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# Standardization of a broth microdilution susceptibility testing method to determine minimum inhibitory concentrations of aquatic bacteria

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ABSTRACT: A multiple laboratory study was conducted in accordance with the standards established by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS), for the development of quality control (QC) ranges using dilution antimicrobial susceptibility testing methods for bacterial isolates from aquatic animal species. QC ranges were established for *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 when testing at 22, 28 and 35°C (*E. coli* only) for 10 different antimicrobial agents (ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, ormetoprim/sulfadimethoxine, oxolinic acid, oxytetracycline and trimethoprim/sulfamethoxazole). Minimum inhibitory concentration (MIC) QC ranges were determined using dry- and frozen-form 96-well plates and cation-adjusted Mueller-Hinton broth. These QC ranges were accepted by the CLSI/NCCLS Subcommittee on Veterinary Antimicrobial Susceptibility Testing in January 2004. This broth microdilution testing method represents the first standardized method for determining MICs of bacterial isolates whose preferred growth temperatures are below 35°C. Methods and QC ranges defined in this study will enable aquatic animal disease researchers to reliably compare quantitative susceptibility testing data between laboratories, and will be used to ensure both precision and inter-laboratory harmonization.

KEY WORDS: Minimum inhibitory concentration · Broth microdilution · Antimicrobial susceptibility testing · Quality control · Antibiotic



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## INTRODUCTION

In the area of anti-infective therapy, researchers and fish disease specialists have made great strides in recent years towards developing standardized methods to determine minimum inhibitory concentrations (MICs) of bacteria isolated from the aquatic environment. A number of studies have provided valuable data assisting in the determination of the most appropriate growth media, incubation temperatures and times, and antimicrobial agent concentrations for testing various bacterial genera found in the aquatic environment (Barnes et al. 1990, Bandin et al. 1991, Inglis & Richards 1991, Hawke & Thune 1992, Martinsen et al. 1992, Alderman & Smith 2001, McGinnis et al. 2003, Michel et al. 2003, Rigos et al. 2003, Samuelsen et al. 2003, Coyne et al. 2004). Some of these studies employed dilution methods of antimicrobial susceptibility testing derived from accepted standards, such as those published by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) (CLSI/NCCLS 2000, 2002b). Because there are no quality control (QC) ranges established for tests conducted at temperatures below 35°C, these studies lacked required internal controls.

Most of these studies recommended using Mueller-Hinton medium for routine susceptibility testing of non-fastidious organisms. Alderman & Smith (2001) published a 'tentative' set of protocols wherein they outlined the problems commonly encountered when comparing data generated by laboratories employing different media and methods. Data generated using these varied protocols differ greatly from laboratory to laboratory, making inter-laboratory correlations of susceptibility results difficult. Thus, there is a pressing need for fish health diagnostic laboratories, veterinarians and researchers to have standardized antimicrobial susceptibility testing methods available for bacterial isolates of aquatic origin.

The CLSI recommend 3 standardized antimicrobial susceptibility methods for testing bacterial pathogens of mammalian origin (CLSI/NCCLS 2002b). These are agar disk diffusion, broth dilution and agar dilution. The E-test® (AB Biodisk) is a commercial proprietary system based on a modified agar diffusion method, which is currently not recommended by the CLSI for use as a standardized antimicrobial susceptibility testing method. To date, only the agar disk diffusion method has been standardized for the testing of aquatic isolates (Miller et al. 2003, CLSI/NCCLS 2005a). Agar disk diffusion test results, however, can be less reliable when testing slower growing organisms. With such slower growing organisms, fairly large zones of inhibition may indicate susceptibility or may simply represent the effect of delayed growth (Acar & Goldstein 1996). These factors help justify the need for susceptible, intermediate and resistant breakpoints for aquaculture drugs and pathogens at the lower temperatures. Disk diffusion tests also yield zones of inhibition which are generally not as useful to the clinician, even when the extrapolation of an MIC value is possible using a linear regression analytical system. Despite its limitations, the agar disk diffusion method is still commonly used in aquatic diagnostic laboratories. In the past decade, however, several studies have been published using dilution susceptibility testing methods on aquatic isolates (Park et al. 1995, Rangdale et al. 1997, Torkildsen et al. 2000, McGinnis et al. 2003, Michel et al. 2003, Rigos et al. 2003, Samuelsen et al. 2003, Coyne et al. 2004).

A standardized dilution susceptibility testing method provides 2 advantages over the disk diffusion test. First, results generated by a dilution testing method may be quantitative (MIC), in addition to qualitative (susceptible, intermediate and resistant). Quantitative results increase the potential for optimizing a dosing regimen based on the pharmacokinetic and pharmacodynamic parameters that drive clinical efficacy. Secondly, broth dilution methods permit the testing of bacteria whose growth characteristics are less amenable to disk diffusion testing (i.e. slower growing or fastidious organisms). Agar dilution, although considered to be the 'gold-standard' for antimicrobial susceptibility testing, can be labor intensive and time-consuming. Therefore, agar dilution tests tend to be performed less frequently than disk diffusion and broth dilution tests. Broth dilution methods offer a preferred choice for quantitatively evaluating slower growing aquatic microorganisms.

To develop a standardized and internationally harmonized dilution susceptibility testing method for aquatic isolates, some members of the CLSI Subcommittee on Veterinary Antimicrobial Susceptibility Testing, Aquaculture Working Group (VAST-AWG) coordinated a multiple laboratory study to standardize a MIC testing method for bacterial isolates that grow at 22 and 28°C. Temperatures chosen for this study were based upon their routine use in aquatic animal disease diagnostic laboratories worldwide, on recommendations of members of the CLSI Subcommittee on VAST-AWG, in an effort to coordinate methodologies with international investigators, and to accommodate temperature optimums for aquatic bacteria isolated from both cold- and warm-water species. The methods used in this study were based on the broth microdilution testing methods described in the CLSI/NCCLS standard M31-A2 (CLSI/NCCLS 2002b). Also incorporated were recommendations of experts in the field of aquatic microbiology, such as incubation temperature and duration, and testing media which were summa-

rized by Alderman & Smith (2001). This method was developed for testing aquatic bacterial isolates which prefer or require temperatures below 35°C, and do not require supplementation of the standard Mueller-Hinton growth medium. Since these aquatic isolates prefer or require these lower temperatures, previously they could not have been tested accurately employing the QC parameters established in the CLSI/NCCLS protocols for testing organisms from mammalian origin whose optimal growth temperatures are ≥35°C (CLSI/NCCLS 2002b). The CLSI Subcommittee on VAST-AWG generated a list of aquatic bacterial pathogens which prefer lower temperatures (Table 1), and to which the standardized susceptibility testing method described herein may apply.

