JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2003, p. 4318–4323 Vol. 41, No. 9
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.9.4318–4323.2003

Antimicrobial Susceptibility Testing of Aquatic Bacteria: Quality Control Disk Diffusion Ranges for *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 at 22 and 28°C

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Received 4 April 2003/Returned for modification 23 May 2003/Accepted 11 June 2003

Quality control (QC) ranges for disk diffusion susceptibility testing of aquatic bacterial isolates were proposed as a result of a multilaboratory study conducted according to procedures established by the National Committee for Clinical Laboratory Standards (NCCLS). Ranges were proposed for *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 at 22 and 28°C for nine different antimicrobial agents (ampicillin, enrofloxacin, erythromycin, florfenicol, gentamicin, oxolinic acid, oxytetracycline, ormetoprim-sulfadimethoxine, and trimethoprim-sulfamethoxazole). All tests were conducted on standard Mueller-Hinton agar. With ≥95% of all data points fitting within the proposed QC ranges, the results from this study comply with NCCLS guidelines and have been accepted by the NCCLS Subcommittee for Veterinary Antimicrobial Susceptibility Testing. These QC guidelines will permit greater accuracy in interpreting results and, for the first time, the ability to reliably compare susceptibility test data between aquatic animal disease diagnostic laboratories.

In 1998, the National Committee for Clinical Laboratory Standards (NCCLS) formed the Subcommittee on Veterinary Antimicrobial Susceptibility Testing-Aquaculture Working Group (VAST-AWG) to produce a guidance document for standardizing methods of antimicrobial susceptibility testing (AST) of bacteria isolated from aquatic animal species. The working group has since relied heavily on the initial work of Barker and Kehoe (2), the efforts of those who organized the Workshop on MIC Methodologies in Aquaculture (Weymouth, United Kingdom, 1998), and Alderman and Smith (1), who published the draft protocols developed at the workshop. Alderman and Smith outlined the problems commonly encountered when comparing data created by aquatic animal disease diagnostic laboratories using different methods. The data generated from these different methods differ greatly from laboratory to laboratory, making it difficult to correlate susceptibility results between labs. The methods published by Alderman and Smith were termed "tentative" by the authors due to a number of "unresolved issues" (1).

Building on the previous efforts, members of the present VAST-AWG have targeted some of these unresolved issues, such as the development of quality control (QC) limits for potential QC strains for antimicrobial agents of interest in aquaculture. Some of these antibiotics, though not yet approved for food source aquaculture purposes, are prescribed for "extralabel" use by veterinarians treating nonfood commercial and hobby aquarium fish. Since many aquatic pathogens require lower incubation temperatures, these organisms cannot be tested accurately using the NCCLS AST methods for organisms whose optimal growth temperature is 35°C.

Although dilution susceptibility tests are becoming more popular, the most commonly used method of susceptibility testing in aquatic diagnostic laboratories is still the disk diffusion method (2). Two QC strains were chosen for this multilaboratory disk diffusion study on the basis of their susceptibility profiles and their international acceptance. *Aeromonas salmonicida* subsp. *salmonicida* (ATCC 33658 and NCIMB 1102) and *Escherichia coli* (ATCC 25922 and NCIMB 12210) are both susceptible to a wide range of antimicrobials, grow well at low temperatures, and have been shown to be stable in the laboratory following multiple passes on artificial media. *Aeromonas salmonicida* subsp. *salmonicida*, in particular, was chosen because it has an optimum growth temperature lower than that of *E. coli* and is representative of aquatic pathogens. It was proposed to the NCCLS Subcommittee on VAST that

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TABLE 1. NCCLS VAST-AWG-recommended grouping for standardizing disk susceptibility tests of various bacteria isolated from fish

