Epidemiologic cutoff values for antimicrobial agents against *Aeromonas salmonicida* isolates determined by frequency distributions of minimal inhibitory concentration and diameter of zone of inhibition data

Ron A. Miller, MS, and Renate Reimschuessel, VMD, PhD

**Objective**—To develop epidemiologic cutoff values by use of frequency distributions for susceptibility to 4 antimicrobial agents when tested against a representative population of a major aquaculture pathogen, *Aeromonas salmonicida*.

**Sample Population**—217 typical and atypical *A. salmonicida* isolates obtained from 20 states and 12 countries.

**Procedures**—Species identification of *A. salmonicida* isolates was confirmed by detection of specific nucleotide sequences by use of a PCR assay. Minimal inhibitory concentration (MIC) and diameter of the zone of inhibition for oxytetracycline, ormetoprim-sulfamethoxine, oxolinic acid, and florfenicol were determined for each isolate in accordance with standardized antimicrobial susceptibility testing methods that have been approved by the Clinical and Laboratory Standards Institute for bacterial isolates from aquatic animals. Susceptibility data were tabulated in a scattergram and analyzed by use of error rate bounding.

**Results**—Susceptibility tests for oxytetracycline, ormetoprim-sulfamethoxine, and oxolinic acid revealed 2 distinct populations of bacteria. Isolates tested against florfenicol clustered into a single population. Oxolinic acid susceptibility data revealed higher MICs in the non–United States *A. salmonicida* isolates. Slow-growing (atypical) *A. salmonicida* isolates were generally more susceptible than typical isolates for all antimicrobials, except oxolinic acid.

**Conclusions and Clinical Relevance**—Use of frequency distributions of susceptibility results to develop epidemiologic cutoff values appears to be applicable to aquatic isolates. Frequency distributions of susceptibility results for *A. salmonicida* revealed clear divisions between isolate susceptibilities. This type of data, considered in conjunction with pharmacokinetic and efficacy data, may be useful for developing clinical breakpoints for use in aquaculture. (Am J Vet Res 2006;67:1837–1843)

Veterinarians are expanding their practices to include exotic species, including fish. In the United States, only a few antimicrobial agents (including ormetoprim-sulfadimethoxine, oxytetracycline, and florfenicol) are approved for use in fish farmed for food production. Legislation such as the Minor Use and Minor Species Animal Health Act of 2004 is fostering the availability of additional therapeutic agents for use in fish. Before such drugs can be used, it is important for clinicians treating fish to become familiar with aquatic bacterial diseases and the susceptibility of those pathogens to various antimicrobial agents. Although, to our knowledge, clinical breakpoints or interpretive criteria (susceptible, intermediate, and resistant) have not been developed for any aquatic pathogen in any aquatic animal species, standardized AST methods for aquatic isolates should improve a clinician’s ability to choose an appropriate antimicrobial agent. Historically, veterinarians and researchers of aquatic diseases have used laboratory-specific clinical breakpoints. These values have had limited application or reliability outside of the regions in which they were generated. These limitations can be attributed to variations among in vitro testing procedures, limited diversity of isolates, and unique environmental conditions that may have affected therapeutic efficacy. Efforts to enhance the probability of therapeutic success when relying on AST results are dependent on interpretive criteria that are as specific as possible for a given bac-

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AST</td>
<td>Antimicrobial susceptibility testing</td>
</tr>
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<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
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<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>(MIC_{50})</td>
<td>Concentration of antimicrobial agent required to inhibit 50% of the isolates</td>
</tr>
<tr>
<td>(MIC_{90})</td>
<td>Concentration of antimicrobial agent required to inhibit 90% of the isolates</td>
</tr>
</tbody>
</table>

Received May 1, 2006.
Accepted June 7, 2006.

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Supported by the Oak Ridge Associated Universities through a grant sponsored by the FDA.


The authors thank Drs. Ana Baya, Rocco Cipriano, Joy Evered, Hui-Min Hsu, Sharon Landin, Lindsay Oaks, Scott Lapatra, Jessica Boyd, David Bruno, Sarah Burr, Maria Figueras, Bjarnhei Gudmundsdottir, Vera Lund, Sarah Maurice, Michael Sinyakov, and Satu Viljamaa-Dirks for provision of bacterial isolates.

