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Quality control ranges for testing broth microdilution susceptibility of *Flavobacterium columnare* and *F. psychrophilum* to nine antimicrobials

Charles M. Gieseker[1,*], Tamara D. Mayer[1], Tina C. Crosby[1], Jeremy Carson[2], Inger Dalsgaard[3], Ahmed M. Darwish[4], Patricia S. Gaunt[5], Dana X. Gao[5], Hui-Min Hsu[6], Tsang L. Lin[9], J. Lindsay Oaks[7], Melissa Pyecroft[2], Charlene Teitzel[7], Temdoung Somsiri[8], Ching C. Wu[9]

[1]Food and Drug Administration, Center for Veterinary Medicine, Office of Research, Laurel, Maryland 20708, USA
[2]Fish Health Unit, Department of Primary Industries, Parks, Water & Environment, Launceston, Tasmania 7249, Australia
[3]National Veterinary Institute, Technical University of Denmark, 1870 Frederiksberg C, Denmark
[4]United States Department of Agriculture, Agriculture Research Service, Harry K. Dupree Stuttgart National Aquaculture Research Center, Stuttgart, Arkansas 72160, USA
[5]Thad Cochran National Warmwater Aquaculture Center, College of Veterinary Medicine, Mississippi State University, Stoneville, Mississippi 38766, USA
[6]Wisconsin Veterinary Diagnostic Laboratory, University of Wisconsin, Madison, Wisconsin 53706, USA
[7]Washington Animal Disease Diagnostic Laboratory, University of Washington, Pullman, Washington 99164, USA
[8]Inland Aquatic Animal Health Research Institute, Kasetsart University, Jatuchak, Bangkok 10900, Thailand
[9]Animal Disease Diagnostic Laboratory, Purdue University, West Lafayette, Indiana 47907, USA

ABSTRACT: A multi-laboratory broth microdilution method trial was performed to standardize the specialized test conditions required for the fish pathogens *Flavobacterium columnare* and *F. psychrophilum.* Nine laboratories tested the quality control (QC) strains *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 against 10 antimicrobials (ampicillin, enrofloxacin, erythromycin, florfenicol, flumequine, gentamicin, ormetoprim/sulfadimethoxine, oxolinic acid, oxytetracycline, and trimethoprim/sulfamethoxazole) in diluted (4 g l^{-1}) cation-adjusted Mueller-Hinton broth incubated at 28 and 18°C for 44–48 and 92–96 h, respectively. QC ranges were set for 9 of the 10 antimicrobials. Most of the minimal inhibitory concentration (MIC) distributions (16 of 18, 9 drugs at both temperatures) for *A. salmonicida* ATCC 33658 were centered on a single median MIC ± 1 two-fold drug dilution resulting in a QC range that spanned 3 dilutions. More of the *E. coli* ATCC 25922 MIC distributions (7 of 16) were centered between 2 MIC dilutions requiring a QC range that spanned 4 dilutions. A QC range could not be determined for *E. coli* ATCC 25922 against 2 antimicrobials at the low temperature. These data and their associated QC ranges have been approved by the Clinical and Laboratory Standards Institute (CLSI), and will be included in the next edition of the CLSI M49-A Guideline. This method represents the first standardized reference method for testing fish pathogenic *Flavobacterium* spp.

KEY WORDS: Standard methods · Antimicrobial susceptibility testing · Fish · *Flavobacteria*

INTRODUCTION

The nutritionally fastidious aquatic gliding bacteria *Flavobacterium columnare* and *F. psychrophilum* are major fish pathogens that cause significant disease losses in freshwater aquaculture worldwide (Wakabayashi & Egusa 1966, Bernardet 1989, Holt et al. 1993, Wagner et al. 2002, Nematollahi et al. 2003, Pulkkinen et al. 2010). They represent an important group that need standard antimicrobial susceptibility testing (AST) methods. The Clinical and Laboratory Standards Institute (CLSI) provides 2 consensus-

*Email: charles.gieseker@fda.hhs.gov

approved guidelines with reference methods and quality control (QC) criteria for disk diffusion and broth dilution susceptibility testing of non-fastidious aquatic bacteria (CLSI 2006 a,b) based on the work of Miller et al. (2003, 2005). CLSI groups fastidious aquatic bacterial pathogens that have similar growth condition requirements, and offers potential media and incubation modifications; however, no standard reference methods or QC criteria have been developed to date.