Commercially-prepared MIC test plates containing dehydrated antimicrobial agents (dry-form plates) were used in this study and validated against a commercially-prepared frozen-form plate, which is the CLSI/NCCLS reference method (CLSI/NCCLS 2001). We chose 10 different antimicrobial agents to represent the major classes, some of which are approved for use in aquaculture in the US and other countries. Some of these antimicrobial agents have been prescribed for 'extra-label' use by veterinarians treating non-food commercial and hobby aquarium fishes. In addition, some of these antimicrobial agents have been identified in the aquatic environment (Capone et al. 1996) and are of growing concern to environmental regulatory agencies (Daughton & Ternes 1999).

The standardized methods established in this work will assist in the more precise monitoring of resistance in bacteria commonly isolated from the environment, as well as aid aquatic disease specialists in the treatment of bacterial infections in aquatic species.

## MATERIALS AND METHODS

**Standardization study.** ***Participating laboratories:*** In this study, data was generated in 10 participating laboratories. These comprised the Food and Drug Administration, Center for Veterinary Medicine (FDA-CVM), Office of Research, Laurel, Maryland; Fish Health Unit, Department of Primary Industries, Water & Environment, Prospect Launceston, Australia; Atlantic Veterinary College, University of Prince Edward Island, Prince Edward Island, Canada; Institute of Marine Research, Department of Aquaculture, Bergen, Norway; Danish Institute for Fisheries Research, Fish Disease Laboratory, Frederiksberg, Denmark; University of Wisconsin, Wisconsin Veterinary Diagnostic Laboratory, Madison, Wisconsin; Alpharma, Animal Health Division, Chicago Heights, Illinois; University of Patras, Laboratory of Public Health, Rio Patras, Greece; Florida Department of Agriculture and Consumer Services, Division of Animal Industry, Bartow, Florida; Washington State University, Washington Animal Disease Diagnostic Laboratory, Pullman, Washington.

While the study was initiated with 10 participating laboratories, QC ranges presented here are based on data from 9 testing laboratories for *Escherichia coli* ATCC 25922 and 7 laboratories for *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. Data from 1 laboratory was consistently out of line with the values observed in the other 9 laboratories for both QC strains, and thus the data from this laboratory was eliminated from the entire study. In the case of the *A. salmonicida* subsp. *salmonicida* strain, 1 laboratory was unable to receive the *A. salmonicida* subsp. *salmonicida* QC strain due to import restrictions, and the other generated data for all antimicrobial agents consistently out of line with the values obtained in the other 7 laboratories. As a result, the maximum total QC data points per organism/antimicrobial agent/temperature/incubation time condition were reduced from 300 to 270 for *E. coli*, and to 210 for *A. salmonicida* subsp. *salmonicida*. Previous CLSI/NCCLS methods standardization and QC studies have eliminated data from laboratories based on inconsistent data (Jorgensen et al. 1996, Marshall et al. 1996, McDermott et al. 2001, Miller et al. 2003). In this study the number of data points produced in the 9 and 7 laboratories for *E. coli* and *A. salmonicida* subsp. *salmonicida*, respec-

Table 1. Broth dilution susceptibility testing conditions for Group 1 organisms, as recommended by the Clinical and Laboratory Standards Institute, CLSI (formerly the National Committee for Clinical Laboratory Standards, NCCLS) Subcommittee on Veterinary Antimicrobial Susceptibility Testing–Aquaculture Working Group (VAST–AWG). CAMHB: cation-adjusted Mueller-Hinton broth

| Aquatic pathogens | Incubation | Suggested media |
|---|---|---|
| *Enterobacteriaceae*<br>*Aeromonas salmonicida* (non-psychrophilic strains)<br>*Aeromonas hydrophila* and other mesophilic Aeromonads<br>*Pseudomonas* spp., *Plesiomonas shigelloides*, *Shewanella* spp.<br>*Vibrio* spp. (non-obligate halophilic strains)<br>*Listonella anguillarum* | 22°C (24–28 h and/or 44–48 h)<br>plus 28°C (24–28 h) | CAMHB |

tively, satisfied the requirements of the CLSI/NCCLS for establishing QC ranges (CLSI/NCCLS 2002a).

***MIC test plates:*** All plates used in the multiple laboratory trial were dry-form plates manufactured by Trek Diagnostic Systems (Lot 3222) in the standard 96 well format. This custom plate consisted of 2-fold dilutions centering on 1 µg $ml^{-1}$, of the following antimicrobial agents: ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, ormetoprim/sulfadimethoxine, oxolinic acid, oxytetracycline, and trimethoprim/sulfamethoxazole (see Tables 2 to 8 for the concentration range tested for each antimicrobial agent; 2 wells in each MIC test plate were used as positive controls).

***Test strains and growth conditions:*** American Type Culture Collection (Manassas) reference strains *Escherichia coli* ATCC 25922; NCIB 12210; DSM 1103 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658; NCMB 1102 were incubated at both 22 ± 2°C for 24 to 28 h and 44 to 48 h, and at 28 ± 2°C for 24 to 28 h in cation-adjusted Mueller-Hinton broth (CAMHB).

***Quality control:*** Following guidelines for QC described in the CLSI/NCCLS standard M31-A2 (CLSI/NCCLS 2002b) *Escherichia coli* ATCC 25922 was incubated at 35°C for 16 to 20 h in CAMHB.

***Broth microdilution susceptibility testing:*** This study was designed in accordance with guidelines described in the CLSI/NCCLS guideline M37-A2 (CLSI/NCCLS 2002a) for conducting QC studies, and followed procedures outlined in the CLSI/NCCLS standard M31-A2 (CLSI/NCCLS 2002b). On 10 testing days, each laboratory tested *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 in 3 lots of CAMHB. CAMHB was prepared by Trek Diagnostic Systems using powders from 3 lots from 3 different sources: BD Diagnostic Systems 212322, Lot 1254009; Hardy Diagnostics C7521, Lot 2049; and Difco 275710, Lot 2218968. Sterility and pH measurements were taken, and cation supplementation was made based on the certificate of analysis for each lot of powder, and adjusted in accordance with the CLSI/NCCLS standard M7-A6 (CLSI/NCCLS 2000). Trek Diagnostic Systems distributed all media in liquid form to the 10 participating laboratories.