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aterial pathogen in a given animal species. The reliabil-
ity of such interpretive criteria is enhanced when stan-
dardized AST methods, such as those published by the
CLSI, are used. Two CLSI guidance documents, M42
and M49, provide standardized test conditions for
nonfastidious aquatic bacterial isolates and provide
details on methods for quality control and quality
assurance.

Frequency distributions of MICs can be used to
delineate epidemiologic cutoff values (also known as
species-specific microbiologic breakpoints), as defined
by the European Committee on AST. These cutoff
values can be used to discriminate wild-type (ie, origi-
nally susceptible bacterial populations) from non–wild-type (ie, populations with acquired and
mutational resistance mechanisms) isolates. These cut-
of values are not to be confused with clinical break-
points, which are used primarily for predicting clinical
outcomes.

The purpose of the study reported here was to
develop epidemiologic cutoff values by use of frequen-
cy distributions of MICs and diameters of zones of
inhibition for 217 typical and atypical (slow growing)
isolates of *Aeromonas salmonicida* (causative agents of
furunculosis, goldfish ulcer disease, and carp erythro-
dermatitis) against 3 FDA-approved antimicrobials and
1 antimicrobial commonly used in some European
countries. These distributions may be useful in de-
veloping clinical breakpoints when combined with data
from pharmacokinetic-pharmacodynamic studies in
targeted fish species and, if possible, clinical outcome
data from fish with furunculosis or outbreaks of asso-
ciated disease.

Materials and Methods

Sample population—Isolates of *A salmonicida* were
obtained from 16 contributors located in various countries (8
in the United States; 2 in Israel; and 1 each in Canada, the
United Kingdom, Switzerland, Spain, Norway, and Finland).
Contributors were contacted by the authors and requested to
provide typical and atypical *A salmonicida* isolates from a
wide geographic region that included clinical and wild-type
strains representing a wide range of susceptibilities.

A total of 217 *A salmonicida* isolates were used for AST,
including 112 isolates from the United States, representing
20 states; 92 isolates from 11 other countries; and 6 isolates
from an unknown origin. Strains were originally isolated
from 28 fish species. The year of original isolation for the iso-
lates ranged from 1955 to 2004 (median year of original iso-
lation, 1995).

All isolates were stored in tryptic soy broth with 20%
glycerol at −80°C and then cultured on tryptic soy agar sup-
plemented with 5% sheep blood at 22°C for 48 hours. After
culture, cells were harvested for DNA extraction and AST.
Six isolates (1 from the National Collections of Industrial,
Food and Marine Bacteria and 5 from the ATCC) served as
control isolates in the PCR assays. These isolates were not
included in the sample population used for AST.

PCR assay—Genomic DNA was extracted from all bac-
terial strains by use of a commercially available kit.5
Extraction was conducted in accordance with the manufac-
turer’s instructions.

The PCR assays were performed in 0.2-mL thin-walled
PCR tubes in a thermal cycler.6 Genomic DNA from *A salmonicida* subsp salmonicida ATCC 33658 was used as a
positive control sample for each of the 2 PCR assays (MIY
and AP). Nuclease-free water was used as a negative control
sample. Template DNA (10 to 100 ng) was added for each
reaction, and a 1-kilobase DNA ladder7 was used. Products
were separated by use of electrophoresis on 1.5% agarose gels
and developed with ethidium bromide staining and UV illu-
mination in a gel documentation system.8

The MIY primer set, which is specific for only typical
strains of *A salmonicida* subsp salmonicida, was used. The
MIY primer set comprises MIY1 (5′–AGGCTTCCAGCCGT-
CACACG–3′) and MIY2 (5′–AAGAGGCCCATACTGT-
GGGG–3′). Each reaction (volume, 25 µL) contained 0.6
units of Taq DNA polymerase, 2.5 µL of 10X PCR buffer,
1.5mM MgCl₂, 16 pmol of each amplification primer (ie,
MIY1 and MIY2), and 0.2mM of each of the 4 deoxynu-
cleotide triphosphates.9 Reaction mixtures were maintained
at 94°C for 2 minutes and amplified for 35 cycles with dena-
tration at 94°C for 30 seconds, annealing at 68°C for 90 sec-
onds, and elongation at 68°C for 90 seconds. A final exten-
sion was performed at 68°C for 3 minutes. Expected size of
the PCR product was 312 bp.