In general, 3 AST methods can be developed as standard CLSI reference methods: disk diffusion, broth dilution, and agar dilution. Dilution techniques (agar and broth) are preferable to disk diffusion since these tests yield a minimal inhibitory concentration (MIC) that can be directly compared to the pharmacokinetics of the drug in the animal. However, disk diffusion is popular among fish disease diagnosticians because the method is inexpensive, easy to perform, and better suits laboratories that test only a small number of isolates infrequently. Disk diffusion is not as practical for testing the fish-pathogenic *Flavobacteria*, in particular *Flavobacterium columnare*, since the gliding motility of the bacterium distorts the margin of the inhibitory zone (Farmer 2004). Alternatively, agar dilution, the 'gold standard' method, has been used to test *F. psychrophilum* (Bruun et al. 2000, Schmidt et al. 2000, Michel et al. 2003), but the method is fairly labor intensive and does not lend itself well to ad hoc testing. Therefore, we determined that broth dilution, specifically broth microdilution, was the best option to develop as a standard reference AST method.

The CLSI broth dilution testing guideline for aquatic bacteria (M49-A) suggests using diluted Mueller-Hinton broth (MHB; 3 g l^{-1}) for testing *Flavobacterium columnare*, *F. psychrophilum*, and *F. branchiophilum*. The guide also suggests that *F. psychrophilum* and *F. branchiophilum* may need additional supplements of 5% horse or fetal calf serum and/or NaCl (CLSI 2006b). These recommendations were partly based on the disk diffusion methods developed by Hawke & Thune (1992) for testing *F. columnare*. However, more recent research has determined that only diluted MHB (4 g l^{-1}) is needed for broth microdilution testing of *F. columnare*, and that Ca^{++} and Mg^{++}, typically added to MHB for broth dilution susceptibility testing, may not be needed (Farmer 2004, Darwish et al. 2008). Preliminary work at the Food and Drug Administration, Center for Veterinary Medicine, Office of Research (Laurel, MD, USA) found diluted MHB (4 g l^{-1}) yielded consistent MICs for *F. columnare* and *F. psychrophilum* isolates, as well as for both QC strains referenced in CLSI M42-A and M49-A guidelines. We had too few isolates to test *F. branchiophilum* adequately. We also found that Ca^{++} (4 mg l^{-1}) and Mg^{++} (2 mg l^{-1}) were needed for testing *F. psychrophilum,* and serum supplementation was not necessary for testing of *F. columnare* or *F. psychrophilum*. NaCl supplementation was not considered, since *F. columnare* and *F. psychrophilum* generally do not grow in media containing more than 0.5% NaCl (Bernardet & Grimont 1989). Ultimately, we found diluted MHB (4 g l^{-1}) with Ca^{++} (4 mg l^{-1}) and Mg^{++} (2 mg l^{-1}) was a good medium for broth dilution testing of both *F. columnare* and *F. psychrophilum* isolates.

Optimal incubation conditions for *Flavobacterium columnare* and *F. psychrophilum* also needed to be established. We found that incubating *F. columnare* at 28°C for 44 to 48 h as recommended by the CLSI (2006b) and Darwish et al. (2008) worked well. In contrast, a slightly higher incubation temperature (18°C) and longer incubation time (96 h) than recommended by the CLSI was needed for *F. psychrophilum* (C. M. Gieseker et al. unpubl.). Although 18°C is just above the recommended growth temperature of 15°C for *F. psychrophilum* (CLSI 2006b), the bacterium grows similarly at both temperatures (Holt et al. 1989). A longer incubation time is needed as the shorter incubation times of 44 to 72 h, as recommended by the CLSI, yielded misleadingly low MICs in preliminary testing (C. M. Gieseker et al. unpubl.).

To establish a standard AST method for fish-pathogenic *Flavobacteria*, drug susceptibility testing ranges were needed for the QC strains under the altered test conditions optimized for these bacteria. Herein, we report the results of a multi-laboratory standardization trial that established MIC QC ranges of 9 antimicrobials for the QC strains *Escherichia coli* ATCC 25922, NCIMB 12210 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, NCIMB 1102. The CLSI Subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST) — Aquaculture Working Group analyzed the data and proposed QC ranges to the VAST Subcommittee. The ranges presented here were unanimously accepted for inclusion in the next edition of the CLSI M49-A Guideline. These QC ranges and testing methods provide the first standard AST reference methods for the important fish pathogens *Flavobacterium columnare* and *F. psychrophilum*.