On each 'testing day', 1 suspension was prepared for each QC strain in demineralized water. The CLSI guideline M31-A2 states that sterile water, Mueller-Hinton broth, or 0.9% saline may be used to prepare inocula of some fastidious pathogens (CLSI/NCCLS 2002b). Turbidities were measured using 1 of the following; colorimeter (0.5 McFarland suspension), spectrophotometer (0.08 to 0.10 at $OD_{625}$), turbidimeter (60 to 70 nephelometer turbidity units), or the line method (CLSI/NCCLS 2005a). Suspensions targeted an inoculum density equivalent to approximately $1.0 \times 10^8$ colony-forming units (CFU $ml^{-1}$). Bacterial suspensions were diluted 1:200 in CAMHB to target an inoculum concentration of approximately $5.0 \times 10^5$ CFU $ml^{-1}$. Dry-form MIC test plates were inoculated with 100 µl per well using either a Trek Autoinoculator® apparatus (Trek Diagnostic Systems) or a multichannel pipetter.

Test plates were covered with plastic adhesive seals and incubated within 15 min of inoculation at 22 and 28°C (*Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658) and 35°C (*E. coli* ATCC 25922). Plates were stacked no more than 2 plates high to ensure proper humidity and air circulation. Test plates were read by removing the seal. This permitted the detection of slight growth detectable with the unaided eye. The seals were carefully replaced for those plates which required incubation at 22°C for an additional 20 to 24 h.

Immediately following inoculation, colony counts were performed for each isolate on each test day, from a positive control well from 1 MIC test plate. To perform the colony counts a 1:1000 dilution was made in demineralized water. A 100 µl aliquot was used to inoculate a tryptic soy agar plate supplemented with 5% sheep blood. The inoculum was uniformly spread across the surface of the agar using an L-shaped spreader. Colony count plates were incubated at 28°C for 24 to 28 h and the number of CFUs counted.

***Definition of minimum inhibitory concentration:*** MICs were defined as the lowest concentration of antimicrobial agent that prevented visible growth of the microorganism. Following recommendations detailed in the CLSI/NCCLS standard M7-A6 (CLSI/NCCLS 2000), when a single skipped well occurred, the highest MIC was read (i.e. the first well with no growth after the skipped well), and when 2 skipped wells occurred, the test was repeated.

***Definition of quality control ranges:*** In accordance with CLSI/NCCLS guideline M37-A2 (CLSI/NCCLS 2002a), the organisms were tested using 3 lots of media tested in at least 7 laboratories on 10 test days. The percentage of participant MICs that fell within the approved QC ranges for *Escherichia coli* ATCC 25922 exceeded 95% for all antimicrobial agents tested (see Tables 2 to 5). In tests on *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, trailing endpoints were observed by researchers in 2 laboratories (see Figs. 4 & 5), which caused the percentage of participant MICs within the approved QC ranges to be slightly lower than the targeted 95% for 4 of the antimicrobial agents (ampicillin, florfenicol, flumequine, and oxolinic acid) at 22°C, 44 to 48 h and 28°C, 24 to 28 h (see Tables 7 & 8).

**Validation study.** To comply with the CLSI/NCCLS guidelines M37-A2 (CLSI/NCCLS 2002a) and M23-A2 (CLSI/NCCLS 2001), when establishing QC criteria, a single laboratory (FDA-CVM) study was required to

show comparability between MIC results generated using the dry-form and frozen-form reference plates.

Test plates consisted of 2-fold dilutions of the following antimicrobial agents: ampicillin (0.03 to 16 µg $ml^{-1}$), enrofloxacin (0.002 to 1 µg $ml^{-1}$), erythromycin (0.25 to 128 µg $ml^{-1}$), florfenicol (0.03 to 16 µg $ml^{-1}$), flumequine (0.008 to 4 µg $ml^{-1}$), gentamicin (0.06 to 4 µg $ml^{-1}$), ormetoprim/sulfadimethoxine (0.008/0.15 to 4/76 µg $ml^{-1}$; first value = ormetoprim, second value = sulfadimethoxine), oxolinic acid (0.004 to 2 µg $ml^{-1}$), oxytetracycline (0.015 to 8 µg $ml^{-1}$) and trimethoprim/sulfamethoxazole (0.015/0.3 to 1/19 µg $ml^{-1}$; first value = trimethoprim, second value = sulfamethoxazole); 2 wells in each MIC test plate were used as positive controls.

The CLSI/NCCLS recommends that a minimum of 100 isolates should be tested to validate MIC test results using the reference frozen-form plates against those obtained in dry-form plates (CLSI/NCCLS 2002a). In this study, over 100 distinct isolates of *Escherichia coli* and *Aeromonas salmonicida* combined were tested at all temperatures and times for which MIC QC ranges were proposed. *E. coli* isolates were obtained from the FDA-CVM culture collection originating from non-piscine host species. *A. salmonicida* isolates were obtained from various aquatic disease research laboratories in the United States, Canada, and the United Kingdom. Both *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658 were used as QC organisms at 22 ± 2°C and 28 ± 2°C and only *E. coli* at 35 ± 2°C, using the ranges approved by members of the CLSI Subcommittee on VAST for testing in dry-form plates as a result of the standardization study described above.

Tests were conducted in CAMHB (Trek Diagnostic Systems) using the same procedure employed in the multiple laboratory trial. Bacterial suspensions equivalent to a 0.5 McFarland suspension were prepared and diluted in CAMHB to a standardized inoculum concentration of approximately $5.0 \times 10^5$ CFU $ml^{-1}$ for the dehydrated plates, and $1.0 \times 10^6$ CFU $ml^{-1}$ for the frozen plates. Dry-form plates were inoculated with 100 µl $well^{-1}$, and frozen plates with 50 µl $well^{-1}$ (making a 1:2 dilution with the thawed antimicrobial agent solution in each well) using a Trek Autoinoculator®.

## RESULTS AND DISCUSSION

### Standardization study

An obligatory component of all antimicrobial susceptibility tests is the establishment of QC ranges for a given QC strain for each antimicrobial agent it is tested against. In this standardization study, QC ranges for 10 different antimicrobial agents were established for broth microdilution susceptibility testing at 22°C (24 to 28 h and 44 to 48 h) and 28°C (24 to 28 h) for *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, and at 35°C (16 to 20 h) for *E. coli* ATCC 25922. These QC strains are well characterized and have been approved by the CLSI for use in disk diffusion tests (CLSI/NCCLS 2005a).

