



The performance of antimicrobial susceptibility testing programmes relevant to aquaculture and aquaculture products

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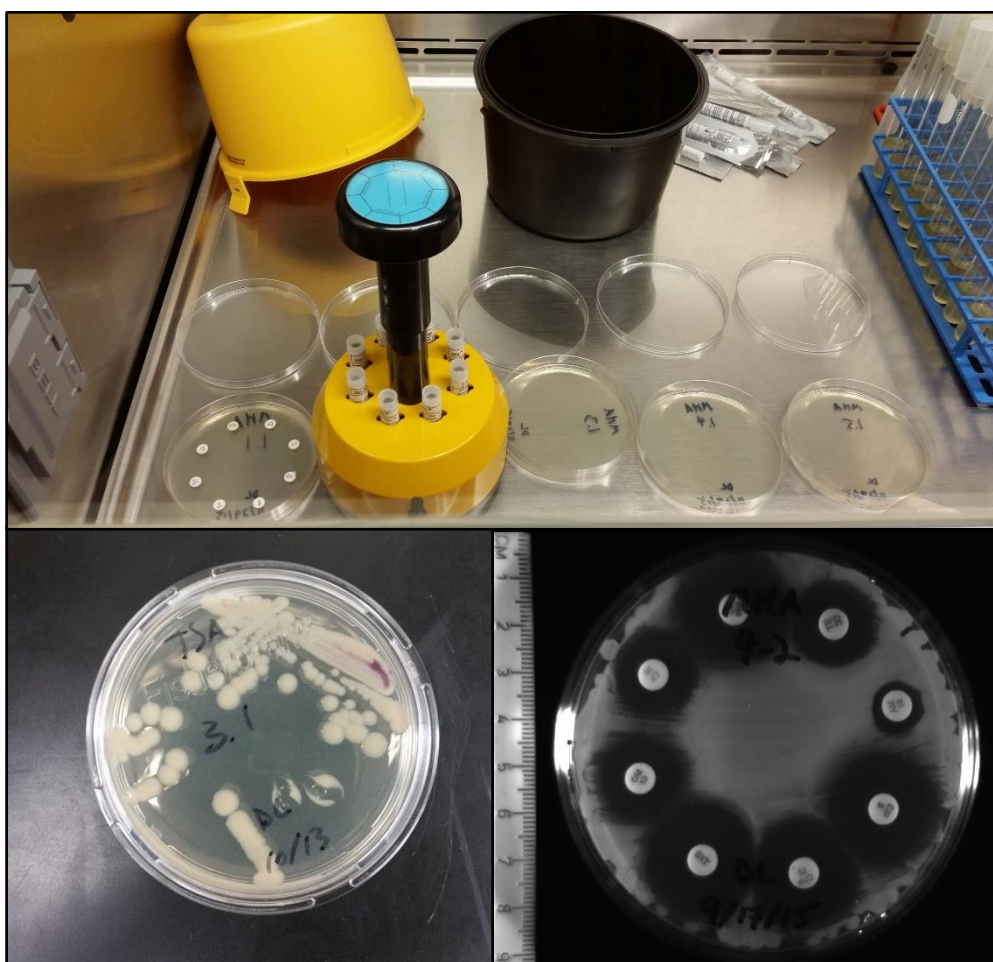
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THE PERFORMANCE OF ANTIMICROBIAL SUSCEPTIBILITY TESTING PROGRAMMES RELEVANT TO AQUACULTURE AND AQUACULTURE PRODUCTS



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Top: BD BBL™ Sensi-Disc Stamp Dispenser™ used for antimicrobial susceptibility testing. Photo credit: ©FAO/O. Elhassan.

Bottom left: *Escherichia coli* culturing and isolation on tryptic soy agar (TSA) plates. Photo credit: ©FAO/O. Elhassan.

Bottom right: *Escherichia coli* lawn stamped with susceptibility discs, showing resistance to ampicillin and intermediate resistance to tetracycline. Photo credit: ©FAO/O. Elhassan.

**THE PERFORMANCE OF ANTIMICROBIAL SUSCEPTIBILITY
TESTING PROGRAMMES RELEVANT TO AQUACULTURE
AND AQUACULTURE PRODUCTS**

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PREPARATION OF THIS DOCUMENT

An initial draft of this paper was produced as a discussion document for a series of regional workshops organized by FAO under the project FAO FMM/RAS/298: Strengthening capacities, policies and national action plans on prudent and responsible use of antimicrobials in fisheries.

The general comment received was that this will be a quite useful document that will provide good guidance to countries with respect to standard susceptibility testing protocols, the quality control requirements and the respective interpretive criteria for bacteria isolated from aquatic animals. It is also considered timely with the current attention given to antimicrobial resistance (AMR).

This was prepared under the auspices of FAO's Strategic Programme 4: Enable more inclusive and efficient agricultural and food systems and specifically 4.1.1: Public sector institutions are supported to improve their capacity to design and implement better policies and regulatory frameworks, and to provide public services related to plant and animal health, food safety and quality.

ABSTRACT

Antimicrobial susceptibility testing of bacteria isolated from aquatic animals and their environments may be performed either as part of a monitoring or surveillance programme or to provide guidance for clinical treatments of diseased animals. This technical paper addresses best practice guidelines for the performance of these susceptibility tests. Section 1 discusses the relevance of this document to *The FAO Action Plan on Antimicrobial Resistance 2016-2020*. Section 2 provides a general background to the principles of antimicrobial susceptibility testing. It stresses the absolute need for the use of internationally agreed standardized test protocols and the adherence to the quality control requirement of those protocols. It also stresses the importance of the use of consensus-based, internationally harmonized criteria in the interpretation of the meanings that can be given to *in-vitro* susceptibility data. It provides a discussion of the theory of interpretive criteria and the methods by which they can be calculated. Section 3 discusses the current status of the standard protocols that can be recommended for use in antimicrobial susceptibility testing of bacteria isolated from aquatic animals. Following a consideration of 44 species of bacteria that represent those most frequently isolated from aquatic animals, it demonstrates that the currently available standardized protocols are adequate for the determination of the antimicrobial susceptibility of 37 of them (84 percent). Section 4 discusses the importance of the design of programmes aimed at monitoring or surveillance of antimicrobial resistance associated with the use of antimicrobial agents in the rearing of aquatic animals. These programmes may be performed to address various questions. It is important that the design adopted in any programme generates data appropriate to the question being asked. In this paper four designs are outlined, each of which will provide data for programmes aimed at answering different questions. Section 5 provides some conclusions, while Section 6 gives a list of references. The technical paper is supported by four annexes that provide: (i) a listing of Clinical and Laboratory Standards Institute (CLSI) documents cited in the paper; (ii) a list of the antimicrobial agents most commonly used in aquaculture; (iii) notes on the selection of test protocols for selected Gram-positive cocci; and (iv) guidance on the possible use of epidemiological cut-off values in a clinical context.

CONTENTS

Preparation of this document	iii
Abstract	iv
Acknowledgements	vii
Abbreviations and acronyms	viii
1. Background	1
2. General susceptibility considerations	1
2.1. Introduction	1
2.2. The use of internationally standardized susceptibility testing protocols	2
2.3. Quality control	3
2.4. Susceptibility testing methods	4
2.4.1. Choice of MIC or disc methods	4
2.4.2. Choice of agent concentrations to be used in MIC tests	5
2.4.3. Choice of discs and disc contents to be used in disc diffusion tests	5
2.5. Interpretive criteria	6
2.5.1. Species specificity of interpretive criteria	6
2.5.2. Protocol specificity of interpretive criteria	6
2.5.3. Use of standardized terminology	7
2.6. Clinical breakpoints	7
2.7. Epidemiological cut-off values	9
2.8. Setting interpretive criteria	10
2.8.1. Setting new clinical breakpoints	10
2.8.2. Setting new ECVs	10
2.8.3. Epidemiological cut-off values for data produced in a single laboratory	11
2.9. Precision of susceptibility test data and precision limits	11
2.9.1. Measurement of MIC data set precision	12
2.9.2. Precision limits of MIC data sets	12
2.9.3. Precision limits of disc diffusion data sets	13
3. Susceptibility testing protocols suitable for bacterial species isolated from aquatic animals	13
3.1. Introduction	13
3.2. Bacterial species	14
3.3. Non-fastidious Gram-negative bacteria	14
3.4. Halophilic Gram-negative bacteria (facultative and obligate halophiles)	15
3.5. Flavobacteria and related species	16
3.6. Gram-positive cocci	17
3.7. Mycobacteria and related species	20
3.8. Miscellaneous species	21
4. The aims and design of studies to investigate antimicrobial agent susceptibility of bacteria isolated from aquatic animals	21
4.1. Introduction	21
4.2. Investigations of the susceptibility of pathogens of aquatic animals	21
4.3. Investigations of the public health implications of the presence in aquacultural products of bacteria with reduced susceptibility to antimicrobial agents	23