MATERIALS AND METHODS

The design of this study was based on CLSI guideline M37-A3 (CLSI 2008). The QC strains *Escherichia coli* (ATCC 25922; NCIMB 12210) and *Aeromonas salmonicida* (ATCC 33658; NCIMB 1102) were tested by 9 laboratories using broth microdilution methods developed for testing *Flavobacterium psychrophilum* and *F. columnare* (Darwish et al. 2008, C. M. Gieseker et al. unpubl.). Both QC strains were tested at 18°C (92–96 h) and 28°C (44–48 h) using 3 different lots of diluted (4 g l^{-1}) cation-adjusted MHB (CAMHB). All laboratories repeated each test 10 times. Each replicate had 3 panels (media lots 1, 2, and 3; 3 panels × 10 replicates = 30 replicates lab^{-1} for each drug/strain/temperature combination).

Participating laboratories

Nine laboratories participated in the standardization trial. The laboratories were the Food and Drug Administration, Center for Veterinary Medicine, Office of Research (Laurel, MD, USA; FDA/CVM/ OR); Animal Health Laboratory, Department of Primary Industries, Parks, Water & Environment (Launceston, Tasmania, Australia); National Veterinary Institute, Technical University of Denmark (Frederiksberg, Denmark); Harry K. Dupree Stuttgart National Aquaculture Research Center (Stuttgart, AR, USA); Mississippi State University, College of Veterinary Medicine (Stoneville, MS, USA); University of Wisconsin Veterinary Diagnostic Laboratory (Madison, WI, USA); Washington Animal Disease Laboratory (Pullman, WA, USA); Inland Aquatic Animal Health Research Institute, Department of Fisheries, Kasetsart University Campus (Jaktujak, Bangkok, Thailand); and the Animal Disease Diagnostic Laboratory, Purdue University (West Lafayette, IN, USA). All 9 laboratories completed testing for the trial. The data presented are based on 8 laboratories for *Escherichia coli* ATCC 25922 and 7 labs for *Aeromonas salmonicida* ATCC 33658. One laboratory admitted making mistakes on their MIC interpretations; therefore, the data from that laboratory were excluded from the analysis. In addition, another laboratory could only test *E. coli* due to import restrictions against *A. salmonicida*.

Broth microdilution panels and test media

Custom, commercially prepared dry-form 96-well broth microdilution panels were purchased from Trek Diagnostic Systems. Each well had dried residue of a separate 2-fold dilution of 1 of 10 antimicrobials. Ten dilutions were tested for each of the following antimicrobials: ampicillin (0.03–16 µg ml^{-1}), enrofloxacin (0.001–0.5 µg ml^{-1}), erythromycin (0.25–128 µg ml^{-1}), florfenicol (0.12–64 µg ml^{-1}), flumequine (0.004–2 µg ml^{-1}), oxolinic acid (0.002–1 µg ml^{-1}), oxytetracycline (0.015–8 µg ml^{-1}), and ormetoprim/sulfadimethoxine (0.008/0.15–4/76 µg ml^{-1}). Seven dilutions were tested of gentamicin (0.03–2 µg ml^{-1}) and trimethoprim/sulfamethoxazole (0.008/0.15–0.5/9.5 µg ml^{-1}).

Three separate lots of broth were made from dehydrated powders manufactured by BBL™ (212322, lot 7331751) and Difco™ (275710, lot 8127115) (Becton-Dickinson), and Oxoid (CM0405, lot 597351). Aliquots (11 ml) of each broth were prepared by Trek Diagnostics Systems. Calcium (4 mg l^{-1}) and magnesium (2 mg l^{-1}) cations were added as per CLSI guidelines if the prepared media were not already supplemented.

Test bacteria and test conditions

American Type Culture Collection (ATCC, Manassas, Virginia, USA) reference strains of *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658 were incubated at 18°C for 92 to 96 h and at 28°C for 44 to 48 h in diluted (4 g l^{-1}) CAMHB.

Broth microdilution susceptibility testing

Tests were conducted in each laboratory over a minimum of 3 d with a maximum of 4 replicates d^{-1}. Each laboratory (besides the one mentioned above) tested 10 replicates of each strain at both temperatures. Each replicate consisted of three 96-well panels, 1 panel for each lot of broth.

Prior to each test day, strains were subcultured twice on tryptic soy agar supplemented with 5% sheep blood. On each test day, a single suspension was prepared of each strain to a predetermined turbidity measured by colorimeter (0.5 McFarland), spectrophotometer (0.08 to 0.13 at OD_{625}), or turbidimeter (20–30 NTUs).