The AP primer set, which is specific for all strains of
*A salmonicida* was used. The primer set comprised AP1
(5′–GCTGTTCTCTTCTCTCTCACC–3′) and AP2 (5′–CAGAGTGAAGATCAGCCGGTG–3′). Each reaction
(volume, 25 µL) contained 0.25 units of Taq DNA poly-
merase, 2.5 µL of 10X PCR buffer, 2.5mM MgCl₂, 8 pmol
of each amplification primer (ie, AP1 and AP2), and 0.2mM of
each of the 4 deoxynucleotide triphosphates.10 Reaction mix-
tures were maintained at 94°C for 2 minutes and amplified
for 30 cycles with denaturation at 94°C for 15 seconds,
annealing at 57°C for 30 seconds, and elongation at 72°C for
90 seconds. A final extension was performed at 72°C for 3
minutes. Expected size of the PCR product was 421 bp.

Disk diffusion testing—Disk diffusion tests were con-
ducted in accordance with CLSI guidelines.11 Escherichia coli
ATCC 25922 or *A salmonicida* subsp salmonicida ATCC
33658, or both, were used as quality-control isolates. All tests
were conducted on Mueller-Hinton agar,12 with incubation at
22°C for 44 to 48 hours. Disks containing florfenicol (30
µg), oxolinic acid (2 µg), oxytetracycline (30 µg), and
ormetoprim-sulfadimethoxine (1.25 and 2.375 µg of orme-
toprim and sulfadimethoxine, respectively) were used.
Diameters of the zones of inhibition were measured with a
ruler and rounded to the nearest millimeter. Bacterial inocu-
la were standardized and monitored for cell densities in the
range of 1 × 10⁷ to 10⁸ CFUs/mL.

MIC testing—Broth microdilution tests were conduct-
ed in accordance with CLSI guidelines.13 Escherichia coli
ATCC 25922 or *A salmonicida* subsp salmonicida ATCC
33658, or both, were used as quality-control organisms. All
tests were conducted in 96-well plates; plates were incu-
bated at 22°C for 44 to 48 hours. Plates contained dehy-
drated antimicrobial agent in each well and were formatted
in 2 identical series of twelve 2-fold dilutions for florfenicol
(32 to 0.015 µg/mL), oxolinic acid (4 to 0.002 µg/mL), and
oxytetracycline (32 to 0.015 µg/mL) and eleven 2-fold dilu-
tions for ormetoprim-sulfadimethoxine (8/152 to
0.008/0.15 µg/mL). Two wells were used as positive control
wells. An autoclaved inoculum was used to place 100 µL of
standardized inoculum prepared in cation-adjusted
Mueller-Hinton broth into each well. Bacterial inocula
were standardized and monitored for cell densities of
approximately 5 × 10⁸ CFUs/mL.

Scattergram analysis—The MIC and corresponding
diameter of the zone of inhibition for each isolate were tabu-
lated to generate a frequency distribution for each antimicro-
bial agent in the form of a scattergram.14 As recommended by
the CLSI, an error rate bounding method initially described elsewhere was modified to calculate discrepancy rates on the basis of MICs and diameters of zones of inhibition for all *A. salmonicida* isolates, typical *A. salmonicida* isolates, and atypical *A. salmonicida* isolates. Discrepancy rates were calculated for use in selecting epidemiologic cutoff values for the diameters of the zones of inhibition. The MIC\(_{50}\) and MIC\(_{90}\) values were also calculated for all isolates, isolates from the United States, and isolates from other countries.