4.4. Investigations of the public health implications of antimicrobial agent use in aquaculture mediated through aquacultural products	24
4.5. Investigations of the public health implications of antimicrobial agent use in aquaculture mediated through the environmental resistome	25
5. Conclusions.....	25
6. References.....	26
Annex 1. CLSI documents cited in this Circular.....	28
Annex 2. Antimicrobial agents used in aquaculture.....	29
Annex 3. Notes on selection of test protocols for selected Gram-positive Cocci	31
Annex 4. On the Possible use of epidemiological cut-off values in a clinical context	35

TABLES

Table 1. Precision limits for disc diffusion zone data sets	13
Table 2. Availability of quality control (QC) data for susceptibility tests performed on unmodified Mueller-Hinton agar (MHA) or cation adjusted Mueller-Hinton broth (CAMHB) with respect to the agents most commonly used in aquaculture.....	15
Table 3. Availability of quality control (QC) data for minimum inhibitory concentration (MIC) susceptibility tests performed on dilute Mueller-Hinton agar (MHA) or cation adjusted Mueller-Hinton broth (CAMHB) with respect to the agents most commonly used in aquaculture.....	17
Table 4. Availability of quality control (QC) data for susceptibility tests performed at 35 °C with and without additions.	19
Table 5. Availability of QC data for susceptibility tests performed at 28 °C with and without additions.....	20

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ABBREVIATIONS AND ACRONYMS

AMR	Antimicrobial Resistance
CAMHB	Cation adjusted Mueller-Hinton broth
CLSI	Clinical and Laboratory Standards Institute
COwt	Wild type cut-off value
ECV	Epidemiological cut-off value
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
G+ve	Gram-positive
LHB	Lysed horse blood
MDR	Multiple drug resistance
MH	Mueller-Hinton (media)
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
NRI	Normalised resistance interpretation
NWT	Non-wild type
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
PK/PD	Pharmacokinetics and pharmacodynamics
QC	Quality control
<i>sd</i>	Standard deviation
S/I/R	Sensitive/intermediate/resistance
SRB	Sheep red blood cells
TSA	Tryptic soy agar
WHO	World Health Organization
WT	Wild type

1. BACKGROUND

In 2016, the Food and Agriculture Organization of the United Nations (FAO) published *The FAO Action Plan on Antimicrobial Resistance 2016-2020* (FAO, 2016), which was designed to implement the resolution 4-2015 of the Thirty-ninth Session of the FAO Conference in June 2015. Fundamental to this action plan was the “One Health” concept, that the health of humans, animals and the environment are interconnected. Therefore, with respect to antimicrobial resistances (AMR), the plan envisaged that there was a need to consider in a coordinated manner all areas where resistances might be selected, including those associated with the use of antimicrobial agents in humans, terrestrial animals and plants and aquatic animals. In conjunction with the World Health Organization (WHO) and the World Organisation for Animal Health (OIE), the plan proposed the development of capacity for the surveillance and monitoring of antimicrobial resistance in all these areas.

In Focus area 2, the FAO plan has the aim of helping strengthen the capacity of national laboratories to monitor AMR. With respect to terrestrial bacteria, this aim faces multiple difficulties, many financial or logistical, and these are particularly severe in some low-income countries and in countries with as yet underdeveloped regulatory infrastructures. However, the surveillance and monitoring of AMR in bacteria isolated from aquatic animals presents, in addition, some unique difficulties associated with the laboratory procedures to be employed in any monitoring programme.

The standard protocols for susceptibility testing of terrestrial bacteria are, to a large extent, fully developed, and have been widely disseminated and are generally adopted. Thus, with respect to the design of national monitoring programmes of AMR in these terrestrial bacteria, there are very few issues concerning the laboratory protocols to be employed.

In contrast, standard protocols for the susceptibility testing of aquatic bacteria are less developed, particularly with respect to consensus and internationally harmonized criteria for interpreting the meaning of laboratory-generated *in-vitro* susceptibility data. In addition, the standard protocols that do exist for the susceptibility testing of aquatic bacteria have not been widely disseminated or adopted. Thus, with respect to national monitoring programmes of AMR in these aquatic bacteria, there are methodological issues to be resolved if these programmes are to generate commensurate data.

This technical paper provides a review of the current state, as of 2018, of standard susceptibility testing protocols, their quality control requirements and their respective interpretive criteria for bacteria isolated from aquatic animals. It was drafted to prepare the ground for harmonization of the design and laboratory protocols to be used in national AMR monitoring programmes for these bacteria. Such harmonization is essential if the data from the various national programmes are to be commensurate and capable of being meaningfully compared and integrated in a “One Health” approach to the problem presented by AMR.

2. GENERAL SUSCEPTIBILITY CONSIDERATIONS

2.1. Introduction

This section provides a general introduction to the antimicrobial susceptibility testing of bacteria isolated from aquatic animals. In particular it discusses the protocols appropriate for the *in-vitro* determination of susceptibility and the quality control (QC) requirements that

represent an essential component of those protocols. It also discusses the protocol-specific interpretive criteria that can provide a meaning for the laboratory-generated susceptibility data.

In this section and throughout this technical paper, references are made to standardized susceptibility testing protocols that have been developed and published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)¹ and more frequently, the Clinical and Laboratory Standards Institute (CLSI)². It must be stressed that the documents issued by these organizations present the definitive versions of these standard protocols. The CLSI and EUCAST standardized protocols provide detailed instructions of how to perform each component of the susceptibility tests. As strict adherence to these protocols is essential, workers are strongly advised to consult the relevant CLSI or EUCAST document prior to designing or performing any susceptibility testing.

This paper aims to provide a guide as to which standard testing protocol would be most appropriate in any situation. It cannot and does not attempt to provide details of how to perform those protocols. In the text, these CLSI protocols are referred to by the codes of the documents in which they were published. Annex 1 provides a full list of the CLSI documents codes and corresponding documents.

2.2. The use of internationally standardized susceptibility testing protocols

Although they are relatively simple to perform, susceptibility tests are not robust. The numerical value of the *in vitro* susceptibility measure of any isolate is strongly influenced by the details of the test protocol used to establish it. Thus, the susceptibility data produced by different laboratories will be commensurate only if they have used identical protocols to generate those data. If studies by different laboratories or in different regions or countries are to be combined or compared, it is essential that the data they produce are commensurate.

A central position taken in this paper is that recommended in the OIE *Aquatic Animal Health Code 2018* (OIE 2018a). In studies of antimicrobial agent susceptibility of bacteria isolated from aquatic animals, the use of standardized laboratory protocols is essential. Equally it is essential that the meaning of the susceptibility data generated be established using internationally harmonized, consensus interpretive criteria (Smith *et al.*, 2013).

In this paper, the standard protocols produced by only CLSI and EUCAST have been considered. Both these agencies have produced standard protocols for the susceptibility determinations of bacterial species that can be tested at 35 °C. These cover the testing of the majority of species that can infect humans. CLSI has also produced standard protocols that cover most of the important terrestrial veterinary pathogens (CLSI, 2017a) that can also be tested at 35 °C. CLSI is the only agency that has developed standard protocols that specify testing at temperatures <35 °C that are frequently required by species isolated from aquatic animals (CLSI, 2006, 2014a).