Group	Bacterial species	Temperature (°C)	Incubation time(s) (h)	Suggested medium
1	*Enterobacteriaceae*	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	MH agar
	Aeromonas salmonicida (nonpsychrophilic strains)	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	MH agar
	Aeromonas hydrophila and other mesophilic aeromonads	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	MH agar
	Pseudomonas sp.	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	MH agar
	Plesiomonas shigelloides	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	MH agar
	Shewanella sp.	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	MH agar
	Vibrio sp. (nonobligate halophilic strains)	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	MH agar
	Listonella anguillarum	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	MH agar
2	*Vibrio* sp. (obligate halophilic strains)	22 ± 2 and/or 28 ± 2	24–28 and/or 44–48	1.5% NaCl addition where basal medium NaCl content was not known; 1.5% NaCl (final concn) where basal NaCl content was known[a]
	Photobacterium damselae subsp. *piscicida* *Photobacterium damselae* subsp. *damselae*	28 ± 2	44–48	1.5% NaCl addition where basal medium NaCl content was not known; 1.5% NaCl (final concn) where basal NaCl content was known[a]
3	*Flavobacterium columnare*	28 ± 2	24–28 and 44–48	Dilute MH agar[b]
	Flavobacterium branchiophilum	28 ± 2	24–28 and 44–48	Dilute MH agar[b]
	Flavobacterium psychrophilum	15 ± 2	44–48 and 68–72	Dilute MH agar with 5% serum[c]
4	*Streptococcus iniae*	35	16–18	MH agar with 5% sheep blood
	Streptococcus dysgalactiae	35	16–18	MH agar with 5% sheep blood
	Lactococcus garvieae	35	16–18	MH agar
	Vagococcus salmoninarum	35	16–18	MH agar
	Other gram-positive cocci	35	16–18	MH agar
5	Psychrophilic *Aeromonas salmonicida* strains	15 ± 2	44–48	MH agar
	Vibrio salmonicida	15 ± 2	44–48	MH agar with 1.5% NaCl
	Streptococcus difficilis	28 ± 2	44–48	MH agar with 5% sheep blood
	Gram-positive rods (*Renibacterium salmoninarum*, *Mycobacterium* sp., *Nocardia* sp., *Erysipelothrix rhusiopathiae*, and *Corynebacterium* sp.)	Multiple variations	Multiple variations	Multiple variations

[a] Conditions recommended by Alderman and Smith (1) and Ottaviani et al. (15).
[b] Conditions recommended by Hawke and Thune (7).
[c] Conditions recommended by Michel et al. (8).

both of these organisms be used as QC organisms for disk diffusion susceptibility testing of those aquatic isolates found in group 1 (Table 1). Since there is a ban on importation of *A. salmonicida* subsp. *salmonicida* strains in some nations, *E. coli* should be used instead in those nations.

QC ranges were defined at two different temperatures, 22 and 28°C, to accommodate optimum temperatures for aquatic bacteria isolated from both warm-water and cold-water species. The specific temperatures were chosen on the basis of routine use in aquatic animal disease diagnostic laboratories worldwide, recommendations of the AWG, and an effort to coordinate methodologies with international investigators.

TABLE 2. Disk diffusion QC results for *E. coli* ATCC 25922 at 22°C and 24 to 28 h with MH agar[a]

Antimicrobial agent	Disk content of antimicrobial (μg)	Zone diam (mm)			No. of data points	% of data points within QC range
		Interlaboratory range	Median	NCCLS-approved QC range		
Ampicillin	10	12–26	20	16–23	540	98
Enrofloxacin	5	35–52	44		540	
Erythromycin	15	12–22	16	13–21	540	99
Florfenicol	30	15–35	26	20–32	540	96
Gentamicin	10	21–39	28	24–32	540	97
Oxolinic acid	2	24–39	33	28–37	297	96
Oxytetracycline	30	18–37	30	26–35	540	95
Ormetoprim-sulfadimethoxine	1.25, 23.75[b]	12–33	20	14–26	540	96
Trimethoprim-sulfamethoxazole	1.25, 23.75[c]	24–44	34	27–40	540	95

[a] MH agar was made with three lots of media common to all nine laboratories.
[b] First value indicates disk content of ormetoprim; second value indicates disk content of sulfadimethoxine.
[c] First value indicates disk content of trimethoprim; second value indicates disk content of sulfamethoxazole.