**Results**

Analysis of PCR results obtained by use of AP (*Salmonicida* species specific for typical or atypical isolates) and MIY (*Salmonicida* subspecies specific for typical isolates) primer sets revealed a pool of isolates consisting of 163 typical and 54 atypical *A. salmonicida* isolates; these results did not include the 6 reference isolates. Of the 163 typical isolates, 110 were from the United States, 49 were from other countries, and 4 were from an unknown origin. Of the 54 atypical isolates, 2 were from the United States, 50 were from other countries, and 2 were from an unknown origin.

Assay of a subset of the population revealed species-specific AP primers yielded positive results for only *A. salmonicida* isolates (Figure 1). One atypical isolate (*A. salmonicida subsp. pectinolytica*) yielded negative results in PCR assays for both primers (data not shown). As expected, all atypical *A. salmonicida* isolates yielded negative results in the PCR assay for the MIY primer set. Some background banding was observed, but PCR products with intensely positive results made identification by use of PCR assays unambiguous.

On the basis of evaluation of scattergrams that contained plots of the MICs versus the diameters of the zones of inhibition for oxytetracycline, ormetoprim-sulfadimethoxine, and oxolinic acid, 2 clearly discernible populations of isolates were observed (wild type [susceptible to antimicrobials; no resistance mechanisms] and non–wild type [acquired and mutational resistance mechanisms]; Figure 2). A wide range of diameters of the zones of inhibition for oxytetracycline (13 to 30 mm) was observed between the 2 populations. Similar separation was evident for ormetoprim-sulfadimethoxine (7 to 19 mm) and oxolinic acid (22 to 34 mm).

Distribution of the plotted points for florfenicol revealed a single wild-type population with all isolates having MICs ≤ 2 µg/mL and zone diameters ≥ 34 mm.

Discrepancy rates and error rate bounding were used as recommended by the CLSI to determine epidemiologic cutoff values (Tables 1 and 2). The epidemiologic cutoff values were adjusted until the number of false wild-type results on disk diffusion tests (very major discrepancies; type I errors) and false non–wild-type results (major discrepancies; type II errors) were held to a minimum. As specified by the CLSI for collections of clinical isolates, all rates for major and very major discrepancies were held at < 1.5% and < 3%, respectively. Minor discrepancies (ie, when 1 test result was classified as intermediate and the other was wild type or non–wild type) were also considered in the calculations.

Analysis of MIC\(_{50}\) and MIC\(_{90}\) values calculated for all isolates, isolates from the United States, and isolates from other countries revealed a pattern only for oxolinic acid (Table 3). Isolates from the United States had considerably lower MICs for oxolinic acid, compared with the MICs for isolates from other countries. Isolates from the United States had slightly higher MICs for oxytetracycline, compared with the MICs for isolates from other countries (Table 4). Isolates from other countries had slightly higher MICs for ormetoprim-sulfadimethoxine, compared with the MICs for isolates from the United States (Table 5). On the basis of geographic origin of the isolates, no difference was observed with regard to MICs for florfenicol (Table 6).

Gross observations of values for MICs and diameters of the zones of inhibition revealed that typical *A. salmonicida* isolates had slightly higher MICs for oxytetracycline, ormetoprim-sulfadimethoxine, and florfenicol than were evident for the atypical isolates (data not shown). Slower growth rate, characteristic of atypical *A. salmonicida* isolates, and subsequent increased growth inhibition may help explain this increased susceptibility.

Analysis of frequency distributions of susceptibility results for all 4 antimicrobial agents revealed a wider range of zones of inhibition for most MICs than the range of MICs at specific diameters of zones of inhibition. These noticeable variations may be explained by a decreased robustness of disk diffusion tests for slower growing (atypical *A. salmonicida*) and fastidious organisms. However, the distinct separation of wild-type and non–wild-type isolates on the basis of diameters of the zones of inhibition alone should still provide accurate and useful epidemiologic cutoff values for isolates of this pathogen. Susceptibility data revealed that both disk diffusion and broth microdilution testing methods may be used.