Fifty-five µl of the cell suspension was added to each 11 ml aliquot of broth (1:200 dilution). Each 11 ml aliquot of broth was used to inoculate into a 96-well broth microdilution panel. A Sensititre® autoinoculator (Trek Diagnostic Systems, 3 labs) or a multichannel pipette (6 labs) was used to add 100 µl

of the inoculated broth to each well. To prevent contamination and dehydration, plastic adhesive seals were immediately attached to the panels covering the top of the wells. Within 15 min of inoculation, the panels were placed in their respective incubators. No more than 4 panels were stacked together to achieve a uniform temperature across the panels.

Actual cell densities were determined using a colony count procedure whereby 2 panels on each test day were arbitrarily chosen for each QC strain (one each at 18 and 28°C). Ten µl was removed from the positive control well and placed in 10 ml sterile saline (1:1000 dilution). A 100 µl aliquot was evenly spread on a tryptic soy agar plate supplemented with 5% sheep blood. Colony count plates were incubated under the same conditions (temperature and time) as the 96-well panels, and colony-forming units (CFUs) were counted at the same time MICs were determined. Means and standard deviations (SD) of CFU ml^{-1} were calculated for each drug/strain/temperature combination across all replicates.

After the appropriate incubation period, the panel seal was removed to read panels. Panels were examined for growth in the wells, either as a pellet or turbidity. The MIC was the first well in the dilution series without growth. When interpreting results in wells with the bacteriostatic sulfonamides, growth <80% of the pellet density in the positive control well was recorded as growth limited, indicative of a bacteriostatic effect evident as a trailing endpoint. Where this occurred, the MIC was established by identifying the well with 50% growth when compared with the positive control well. The next highest dilution well adjacent to the 50% growth well was assumed to have approximately 80% growth; therefore, the concentration in the well with 50% growth inhibition was considered the MIC.

Data analysis

The data were pooled into frequency distributions by broth lot, and also by laboratory. Each drug/strain/temperature combination was analyzed separately for MIC mean, mode, median, minimum, maximum, and range.

All MIC data were entered into RangeFinder; a specialized Excel spreadsheet program that analyzes antimicrobial susceptibility testing data from CLSI standardization trials (Turnidge & Bordash 2007). RangeFinder calculated a QC range, number of 2-fold dilutions in the range, and the percent of observations captured by the calculated range.

Frequency distributions and histograms for each drug/strain/temperature combination were used to also determine QC ranges using the median method developed by Gavan et al. (1981). Ideally, the QC ranges encompassed 95% of the observations and spanned 3 drug concentrations centered on the mode of the distribution. If data were skewed in one direction, where the MIC frequency at the 2-fold dilution immediately adjacent to the mode was ≥60% of MIC frequency at the mode, an additional 2-fold dilution was added to the final proposed QC range. The CLSI's VAST — Aquaculture Working Group compared the QC ranges estimated by the Gavan and RangeFinder methods to determine proposed QC ranges for each drug/strain/temperature combination. Both methods use arbitrary limits that require expert opinion.

Confirmation of the drug concentration on the custom dry-form broth microdilution panels

One laboratory (FDA/CVM/OR) followed the same procedures used in the standardization trial to test 5 subcultures of *Escherichia coli* ATCC 25922 and 5 subcultures of *Aeromonas salmonicida* ATCC 33658 in undiluted CAMHB to confirm the potency of the drugs on the panels. The testing was repeated 3 times, and each replicate was prepared on a separate day. Tests were conducted at 22 and 28°C according to the CLSI M49-A guideline. Spread plate colony counts were also prepared identically as described previously to confirm cell densities on each day testing was conducted. Drug potencies were considered acceptable if MIC test results from the 2 QC strains on the custom dry-form panels were within the acceptable ranges listed in the M49-A guideline.

