breakpoints for aquatic bacteria, the Clinical and Laboratory Standards Institute (CLSI) has published guidelines for the methodology of minimal inhibitory concentration (MIC) assays.[2] The MIC technique is a quantitative antimicrobial susceptibility assay.[2,3] MIC assays can be conducted using either large volumes (1 mL) of inoculum (broth-macrodilution assay) or small volumes (100 µl) of inoculum (broth-microdilution assay). Most laboratories prefer the broth-microdilution assay because it requires smaller inocula volumes, is less labor intensive and is contained within a single tray or plate.

Although CLSI provides the procedures for in-house preparation of broth microdilution plates with serial dilutions of antimicrobial standards, microdilution plates are commercially available with either frozen or lyophilized antimicrobials. For illustration purposes, the following discussion will focus on the commercially prepared microdilution plates (Sensititre® Plate, Trek Diagnostic Systems Ltd., Cleveland, OH) containing the antibiotic florfenicol (Figure 1). Florfenicol (FFC), the active ingredient in AQUAFLOR, is approved in the USA for control of mortality due to *Edwardsiella ictaluri* in catfish,[6] *Flavobacterium psychrophilum*[7] and *Aeromonas salmonicida*[8] in freshwater-reared salmonids, *Flavobacterium columnare* in freshwater-reared finfish, and *Streptococcus iniae* in freshwater-reared warmwater finfish.

Determining Florfenicol MICs with the broth-microdilution assay

Prior to *in vitro* susceptibility testing, the bacteria must be isolated in pure culture then identified using routine morphological, biochemical and/or molecular-based assays. Sensititre® microdilution MIC assays utilize sterile 96-well microdilution plates arranged in rows and columns (Figure 1). For illustration purposes, the last column of wells in this plate contains 0 µg/mL FFC and serves as a positive control to assess the viability of organisms (Figure 2). Each of the following columns contains FFC in a series of two-fold dilutions that begin with 0.125 µg/mL FFC; the concentration then doubles in each subsequent column to a maximum FFC concentration of 128 µg/mL. Following is the procedure for setting up the MIC assay according to CLSI guidelines.[2,5]

Prior to *in vitro* susceptibility testing, the bacteria must be isolated in pure culture and identified using routine morphological, biochemical and/or molecular-based assays.

1 For most organisms, the the colony suspension method is used. Three to five (3 to 5) isolated bacterial colonies are removed from a 24- to 48-hour non-selective agar plate and are inoculated directly into broth or sterile saline. To ensure uniformity in methodology, cation-adjusted Mueller Hinton broth (CAMHB) is the broth of choice for culturing most aquatic pathogens. For flavobacterium, the broth culture method is preferred for inoculum preparation.[2] The colonies are suspended in diluted CAMHB and bacterial clumps are allowed to settle. An aliquot from the upper homogeneous portion of the culture is removed and is transferred to a sterile culture tube.

2 For both, the colony suspension method and the broth culture method, the density of testing inocula is standardized to a 0.5 McFarland standard. This is equivalent to a cell density of ~1-2 x 10^8 cfu/mL, or to an absorbency of 0.08-0.13 at 625 nm on a spectrophotometer, which should be verified periodically using plate counts.

3 The standardized inocula suspensions are diluted in CAMHB so that each well on the plate contains ~5 x 10^5 cfu/mL. A known inoculum volume per well is used to make this calculation. For example, if 100 µL of medium and an inoculum volume of 5 µL are used, the 1 x 10^8 dilution suspension must be diluted to yield 10^7 cfu/mL. This will yield a final test concentration of bacteria in the well of ~5 x 10^4 cfu/well (~5 x 10^5 cfu/mL).

Each test organism is inoculated in a separate row. Included in each tray should be a column of wells containing no antimicrobial agent (positive control wells) to assess the viability of organisms. The test inoculum suspension should be subcultured on agar plates to check for purity.

4 One row of wells per plate is inoculated with a quality-control organism (Figure 2). *Escherichia coli* ATCC 25922 or *Aeromonas salmonicida* subsp *salmonicida* ATCC 33658 is recommended by CLSI as the control organism for most aquatic pathogens tested between 22° C to 28° C (71.6° F to 82.4° F). For organisms tested at lower or higher temperatures or other special requirements, the reader is referred to CLSI VET03 for additional quality control strain recommendations.

5 One row of wells per plate is filled with un-inoculated broth to serve as a negative control.

6 To prevent drying during incubation, each plate is sealed with a tight-fitting plastic cover before incubation. The plates are stacked no more than four plates high in the incubator.