Tables 2 to 5 summarize the MICs and QC limits for the 9 antimicrobial agents tested for *Escherichia coli* ATCC 25922. Similarly, Table 6 to 8 summarize the MICs and QC limits for *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. The CLSI Subcommittee on VAST approved these MIC QC ranges

Table 2. *Escherichia coli* ATCC 25922. Minimum inhibitory concentration (MIC) quality control (QC) results at 22°C and 24 to 28 h with CAMHB made from 3 lots of media, common to all 9 laboratories

| Antimicrobial agent | Testing range (µg $ml^{-1}$) | MIC (µg $ml^{-1}$) | | | No. of data points | % within QC range |
|---|---|---|---|---|---|---|
| | | Inter-laboratory range | Median | CLSI-approved QC range | | |
| Ampicillin | 0.06–32 | 2–16 | 4 | 2–16 | 267 | 100 |
| Enrofloxacin | 0.002–1 | 0.004–0.03 | 0.008 | 0.004–0.015 | 266 | 98.1 |
| Florfenicol | 0.12–64 | 2–16 | 8 | 2–16 | 266 | 100 |
| Flumequine | 0.015–8 | 0.12–0.5 | 0.25 | 0.06–0.5 | 266 | 100 |
| Gentamicin | 0.12–8 | ≤0.12–2 | 0.25 | 0.12–0.5 | 266 | 95.1 |
| Ormetoprim/ sulfadimethoxine[a] | 0.008/0.15–4/76 | 0.12/2.4–2/38 | 0.5/9.5 | 0.12/2.4–1/19 | 267 | 99.6 |
| Oxolinic acid | 0.004–2 | 0.06–0.25 | 0.06 | 0.03–0.25 | 266 | 100 |
| Oxytetracycline | 0.03–16 | 0.25–2 | 0.5 | 0.25–1 | 267 | 99.3 |
| Trimethoprim/ sulfamethoxazole[b] | 0.03/0.6–2/38 | ≤0.03/0.6–0.25/4.8 | 0.06/1.2 | 0.03/0.6–0.12/2.4 | 267 | 99.6 |

[a]First value indicates concentration of ormetoprim; second value concentration of sulfadimethoxine
[b]First value indicates concentration of trimethoprim; second value concentration of sulfamethoxazole

Table 3. *Escherichia coli* ATCC 25922. MIC QC results at 22°C and 44 to 48 h with CAMHB made from 3 lots of media, common to all 9 laboratories. Footnotes [a] and [b] as in Table 2

| Antimicrobial agent | Testing range (µg ml$^{-1}$) | MIC (µg ml$^{-1}$) | | | No. of data points | % within QC range |
|---|---|---|---|---|---|---|
| | | Inter-laboratory range | Median | CLSI-approved QC range | | |
| Ampicillin | 0.06–32 | 4–>32 | 8 | 4–16 | 267 | 99.6 |
| Enrofloxacin | 0.002–1 | 0.004–0.03 | 0.008 | 0.004–0.015 | 265 | 97.4 |
| Florfenicol | 0.12–64 | 4–16 | 8 | 4–16 | 264 | 100 |
| Flumequine | 0.015–8 | 0.12–1 | 0.25 | 0.12–0.5 | 264 | 99.2 |
| Gentamicin | 0.12–8 | 0.25–4 | 0.5 | 0.25–1 | 264 | 96.2 |
| Ormetoprim/ sulfadimethoxine[a] | 0.008/0.15–4/76 | 0.12/2.4–2/38 | 0.5/9.5 | 0.25/4.8–2/38 | 266 | 95.5 |
| Oxolinic acid | 0.004–2 | 0.06–0.5 | 0.12 | 0.06–0.25 | 264 | 99.2 |
| Oxytetracycline | 0.03–16 | 0.5–4 | 1 | 0.5–2 | 267 | 99.3 |
| Trimethoprim/ sulfamethoxazole[b] | 0.03/0.6–2/38 | ≤0.03/0.6–0.25/4.8 | 0.06/1.2 | 0.03/0.6–0.25/4.8 | 265 | 100 |

Table 4. *Escherichia coli* ATCC 25922. MIC QC results at 28°C and 24 to 28 h with CAMHB made from 3 lots of media, common to all 9 laboratories. Footnotes [a] and [b] as in Table 2

| Antimicrobial agent | Testing range (µg ml$^{-1}$) | MIC (µg ml$^{-1}$) | | | No. of data points | % within QC range |
|---|---|---|---|---|---|---|
| | | Inter-laboratory range | Median | CLSI-approved QC range | | |
| Ampicillin | 0.06–32 | 2–32 | 4 | 2–16 | 265 | 99.6 |
| Enrofloxacin | 0.002–1 | 0.008–0.03 | 0.015 | 0.008–0.03 | 263 | 100 |
| Florfenicol | 0.12–64 | 4–16 | 8 | 4–16 | 264 | 100 |
| Flumequine | 0.015–8 | 0.12–1 | 0.25 | 0.12–0.5 | 264 | 99.6 |
| Gentamicin | 0.12–8 | 0.25–4 | 0.5 | 0.25–1 | 260 | 97.7 |
| Ormetoprim/ sulfadimethoxine[a] | 0.008/0.15–4/76 | 0.06/1.2–2/38 | 0.5/9.5 | 0.12/2.4–1/19 | 265 | 98.5 |
| Oxolinic acid | 0.004–2 | 0.06–0.5 | 0.12 | 0.06–0.25 | 262 | 99.6 |
| Oxytetracycline | 0.03–16 | 0.5–8 | 1 | 0.5–2 | 264 | 99.2 |
| Trimethoprim/ sulfamethoxazole[b] | 0.03/0.6–2/38 | ≤0.03/0.6–0.25/4.8 | 0.06/1.2 | 0.03/0.6–0.25/4.8 | 263 | 100 |