TABLE 3. Disk diffusion QC results for *E. coli* ATCC 25922 at 22°C and 44 to 48 h with MH agar[a]

Antimicrobial agent	Disk content of antimicrobial (μg)	Zone diam (mm)			No. of data points	% of data points within QC range
		Interlaboratory range	Median	NCCLS-approved QC range		
Ampicillin	10	11–25	17	13–22	540	97
Enrofloxacin	5	34–55	46		540	
Erythromycin	15	11–22	16	13–20	540	97
Florfenicol	30	17–36	26	20–32	540	96
Gentamicin	10	20–38	30	23–34	540	97
Oxolinic acid	2	22–44	35	28–40	297	96
Oxytetracycline	30	22–39	30	25–35	540	96
Ormetoprim-sulfadimethoxine	1.25, 23.75[b]	12–30	16	13–22	540	96
Trimethoprim-sulfamethoxazole	1.25, 23.75[c]	21–44	31	26–36	540	95

[a] MH agar was made with three lots of media common to all nine laboratories.
[b] First value indicates disk content of ormetoprim; second value indicates disk content of sulfadimethoxine.
[c] First value indicates disk content of trimethoprim; second value indicates disk content of sulfamethoxazole.

The multilaboratory trial established QC ranges for nine different antimicrobial agents: ampicillin, enrofloxacin, erythromycin, florfenicol, gentamicin, oxytetracycline, ormetoprim-sulfadimethoxine, trimethoprim-sulfamethoxazole, and oxolinic acid. These drugs were chosen to represent major classes of antimicrobial agents, some of which are approved for use in the United States and/or abroad. In addition, some of these drugs have been identified in the environment (3, 4) and are of growing concern to environmental regulatory agencies. Results from this study will assist in the accurate monitoring of resistance in bacteria commonly isolated from the environment.

MATERIALS AND METHODS

Participating laboratories. In this study, data were generated in 10 participating laboratories. These included the Animal Health Diagnostic Laboratory, Maryland Department of Agriculture, College Park, Md.; Idaho Fish Health Center, U.S. Fish and Wildlife Service, Ahsahka, Idaho; Atlantic Veterinary College, University of Prince Edward Island, Prince Edward Island, Canada; School of Veterinary Medicine, Louisiana State University, Baton Rouge, La.; Virginia Department of Agriculture and Consumer Services, Warrenton, Va.; Animal Health Division, Alpharma, Chicago Heights, Ill.; Wisconsin Veterinary Diagnostic Laboratory, University of Wisconsin, Madison, Wis.; Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman, Wash.; Laboratory of Public Health, University of Patras, Rio Patras, Greece; and the Center for Veterinary Medicine, Division of Animal and Food Microbiology, Food and Drug Administration, Laurel, Md.

Despite the fact that this study was initiated with 10 participating laboratories, QC ranges were chosen on the basis of data from nine testing sites. One laboratory's values were consistently lower than those generated in the other participating laboratories due to the use of a magnifying lens rather than the unaided eye to interpret the zones of inhibition. As a result, the total QC data points per organism per drug per temperature per incubation time condition were reduced from 600 to 540.

Antimicrobial compounds. All antimicrobial disks were obtained from BD Diagnostic Systems (Sparks, Md.). The nine antimicrobials tested and their corresponding disk quantities and disk lot numbers were as follows: ampicillin, 10 μg (lot no. 1179720 and 1277720); enrofloxacin, 5 μg (1346726 and 1332725); erythromycin, 15 μg (1267734 and 1297736); florfenicol, 30 μg (1052735 and 1129733); gentamicin, 10 μg (1191729 and 1276721); oxytetracycline, 30 μg (0054734 and 1309724); ormetoprim-sulfadimethoxine, 1.25 and 23.75 μg (1254725 and 1317725); trimethoprim-sulfamethoxazole, 1.25 and 23.75 μg (1267736 and 1306720); and oxolinic acid, 2 μg (1302722). In this study, only one lot of oxolinic acid disks was available to all laboratories, but a limited supply of another lot (1046728) was made available to one of the participating laboratories.

Test strains and growth conditions. *E. coli* reference strain ATCC 25922 and *A. salmonicida* subsp. *salmonicida* reference strain ATCC 33658 were used to establish QC ranges at both 22 ± 2 and 28 ± 2°C. The Center for Veterinary Medicine at the Food and Drug Administration conducted an internal QC study with *E. coli* ATCC 25922 (using enrofloxacin, ampicillin, gentamicin, trimethoprim-sulfamethoxazole, oxytetracycline, and florfenicol), *Pseudomonas aeruginosa* ATCC 27853 (using gentamicin), and *Enterococcus faecalis* ATCC 29212 (using trimethoprim-sulfamethoxazole) as reference strains at 35°C with the three lots of Mueller-Hinton (MH) agar used in this study. All QC testing data points obtained from the reference strains at 35°C fell within the established QC limits found in NCCLS document M31-A2 (13).