7 The microdilution trays are incubated at 22° C to 28° C (71.6° F to 82.4° F) ± 2° C for either 24 to 28 hours or 44 to 48 hours for bacteria such as *E. ictaluri*, *F. columnare*, *A. hydrophila* and *A. salmonicida*. Extended incubation of inoculated plates is discouraged because antimicrobial deterioration could result in falsely elevated MICs. For assaying FFC MIC against *F. psychrophilum*, which requires a lower incubation temperature at 18° C ± 2° C for 92-96 hours, the reader is referred to CLSI VET03 and CLSI VET04.

8 Bacterial growth appears as a dense button in the well bottom. The well with the lowest concentration of FFC exhibiting no observable growth is the MIC against the test organism. For the results to be valid, a button of bacteria ≥ 2 mm in diameter must grow in the positive control well.

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Figure 1

- **Uninoculated microdilution plate for broth dilution susceptibility testing of bacteria**

  Florfenicol (FFC) dilutions double in each vertical column of wells with the exception of the last column, which contains no FFC (see arrow).

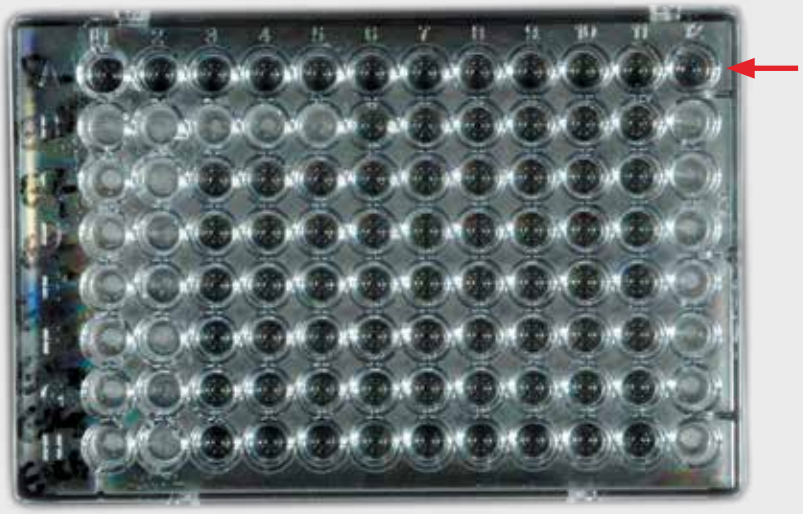
Figure 2

- **Sensititre Plate inoculated with *Edwardsiella ictaluri* to determine the florfenicol (FFC) minimal inhibitory concentration (MIC)**

  Row A (see arrow) contained uninoculated broth (negative control). Note the clear appearance in the wells, indicating no bacterial growth.

  The wells in column #12 contain no FFC. Without the presence of the antibiotic, there was bacterial growth appearing as a cloudy dense button in the well bottom (positive control).

  The remaining wells contained FFC in dilutions beginning at 0.125 µg/mL and doubling in each column, from left to right, to a final concentration of 128 µg/mL.



Row B was inoculated with *E. coli* ATCC 25922 to serve as the quality control. The **first five wells in Row B**, which contained FFC concentrations ranging from 0.125 µg/mL - 2 µg/mL, showed growth of the bacterium. The MIC for FFC against *E. coli* ATCC 25922 was 4 µg/mL. This MIC value is in agreement with the known FFC MIC for this specific organism indicating the plate has performed as expected.

Rows C-H were inoculated with *E. ictaluri*. The **first two wells in each row** showed growth of the bacterium. The MIC for FFC against the *E. ictaluri* isolate was 0.5 µg/mL.

DISCUSSION

When conducted according to standardized methods, the MIC assay will reliably assess the *in vitro* susceptibility of bacteria to an antibiotic.[2,3,4] The culture of the same organism by different personnel and laboratories using different media, antibiotic strengths, incubation temperatures and durations has produced highly variable results,[4] which will confound the usefulness of *in vitro* testing.

In an effort to establish reliable clinical breakpoints for aquatic bacteria, the CLSI has approved guidelines for determining MIC assays for nonfastidious bacteria such as *E. ictaluri, F. columnare, A. hydrophila, A. salmonicida, S. iniae, F. psychrophilum* and *A. salmonicida*.[2] Standards are provided for media preparation, inoculation densities, incubation temperature, quality-control organisms and interpretation of results to ensure these procedures are rigorous and reproducible between laboratories.