When references to CLSI documents are made, the latest edition available at the time of writing is cited. CLSI documents are, however, regularly updated. Readers should be aware that the

¹www.eucast.org

²<https://clsi.org>

documents relating to aquatic animals (CLSI, 2006, 2014a, 2014b) are currently undergoing revision and new editions are expected by 2019–2020.

When using any standard testing protocol, it is important to remember that no alterations can be made to any of the parameters of that protocol. If, for example, the incubation temperature or time used in a study are not those specified in the standard protocol, then the data generated must be considered as having been generated by a new, non-standard protocol.

2.3. Quality control

An essential property of all CLSI standard protocols is that they also specify the quality control criteria that must be met if any work is to claim compliance with those protocols. It is important to note that unless work is performed in compliance with a specific protocol it is not legitimate to apply any interpretive criteria developed for that protocol to the data obtained.

For each standard protocol, the range of acceptable results for one or more quality control reference strains is established by CLSI and published in association with the standard protocol. Laboratories must test their performance of the protocol they are following by regularly checking that the results they obtain with the reference strain are within the specified acceptable range. It should be noted that reference strain quality control checks should be run for each antimicrobial agent for which susceptibility data is sought.

CLSI also specify the frequency with which these quality control checks must be performed. Many laboratories involved with susceptibility determinations of isolates made from aquatic animals will not be performing tests on a regular, daily basis. For these laboratories, it is recommended that reference strains be tested on each and every day that the protocol is used.

CLSI have tended to limit the number of quality control reference strains for which acceptable ranges are established. The most frequently used are *E. coli* ATCC25922, *S. aureus* ATCC25923, *P. aeruginosa* ACC27853 and *S. pneumoniae* ATCC49619. It should be noted that the selection of the reference strains recommended for testing susceptibility to a specific antimicrobial agent by a specific protocol is influenced by the test conditions of the protocol and occasionally the nature of the antimicrobial agent. It is not, however, influenced by the nature of the species being tested. The number of antimicrobial agents for which acceptable ranges have been established for protocols that specify testing at 35 °C is generally ≈ 100 and unlikely to be limiting in any study.

The quality control reference strains recommended for all the CLSI protocols that specify temperatures ≤ 28 °C have been *E. coli* ATCC25922 and/or *A. salmonicida* subsp. *salmonicida* ATCC33658. However, the number of antimicrobial agents for which acceptable ranges have been established for these protocols is, so far, small and does not include all the antimicrobial agents reported to be used in global aquaculture (see Annex 2). There is a need to expand the number of antimicrobial agents for which acceptable ranges have been established for these protocols. CLSI have published the procedures by which this can be achieved (see M37-A3).

There are acceptable ranges for reference strains for more antimicrobial agents when testing is performed at 35 °C than when testing is at 28 °C. When the species being investigated can be tested at both temperatures this consideration may influence the choice of protocols to adopt in any study.

It is important to note that the acceptable ranges are protocol specific. They can serve as quality control criteria for susceptibility tests only if those tests were performed by strict adherence to the standard protocol for which the acceptable ranges were developed. If in any study alterations are made, for example, in the incubation conditions (time or temperature) or by the addition of supplements to the media, they must be considered to have used a new and non-standard protocol. Before it could be standardized such a new protocol would require the establishment of a new set of quality control criteria.

In reporting the results of any susceptibility tests, it is essential that for each antimicrobial agent studied, laboratories provide explicit evidence of their compliance with the quality control requirements of the testing protocol used.

2.4. Susceptibility testing methods

A variety of methods are available for performing *in-vitro* phenotypic susceptibility tests. The two most commonly used are disc diffusion or minimum inhibitory concentration (MIC) tests. MIC tests can be performed using broth micro-dilution, broth macro-dilution or agar dilution. Hybrid gradient diffusion/MIC methods, such as the E-test, have occasionally been used for bacteria isolated from aquatic animals.

It should be noted that for the protocols specified in VET03-A and VET04-A2 (CLSI, 2006, 2014a) QC acceptable range data and interpretive criteria (see Section 3) are available only for disc diffusion and broth dilution MIC data. For this reason preference should be given to these methods.

2.4.1. Choice of MIC or disc methods

Logistical and cost factors as well as the technical facilities of the laboratories involved are frequently important factors in deciding whether disc diffusion or MIC is the most appropriate method in any study. The number of isolates to be analysed and the number of agents to which susceptibility needs to be determined will also often play a major role in deciding which method to adopt.

The relative precision of the methods should also be considered. The inherent precision of MIC test data is unaffected by either the temperature or duration of the incubation required by the species under test. However, the inherent precision of disc diffusion data decreases as the temperature decreases and the time of incubation increases (Smith *et al.*, 2018).

When tests are performed at ≥ 28 °C, both MIC and disc diffusion methods are capable of generating data sets of sufficient and roughly equal precision. However, when tests are performed at temperatures ≤ 22 °C, MIC methods are capable of generating data with greater precision than disc diffusion methods. When testing bacterial species that require incubation at these temperatures, preference should be given to the use of MIC methods.

When tests are performed at temperature ≤ 18 °C with prolonged (>48 h) incubation times, the use of the disc diffusion method is not recommended.

2.4.2. Choice of agent concentrations to be used in MIC tests

When MIC methods are to be employed, a choice must be made as to the range of agent concentrations to be included in the tests. In part this choice will depend on whether an internationally agreed epidemiological cut-off value (ECV) has been set for the species under test.

Some tests will be performed in situations where such relevant species and protocol-specific ECV exist. When the aim of the study is to determine whether the susceptibilities of the isolates are above or below this cut-off value, it is only necessary for the range to span the ECV concentration. A minimum a five-dilution series with ECV as the mid-point would generally be adequate.

When there is no ECV available, slightly different criteria will govern the selection of concentrations. In these situations, a minimally adequate range would be one that allows the quantitation of the MIC values of all fully susceptible wild type (WT) isolates.

Commercially available 96-well micro-dilution trays are frequently used in MIC tests. Logistical arguments may suggest purchasing these, and they have the additional advantage that the accuracy of their dilutions will have been subject to quality control by their manufacturers. However, the range of concentrations in many of the trays that are available may have been established for different species and protocols. As a consequence, the range of concentrations they include may not be appropriate for the study of bacteria isolated from aquatic animals.

2.4.3. Choice of discs and disc contents to be used in disc diffusion tests

The choice of the range of agents for susceptibility testing of bacteria isolated from aquatic animals will depend on the reasons why the testing is being performed. In studies primarily addressing food safety issues, the antimicrobial agents of interest will be dominated by those used in therapy of human infections by the bacterial species under consideration. However, if the primary interest is in aquatic animal therapies, the agents will be those used to treat aquatic animals. For many species, there is considerable overlap between the agents used in humans and in aquatic animals. It is important to note, however, there are some significant differences.

In designing studies that include investigations of multiple drug resistance (MDR), it is important to remember that some resistance mechanisms may confer reduced susceptibility to more than one antimicrobial agent in the same class. Analysing the frequency of MDR data for multiple members of a class of antimicrobial agents should only be considered if it is known that they do not demonstrate cross-resistance. Some examples of agents that show cross-resistance are listed in Annex 2.

The zone sizes obtained in any susceptibility test will be a function of the content of the disc used. For this reason, the specification of the appropriate disc contents is an essential component of any standardized disc diffusion protocol. Both the quality control (QC) requirements and the interpretive criteria of that protocol can be applied only if the correct discs are used. The CLSI guideline M100-S27 (CLSI, 2017b) provides details of the disc content to be used in determining susceptibility to those agents most frequently used in the therapy of

humans. The recommended contents of the discs for the agents most frequently used in aquatic animal therapies are given in VET03-A (CLSI, 2006) and Annex 2.

2.5. Interpretive criteria

It is essential that when the results of any susceptibility testing programme are published or placed in the public domain that the raw, unprocessed data (MIC values or disc zone sizes) are made available, however, it is often also an advantage to present the meaning that can be attributed to these raw data.

The meaning of the MIC data or zone sizes generated by standardized susceptibility tests must be established by the application of standardized and internationally harmonized, consensus interpretive criteria when these are available. When internationally harmonized criteria have not been set the meanings should be established using “local” epidemiological cut-off values calculated by one of the objective and statistically based methods discussed in Section 2.8.2.