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**Figure 1**—Agarose gel revealing PCR products obtained by use of the *Salmonicida* subspecies-specific MIY primer set (612-bp product) for typical and atypical isolates only (lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18) and the *Salmonicida* species-specific AP primer set (421-bp product) for typical and atypical isolates (lanes 3, 5, 7, 9, 11, 13, 15, 17, and 19). Lanes were as follows: 1 and 20, 1-kilobase DNA ladder; 2 and 3, *Aeromonas salmonicida* subsp. salmonicida ATCC 33658; 4 and 5, *A. salmonicida* subsp. masoucida ATCC 27013; 6 and 7, *A. salmonicida* subsp. *achromogenes* ATCC 33659; 8 and 9, *A. salmonicida* subsp. *smithia* ATCC 49393; 10 and 11, *Maine91* (atypical); 12 and 13, 4059 (atypical); 14 and 15, *A. caviae* ATCC 15468; 16 and 17, *A. veronii* ATCC 90071; and 18 and 19, negative control samples. Values on the left represent molecular size in number of bp. Notice that the MIY primer set did not generate a band at 421 bp in lanes 4, 6, 8, and 12, which is as expected for atypical *A. salmonicida* isolates.
Figure 2—Frequency distribution for MICs and diameters of the zone of inhibition for isolates of *A. salmonicida* when tested against oxytetracycline (30 µg; A), ormetoprim-sulfadimethoxine (1.25 and 23.75 µg, respectively; B), oxolinic acid (2 µg; C) and florfenicol (30 µg; D). Epidemiologic cutoff values are indicated for MICs (vertical dashed lines) and diameters of the zones of inhibition (horizontal dashed lines) for each antimicrobial. Notice that there are 2 clusters of isolates for oxytetracycline, ormetoprim-sulfadimethoxine, and oxolinic acid but only 1 cluster of isolates for florfenicol.
Table 1—Discrepancy between MICs and diameters of the zones of inhibition for *Aeromonas salmonicida* when tested against various antimicrobials.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Isolates</th>
<th>Diameter of zone of inhibition (mm)</th>
<th>MIC (µg/mL)</th>
<th>Discrepancy*†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td></td>
<td>Very major</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>All isolates</td>
<td>≥ Ip + 2</td>
<td>217</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip + 1 to Ip low – 1</td>
<td>217</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip low – 2</td>
<td>217</td>
<td>NA</td>
</tr>
<tr>
<td>Typical isolates</td>
<td></td>
<td>≥ Ip + 2</td>
<td>163</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip + 1 to Ip low – 1</td>
<td>163</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip low – 2</td>
<td>163</td>
<td>NA</td>
</tr>
<tr>
<td>Atypical isolates</td>
<td></td>
<td>≥ Ip + 2</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip + 1 to Ip low – 1</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip low – 2</td>
<td>54</td>
<td>NA</td>
</tr>
<tr>
<td>Ormetoprim-sulfadimethoxine</td>
<td>All isolates</td>
<td>≥ 1 + 2</td>
<td>217</td>
<td>1 (&lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 + 1 to 1 – 1</td>
<td>217</td>
<td>1 (&lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥ 1 – 2</td>
<td>217</td>
<td>NA</td>
</tr>
<tr>
<td>Typical isolates</td>
<td></td>
<td>≥ 1 + 2</td>
<td>163</td>
<td>1 (0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 + 1 to 1 – 1</td>
<td>163</td>
<td>1 (0.01)</td>
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<tr>
<td></td>
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<td>Atypical isolates</td>
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<tr>
<td></td>
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<td>Ip low – 2</td>
<td>217</td>
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<tr>
<td>Typical isolates</td>
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<td>≥ Ip + 2</td>
<td>163</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip + 1 to Ip low – 1</td>
<td>163</td>
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<tr>
<td></td>
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<td>Ip low – 2</td>
<td>163</td>
<td>NA</td>
</tr>
<tr>
<td>Atypical isolates</td>
<td></td>
<td>≥ Ip + 2</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip + 1 to Ip low – 1</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ip low – 2</td>
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<td>NA</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>All isolates</td>
<td>≥ NWT + 1</td>
<td>217</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NWT + WT</td>
<td>217</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥ WT – 1</td>
<td>217</td>
<td>NA</td>
</tr>
<tr>
<td>Typical isolates</td>
<td></td>
<td>≥ NWT + 1</td>
<td>163</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NWT + WT</td>
<td>163</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥ WT – 1</td>
<td>163</td>
<td>NA</td>
</tr>
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<td>Atypical isolates</td>
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<td>≥ NWT + 1</td>
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<td>0</td>
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<td>NWT + WT</td>
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<tr>
<td></td>
<td></td>
<td>≥ WT – 1</td>
<td>54</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Very major discrepancies represent the number of false wild-type (WT) results on disk diffusion tests (type I errors), major discrepancies represent the number of false non–wild-type (NWT) results on disk diffusion tests (type II errors), and minor discrepancies represent when 1 test result was classified as intermediate and the other was WT or NWT. Values reported are number (%). Ip + 1 and Ip + 2 represent 1 and 2 dilutions above the highest MIC within the intermediate range, respectively, and Ip low – 1 and Ip low – 2 represent 1 and 2 dilutions below the lowest MIC within the intermediate range, respectively. The I + 1 and I + 2 represent 1 and 2 dilutions above the intermediate MIC value, respectively, and I – 1 and I – 2 represent 1 and 2 dilutions below the intermediate MIC value, respectively. The NWT represents results for the population of isolates susceptible to antimicrobials (no resistance mechanisms). The MIC range for florfenicol was defined such that NWT + 1, NWT + WT, and WT – 1 represent 1 dilution above the NWT cutoff value, the NWT cutoff value and WT cutoff value, and 1 dilution below the WT cutoff value, respectively.