RESULTS AND DISCUSSION

Standardization trial

Using standard AST methods makes it easier for laboratories to reliably share data, monitor development of drug resistance, and provide consistent clinical recommendations. Proper QC procedures with expected test results are required to confirm method performance. Miller et al. (2003, 2005) established the first standard reference disk diffusion and MIC testing methods for non-fastidious aquatic bacterial pathogens (CLSI 2006a,b). These organisms include

members of *Enterobacteriaceae* and *Vibrionaceae*, and *Aeromonas salmonicida, A. hydrophila*, and other mesophilic aeromonads, *Pseudomonas* spp., *Plesiomonas shigelloides*, and *Shewanella* spp. Many fastidious aquatic bacteria are also important fish pathogens. In particular, the pathogenic gliding bacteria *Flavobacterium columnare* and *F. psychrophilum* cause disease and major economic loss for a wide variety of freshwater aquaculture industries. Recent approval of antimicrobials in the US to control mortality of fish infected with *F. columnare* and *F. psychrophilum* have further fueled a need for standard AST methods for these fastidious organisms. Our research directly addresses this issue and reports the results of a standardization trial to set MIC QC ranges for testing *F. columnare* and *F. psychrophilum.*

The proposed QC ranges determined by the CLSI VAST — Aquaculture Working Group were presented to the VAST Subcommittee and accepted with minor revisions to be included in the next edition of the M49-A guideline for dilution susceptibility testing of bacteria isolated from aquatic animals (CLSI 2006b). Table 1 provides the lot-by-lot comparison of the MIC results from our standardization trial. No laboratory was consistently different, although in some cases a laboratory had some MIC results outside of the majority (~95%) of the data distribution. A visual analysis comparing data by broth lot showed that all lots consistently agreed across the laboratories. Tables 2 to 5 summarize the MIC results and QC limits approved by the CLSI VAST Subcommittee for *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658. Most of the MICs observed at both temperatures for *A. salmonicida* ATCC 33658 were centered on a single median MIC ± 1 two-fold drug dilution (e.g. Fig. 1). In 2 cases, enrofloxacin at 18°C and ormetoprim/sulfadimethoxine at 18°C (Fig. 2), a QC range that spanned 4 dilutions was approved since the MIC distribution was asymmetric and had a frequency adjacent to the median that was >60% of the frequency of the median MIC. More of the approved QC ranges for *E. coli* ATCC 25922 were 4 dilutions. This was the case at 28°C for 4 of the 9 drugs, and at 18°C for 3 of 7 drugs; in addition, because of inadequate data, QC ranges could not be established for enrofloxacin and ormetoprim/sulfadimethoxine at 18°C.

No MIC QC ranges could be set for gentamicin since a majority of laboratories observed MICs below the test range (≤0.03 µg ml^{-1}). When compared to frozen-form panels with the same drug format (performed by 1 laboratory, FDA/CVM/OR), genta-

Table 1. Comparison by broth lot of minimal inhibitory concentration (MIC) results against oxytetracycline across each laboratory for *Escherichia coli* ATCC 25922 incubated at 18°C for 92 to 96 h. Number of tests out of 10 replicates per MIC and summary statistics (number of tests, geomean, mode, minimum, maximum and range). MIC range tested: 0.015 to 8 µg ml^{-1}. The grey shaded area denotes the CLSI-approved quality control range. For all labs, median = 0.5

	Oxytetracycline *E. coli* 25922 18°C/96 h, 8 laboratories																																							
	Lab 1			Lab 2			Lab 3			Lab 4			Lab 5			Lab 6			Lab 7			Lab 8			All labs		
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
0.06																											
0.12																											
0.25	2			4			4	2		2			1	2		2	8	6	3	10	8	7	7	2	6	39	18	19
0.5	6	10	10	2	8	6	6	8	10	5	7	8	8	10	8	2	4	7		2	3	3	8	4	32	57	56
1	2			3		2				3	3	1													8	3	3
2				1	2	2																			1	2	2
N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	80	80	80
Geomean	0.50	0.50	0.50	0.54	0.66	0.76	0.38	0.44	0.50	0.54	0.62	0.50	0.44	0.50	0.44	0.29	0.33	0.41	0.25	0.29	0.31	0.31	0.44	0.33	0.39	0.45	0.45
Mode	0.5	0.5	0.5	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.25	0.25	0.25	0.25	0.5	0.25	0.25	0.5	0.5
Min.	0.25	0.5	0.5	0.25	0.5	0.5	0.25	0.25	0.5	0.25	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Max.	1	0.5	0.5	2	2	2	0.5	0.5	0.5	1	1	1	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.5	0.5	0.5	0.5	2	2	2
Range	3	1	1	4	3	3	2	2	1	3	2	3	2	1	2	2	2	2	1	2	2	2	2	2	4	4	4

Table 2. Minimal inhibitory concentration (MIC) results for *Escherichia coli* ATCC 25922 incubated at 28 ± 2°C for 44 to 48 h in diluted (4 g l^{-1}) cation-adjusted Mueller-Hinton broth against 9 antimicrobials. QC: quality control