2.5.1. Species specificity of interpretive criteria

It has been the practice to generate separate interpretive criteria for each species. Currently the criteria given in VET03-VET04-S2 (CLSI, 2014b) are all species specific, as are those published by EUCAST. At the present state of our understanding, it is recommended that for bacteria isolated from aquatic animals the use of species-specific criteria should be considered as best practice. As a consequence, there is a requirement to determine accurately the taxonomic status of any isolate prior to applying any interpretive criteria.

The use of molecular data in taxonomic studies is, however, resulting in a rapid proliferation of new species. For example, the application of these methods has resulted in over 100 species currently being identified within the genus *Vibrio* (Romalde *et al.*, 2014). The development of anything like 100 sets of species-specific interpretive criteria for this genus would represent an enormous task. As a consequence, it may be necessary to investigate the validity of interpretive criteria developed for and applied to multispecies groupings. The work of Baron *et al.* (2017), who developed epidemiological cut-off values for *Aeromonas* spp., suggests that this approach might not result in an unacceptable reduction in the precision of the criteria. It should be noted that interpretive criteria for multispecies groups have been published by CLSI in M100-S27 and M45-A3 (CLSI 2016, 2017b).

2.5.2. Protocol specificity of interpretive criteria

The setting of all interpretive criteria involves a consideration of the distribution of the laboratory-determined, *in-vitro* susceptibility measures (zone size or MIC) for the bacterial species under consideration. The numerical value of these susceptibility measures will depend on the test protocol used to generate them. It follows that the numerical values of the interpretive criteria will also be dependent on that protocol.

Thus, all interpretive criteria must be treated as protocol specific. They can only be used to interpret the meaning of experimental susceptibility data if that data were generated using the same standard protocol that was used to establish the criteria themselves.

2.5.3. Use of standardized terminology

Interpretive criteria can be of two types, clinical breakpoints or epidemiological cut-off values. These differ both in how they are set and in the meanings they are intended to generate. There has been much confusion in the literature about the descriptive terms that should be applied to the categories generated by interpretive criteria. In particular, the term “resistant” is frequently used without any consideration of its meaning or its definition. Silley (2012) attempted to establish a set of definitions of the various terms that have been used and to recommend a standardized terminology. In this technical paper, this terminology has been used, and it is strongly recommended that it should be used in all future publications and reports in the area.

2.6. Clinical breakpoints

The aim of a clinical breakpoint is to categorize an isolate on the basis of the probable outcome of the administration of an antimicrobial agent to a host infected by that bacterium. These breakpoints categorize isolates as either sensitive (S), intermediate (I) or resistant (R). The categorization of an isolate as S with respect to an agent implies that an administration of that agent, using a standard dose regime, could be expected to have a positive therapeutic outcome for a specified host infected by that isolate. A categorization as R would imply that it was improbable that such a therapy would have any beneficial outcome. The categorization of an isolate as I is used to indicate an uncertainty as to the probable clinical outcome or, in some cases, that the infection might be controllable if a higher dose was administered.

The CLSI guideline M37-A3 (CLSI, 2008) describes the general approach to the setting of clinical breakpoints. This is a complex process involving the consideration of a variety of data (Turnidge and Paterson, 2007). These include *in-vitro* susceptibility of the bacterium, the pharmacokinetics and pharmacodynamics (PK/PD) of the agent in the host and the historical records of the clinical outcomes of previous treatments of the host. Both the nature of the host and dose regime used in the administration of the agent will influence the PK/PD and the resultant clinical outcomes. As a consequence, clinical breakpoints will be dose and host specific. Their validity is always limited to situations where the therapeutic administration of a specified, standard dose of an agent is given to a specified host.

The setting of clinical breakpoints relevant to aquaculture encounters additional problems. Some of these are associated with the fact that most administrations to an aquatic animal cannot in a strict sense be classified as therapeutic. In the context of infections of humans, and any other animal treated individually, agents are frequently administered to an infected individual and maybe, therefore, be classified as therapeutic. The majority of antimicrobial agent administrations in aquaculture are, however, given to a population of aquatic animals, only some of which are infected. These treatments are, therefore classified as metaphylactic rather than therapeutic. When metaphylactic treatments are under consideration, clinical resistance can be defined in the following way:

A bacterium should be considered as clinically resistant if, as a result of its reduced susceptibility to an antimicrobial agent, it would be able to continue to contribute to morbidity and mortality in a population during and after a particular administration of that agent to that population.

Inherent in this definition is the recognition that the clinical outcome of a particular metaphylactic administration is influenced by a number of factors, only one of which is the susceptibility of the infecting bacterium. As is the case with therapeutic administrations, these other factors include the dose of the agent administered, its pharmacokinetics in the treated host and the nature of the infective process itself.

The metaphylactic nature of most aquaculture administrations raises serious problems, as yet unresolved, as to which subgroup of the treated population can be used to generate relevant PK data. As these treatments are frequently administered by presenting medicated feed, should the PK data be collected from the healthy subpopulation, which can be assumed to be still feeding, or the infected subpopulation, which may either be not feeding or feeding at a lower rate (Smith, 2008)?

With respect to antimicrobial use in global aquaculture, there are wide variations in treated species, treatment regimens employed and environments within which those treatments occur. These factors will influence the PD/PK of the agent and the clinical outcomes and necessitate the generation of a large number of clinical breakpoints. Further, in many situations, it has proved very difficult to measure accurately the clinical outcome of on-farm therapies. Thus, it would appear to be very difficult to generate clinical breakpoints that can be validly applied in the context of aquacultural therapies.

What is certain is that it will not be possible to generate globally applicable *clinical breakpoints* as a basis for interpretation of susceptibility test data for bacteria isolated from aquatic animals. The achievement of clinical breakpoints relating to specific therapies of specific hosts in a specific clinical and environmental context may be possible, but application of these breakpoints will always be of local and not general validity.

CLSI have produced some clinical breakpoints relevant to some species that are isolated from aquatic animals. The CLSI guideline M45-A3 (CLSI, 2016) provides some clinical breakpoints applicable to *Aeromonas* spp. and *Vibrio* spp. data obtained at 35 °C with 16–18 h incubation using unmodified Mueller-Hinton (MH) media. It should be noted, however, that it is stated in this guideline that the empirical evidence supporting these breakpoints is weak. The CLSI document M100-S27 (CLSI, 2017b) provides some clinical breakpoints applicable to data for β -haemolytic and viridans streptococci obtained at 35 °C with 16–18 h incubation using MH media modified by the addition of blood products.

These interpretive criteria for *Aeromonas* spp., *Streptococcus* spp. and *Vibrio* spp. are clinical breakpoints derived from human data. Therefore, they could only have a valid application when the aim of the study is to investigate susceptibility in terms of therapies of human patients. They cannot and should not be used to predict the outcomes of therapies of aquatic animals.

CLSI have presented a very limited number of clinical breakpoints relevant of the therapy of aquatic animals. VET03/04-S2 (CLSI, 2014b) gives breakpoints relevant to the therapy of *Aeromonas salmonicida* infections. The limited data supporting these breakpoints were derived for studies of salmon in freshwater at low temperatures.

2.7. Epidemiological cut-off values

Epidemiological cut-off values (ECVs) are, in common with clinical breakpoints, agent-specific, protocol-specific and species-specific interpretive criteria. Their aim is, however, different. The aim of the application of an ECV is to facilitate the categorizing of an isolate as a fully susceptible member of its species or as manifesting a reduced susceptibility. The only data needed to set these cut-off values are those concerning *in-vitro* susceptibility measurements.

Epidemiological cut-off values represent the upper (MIC) or lower (disc zone) limit of the distribution of experimentally determined values for fully susceptible members of the species under examination. Historically, these cut-off values were set subjectively following visual examination of relevant data sets using the so-called “eyeball technology”. However, observations have shown that the distribution of zone sizes for fully susceptible isolates approximated well to a normal Gaussian curve and the distribution of log₂ transformed MIC values for fully susceptible isolates shows a similar normality. These normal distributions have presented the possibility of developing statistically based method for calculating the limit values of the susceptibility measures of fully susceptible isolates and thereby objectively calculating epidemiological cut-off values.