NA = Not applicable.

Table 2—Epidemiologic cutoff values for diameters of the zones of inhibition and MICs for all *Aeromonas salmonicida* isolates when tested against various antimicrobials.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Diameter of zone of inhibition (mm)</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intermediate range</td>
<td>WT</td>
</tr>
<tr>
<td>Oxytetracycline (30 µg)</td>
<td>≥ 28</td>
<td>24–27</td>
</tr>
<tr>
<td>Ormetoprim-sulfadimethoxine</td>
<td>(1.25 and 23.75 µg)*</td>
<td>≥ 20</td>
</tr>
<tr>
<td>Oxolinic acid (2 µg)</td>
<td>≥ 30</td>
<td>26–29</td>
</tr>
<tr>
<td>Florfenicol (30 µg)</td>
<td>≥ 31</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Values reported are for ormetoprin and sulfadimethoxine, respectively.

Table 1 for remainder of key.

to monitor for the development of antimicrobial resistance in *Aeromonas salmonicida* isolates.

On the basis of the epidemiologic cutoff values developed in the study, 6 (2.7%) isolates were clasi-
These antimicrobials; and 56 (25.1%) isolates obtained from other countries, and 6 isolates obtained from an unknown origin when tested against oxolinic acid.

Table 3—Cumulative percentage of MICs for 217 isolates of _A. salmonicida_ (112 isolates obtained from the United States, 99 isolates obtained from other countries, and 6 isolates obtained from an unknown origin) when tested against oxolinic acid.

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th>All isolates</th>
<th>United States</th>
<th>Other countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 4</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>96.6</td>
<td>96.1</td>
<td>97.1</td>
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<tr>
<td>2</td>
<td>95.9</td>
<td>100</td>
<td>91.2*</td>
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<td>1</td>
<td>94.0*</td>
<td>100</td>
<td>97.3</td>
</tr>
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<td>0.5</td>
<td>89.9</td>
<td>100</td>
<td>78.4</td>
</tr>
<tr>
<td>0.25</td>
<td>89.9</td>
<td>100</td>
<td>78.4</td>
</tr>
<tr>
<td>0.12</td>
<td>89.9</td>
<td>100</td>
<td>76.4</td>
</tr>
<tr>
<td>0.06</td>
<td>88.0</td>
<td>98.3</td>
<td>76.5</td>
</tr>
<tr>
<td>0.03</td>
<td>82.0*1</td>
<td>93.9*1</td>
<td>68.6</td>
</tr>
<tr>
<td>0.015</td>
<td>9.7</td>
<td>8.7</td>
<td>10.9</td>
</tr>
<tr>
<td>0.008</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.004</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.002</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>≤ 0.002</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Represents the MIC<sub>IC</sub> †Represents the MIC<sub>IC</sub>

Table 4—Cumulative percentage of MICs for 217 isolates of _A. salmonicida_ (112 isolates obtained from the United States, 99 isolates obtained from other countries, and 6 isolates obtained from an unknown origin) when tested against ormetoprim-sulfadimethoxine.