Table 5. *Escherichia coli* ATCC 25922: MIC QC results at 35°C and 16 to 20 h with CAMHB made from 3 lots of media, common to all 9 laboratories. Footnotes [a] and [b] as in Table 2

| Antimicrobial agent | Testing range (µg ml$^{-1}$) | MIC (µg ml$^{-1}$) | | | No. of data points | % within QC range |
|---|---|---|---|---|---|---|
| | | Inter-laboratory range | Median | CLSI-approved QC range | | |
| Ampicillin[c] | 0.06–32 | 1–>32 | 4 | 2–8 | 266 | 98.1 |
| Enrofloxacin[c] | 0.002–1 | ≤0.002–0.12 | 0.015 | 0.008–0.03 | 267 | 95.9 |
| Florfenicol[c] | 0.12–64 | 4–16 | 8 | 2–8 | 267 | 98.9 |
| Flumequine[d] | 0.015–8 | 0.25–1 | 0.5 | 0.25–1 | 267 | 100 |
| Gentamicin[c] | 0.12–8 | 0.25–4 | 0.5 | 0.25–1 | 266 | 95.5 |
| Ormetoprim/ sulfadimethoxine[a,c,d] | 0.008/0.15–4/76 | 0.06/1.2–4/76 | 0.25/4.8 | 0.06/1.2–1/19 | 267 | 99.6 |
| Oxolinic acid[d] | 0.004–2 | 0.06–0.5 | 0.12 | 0.06–0.25 | 266 | 98.1 |
| Oxytetracycline[c,d] | 0.03–16 | 0.5–16 | 1 | 0.5–4 | 266 | 99.6 |
| Trimethoprim/ sulfamethoxazole[b,c,d] | 0.03/0.6–2/38 | ≤0.03/0.6–0.12/2.4 | 0.06/1.2 | 0.03/0.6–0.12/2.4 | 266 | 100 |

[c]A CLSI/NCCLS QC range was already approved for this drug (CLSI/NCCLS 2002b)
[d]A new CLDSI/NCCLS QC range was approved for this drug

Table 6. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. MIC QC results at 22°C and 24 to 28 h with CAMHB made from 3 lots of media, common to all 7 laboratories. Footnotes [a] and [b] as in Table 2

| Antimicrobial agent | Testing range (µg ml$^{-1}$) | MIC (µg ml$^{-1}$) | | | No. of data points | % within QC range |
|---|---|---|---|---|---|---|
| | | Inter-laboratory range | Median | CLSI-approved QC range | | |
| Ampicillin | 0.06–32 | 0.12–4 | 0.25 | 0.12–1 | 206 | 97.1 |
| Enrofloxacin | 0.002–1 | 0.004–0.06 | 0.015 | 0.008–0.03 | 207 | 98.6 |
| Erythromycin | 0.03–16 | 4–16 | 8 | 4–16 | 206 | 100 |
| Florfenicol | 0.12–64 | 0.25–4 | 0.5 | 0.25–1 | 205 | 99.0 |
| Flumequine | 0.015–8 | ≤0.015–0.25 | 0.06 | 0.015–0.12 | 207 | 98.6 |
| Gentamicin | 0.12–8 | 0.25–4 | 0.5 | 0.25–1 | 207 | 98.1 |
| Ormetoprim/ sulfadimethoxine[a] | 0.008/0.15–4/76 | 0.03/0.6–0.5/9.5 | 0.12/2.4 | 0.06/1.2–0.25/4.8 | 205 | 97.1 |
| Oxolinic acid | 0.004–2 | ≤0.004–0.06 | 0.015 | 0.008–0.03 | 205 | 99.0 |
| Oxytetracycline | 0.03–16 | 0.06–1 | 0.12 | 0.06–0.25 | 207 | 99.5 |
| Trimethoprim/ sulfamethoxazole[b] | 0.03/0.6–2/38 | ≤0.03/0.6–0.12/2.4 | 0.06/1.2 | 0.03/0.6–0.12/2.4 | 207 | 100 |

Table 7. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. MIC QC results at 22°C and 44 to 48 h with CAMHB made from 3 lots of media, common to all 7 laboratories. Footnotes [a] and [b] as in Table 2

| Antimicrobial agent | Testing range (µg ml$^{-1}$) | MIC (µg ml$^{-1}$) | | | No. of data points | % within QC range |
|---|---|---|---|---|---|---|
| | | Inter-laboratory range | Median | CLSI-approved QC range | | |
| Ampicillin | 0.06–32 | 0.25–8 | 0.5 | 0.25–1 | 201 | 90.5 |
| Enrofloxacin | 0.002–1 | 0.008–0.06 | 0.015 | 0.008–0.03 | 205 | 99.0 |
| Erythromycin | 0.03–16 | 8–>16 | 16 | 4–32 | 205 | 100 |
| Florfenicol | 0.12–64 | 0.25–8 | 1 | 0.5–2 | 203 | 93.6 |
| Flumequine | 0.015–8 | ≤0.015–0.5 | 0.06 | 0.03–0.12 | 202 | 94.1 |
| Gentamicin | 0.12–8 | 0.5–4 | 0.5 | 0.25–2 | 203 | 98.0 |
| Ormetoprim/ sulfadimethoxine[a] | 0.008/0.15–4/76 | 0.06/1.2–2/38 | 0.25/4.8 | 0.06/1.2–0.5/9.5 | 202 | 98.0 |
| Oxolinic acid | 0.004–2 | 0.008–0.12 | 0.015 | 0.008–0.03 | 197 | 90.4 |
| Oxytetracycline | 0.03–16 | 0.12–1 | 0.25 | 0.12–1 | 205 | 100 |
| Trimethoprim/ sulfamethoxazole[b] | 0.03/0.6–2/38 | ≤0.03/0.6–0.25/4.8 | 0.12/2.4 | 0.03/0.6–0.25/4.8 | 202 | 100 |

Table 8. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. MIC QC results at 28°C and 24 to 28 h with CAMHB made from 3 lots of media, common to all 7 laboratories. Footnotes [a] and [b] as in Table 2

| Antimicrobial agent | Testing range (µg ml$^{-1}$) | MIC (µg ml$^{-1}$) | | | No. of data points | % within QC range |
|---|---|---|---|---|---|---|
| | | Inter-laboratory range | Median | CLSI-approved QC range | | |
| Ampicillin | 0.06–32 | 0.12–8 | 0.5 | 0.12–1 | 205 | 91.7 |
| Enrofloxacin | 0.002–1 | 0.004–0.25 | 0.008 | 0.004–0.03 | 207 | 99.5 |
| Erythromycin | 0.03–16 | 4–>16 | 8 | 4–32 | 207 | 100 |
| Florfenicol | 0.12–64 | 0.25–8 | 1 | 0.5–2 | 205 | 91.7 |
| Flumequine | 0.015–8 | 0.03–>8 | 0.06 | 0.015–0.12 | 206 | 92.7 |
| Gentamicin | 0.12–8 | 0.25–>8 | 0.5 | 0.25–1 | 204 | 98.0 |
| Ormetoprim/ sulfadimethoxine[a] | 0.008/0.15–4/76 | 0.06/1.2–1/19 | 0.25/4.8 | 0.06/1.2–0.5/9.5 | 206 | 97.6 |
| Oxolinic acid | 0.004–2 | 0.008–0.12 | 0.015 | 0.008–0.03 | 206 | 92.7 |
| Oxytetracycline | 0.03–16 | 0.12–8 | 0.25 | 0.12–1 | 207 | 99.5 |
| Trimethoprim/ sulfamethoxazole[b] | 0.03/0.6–2/38 | ≤0.03/0.6–0.5/9.5 | 0.12/2.4 | 0.03/0.6–0.25/4.8 | 204 | 99.5 |