Thus, in contrast to the possibly insurmountable difficulties in obtaining the data required to set harmonized clinical breakpoints, it is relatively easy and cheap to establish cut-off values.

ECVs allow the categorization of an isolate of a species as wild type (WT) or non-wild type (NWT) to a specific antimicrobial agent.

The terms “resistant” and “sensitive” should never be used to describe the categories generated by applying ECVs. Silley (2012) has argued that the terms “resistant” and “sensitive” should be used only when they relate to clinical therapies. The sensitive/intermediate/resistance (S/I/R) nomenclature should only be applied to categories identified by the application of *clinical breakpoints*.

The meanings of the terms WT and NWT can be defined as follows:

- **WT** isolates of a species are those for which the susceptibility measures (MIC or zone size) for a particular antimicrobial agent **are not** significantly different from those of fully susceptible members of that species. It can be assumed that isolates categorized as WT with respect to an antimicrobial agent do not possess any functional resistance mechanism(s) with respect to that agent.
- **NWT** isolates are those for which the susceptibility measures (MIC or zone size) for a particular antimicrobial agent **are** significantly different from those of fully susceptible members of that species. It can be assumed that isolates categorized as NWT with respect to an antimicrobial agent do possess some functional resistance mechanism that reduces their *in-vitro* susceptibility to the agent.

With respect to the application of ECVs, the OIE *Aquatic Animal Health Code (2018)* (OIE, 2018a) recommends that, in programmes that aim to monitor or survey the susceptibility of bacteria isolated from aquatic animals, the meaning of the data should be established by the application of internationally harmonized consensus ECVs.

It is clear that the categories WT and NWT do not have any inherent clinical significance. Possible ways in which ECVs can be used to guide appropriate selection of which antimicrobial agent should be used in a clinical context are presented in Annex 4. Currently very few ECVs have been established by CLSI for bacteria isolated from aquatic animals. The supplement VET03-VET04-S2 (CLSI, 2014b) presents ECVs that can be applied to *A. salmonicida* disc and MIC data obtained at 22 °C with 44–48 h incubation. It is expected that the forthcoming edition of this document will also include:

- a. ECVs for *A. hydrophila* disc and MIC data obtained at 28 °C with 24–28 h incubation.
- b. ECVs for *F. columnare* MIC data obtained using dilute cation adjusted Mueller-Hinton broth (CAMHB) at 28 °C with 24–28 h incubation.
- c. ECVs for *F. psychrophilum* MIC data obtained using dilute CAMHB at 18 °C with 93–96 h incubation.

2.8. Setting interpretive criteria

2.8.1. Setting new clinical breakpoints

As argued above, there are many difficulties and unresolved problems encountered in attempting to set clinical breakpoints relevant to aquacultural therapies. This issue will not be discussed further in this document.

2.8.2. Setting new ECVs

ECV are the limit values of the distribution of the susceptibility measures obtained from the fully susceptible or WT members of a species. Setting new ECVs is a relatively simple task. All that is required is *in-vitro* susceptibility data of sufficient quantity and quality.

Source of strains sets: The susceptibility measures, MIC or zone size, distributions for WT members of a species are assumed to be the same independent of the geographical location or the host from which they were obtained. A considerable body of evidence has demonstrated that this assumption is reasonable. As a consequence, globally applicable ECVs can be set from data obtained from national or regional studies.

The quantity of data required: To set ECVs for any antimicrobial agent/species combination, all that is required are susceptibility measures (MIC or zone sizes) for a sufficient number of WT isolates. In order to take account of interlaboratory variations these should be obtained from a number of independent laboratories. At present no agency has published a formal position on how many WT isolates or how many laboratories should be considered as being sufficient. The Aquatic Working Group of CLSI is currently considering a proposal that 100 WT isolates generated from at least three independent laboratories with no one laboratory contributing over 50 percent of the observations would be sufficient. Discussions within EUCAST are centred on similar quantitative criteria with possibly data from five laboratories being required. ECVs can be continuously updated. After an ECV has been initially set on the basis of sufficient data, there is no problem in incorporating additional new relevant data (that meets the qualitative and quantitative requirements) and continuously updating the ECV and improving its precision.

The quality of data required: Any laboratory can contribute data to be included in the calculations towards setting an ECV provided that they meet a few simple quality criteria. All susceptibility data must have been established by use of the relevant standard test protocol, and the laboratories should have met the QC requirements for the antimicrobial agent specified within that protocol for at least one reference strain. The taxonomic status of the bacteria studied must have been confirmed using a recognized method. The data set from any individual laboratory, which should comprise at least 15 WT observations, should show a single peak in the putative WT observations. Analysis of the data from each laboratory should have demonstrated it possesses sufficient precision (see Section 2.6).

Data analysis: Excel® spreadsheets that allow ECVs to be calculated automatically by statistically based methods have now been produced.

- For MIC data sets, two automatic validated statistical methods are available:
 ECOFFinder @ clsi.org/standards/micro/ecoffinder/
 NRI @ <http://www.bioscand.se/nri/>
- For disc data, there is only one automatic statistical method:
 NRI @ <http://www.bioscand.se/nri/>

Caution should be taken when using any of these automatic programmes with data that do not show evidence of a peak in the frequencies for putative WT isolates or that have a distribution of putative WT values that is very far from normal. It is always possible that in some cases the programmes will calculate a cut-off value from data that does not have a distribution that justifies such an action. In order to detect errors of this type, it is always recommended that the users of these software programmes examine the graphical representation they provide together with the numerical values they have calculated for the cut-off value.

2.8.3. Epidemiological cut-off values for data produced in a single laboratory

A laboratory may generate susceptibility data using a specific protocol for a bacterial species/agent combination for which no international consensus ECV has been developed. In such situations that laboratory may employ NRI (normalised resistance interpretation) or ECOFFinder to develop provisional and “local” cut-off values. It is strongly recommended that the acronym ECV (or the acronym ECOFF used by EUCAST) should not be used when referring to “local” cut-off values that have not been set by an international agency. When referring to these values, the acronym COWt (Wild type cut-off value) is more appropriate.

2.9. Precision of susceptibility test data and precision limits

In performing susceptibility tests, a critical factor is the precision of the data sets achieved in any laboratory. The use of low-precision data sets can result in errors in setting ECVs or errors in categorizing isolates.

Low precision in the data obtained may derive from an excessive taxonomic diversity in the isolates examined or from the performance of the test itself.

It has been observed that low precision has frequently been associated with the inexperience of the operator with the test or the species being analysed. As is common with nearly all laboratory procedures, the only short-term corrective action when imprecise data is obtained is for the operator to repeat the analyses until the requisite level of precision is obtained. In the longer term, laboratories should recognize that the performance of these test is a skill that is learnt only by experience and that an experienced operator is an invaluable asset.

With respect to disc zone data, low precision may also arise as a consequence of differences in the growth rates of isolates of different members of the same species that share the same susceptibility. These differences are most frequently encountered when isolates are initially recultured after long-term storage. Some isolates when initially recultured may manifest a reduced growth rate. As disc diffusion zones are growth rate dependent, isolates with this phenotype will characteristically show abnormally large zones. To minimize the occurrence of this phenomenon, it is recommended that isolates taken from storage should be subcultured two or three times before their susceptibility is measured.

2.9.1. Measurement of MIC data set precision

Analysis of an MIC data set can be performed using NRI or ECOFFinder (see Section 2.8.2.). Both these analytical methods generate the standard deviation (*sd*) of the distributions they calculate for the \log_2 -transformed measures for WT isolates. These *sd* values provide proxy measures of the precision of data sets from which they are calculated.

The distribution of the *sd* values calculated for a number of data sets is normally distributed. A data set from a single laboratory should be considered as insufficiently precise if its *sd* is greater than the mean plus two standard deviations calculated from the analysis of the distribution of the *sd* for a sufficiently large number of previously accepted data sets generated by single laboratories.