Disk diffusion susceptibility testing. The study was designed in accordance with NCCLS guidelines M23-A2 (11) and M37-A2 (12). On 10 testing days, each laboratory inoculated each of the control strains onto 12 MH agar plates. The MH agar was prepared by Prepared Media Laboratory (Wilsonville, Oreg.), as

TABLE 4. Disk diffusion QC results for *E. coli* ATCC 25922 at 28°C and 24 to 28 h with MH agar[a]

Antimicrobial agent	Disk content of antimicrobial (μg)	Zone diam (mm)			No. of data points	% of data points within QC range
		Interlaboratory range	Median	NCCLS-approved QC range		
Ampicillin	10	10–24	19	14–23	540	99
Enrofloxacin	5	31–45	39		540	
Erythromycin	15	9–17	12	10–15	540	97
Florfenicol	30	18–33	25	20–30	540	97
Gentamicin	10	19–37	26	22–29	540	97
Oxolinic acid	2	24–35	29	25–32	297	96
Oxytetracycline	30	22–30	26	23–29	540	97
Ormetoprim-sulfadimethoxine	1.25, 23.75[b]	15–28	20	17–23	538	96
Trimethoprim-sulfamethoxazole	1.25, 23.75[c]	23–34	28	25–32	539	98

[a] MH agar was made with three lots of media common to all nine laboratories.
[b] First value indicates disk content of ormetoprim; second value indicates disk content of sulfadimethoxine.
[c] First value indicates disk content of trimethoprim; second value indicates disk content of sulfamethoxazole.

TABLE 5. Disk diffusion QC results for *A. salmonicida* subsp. *salmonicida* ATCC 33658 at 22°C and 24 to 28 h with MH agar[a]

Antimicrobial agent	Disk content of antimicrobial (μg)	Zone diam (mm)			No. of data points	% of data points within QC range
		Interlaboratory range	Median	NCCLS-approved QC range		
Ampicillin	10	31–44	38	34–42	540	97
Enrofloxacin	5	29–48	41	36–46	540	97
Erythromycin	15	16–30	23	17–28	540	98
Florfenicol	30	27–46	38	32–44	539	95
Gentamicin	10	18–39	26	23–29	540	97
Oxolinic acid	2	30–44	38	34–43	297	95
Oxytetracycline	30	27–42	34	30–39	539	97
Ormetoprim-sulfadimethoxine	1.25, 23.75[b]	19–40	32	24–38	540	96
Trimethoprim-sulfamethoxazole	1.25, 23.75[c]	22–43	35	27–40	540	96

[a] MH agar was made with three lots of media common to all nine laboratories.
[b] First value indicates disk content of ormetoprim; second value indicates disk content of sulfadimethoxine.
[c] First value indicates disk content of trimethoprim; second value indicates disk content of sulfamethoxazole.

described in the NCCLS document M31-A2 (13) and distributed to the participating laboratories. Three different MH agar lots were used: Acumedia (Baltimore, Md.) catalog no. 0109-126, Difco (Sparks, Md.) catalog no. 112185-1, and BBL (Baltimore, Md.) catalog no. 112372-1. The nine antimicrobial disks were applied to the media. Zones of inhibition were read after 24 to 28 h and 44 to 48 h (22°C) and after 24 to 28 h (28°C). Laboratories tested the strains in parallel on each of the 10 test days under the conditions indicated above. Four independent suspensions were taken from four separate culture plates (*E. coli* [22 and 28°C] and *A. salmonicida* subsp. *salmonicida* [22 and 28°C]), resulting in a total of 540 QC data points (1 suspension × 3 lots of media × 2 lots of disks × 10 test days × 9 laboratories) for each condition.

Proposed QC strains were each tested 20 times with each agar lot for all 10 antimicrobial agents assayed. This resulted in a total of 60 possible tests by each laboratory for each antimicrobial agent and a target of 540 test values for each organism per drug per temperature per incubation time condition. For oxolinic acid, 297 total test values were recorded (30 tests per laboratory plus an additional 27 tests performed by one laboratory which had a limited number of disks from another lot).