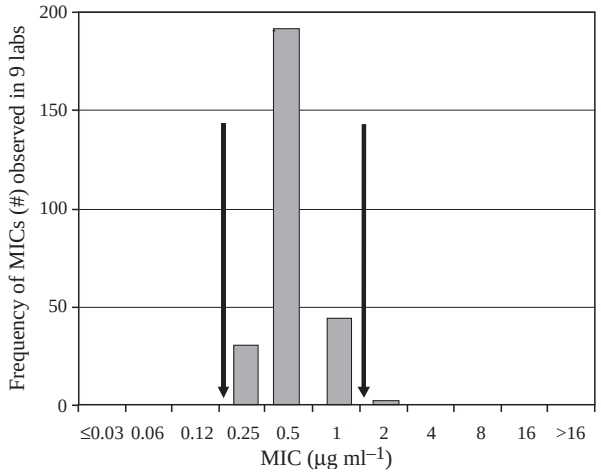


Fig. 1. *Escherichia coli* ATCC 25922. Oxytetracycline at 22°C, 24 to 28 h. MIC distribution from 9 laboratories with a distinct median MIC frequency, which was used to calculate the approved 3 dilution quality control (QC) range of 0.25 to 1 µg ml$^{-1}$ (arrowed)

using a modification of the median method described by Gavan et al. (1981) for disk diffusion testing. In many cases there was a single defined median MIC, in which case the QC range was defined as ±1 dilution from the median MIC (Fig. 1). There were some cases where an underlying distribution of MICs appeared to be asymmetric (Fig. 2). In these cases, the QC range was expanded 1 dilution above or below any shoulder ≥66.7% of the peak MIC frequency. There was 1 instance of an asymmetric distribution (*E. coli* ATCC 25922, ormetoprim/sulfadimethoxine at 35°C), where a dilution range of 5 was approved (Fig. 3).

Minimal variability in MIC results was observed with the 3 lots of CAMHB within and between the laboratories for both QC strains (data not shown). However, trailing endpoints (Figs. 4 & 5) in tests on both organisms were observed by 8 of 10 laboratories in 1 of the lots of media for all antimicrobial agents tested.

Colony-count data generated by laboratories were between $5.0 \times 10^4$ and $1.1 \times 10^6$ CFU ml$^{-1}$. While the cell concentrations in some cases were slightly lower or higher than the desired $5.0 \times 10^5$ CFU ml$^{-1}$ concentration, this did not affect the results, as the MICs were within the approved QC range for *Escherichia coli* ATCC 25922 at 35°C.

Approved CLSI/NCCLS guidelines for testing bacterial isolates at 35°C suggest that equivalent MIC results should be achieved by tests using tetracycline in place of oxytetracycline, and trimethoprim-sulfamethoxazole in place of ormetoprim/sulfadimethoxine (CLSI/NCCLS 2002b). After conducting QC tests using *Escherichia coli* ATCC 25922 at 35°C in each of the 9 laboratories, modifications to these approved QC ranges were necessary. These new modified ranges were approved by members of the CLSI Subcommittee on VAST in January 2004, and will be included along with the QC ranges at 22 and 28°C in a forthcoming CLSI guidance document (CLSI/NCCLS

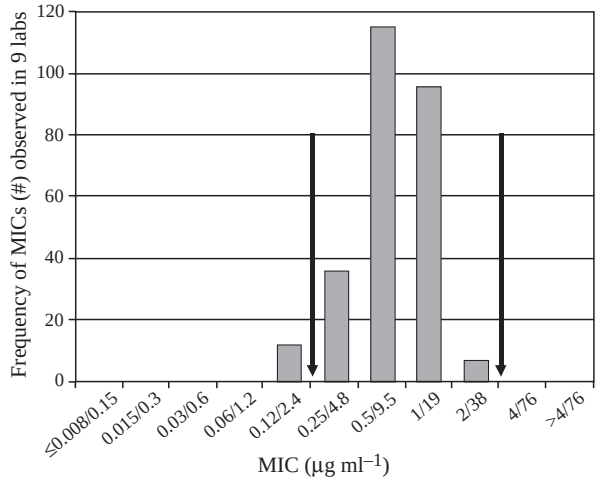


Fig. 2. *Escherichia coli* ATCC 25922. Ormetoprim/sulfadimethoxine at 22°C, 44 to 48 h. Underlying asymmetric distribution from 9 laboratories, with the larger shoulder (1/19 µg ml$^{-1}$) representing 83.5% of peak MIC (0.5/9.5 µg ml$^{-1}$) frequency. This was used to calculate approved 4 dilution QC range of 0.25/4.8 to 2/38 µg ml$^{-1}$ (arrowed). First value: ormetoprim; second value: sulfadimethoxine

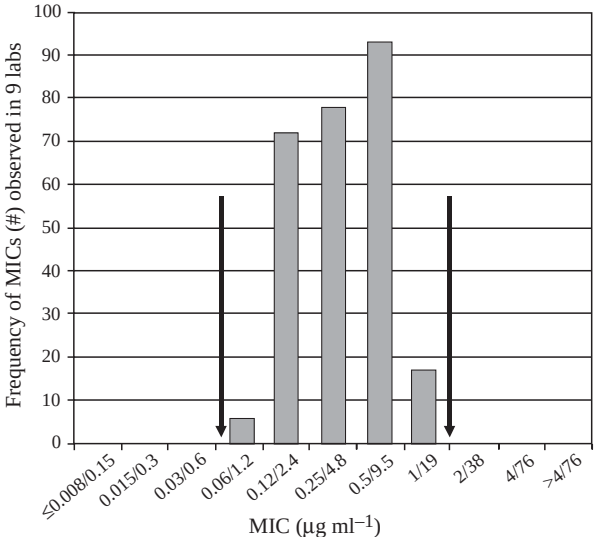


Fig. 3. *Escherichia coli* ATCC 25922. Ormetoprim/sulfadimethoxine at 35°C, 16 to 20 h. Underlying asymmetric distribution from 9 laboratories with 2 large shoulders (0.12/2.4 µg ml$^{-1}$ and 0.25/4.8 µg ml$^{-1}$) representing 77.4 and 83.9% of peak MIC (0.5/9.5 µg ml$^{-1}$) frequency, respectively. This was used to calculate approved 5 dilution QC range of 0.06/1.2 to 1/19 µg ml$^{-1}$ (arrowed)