In addition to assessing bacterial susceptibility, the MIC value is often used to predict therapeutic plasma antibiotic concentrations needed in patients to obtain optimal efficacy.[9,10] The therapeutic plasma concentration of an antibiotic should exceed its MIC value against a particular pathogen for the interdose interval or by a concentration factor depending on whether the mechanism of antibiotic acts via a time-dependent or concentration-dependent fashion, respectively.[11]

However, there are limitations when using this calculation to predict therapeutic outcomes because marked dissimilarities occur between *in vitro* and *in vivo* conditions.[3,4] Internal and external factors influence the antimicrobial plasma concentration calculated from *in vitro* methods and the concentration of antibiotic at the site of infection in a piscine patient. These factors include: drug administration route and pharmacokinetics, species and disease state of fish, water temperature and salinity, virulence of the pathogen and its susceptibility to antimicrobial treatment, as well as the presence of multiple pathogens.[3,4,10]

Before predicting the therapeutic outcome with an antibiotic administered to diseased fish, data from both *in vitro* and *in vivo* factors should be assessed. *In vitro* antimicrobial data obtained by standardized MIC methodology should be correlated with both piscine pharmacokinetic and field efficacy data, allowing universal clinical breakpoints to be established for aquatic bacterial pathogens. This will enable diagnosticians to reliably monitor antimicrobial susceptibility between laboratories and choose the appropriate antimicrobial therapy when needed.

- **When conducted according to standardized methods, the MIC assay will reliably assess the *in vitro* susceptibility of bacteria to an antibiotic.**

- **The MIC value, coupled with pharmacokinetic and field efficacy data, can be used to predict the therapeutic plasma antibiotic concentrations needed to obtain optimal efficacy.**

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REFERENCES

[1] Plumb J and Hanson L. Epizootiology of Fish Diseases. *Health Maintenance and Principal Microbial Diseases of Cultured Fishes*. Iowa State University, Ames, Iowa, USA, 2011:31-38.

[2] Clinical and Laboratory Standards Institute (CLSI). *Methods for Antimicrobial Broth Dilution and Disk Diffusion Susceptibility Testing of Bacteria Isolated From Aquatic Animals*. 2nd ed. CLSI guideline VET03. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.

[3] Forbes BA, Sahm DF, Weissfeld AS. Laboratory Methods and Strategies for Antimicrobial Susceptibility Testing. *Bailey & Scott's Diagnostic Microbiology*. St. Louis, Missouri: Mosby Elsevier, 2007:187-215.

[4] Miller R, Reimschuessel R. Epidemiologic cutoff values for antimicrobial agents against *Aeromonas salmonicida* isolates determined by frequency distributions of minimal inhibitory concentration and diameter of zone of inhibition data. *American Journal of Veterinary Research* 2006;67:1837-1843.

[5] Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated From Aquatic Animals*. 3rd ed. CLSI supplement VET04. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.

[6] United States Food and Drug Administration, Center for Veterinary Medicine. FREEDOM OF INFORMATION SUMMARY ORIGINAL NEW ANIMAL DRUG APPLICATION NADA 141-246 AQUAFLOR Type A Medicated Article (florfenicol), An Antibiotic "for the control of mortality in catfish due to enteric septicemia of catfish associated with *Edwardsiella ictaluri*." October 24, 2005 Available at: https://animaldrugsatfda.fda.gov/adafda/app/search/public/document/downloadFoi/796 Accessed February 24, 2021.

[7] United States Food and Drug Administration. FREEDOM OF INFORMATION SUMMARY SUPPLEMENTAL NEW ANIMAL DRUG APPLICATION NADA 141-246 AQUAFLOR Florfenicol Type A medicated article Freshwater-reared salmonids "for the control of mortality in freshwater-reared salmonids due to coldwater disease associated with *Flavobacterium psychrophilum*." March 19, 2007. Available at: Florfenicol (fda.gov) Accessed February 24, 2021.

[8] United States Food and Drug Administration. FREEDOM OF INFORMATION SUMMARY SUPPLEMENTAL NEW ANIMAL DRUG APPLICATION NADA 141-246 AQUAFLOR Florfenicol Type A medicated article Freshwater-reared salmonids "for the control of mortality in freshwater-reared salmonids due to furunculosis associated with *Aeromonas salmonicida*." October 26, 2007. Available at: https://animaldrugsatfda.fda.gov/adafda/app/search/public/document/downloadFoi/798 Accessed February 24, 2021.

[9] Samuelsen OB, Bergh Ø, Arne E. Pharmacokinetics of florfenicol in cod *Gadus morhua and in vitro* antibacterial activity against *Vibrio anguillarum*. *Diseases of Aquatic Organisms* 2003;56:127-133.

[10] Stamm JM. *In vitro* resistance by fish pathogens to aquacultural antibacterials, including the quinolones difloxacin (A-56619) and sarafloxacin (A-56620). *Journal of Aquatic Animal Health* 1989;1:135-141.

[11] AliAbadi FS and Lees P. Antibiotic treatment for animals: effect on bacterial population and dosage regimen optimization. *International Journal of Antimicrobial Agents* 2000;14:307-313.

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