Testing protocol. The study was performed according to the methods described in NCCLS report M42-R (9). Using a Sensi-disk self-tamping 12-place dispenser (BBL), nine antimicrobial disks were applied to each 15- by 150-mm MH agar plate. Plates were stacked no more than four high and placed in ambient air incubators at 22 or 28°C.

Definition of zones of inhibition. Each area that was detected with the unaided eye as showing no obvious, visible growth was recorded as a zone of inhibition. Faint growth or tiny colonies that were detected only with difficulty at the edge of a zone of inhibited growth were not considered. When the potentiated sulfonamides were tested, antagonists in the medium allowed some growth (6); therefore, slight growth was disregarded and the margin of heavy growth (>80% of a lawn) was used to determine the zone diameter.

RESULTS AND DISCUSSION

QC ranges were developed at 22°C (24 to 28 h and 44 to 48 h) and 28°C (24 to 28 h) for *E. coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658. The organisms were tested 540 times per drug per temperature condition. In accordance with NCCLS guidelines M23-A2 (11) and M37-A2 (12), the percentage of participant zone diameters that fell within the approved QC ranges exceeded 95% for each antibacterial agent tested.

MH agar was selected as the growth medium on the basis of its ability to support the growth of many aquatic isolates as well as of the QC strains. It is also specified for aerobic disk susceptibility testing methods (10) and is ion adjusted and therefore standardized. Additionally, this medium has shown good intralaboratory reproducibility of susceptibility results of tests using the QC strains as well as various aquatic isolates. The three lots of MH agar used in this study yielded minimal lot-to-lot variation within and between the laboratories for both QC organisms tested. Similarly, very little variation was observed in the zones of inhibition measured around the two different lots of antimicrobial disks tested.

Table 2, Table 3, and Table 4 summarize the zones of inhibition and QC limits for the nine antimicrobial agents tested for *E. coli.* The NCCLS-approved zone diameter QC ranges

TABLE 6. Disk diffusion QC results for *A. salmonicida* subsp. *salmonicida* ATCC 33658 at 22°C and 44 to 48 h with MH agar[a]

Antimicrobial agent	Disk content of antimicrobial (μg)	Zone diam (mm)			No. of data points	% of data points within QC range
		Interlaboratory range	Median	NCCLS-approved QC range		
Ampicillin	10	25–46	39	35–44	540	96
Enrofloxacin	5	30–54	43	37–49	540	95
Erythromycin	15	15–34	26	19–31	540	95
Florfenicol	30	26–50	40	34–47	539	96
Gentamicin	10	15–38	27	22–32	540	96
Oxolinic acid	2	28–48	39	33–45	297	95
Oxytetracycline	30	20–42	33	28–38	539	96
Ormetoprim-sulfadimethoxine	1.25, 23.75[b]	17–39	30	21–35	540	96
Trimethoprim-sulfamethoxazole	1.25, 23.75[c]	19–43	33	24–39	540	96

[a] MH agar was made with three lots of media common to all nine laboratories.
[b] First value indicates disk content of ormetoprim; second value indicates disk content of sulfadimethoxine.
[c] First value indicates disk content of trimethoprim; second value indicates disk content of sulfamethoxazole.

TABLE 7. Disk diffusion QC results for *A. salmonicida* subsp. *salmonicida* ATCC 33658 at 28°C and 24 to 28 h with MH agar[a]

Antimicrobial agent	Disk content of antimicrobial (μg)	Zone diam (mm)			No. of data points	% of data points within QC range
		Interlaboratory range	Median	NCCLS-approved QC range		
Ampicillin	10	31–43	36	33–41	540	97
Enrofloxacin	5	32–47	40	35–45	540	96
Erythromycin	15	16–31	25	21–29	532	96
Florfenicol	30	26–45	37	33–41	539	96
Gentamicin	10	21–31	27	24–30	540	97
Oxolinic acid	2	30–43	36	32–41	295	98
Oxytetracycline	30	26–37	31	28–34	540	97
Ormetoprim-sulfadimethoxine	1.25, 23.75[b]	18–38	28	21–32	540	96
Trimethoprim-sulfamethoxazole	1.25, 23.75[c]	20–40	31	26–36	540	95

[a] MH agar was made with three lots of media common to all nine laboratories.
[b] First value indicates disk content of ormetoprim; second value indicates disk content of sulfadimethoxine.
[c] First value indicates disk content of trimethoprim; second value indicates disk content of sulfamethoxazole.

for the organism per drug per temperature per incubation time condition were determined by using a modification of the median method described by Gavan et al. (5). Where appropriate, the calculated ranges were adjusted to incorporate more or fewer of the data points as long as ≥95% were included.

