

## Determining antimicrobial MICs against aquaculture pathogens using Sensititre® plates

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### S U M M A R Y

- In US aquaculture, where bacterial infections are a leading cause of death, monitoring bacterial pathogen susceptibility to antibiotics is important because there are only three commercially available antimicrobials for food-fish use.
- When conducted according to standardized methods, the minimal inhibitory concentration (MIC) assay can 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.

### Introduction

Bacterial pathogens are a leading cause of mortality in aquacultured fish.<sup>1</sup> However, only three antimicrobials — florfenicol (Aquaflor®), oxytetracycline (Terramycin®) and sulfadimethoxine/ormetoprim (Romet 30®) — are commercially available for treating bacterial infections 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, intermediately susceptible or resistant to an antibiotic, and their values are derived from extensive *in vitro*, pharmacokinetic and field trial studies.<sup>2,3</sup> While clinical breakpoints are in place for many bacterial pathogens of terrestrial animals, the only aquaculture pathogen with established clinical breakpoints is *Aeromonas salmonicida*.<sup>4,5</sup> 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).<sup>4</sup> Diagnostic laboratories that routinely test aquatic bacteria have established their own laboratory-specific clinical breakpoints based on two parameters: specific *in vitro* assays and the clinical field response to antimicrobial use.

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.<sup>2</sup> The MIC technique is a

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quantitative antimicrobial susceptibility assay.<sup>2,3</sup> MIC assays can be conducted using either large volumes ( $\geq 1$  mL) of inoculum (broth-macrodilution assay) or small volumes (50-100  $\mu$ L) 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 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, was recently approved in the US for control of mortality associated with *Edwardsiella ictaluri* in catfish and *Flavobacterium psychrophilum* and *Aeromonas salmonicida* in freshwater-reared salmonids.<sup>6,7,8</sup> In

addition, Aquaflor®-CA1 (florfenicol) is conditionally approved for control of mortality in catfish due to columnaris disease associated with *Flavobacterium columnare*.<sup>9</sup>

#### **Determining FFC MICs with the broth-microdilution assay**

Prior to *in vitro* susceptibility testing, the bacteria must be purified and identified using routine morphological, biochemical and/or polymerase chain reaction assays. Sensititre microdilution MIC assays utilize sterile 96-well microdilution plates arranged in rows and columns. The last column of wells contains 0  $\mu$ g/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  $\mu$ g/mL FFC; the concentration then doubles in each subsequent column to a maximum FFC concentration of 128  $\mu$ g/mL. Following is the procedure for setting up the MIC assay according to CLSI guidelines.<sup>2</sup>

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1 Three to five (3 to 5) 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. Some fastidious organisms require supplemented media, and information on these special requirements is provided by CLSI M49-A.<sup>2</sup>

10<sup>7</sup> cfu/mL. This will yield a final test concentration of bacteria in the well of ~5 x 10<sup>4</sup> cfu/well (~5 x 10<sup>5</sup> cfu/mL).

Each test organism is inoculated in a separate row. The test inoculum suspension should be subcultured on agar plates to check for purity.

Included in each tray should be a column of wells containing no antimicrobial agent (positive control wells) to assess the viability of organisms.

6 To prevent drying during incubation, each plate is sealed with a tight-fitting plastic cover before incubation. The plates are stacked ≤ 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* and *A. salmonicida*. Extended incubation of inoculated plates is discouraged because antimicrobial deterioration could result in falsely elevated MICs. For assaying FFC's MIC against *F. psychrophilum*, which requires a lower incubation temperature, the reader is referred to CLSI M49-A.<sup>2</sup>

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2 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<sup>8</sup> cfu/mL, which should be verified periodically using plate counts.

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 higher temperatures or other special requirements, the reader is referred to CLSI M-49 for additional quality-control strain recommendations.