Antimicrobial agent	Testing range (µg ml^{-1})	MIC (µg ml^{-1})			No. of dilutions in QC range	% within QC range (n = 239)
		Inter-laboratory range	Median	CLSI-approved QC range		
Ampicillin	0.03–16	1–4	2	1–4	3	100.0
Enrofloxacin	0.001–0.5	0.004–0.015	0.008	0.002–0.015	4	100.0
Erythromycin	0.25–128	4–64	32	16–64	3	94.6
Florfenicol	0.12–64	1–8	4	2–8	3	99.6
Flumequine	0.004–2	0.12–0.5	0.25	0.06–0.5	4	100.0
Ormetoprim/ sulfadimethoxine[a]	0.008/0.15 – 4/76	0.06/1.19 – 0.5/9.5	0.25/4.75	0.12/2.38 – 0.5/9.5	3	97.1
Oxolinic acid	0.002–1	0.06–0.25	0.06	0.03–0.12	3	99.6
Oxytetracycline	0.015–8	0.12–1	0.5	0.12–1	4	100.0
Trimethoprim/ sulfamethoxazole[b]	0.008/0.15 – 0.5/9.5	0.015/0.30 – 0.06/1.19	0.06/1.19	0.015/0.30 – 0.12/2.38	4	100.0[c]

[a]First (second) value indicates concentration of ormetoprim (sulfadimethoxine)
[b]First (second) value indicates concentration of trimethoprim (sulfamethoxazole)
[c]n = 238

Table 3. Minimal inhibitory concentration (MIC) results of *Escherichia coli* ATCC 25922 incubated at 18 ± 2°C for 92 to 96 h in diluted (4 g l^{-1}) cation-adjusted Mueller-Hinton broth against 9 antimicrobials. QC: quality control

Antimicrobial agent	Testing range (µg ml^{-1})	MIC (µg ml^{-1})			No. of dilutions in QC range	% within QC range (n = 239)
		Inter-laboratory range	Median	CLSI-approved QC range		
Ampicillin	0.03–16	0.25–>16	4	2–8	3	95.8
Enrofloxacin	0.001–0.5	≤0.001–0.015	0.002	No ranges proposed		
Erythromycin	0.25–128	2–32	8	4–16	3	97.5
Florfenicol	0.12–64	≤0.12–32	8	4–32	4	97.9
Flumequine	0.004–2	≤0.004–0.5	0.12	0.06–0.25	3	96.3
Ormetoprim/ sulfadimethoxine[a]	0.008/0.15 – 4/76	0.015/0.30 – 1/19	0.5/9.5	Ranges not accepted		
Oxolinic acid	0.002–1	0.015–0.12	0.06	0.03–0.12	3	97.1
Oxytetracycline	0.015–8	0.25–2	0.5	0.12–1	4	97.9
Trimethoprim/ sulfamethoxazole[b]	0.008/0.15 – 0.5/9.5	≤0.008/0.15 – 0.12/2.38	0.03/0.59	0.015/0.30 – 0.12/2.38	4	97.5

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

micin MICs from the dry-form panels were still observed to be below the test concentration range (data not shown). However, gentamicin MIC results were in range when both QC strains were tested with the dry-form panels in full strength CAMHB in this study (below) and by the manufacturer. We opted to use dry-form panels since these panels could withstand potentially longer transit times, and were shown to perform well in a similar standardization trial (Miller et al. 2005). Miller et al. (2005) also found that MIC results on dry-form and frozen-form broth microdilution panels agreed for the same QC strains.

Colony count data tracking approximated the final target concentration of approximately 5×10^5 CFU ml^{-1} bacteria as recommended by the CLSI (2006b). The mean (SD) CFU ml^{-1} for *Aeromonas salmonicida* ATCC 33658 was 4.0×10^5 (1.8×10^5) at 18°C and 4.3×10^5 (1.7×10^5) at 28°C. The mean (SD) CFU ml^{-1} for *Escherichia coli* ATCC 25922 was 3.3×10^5 (1.9×10^5) at 18°C and 4.5×10^5 (1.6×10^5) at 28°C. No trends were observed between MIC results and low/high cell densities, indicating that the variability in the cell densities did not markedly affect MIC results.