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th>All isolates</th>
<th>United States</th>
<th>Other countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 32</td>
<td>100</td>
<td>100*</td>
<td>100</td>
</tr>
<tr>
<td>32</td>
<td>92.2*</td>
<td>99.6</td>
<td>95.1*</td>
</tr>
<tr>
<td>16</td>
<td>81.1</td>
<td>75.7</td>
<td>87.3</td>
</tr>
<tr>
<td>8</td>
<td>71.0</td>
<td>67.0</td>
<td>75.5</td>
</tr>
<tr>
<td>4</td>
<td>69.1</td>
<td>63.5</td>
<td>75.5</td>
</tr>
<tr>
<td>2</td>
<td>69.1</td>
<td>63.5</td>
<td>75.5</td>
</tr>
<tr>
<td>1</td>
<td>68.7</td>
<td>63.5</td>
<td>74.5</td>
</tr>
<tr>
<td>0.5</td>
<td>66.2</td>
<td>62.6</td>
<td>74.5</td>
</tr>
<tr>
<td>0.25</td>
<td>50.71</td>
<td>48.7</td>
<td>52.91</td>
</tr>
<tr>
<td>0.12</td>
<td>8.8</td>
<td>10.4</td>
<td>6.9</td>
</tr>
<tr>
<td>0.06</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.03</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.015</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.002</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>≤ 0.002</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 5—Cumulative percentage of MICs for 217 isolates of _A. salmonicida_ (112 isolates obtained from the United States, 99 isolates obtained from other countries, and 6 isolates obtained from an unknown origin) when tested against ormetoprim-sulfadimethoxine.

<table>
<thead>
<tr>
<th>MIC (µg/mL)*</th>
<th>All isolates</th>
<th>United States</th>
<th>Other countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 8/152</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>8/152</td>
<td>94.0</td>
<td>96.5</td>
<td>91.2</td>
</tr>
<tr>
<td>8/6</td>
<td>94.0</td>
<td>96.5</td>
<td>91.2</td>
</tr>
<tr>
<td>8/2</td>
<td>94.0</td>
<td>96.5</td>
<td>91.2</td>
</tr>
<tr>
<td>1/128</td>
<td>92.6</td>
<td>94.6</td>
<td>90.2</td>
</tr>
<tr>
<td>0.5/64</td>
<td>90.31</td>
<td>93.91</td>
<td>86.3</td>
</tr>
<tr>
<td>0.25/32</td>
<td>88.6</td>
<td>88.7</td>
<td>84.3</td>
</tr>
<tr>
<td>0.12/16</td>
<td>74.71</td>
<td>76.51</td>
<td>72.54</td>
</tr>
<tr>
<td>0.06/2</td>
<td>11.1</td>
<td>6.1</td>
<td>16.7</td>
</tr>
<tr>
<td>0.03/0.5</td>
<td>0.5</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.015/0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.008/0.015</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>≤ 0.008/0.015</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Values represent concentrations for ormetoprim and sulfadimethoxine, respectively. †Represents the MIC<sub>IC</sub> ‡Represents the MIC<sub>IC</sub>

Table 6—Cumulative percentage of MICs for 217 isolates of _A. salmonicida_ (112 isolates obtained from the United States, 99 isolates obtained from other countries, and 6 isolates obtained from an unknown origin) when tested against florfenicol.

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th>All isolates</th>
<th>United States</th>
<th>Other countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 32</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>32</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
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<td>100</td>
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</tr>
<tr>
<td>4</td>
<td>100</td>
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<td>100</td>
</tr>
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<td>2</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>98.2*</td>
<td>98.3*</td>
<td>98.0*</td>
</tr>
<tr>
<td>0.5</td>
<td>78.8</td>
<td>72.2</td>
<td>86.3</td>
</tr>
<tr>
<td>0.25</td>
<td>23.5</td>
<td>15.7</td>
<td>32.4</td>
</tr>
<tr>
<td>0.12</td>
<td>3.7</td>
<td>0.9</td>
<td>6.9</td>
</tr>
<tr>
<td>0.06</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.03</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.015</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>≤ 0.015</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

See Table 3 for remainder of key.