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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3 The standardized inocula suspensions are diluted in CAMHB so that each well on the plate contains ~5 x 10<sup>5</sup> 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<sup>8</sup> dilution suspension must be diluted to yield

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

## Discussion

When conducted according to standardized methods, the MIC assay will reliably assess the *in vitro* susceptibility of bacteria to an antibiotic.<sup>2,3,4</sup> 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,<sup>4</sup> 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 non-fastidious bacteria such as *E. ictaluri* and *A. salmonicida*.<sup>2</sup> 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. However, guidelines for certain groups of bacteria, e.g., gliding bacteria such as *F. columnare* and *F. psychrophilum*, are only provisional.

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.<sup>10,11</sup> 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 antibiotic acts via a time-dependent or concentration-dependent mechanism, respectively.<sup>12</sup>

However, there are limitations when using this calculation to predict therapeutic outcomes because marked dissimilarities occur between *in vitro* and *in vivo* conditions.<sup>3,4</sup> 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

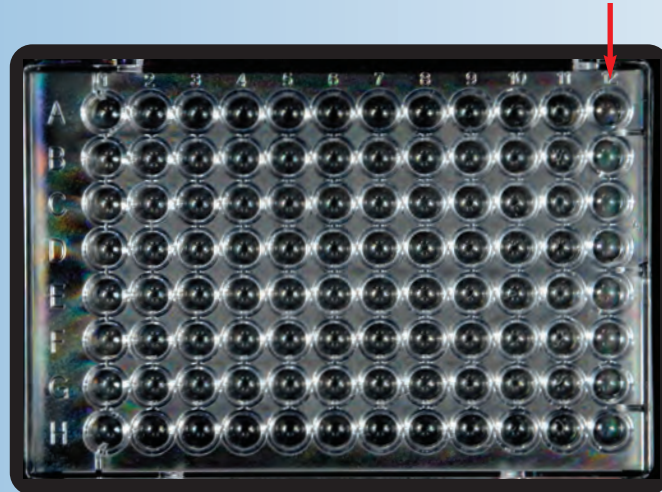
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**Figure 1**  
Microdilution plate for broth dilution susceptibility testing of bacteria

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

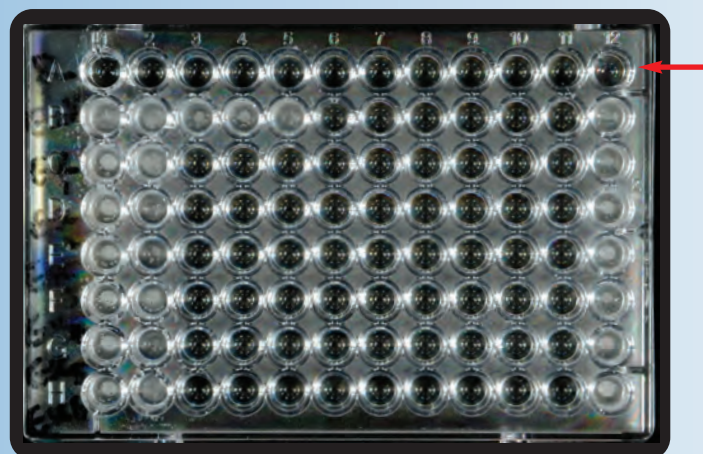


**Figure 2**  
Sensititre Plate inoculated with *Edwardsiella ictaluri* to determine the florfenicol (FFC) minimal inhibitory concentration (MIC)

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

**Rows B-H of the last column** contained 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, showed growth of the bacterium. The MIC for FFC against *E. coli* ATCC 25922 was 4 µg/mL.

**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.

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treatment, as well as the presence of multiple pathogens.<sup>3,4,10</sup>

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.

Regarding the use of Aquaflor in the US:

CAUTION: Federal law limits this drug to use under the professional supervision of a licensed veterinarian. Animal feed bearing or containing this veterinary-feed-directive drug shall be fed to animals only by or upon a lawful Veterinary Feed Directive (VFD) issued by a licensed veterinarian in the course of the veterinarian's professional practice.

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