Table 4. Minimal inhibitory concentration (MIC) results of *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 incubated at 28 ± 2°C for 44 to 48 h in diluted (4 g l^{-1}) cation-adjusted Mueller-Hinton broth against 9 antimicrobials. QC: quality control

Antimicrobial agent	Testing range (µg ml^{-1})	MIC (µg ml^{-1})			No. of dilutions in QC range	% within QC range (n = 239)
		Inter-laboratory range	Median	CLSI-approved QC range		
Ampicillin	0.03–16	0.12–16	0.12	0.06–0.25	3	99.0
Enrofloxacin	0.001–0.5	0.008–0.015	0.008	0.004–0.015	3	100.0
Erythromycin	0.25–128	1–16	8	4–16	3	99.5
Florfenicol	0.12–64	0.5–2	0.5	0.25–1	3	99.5
Flumequine	0.004–2	0.015–0.06	0.03	0.015–0.06	3	100.0
Ormetoprim/ sulfadimethoxine[a]	0.008/0.15 – 4/76	0.06/1.19 – 0.5/9.5	0.12/2.38	0.06/1.19 – 0.25/4.75	3	99.5
Oxolinic acid	0.002–1	0.008–0.06	0.015	0.008–0.03	3	99.5
Oxytetracycline	0.015–8	0.12–0.5	0.12	0.06–0.25	3	99.5
Trimethoprim/ sulfamethoxazole[b]	0.008/0.15 – 0.5/9.5	≤0.008/0.15 – 0.12/2.38	0.06/1.19	0.03/0.59 – 0.12/2.38	3	99.5

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

Table 5. Minimal inhibitory concentration (MIC) results of *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 incubated at 18 ± 2°C for 92 to 96 h in diluted (4 g l^{-1}) cation-adjusted Mueller-Hinton broth against 9 antimicrobials. QC: quality control

Antimicrobial agent	Testing range (µg ml^{-1})	MIC (µg ml^{-1})			No. of dilutions in QC range	% within QC range (n = 239)
		Inter-laboratory range	Median	CLSI-approved QC range		
Ampicillin	0.03–16	0.12–1	0.12	0.06–0.25	3	98.1
Enrofloxacin	0.001–0.5	0.004–0.015	0.008	0.004–0.03	4	100.0
Erythromycin	0.25–128	4–16	8	4–16	3	100.0
Florfenicol	0.12–64	0.5–2	0.5	0.25–1	3	99.5
Flumequine	0.004–2	0.03–0.12	0.03	0.015–0.06	3	98.1
Ormetoprim/ sulfadimethoxine[a]	0.008/0.15 – 4/76	0.06/1.19 – 0.25/4.75	0.06/1.19	0.03/0.59 – 0.25/4.75	4	99.5
Oxolinic acid	0.002–1	0.008–0.06	0.015	0.008–0.03	3	99.5
Oxytetracycline	0.015–8	0.12–1	0.12	0.06–0.25	3	99.5
Trimethoprim/ sulfamethoxazole[b]	0.008/0.15 – 0.5/9.5	0.015/0.30 – 0.06/1.19	0.03/0.59	0.015/0.30 – 0.06/1.19	3	100.0

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

Validation of drug concentrations with undiluted CAMHB

The established QC ranges from the M49-A CLSI guideline (CLSI 2006b) for broth microdilution testing in full strength CAMHB of *Aeromonas salmonicida* ATCC 33658 and *Escherichia coli* ATCC 25922 at 22°C (24 and 48 h) and 28°C (24 h) were used to validate the drug concentrations of the custom dry-form broth microdilution panels. All MIC results at 22°C were within the accepted QC ranges and were in agreement among the replicates within ±1 two-fold dilution except for erythromycin results for *A. salmonicida*. At 24 h, 9 of 15 erythromycin test results were outside of the accepted parameters, but by 48 h, only 2 of 15 were out of range.

All of the 28°C *Aeromonas salmonicida* erythromycin MIC results were in the QC range, but were concentrated at the lowest MIC. However, in the standardization trial, the median erythromycin MIC data in diluted CAMHB for *A. salmonicida* at 18°C (96 h) was very similar to 28°C (48 h; Tables 4 & 5). At least 99.5% of the MIC data was within the same 2-fold dilution range with the same median at both temperatures.