Discussion

To our knowledge, the study reported here represents the first large-scale study in which standardized AST methods were used to generate frequency distributions of MICs and diameters of the zones of inhibition for a disease-causing bacterium in aquaculture. As recommended by the CLSI for the development of interpretive criteria, more than 100 clinical and wild-type isolates relevant to the class of antimicrobial and representing multiple geographic locations were tested. The study reported here relied on donors providing us with isolates from their own stocks; thus, it did not fully represent a random sample of _A. salmonicida_ isolates. Also it is possible some isolates used in this study may have been derived from the same bacterial clone. Clonality was not addressed in this study. Nevertheless, the large number and diversity of isolates in terms of location and species of origin should contribute to the credibility of these data.

General recommendations can be made on the basis of the distinct separation (or clustering in the case of florfenicol) of the test population with regard to susceptibility. These epidemiologic cutoff values for isolates of _A. salmonicida_ should not be considered in a clinical context because they are based solely on susceptibility distributions determined in vitro. These cutoff values can be used to detect the development of resistance. Discrepancy between in vitro test results of susceptibility and therapeutic effectiveness is a result of the numerous factors that influence the interactions of antimicrobials and bacteria in vivo. To have clinical application, these cutoff values must subsequently be correlated (and adjusted when necessary) with serum kinetics of the antimicrobial agent when administered at therapeutic doses and, if possible, clinical outcome data. In the United States, such clinical breakpoints for antimicrobials used in humans have been determined by panels of experts who review large data sets. The data provided here should assist in efforts to determine clinical breakpoints for antimicrobials used in aquatic animal medicine.

In 1 study, investigators reported the frequency distribution of MICs for 70 isolates of _A. salmonicida_ against oxytetracycline and 5 other antimicrobial agents and sug-
gested a susceptible breakpoint of ≤ 1 µg/mL for oxytetra-
cycline. Data reported here reinforce this recommen-
dation that an oxytetracycline cutoff value of 1 µg/mL
clearly separates the wild-type from the non–wild-type
population (ie, susceptible from resistant). In another
study, investigators evaluated frequency distributions for
MICs and diameters of the zones of inhibition for oxolin-
ic acid against A. salmonicida isolates and postulated clas-
sifying A. salmonicida strains into 3 groups (susceptible,
≤ 0.0625 µg/mL; intermediate, 0.125 to 0.5 µg/mL; and
resistant, ≥ 1 µg/mL). Those results are extremely similar
to the findings of the study reported here.

When veterinarians are faced with a decision to
treat a patient or population, oftentimes the only tools
they possess are susceptibility data for the test isolate,
recommendations from the supplier of the disk diffusion
tests, clinical experience, and information extracted
from published reports. Other important considerations
are the pharmacokinetic and physiologic differences
among species, overall health of the patient or popula-
tion, and route of administration. Data sets collected by
our laboratory group summarize the multitude of external
factors that can alter the pharmacokinetics of
many drugs in piscine patients. Some of these include
route of administration, species, temperature, salinity,
and disease state. Effects of such variables must also be
considered when treating fish.

Clinical aquatic animal medicine is challenging
because of a lack of available antimicrobial agents, mini-
mal efficacy data in many cultured fish species, and little
information regarding frequency distributions of suscepti-
bility results. The study reported here was an attempt to
provide clinicians with some of this much needed data.
These data represent a valuable component in the devel-
oment of interpretive criteria and should be useful as
researchers and clinicians move closer to establishing true
clinical breakpoints for a major aquatic pathogen,
A. salmonicida. Additional high-quality in vivo pharmacoki-
netic-pharmacodynamic and efficacy data will be
required to allow clinicians and researchers to make com-
parisons and correlations with in vitro data on frequency
distributions of susceptibility results reported here.

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