When tested with enrofloxacin, *E. coli* yielded ranges considerably wider than those presently being used in disk diffusion testing of veterinary isolates at 35°C (13). To strengthen the testing method, all ranges for *E. coli* tested with enrofloxacin were deemed unacceptable. When *E. coli* was tested with ampicillin, distinct inner and outer margins, some resembling a halo or ring of growth forming the inner margin, were observed. Some zones had individual resistant colonies inside the inner zone of inhibition. The ranges, however, were narrow and consistent among most of the laboratories, thus permitting ranges to be established for ampicillin.

Aeromonas salmonicida subsp. *salmonicida* produced zones of inhibition with NCCLS-approved ranges of ≤16 mm (Table 5, Table 6, and Table 7), which were considered acceptable QC ranges under the test conditions for all antimicrobial agents tested. When tested against the potentiated sulfonamides, *A. salmonicida* subsp. *salmonicida* yielded a very distinct inner and outer margin of growth. The antagonists in the medium allowed some slight growth inside the outer margin; however, this was not observed with *E. coli*. Measuring zones around these disks with *A. salmonicida* subsp. *salmonicida* as a QC organism may result in some laboratory-to-laboratory variation in the interpretation of the margin, so *E. coli* may be a more suitable QC organism when testing the potentiated sulfonamides. When tested with all drugs except gentamicin and enrofloxacin, larger zones of inhibition were observed at both 22 and 28°C with *A. salmonicida* subsp. *salmonicida* than with *E. coli*. Despite being active against primarily gram-positive and rapidly dividing bacteria, erythromycin was shown to be active against both of the *E. coli* and *A. salmonicida* subsp. *salmonicida* strains under in vitro conditions at the temperatures used in this study.

Results at the lower temperatures used here showed a clear decrease in the precision of the disk diffusion test, with wider ranges resulting for some of the antimicrobials tested compared to those ranges presently used at 35°C (14). The effect of temperature on the intralaboratory and approved QC ranges of most of the test articles was apparent, with narrower ranges resulting when testing at 28 versus 22°C after 24 h. This range narrowing indicates that at 28°C, margins were more defined than at 22°C, probably due to an increased growth rate or to a temperature-dependent decrease in the drug diffusion rate at the lower temperature. Wider ranges were observed in most cases, especially with *A. salmonicida* subsp. *salmonicida*, at 22°C after 48 h versus those measured after 24 h at 22 and 28°C. This suggests that the precision of the method decreased slightly over time.

It is recognized that some of these approved ranges may be wider than those used for the same drugs at higher temperatures, but the investigators believe this is a direct effect of the decreased incubation temperature. The growth kinetics of these two organisms at the temperatures used for testing was not determined but may be a subject of interest in the future.

The methods used and ranges proposed were presented to the NCCLS Subcommittee on VAST. All methods and ranges have been accepted for inclusion in NCCLS report M42-R (9).

This is the first large-scale study to establish QC ranges for AST of aquatic isolates at temperatures lower than the standard 35°C. It is envisioned that these ranges can be a nidus for future studies to establish ranges for more drugs using this core method and to develop additional standardized testing methods for fastidious aquatic pathogens (Table 1).

ACKNOWLEDGMENTS

We extend our sincere thanks to the support staff of the participating laboratories, especially David Melka, Gina Cheuk, Lorraine Lund, Doris Poole, and Marilyn Blair. We also thank Danae Venieri for her hard work during the study.

This study was made possible by financial support from Schering-Plough Inc. (Kenilworth, N.J.), BD Diagnostic Systems (Sparks, Md.), and the Oak Ridge Associated Universities sponsored by the Food and Drug Administration.

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