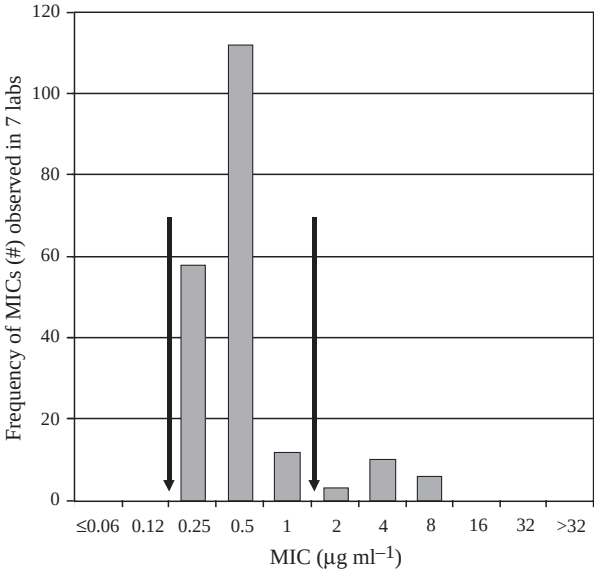


Fig. 4. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658. Ampicillin at 22°C, 44 to 48 h. MIC distribution for 7 laboratories resulting in approved 3 dilution QC range (0.25 to 1 µg ml$^{-1}$) (arrowed) with a distinct median MIC frequency. Approved QC range comprised of <95% of total data points caused by trailing endpoints observed in 2 laboratories

2005a). The previously approved CLSI QC range for *E. coli* ATCC 25922 and tetracycline at 35°C was 0.5 to 2 µg ml$^{-1}$ (CLSI/NCCLS 2002b). However, this range did not correlate with the data observed in this study (QC range of 0.5 to 4 µg ml$^{-1}$). The CLSI/NCCLS-approved QC range for both trimethoprim/sulfamethoxazole and ormetoprim/sulfadimethoxine against *E. coli* ATCC 25922 was ≤0.5/9.5 µg ml$^{-1}$ (CLSI/NCCLS 2002b). Since this value was not a true range, it was important to attempt to establish a QC range with an upper and lower limit. QC ranges were established for trimethoprim/ sulfamethoxazole and ormetoprim/ sulfadimethoxine using *E. coli* ATCC 25922 at 35°C.

CLSI QC ranges for dilution susceptibility testing are typically 3 or 4 dilutions wide (CLSI/NCCLS 2002b), and this was the finding for the majority of the ranges determined in this work. The affect of temperature on the MICs was clearly demonstrated for both organisms. With an increase in temperature from 22 to 28°C after 24 to 28 h incubation, there was a clear increase in the approved ranges of approximately 1 dilution for both QC strains, indicating an amplified growth rate and/or antimicrobial agent metabolism. Additionally, incubation time affected the MICs for both organisms, where a 1 dilution increase in the approved QC range at 22°C was observed with the majority of the antimicrobial agents. The increase in MICs with increased incubation time suggests there may be an amplified antimicrobial agent metabolism and/or degradation during the second 24 h of incubation at 22°C.

### Validation study

MICs were obtained for each isolate from the dry-form and frozen-form plates. MICs were evaluated by comparing the number of $\log_2$ dilution steps from the dry-form plates to the MIC results on the frozen-form plates. Although there were some trends of increasing or decreasing MICs, depending upon the organism, antimicrobial agent and temperature condition, most of the MIC results (>95%) for each condition of organism, antimicrobial agent, temperature and incubation time were within ±1 $\log_2$ dilution step of each other (Table 9). When the percentage agreement within ±1 $\log_2$ dilution fell below 95%, these results were primarily due to trailing endpoints observed in the dry-form plates. Despite using an automated inoculation system, there was a relatively high frequency of occurrence of skipped wells, which contributed in some instances to a >1 $\log_2$ dilution step difference between

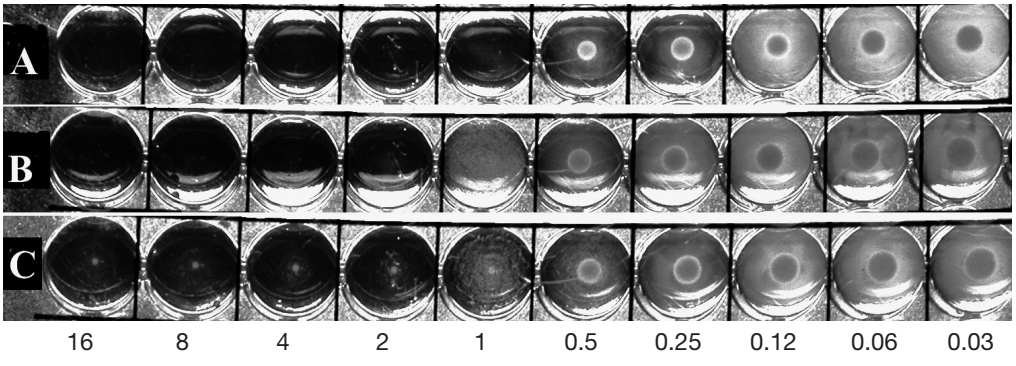


Fig. 5. *Escherichia coli* ATCC 25922. Oxytetracycline 28°C 24 to 28 h. Using 2 lots of CAMHB from 2 different manufacturers, Rows A and B show normal growth in wells yielding MICs of 1 and 2 µg ml$^{-1}$, respectively. Using a third lot of CAMHB, with MIC of 2 µg ml$^{-1}$, Row C shows small pellets of growth or 'trailing endpoints'