2.9.2. Precision limits of MIC data sets

Precision limits for MIC data generated in single laboratories were calculated from the *sd* of 131 published data sets (Smith *et al.*, 2018). Applying NRI to the analysis of these data sets, the mean *sd* was $0.75 \log_2 \mu\text{g/ml}$ with a standard deviation of $0.26 \log_2 \mu\text{g/ml}$. This gives a limit of acceptability of $<1.19 \log_2 \mu\text{g/ml}$.

Applying ECOFFinder to the analysis of the same data sets, the mean *sd* was $0.62 \log_2 \mu\text{g/ml}$ with a standard deviation of $0.26 \log_2 \mu\text{g/ml}$. This gives a limit of acceptability of $<1.14 \log_2 \mu\text{g/ml}$.

It is recommended that MIC data sets for which the calculated *sd* is greater than either of these limits should be treated with extreme caution and should never be used in establishing ECVs.

2.9.3. Precision limits of disc diffusion data sets

Analysis of a disc zone size data set using NRI provides a measure of the standard deviation (*sd*) of the normalized distribution of WT observations that can serve as a proxy measure of the precision of the data.

Evidence has shown that the inherent precision of zone size data decreases as the incubation temperature is reduced. As a consequence, separate, temperature specific, acceptable limits of precision are required (Table 1).

Table 1. Precision limits for disc diffusion zone data sets (Smith *et al.*, 2018)

Temperature	Sets analysed	Upper limit of <i>sd</i> ¹
35 °C	40	3.4 mm
28 °C	43	4.9 mm
22 °C	27	6.5 mm

¹*sd* = standard deviation.

As there are limited disc zone data from single laboratories available to set these limits, they should be treated only as provisional estimates. However, it is recommended that zone data sets for which the calculated *sd* is greater than these temperature-specific limits should be treated with extreme caution and should never be used in establishing ECVs.

3. SUSCEPTIBILITY TESTING PROTOCOLS SUITABLE FOR BACTERIAL SPECIES ISOLATED FROM AQUATIC ANIMALS

3.1. Introduction

This section addresses the standard protocols that have been published that are suitable for the antimicrobial agent susceptibility testing of bacteria that have been associated with disease of aquatic animals.

The bacterial species that have been associated with aquatic animal disease belong to a large number of species and genera (Austin and Austin, 2012). In this section, a list of 44 species are identified and the suitability of the available standardized protocols for their susceptibility testing is discussed. These 44 species do not represent a comprehensive list of all those that have been reported as being associated with disease conditions of aquatic animals, but it is hoped that they include the most significant and the most frequently encountered.

Not included in the 44 species are those bacterial species that are occasionally found in association with aquaculture or aquacultural products that can infect and cause disease in humans but not in aquatic animals. Standard protocols for the susceptibility testing of these bacterial species are well established (CLSI, 2015a, 2015b).

Overall it is suggested there are standard susceptibility test protocols suitable for 37 of the 44 species (84 percent).

3.2. Bacterial species

The 44 species show considerable variation in the media they require for *in vitro* growth and, as a consequence they require a variety of test protocols. In this section, the 44 species are treated in six, roughly physiological groups and the susceptibility testing protocols appropriate for each of these groups are detailed below.

3.3. Non-fastidious Gram-negative bacteria

<i>Aeromonas caviae</i>	<i>Edwardsiella anguillarum</i>
<i>Aeromonas hydrophila</i>	<i>Edwardsiella ictaluri</i>
<i>Aeromonas jandaei</i>	<i>Edwardsiella piscicida</i>
<i>Aeromonas salmonicida</i>	<i>Edwardsiella tarda</i>
<i>Aeromonas sobria</i>	<i>Pseudomonas anguilliseptica</i>
<i>Aeromonas veronii</i>	<i>Pseudomonas fluorescens</i>
<i>Acinetobacter</i> spp.	<i>Yersinia ruckeri</i>
<i>Citrobacter freundii</i> .	

Susceptibility tests for the 15 species in this group can be performed with the standard protocols (CLSI, 2006, 2014a) that specify using unmodified Mueller-Hinton (MH) media incubated at 28 °C for 24–28 h or at 22 °C incubated for 24–48 h or 44–48 h. For 13 of the species, the tests could also be performed using the standard protocols that specify using unmodified MH media incubated at 35 °C (CLSI, 2015a, 2015b, 2016).

However, *A. salmonicida* will not grow at 35 °C, and it has been reported that some serovars of *Y. ruckeri* have failed to grow at this temperature. It is, therefore, recommended that these two species be tested using protocols that use incubation temperatures ≤ 28 °C.

Table 2 lists the agents for which acceptable ranges have been set for reference quality control strains when testing is performed using unmodified Mueller-Hinton (MH) media.

Table 2. Availability of quality control (QC) data for susceptibility tests performed on unmodified Mueller-Hinton agar (MHA) or cation adjusted Mueller-Hinton broth (CAMHB) with respect to the agents most commonly used in aquaculture (see Annex 1)

Temperature (°C)	MIC ¹		Disc	
	35	28 & 22	35	28 & 22
Agents commonly used in aquaculture				
Amoxicillin/ampicillin	Yes	Yes	Yes	Yes
Chloramphenicol	Yes		Yes	
Doxycycline	Yes		Yes	
Enrofloxacin	Yes	Yes	Yes	Yes
Erythromycin	Yes	Yes	Yes	Yes
Florfenicol	Yes	Yes	Yes	Yes
Flumequine	Yes	Yes		Yes
Nitrofurantoin	Yes		Yes	
Josamycin				
Neomycin				
Ormetoprim-sulfadimethoxine		Yes		Yes
Oxolinic acid	Yes	Yes		Yes
Oxytetracycline	Yes	Yes		Yes
Sulphamethoxazole				
Thiamphenicol				
Trimethoprim	Yes		Yes	
Trimethoprim-sulfamethoxazole	Yes	Yes	Yes	Yes
Additional agents used in humans				
Various	92	0	89	0

¹MIC = minimum inhibitory concentration.

3.4. Halophilic Gram-negative bacteria (facultative and obligate halophiles)

Aliivibrio salmonicida

Photobacterium damsela

Vibrio alginolyticus

Vibrio anguillarum

Vibrio harveyi

Vibrio parahaemolyticus

Vibrio vulnificus

Most *Vibrio* spp. are halophilic, however, only a few species are obligate halophiles and considerable evidence has demonstrated that MHA and CAMHB is sufficient for the growth and susceptibility testing of those *Vibrio* and *Photobacterium* species that are most frequently isolated from aquatic animals (Smith and Egan, 2018).

Susceptibility tests for the five *Vibrio* species in this group can be performed with the standard protocols that specify using unmodified MH media incubated at 28 °C for 24 h (CLSI, 2006, 2014a). *Photobacterium damsela* can also be tested at this temperature but may require prolonged (48 h) incubation.

Five of these species (*P. damsela*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus*) have been reported as capable of infecting humans and it is, therefore, reasonable to assume that tests of their susceptibility could also be performed using the standard protocols that specify using unmodified MH media incubated at 35 °C (CLSI, 2015a, 2015b, 2016).

Current information suggests that it would be safer not to recommend testing at 35 °C for *V. anguillarum*.

Aliivibrio salmonicida is a psychrophilic species. Currently no standard susceptibility test protocols suitable for its susceptibility testing have been developed. VET03-A (CLSI, 2006) suggests that MH media, possibly with some supplementation, and incubation at 15 °C for six days may be required.

CLSI have suggested that if obligate halophilic vibrios are encountered, they should be tested using MHA and CAMHB incorporating an additional 1 percent NaCl. However, no standard testing protocol and no acceptable ranges for quality control reference strains have been developed for tests using media with additional NaCl. It is recommended here that, where possible, susceptibility testing of the *Vibrio* species should be performed using MH media without additional NaCl.

The acceptable ranges for reference strains given in Table 2 would provide suitable QC for *Vibrio* and related species tested on unmodified MH media. There are, however, no QC data for protocols that specify the use of MH media supplemented with additional NaCl.