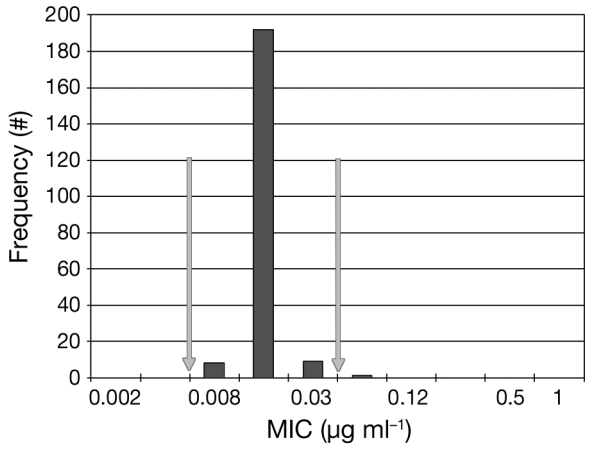

Fig. 1. Minimal inhibitory concentration (MIC) frequency distribution of *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 against oxolinic acid, incubated at 18°C for 96 h. Arrows indicate the 3-dilution CLSI-approved MIC quality control range

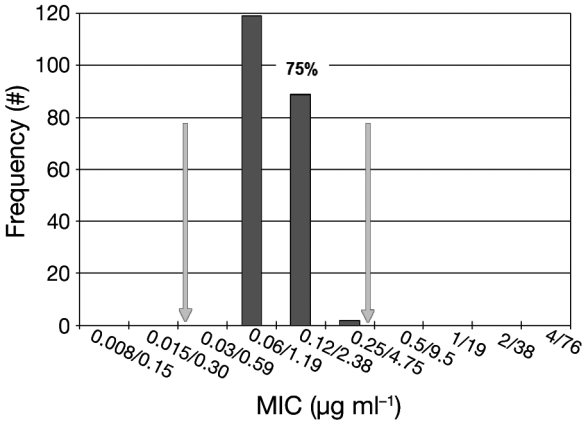

Fig. 2. Minimal inhibitory concentration (MIC) frequency distribution of *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 against ormetoprim/sulfadimethoxine, incubated at 18°C for 96 h. Arrows indicate the CLSI-approved MIC quality control range. A MIC range spanning 4 dilutions was approved, since the number of data points at 0.12/2.38 µg ml^{-1} was 75% of the data points in the mode, 0.06/1.19 µg ml^{-1}

The data from this validation experiment were presented to the CLSI VAST committee concurrently with the standardization trial, and the results confirmed that drug concentration ranges were valid on the dry-form panels used in the standardization trial.

CONCLUSIONS

Consistent MIC results across the laboratories showed that *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658 are reliable QC strains for broth microdilution testing at 18 and 28°C in diluted CAMHB (4 g l^{-1}). These strains can be used for QC testing of clinical isolates of *Flavobacterium columnare* and *F. psychrophilum*.

CLSI testing guidelines are intended to be living documents that are continually updated as new methods and approaches are developed. This newly standardized AST method for *Flavobacteria* and the MIC QC ranges provided in Table 6 will be included in the next edition of the CLSI M49-A guideline. We hope that our work as well as the work of Miller et al. (2003, 2005) will serve as helpful resources to aid in continued development of more standardized AST methods for other important aquatic bacterial pathogens.

Table 6. Summary of CLSI-approved minimal inhibitory concentration quality control ranges (µg ml^{-1}) for broth dilution susceptibility testing in dilute 4 g l^{-1} cation-adjusted Mueller-Hinton broth

Antimicrobial agent	*Escherichia coli* ATCC 25922		*Aeromonas salmonicida* ATCC 33658	
	28°C, 44–48 h	18°C, 92–96 h	28°C, 44–48h	18°C, 92–96 h
Ampicillin	1–4	2–8	0.06–0.25	0.06–0.25
Enrofloxacin	0.002–0.015	No ranges proposed	0.004–0.015	0.004–0.03
Erythromycin	16–64	4–16	4–16	4–16
Florfenicol	2–8	4–32	0.25–1	0.25–1
Flumequine	0.06–0.5	0.06–0.25	0.015–0.06	0.015–0.06
Ormetoprim/ sulfadimethoxine[a]	0.12/2.38 – 0.5/9.5	Ranges not accepted	0.06/1.19 – 0.25/4.75	0.03/0.59 – 0.25/4.75
Oxolinic acid	0.03–0.12	0.03–0.12	0.008–0.03	0.008–0.03
Oxytetracycline	0.12–1	0.12–1	0.06–0.25	0.06–0.25
Trimethoprim/ sulfamethoxazole[b]	0.015/0.3 – 0.12/2.38	0.015/0.3 – 0.12/2.38	0.03/0.59 – 0.12/2.38	0.015/0.3 – 0.06/1.19

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

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