Table 9. *Escherichia coli* and *Aeromonas salmonicida*. Percentage agreement within 1 $\log_2$ dilution between MIC results on dried- and frozen-form panels. AMP: ampicillin; ENRO: enrofloxacin; ERY: erythromycin; FFN: florfenicol; FLUQ: flumequine; GEN: gentamicin; PRI: ormetoprim/sulfadimethoxine; OXO: oxolinic acid; OXY: oxytetracycline; SXT: trimethoprim/sulfamethoxazole

| | N | *T*/time | AMP | ENRO | ERY | FFN | FLUQ | GEN | PRI | OXO | OXY | SXT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *E. coli* isolates | 74 | 22°C/24 h | 98.6 | 75.7 | 100.0 | 100.0 | 95.9 | 95.9 | 98.7 | 93.2 | 98.6 | 98.2 |
| | 74 | 22°C/48 h | 95.9 | 84.3 | 93.2 | 100.0 | 98.6 | 87.8 | 100.0 | 95.9 | 98.6 | 100.0 |
| | 69 | 28°C/24 h | 100.0 | 88.4 | 100.0 | 100.0 | 100.0 | 92.6 | 98.6 | 100.0 | 97.0 | 100.0 |
| | 112 | 35°C/16 h | 98.2 | 91.9 | 99.0 | 100.0 | 99.0 | 87.9 | 98.2 | 96.2 | 78.6 | 99.0 |
| | **Avg.** | | **98.2** | **85.1** | **98.1** | **100.0** | **98.4** | **91.1** | **98.9** | **96.3** | **93.2** | **99.3** |
| *A. salmonicida* isolates | 40 | 22°C/24 h | 100.0 | 97.4 | 97.5 | 100.0 | 97.5 | 100.0 | 97.5 | 100.0 | 94.3 | 100.0 |
| | 40 | 22°C/48 h | 97.5 | 92.3 | 97.5 | 97.6 | 95.0 | 87.5 | 100.0 | 97.5 | 97.1 | 100.0 |
| | 40 | 28°C/24 h | 90.2 | 97.3 | 100.0 | 100.0 | 94.9 | 97.5 | 97.5 | 97.4 | 97.1 | 100.0 |
| | **Avg.** | | **95.9** | **95.7** | **98.3** | **99.2** | **95.8** | **95.0** | **98.3** | **98.3** | **96.2** | **100.0** |

the plate types. Isolates were only retested if 2 skipped wells were observed in 2 or more antimicrobial agent-dilution series (CLSI/NCCLS 2000).

Colony-count data for the *Escherichia coli* and *Aeromonas salmonicida* isolates consistently yielded inocula concentrations in the $2.0 \times 10^5$ to $8.0 \times 10^5$ CFU ml$^{-1}$ range. Counts slightly out of this range, did not appear to impact the validity of the test and were included.

## CONCLUSIONS

Based on these standardization and validation studies, both *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 are acceptable QC strains for broth microdilution tests in dry- and frozen-form (or in-house prepared) MIC plates.

The methods and QC ranges (Table 10) described in this study were presented to members of the CLSI Subcommittee on VAST in January 2004, and accepted for inclusion in a forthcoming CLSI/NCCLS proposed guideline M49-P (CLSI/NCCLS 2005b).

This study represents the first published multiple-laboratory study conducted in accordance with approved guidelines to establish MIC QC ranges at lower temperatures. These standardized methods and approved QC ranges should provide a foundation for the establishment of more ranges for other economically important antimicrobial agents, and will serve as a model for the development of additional standardized testing methods for bacterial pathogens of aquatic animals. The utility of these methods and associated QC ranges should also extend to the development of susceptible, intermediate, and resistant breakpoints for antimicrobial agents used in aquaculture against economically important aquaculture pathogens.

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Table 10. Summary of CLSI/NCCLS-approved MIC QC ranges (µg ml$^{-1}$) for broth dilution susceptibility testing in CAMHB. Values in parentheses: QC ranges established previously (CLSI/NCCLS 2002b)

| Antimicrobial agent | *Escherichia coli* ATCC 25922 | | | | *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 | | |
|---|---|---|---|---|---|---|---|
| | 22°C, 24–28 h | 22°C, 44–48 h | 28°C, 24–28 h | 35°C, 16–20 h | 22°C, 24–28 h | 22°C, 44–48 h | 28°C, 24–28 h |
| Enrofloxacin | 0.004–0.015 | 0.004–0.015 | 0.008–0.03 | (0.008–0.03) | 0.008–0.03 | 0.008–0.03 | 0.004–0.03 |
| Ampicillin | 2–16 | 4–16 | 2–16 | (2–8) | 0.12–1 | 0.25–1 | 0.12–1 |
| Oxytetracycline | 0.25–1 | 0.5–2 | 0.5–2 | 0.5–4 | 0.06–0.25 | 0.12–1 | 0.12–1 |
| Erythromycin | – | – | – | – | 4–16 | 4–32 | 4–32 |
| Florfenicol | 2–16 | 4–16 | 4–16 | (2–8) | 0.25–1 | 0.5–2 | 0.5–2 |
| Flumequine | 0.06–0.5 | 0.12–0.5 | 0.12–0.5 | 0.25–1 | 0.015–0.12 | 0.03–0.12 | 0.015–0.12 |
| Ormetoprim/ sulfadimethoxine | 0.12/2.4–1/19 | 0.25/4.8–2/38 | 0.12/2.4–1/19 | 0.06/1.2–1/19 | 0.06/1.2–0.25/4.8 | 0.06/1.2–0.5/9.5 | 0.06/1.2–0.5/9.5 |
| Oxolinic acid | 0.03–0.25 | 0.06–0.25 | 0.06–0.25 | 0.06–0.25 | 0.008–0.03 | 0.008–0.03 | 0.008–0.03 |
| Gentamicin | 0.12–0.5 | 0.25–1 | 0.25–1 | (0.25–1) | 0.25–1 | 0.25–2 | 0.25–1 |
| Trimethoprim/ sulfamethoxazole | 0.03/0.6–0.12/2.4 | 0.03/0.6–0.25/4.8 | 0.03/0.6–0.25/4.8 | 0.03/0.6–0.12/2.4 | 0.03/0.6–0.12/2.4 | 0.03/0.6–0.25/4.8 | 0.03/0.6–0.25/4.8 |

lates for the validation study, including A. Baya, J. Hawke, D. Bruno, R. Cipriano, S. Landin, H. M. Hsu, J. Evered, and R. Stanek. This study was made possible by financial support from the Oak Ridge Associated Universities through a grant sponsored by the Food and Drug Administration.

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