3.5. *Flavobacteria* and related species

Flavobacterium branchiophilum
Flavobacterium columnare

Flavobacterium psychrophilum
Tenacibaculum maritimum

Standard protocols for MIC susceptibility testing with appropriate QC data (Table 3) have been developed for the three *Flavobacterium* species of importance in aquaculture in this group. Standard protocols have not been developed for *T. maritimum* or for disc diffusion tests for any of the three *Flavobacterium* species.

- *Subgroup 1 – F. columnare*: Standard MIC susceptibility tests have been set using dilute CAMHB with incubation at 28 °C for 44–48 h that are suitable for this species (CLSI, 2014a).
- *Subgroup 2 – F. psychrophilum* and *F. branchiophilum*: *F. branchiophilum* is closely related to *F. psychrophilum* and in the absence of evidence to the contrary it has been assumed that it can be tested under the conditions that have been adopted for *F. psychrophilum*. Standard MIC susceptibility tests have been set using dilute CAMHB with incubation at 18 °C for 92–96 h that are suitable for these two species (CLSI, 2014a). Disc diffusion tests performed at 18 °C for 92–96 h have been shown to generate data of low precision and their use is not recommended for these species.
- *Subgroup 3 – Tenacibaculum maritimum*: No standard susceptibility tests have been set suitable for this subgroup. It has, however, been reported that the susceptibility (MIC) of isolates of *T. maritimum* has been determined using diluted CAMHB (1:7) with inorganic ion supplementation and incubation at 25 °C for 48 h.

The availability of QC data for MIC tests performed at 28 °C and 18 °C employing dilute CAMHB with no additions is shown in Table 3.

Table 3. Availability of quality control (QC) data for minimum inhibitory concentration (MIC) susceptibility tests performed on dilute Mueller-Hinton agar (MHA) or cation adjusted Mueller-Hinton broth (CAMHB) with respect to the agents most commonly used in aquaculture (Annex 2)

Conditions	28 °C for 44–48 h		18 °C for 92–96 h	
Reference strain ¹	<i>E. coli</i>	<i>A. sal</i>	<i>E. coli</i>	<i>A. sal</i>
Agents commonly used in aquaculture				
Amoxicillin/ampicillin	Yes	Yes	Yes	Yes
Chloramphenicol				
Doxycycline				
Enrofloxacin	Yes	Yes		Yes
Erythromycin	Yes	Yes	Yes	Yes
Florfenicol	Yes	Yes	Yes	Yes
Flumequine	Yes	Yes	Yes	Yes
Nitrofurantoin				
Josamycin				
Neomycin				
Ormetoprim/sulfadimethoxine	Yes	Yes		Yes
Oxolinic acid	Yes	Yes	Yes	Yes
Oxytetracycline	Yes	Yes	Yes	Yes
Sulphamethoxazole				
Thiamphenicol				
Trimethoprim				
Trimethoprim/sulfamethoxazole	Yes	Yes	Yes	Yes

¹*E. coli* = *Escherichia coli*, *A. sal* = *Aeromonas salmonicida*.

3.6. Gram-positive cocci

Mesophilic species

Aerococcus viridans

Lactococcus garvieae

Streptococcus agalactiae

Streptococcus dysgalactiae

Streptococcus iniae

Streptococcus phocae

Weissella spp.

Psychrophilic species

Lactococcus piscium

Vagococcus salmoninarum

For susceptibility test on *Streptococcus* spp. isolated from warm-bodied animals and performed at 35 °C, CLSI recommend that for disc tests MHA supplemented with 5 percent defibrinated sheep blood should be used (CLSI, 2015b). For MIC tests, they recommend supplementation of CAMHB with 5 percent lysed horse blood and incubation in an atmosphere with 5 percent CO₂ (CLSI, 2015a).

Standard protocols using these media together with the acceptable ranges for reference strains have been published. Interpretive criteria (human clinical breakpoints) for *Streptococcus* spp. tested using these protocols have also been developed (M100-A27).

In discussing the susceptibility testing of *Streptococcus* spp. isolated from aquatic animals, CLSI documents VET03-A and VET04-A2 (CLSI, 2006, 2014a) have followed the recommendations concerning supplementation with blood products but have suggested that temperatures >35 °C may be necessary. However, no standard testing protocols, acceptable

ranges for reference strains or interpretive criteria have yet been developed for data generated using blood-supplemented MH media at temperatures <35 °C.

There are, however, some unresolved uncertainties with the media and temperature suitable for susceptibility testing of the Gram-positive (G+ve) cocci listed above. The literature relating to these uncertainties is treated in more detail in Annex 3 but a summary is presented here.

With respect to the suggestion in VET03-A and VET04-A2 (CLSI, 2006, 2014a) that some *Streptococcus* spp. isolated from aquatic animals may require incubation temperatures <35 °C, it should be noted that there are reports that all the mesophilic G+ve cocci being considered here have been isolated from humans or other warm-blooded animals. Thus, it is reasonable to infer that their growth may not be impaired at 35 °C. There are also published papers that have reported susceptibility testing of all of the seven species at ≥35 °C.

It would seem reasonable to suggest that the susceptibility of all the mesophilic G+ve cocci listed as of importance in aquaculture could be tested at 35 °C using the standard protocols provided in the CLSI guidelines M02-A12, M07-A10, M45-A3 or VET01-S5 (CLSI, 2015a, 2015b, 2016, 2017a). This temperature would not be suitable for the two psychrophilic species *L. piscium* and *V. salmoninarum*. For these two species, testing at 22 °C would be appropriate.

With respect the appropriate media, VET03-A and VET04-A2 (CLSI, 2006, 2014a) suggest that MHA and CAMHB are inadequate for susceptibility testing of many streptococci. As a consequence, they recommend the supplementation of these media with 5 percent defibrinated sheep blood for disc tests and 5 percent lysed horse blood for MIC tests and incubation in 5 percent CO₂. It should be noted that there are no standard protocols, acceptable ranges for reference strain or interpretive criteria for tests performed with blood product supplementation at temperatures ≤28 °C.

There are, however, published papers that demonstrate that susceptibility testing of six of these G+ve species has been performed using unmodified MHA or CAMHB without any addition of blood derived products.

Conclusions

It is probably unwise at this stage to make definitive recommendations about the most appropriate standard protocols to be employed in susceptibility testing of the G+ve cocci isolated from aquatic animals.

Although it is possible that all the G+ve cocci could be tested using blood-supplemented MH media at 28 °C or 22 °C, there are no standard protocols and no quality control criteria for tests performed under these conditions.

It would appear that all the mesophilic species could be tested using the existing standard protocols that specify the use of blood-supplemented MH media at 35 °C (CLSI, 2015a, 2015b).

The mesophilic species, with the possible exception of *A. viridans* and *S. phocae* could also be tested using standard protocols that specify the use of unmodified MH media and incubation at either 35 °C (CLSI, 2015a, 2015b) or 28 °C (CLSI, 2006, 2014a).

Currently available evidence would suggest that the susceptibility of the psychrophilic species *V. salmoninarum* could be established using the standard protocols that specify unmodified MH media and incubation at 22 °C (CLSI, 2006, 2014a, VET04-A2).

A final decision as to which of the three possible standard protocols to adopt in testing mesophilic G+ve cocci may be influenced by the experience and expertise of the laboratory and the appropriateness of the available quality control (Tables 4 and 5) and interpretive criteria.

Table 4. Availability of quality control (QC) data for susceptibility tests performed at 35 °C with and without additions¹

Additions	MIC		Disc	
	None	5% LHB 5% CO ₂	None	5% SRB 5% CO ₂
Reference strain	<i>E. coli</i>	<i>S. pneumoniae</i>	<i>E. coli</i>	<i>S. pneumoniae</i>
Agents commonly used in aquaculture				
<i>Amoxicillin/ampicillin</i> ²	Yes	Yes	Yes	Yes
Chloramphenicol	Yes	Yes	Yes	Yes
<i>Doxycycline</i>	Yes		Yes	
<i>Enrofloxacin</i>	Yes		Yes	
<i>Erythromycin</i>	Yes	Yes	Yes	Yes
<i>Florfenicol</i>	Yes		Yes	
Flumequine	Yes			
Nitrofurantoin	Yes		Yes	
<i>Josamycin</i>				
<i>Neomycin</i>				
<i>Ormetoprim-sulfadimethoxine</i>				
Oxolinic acid	Yes			
<i>Oxytetracycline</i>	Yes			
Sulphamethoxazole				
Thiamphenicol				
Trimethoprim	Yes		Yes	
<i>Trimethoprim-sulfamethoxazole</i>	Yes	Yes	Yes	Yes
Additional agents used in humans				
Various	92	25	89	5

¹ MIC = minimum inhibitory concentration, LHB = lysed horse blood, SRB = sheep red blood cells, *E. coli* = *Escherichia coli*, *S. pneumoniae* = *Streptococcus pneumoniae*.

²Agents of particular importance in the therapy of Gram-positive infections of aquatic animals are italicized.

Table 5. Availability of QC data for susceptibility tests performed at 28 °C with and without additions¹

Additions	MIC		Disc	
	none	5% LHB 5% CO ₂	none	5% SRB 5% CO ₂
Reference strain	<i>E. coli</i>	<i>S. pneumoniae</i>	<i>E. coli</i>	<i>S. pneumoniae</i>
Agents commonly used in aquaculture				
<i>Amoxicillin/ampicillin</i> ²	Yes		Yes	
Chloramphenicol				
<i>Doxycycline</i>				
<i>Enrofloxacin</i>	Yes		Yes	
<i>Erythromycin</i>	Yes		Yes	
<i>Florfenicol</i>	Yes		Yes	
Flumequine	Yes		Yes	
Nitrofurantoin	Yes		Yes	
<i>Josamycin</i>				
<i>Neomycin</i>				
Ormetoprim-sulfadimethoxine			Yes	
Oxolinic acid	Yes		Yes	
<i>Oxytetracycline</i>	Yes		Yes	
Sulphamethoxazole				
Thiamphenicol				
Trimethoprim				
<i>Trimethoprim-sulfamethoxazole</i>	Yes		Yes	
Additional agents used in humans				
Various	0	0	0	0

¹ MIC = minimum inhibitory concentration, LHB = lysed horse blood, SRB = sheep red blood cells, *E. coli* = *Escherichia coli*, *S. pneumoniae* = *Streptococcus pneumoniae*.

² Agents of particular importance in the therapy of Gram-positive infections of aquatic animals are italicized.

3.7. *Mycobacteria and related species*

There are five mycobacterial species reported as of importance in aquaculture:

<i>Mycobacterium fortuitum</i>	<i>Nocardia crassostreae</i>
<i>Mycobacterium marinum</i>	<i>Nocardia seriolae</i>
<i>Nocardia asteroides</i>	

Susceptibility testing of these species will not be treated in detail here. However, four of the species of interest (*M. fortuitum*, *M. marinum*, *N. asteroides* and *N. crassostreae*) have been reported to infect humans. The CLSI document M24-A2 (CLSI, 2011) provides comprehensive details of the standardized protocols that may be applied to susceptibility testing of acid-fast human pathogens.

Also included in CLSI document M24-A2 (CLSI, 2011) are proposed antimycobacterial agents to be studied and the MIC values indicating resistance of *M. marinum*, as well as broth microdilution breakpoints for testing *Nocardiae*.

3.8. Miscellaneous species

Standard protocols (and the relevant QC requirements) have not been set for five miscellaneous species that are of interest:

Clostridium botulinum

Francisella noatunensis

Hepatobacter penaei

Piscirickettsia salmonis

Renibacterium salmoninarum

Recommended conditions for testing have, however, been suggested in the CLSI VET04-A2 (CLSI, 2014a) for susceptibility testing of *F. noatunensis*, *R. salmoninarum* and *P. salmonis*.

There are at present insufficient data to formulate standard protocols for these species. The reason for publishing suggested testing conditions is to encourage laboratories to focus on these and to generate the data required to formulate a standardized protocol.

4. THE AIMS AND DESIGN OF STUDIES TO INVESTIGATE ANTIMICROBIAL AGENT SUSCEPTIBILITY OF BACTERIA ISOLATED FROM AQUATIC ANIMALS

4.1. Introduction

The aims of any study, why it is being performed, will have a major influence on its design and the experimental protocols that should be adopted. This section addresses the influence of the aims of any study of the antimicrobial susceptibility of bacteria associated with aquaculture or aquacultural products on its design.

Four of the possible aims are considered below:

1. Investigations performed to establish the susceptibility to the antimicrobial agents used as therapeutic agents in aquaculture of the bacterial species that are the primary targets of that use.
2. Investigations performed to investigate the public health implications of the presence of bacteria with reduced antimicrobial susceptibility in aquacultural products.
3. Investigations performed to investigate the public health implications of antimicrobial agent use in aquaculture mediated through aquacultural products.
4. Investigations performed to investigate the impact of antimicrobial agent use in aquaculture on the susceptibility to antimicrobial agents of the microflora in the local environment of aquaculture enterprises.

4.2. Investigations of the susceptibility of pathogens of aquatic animals

Rationale

Information about the susceptibility of the bacterial species that are the primary target of antimicrobial use in aquaculture is of value in:

- informing the selection of the agent to be used in any disease situation. Correct choice of agent will reduce the use of agents in disease situations when, due to the lack of susceptibility of the target species, they cannot have any benefit to the farmer or the aquatic animals;
- monitoring the consequences of antimicrobial agent use in aquaculture in a region or in a specific aquatic animal host; and
- monitoring the changes in susceptibility of specific bacterial species over time.

Relevant bacterial species

The bacterial species to be prioritized in studies of this type are those that are the most frequent target of therapeutic (or prophylactic) antimicrobial agent use in the region or in the host species.

It is important that the taxonomic status of the isolates is confirmed using a recognized (frequently polymerase chain reaction (PCR) based) method.

Relevant antimicrobial agents

The antimicrobial agents to be prioritized in studies of this type are those that are the most frequently used in aquaculture as therapeutic (or prophylactic) treatments in the region or in the treated aquatic host species. A list of the most commonly used antimicrobial agents used in aquaculture is given in Annex 2.

Strain collection protocols

For this class of investigation, the strain set should as far as possible be representative of the strains infecting the aquatic animals under consideration in the particular geographic area and during a particular (defined) time period.

The *OIE Aquatic Animal Health Code (2018)* (OIE, 2018a) has suggested that in investigations of this type, possibly the most effective and cost-efficient method of strain collection would be to access isolates from laboratories involved in disease diagnosis. This method of collection would maximize the chances that the strains collected were capable of infecting aquatic animals and would, therefore, have been the target of antimicrobial therapies.

Additional strains could be collected during investigation-specific sampling of aquatic animals and their immediate environment in the absence of any disease. However, there is a risk that strains isolated in such sampling programmes may not be pathogenic variants of their species.

Susceptibility testing protocols

A discussion of the susceptibility testing protocols appropriate to this class of investigations is presented in Section 3.

Interpretive criteria

Epidemiological cut-off values are most appropriate interpretive criteria for this class of investigations. There is an almost complete lack of clinical breakpoints relevant to the antimicrobial treatment of aquatic animals.

4.3. Investigations of the public health implications of the presence in aquacultural products of bacteria with reduced susceptibility to antimicrobial agents

Rationale

There are some bacterial species that can infect humans after they have consumed, or been in contact with, aquacultural products. Some, but not all, of these infections may require or respond to antimicrobial therapy of the infected humans.

Investigations of this type often aim to establish the risk to human health represented by the presence, in aquacultural products, of bacteria with a reduced susceptibility to those antimicrobial agents important in human medicine.

Relevant bacterial species

The bacterial species to be prioritized in studies of this type are those that are known to cause infections in humans following consumption of or contact with aquatic animals. Those bacterial species should further be prioritized on the basis of whether the infections that they are associated with in humans require or respond to antimicrobial therapy.

It is important that the taxonomic status of the isolates is confirmed using a recognized (frequently PCR based) method.

Relevant antimicrobial agents

The antimicrobial agents to be prioritized in studies of this type are those that are the most frequently used in humans as therapeutic treatments of the food-borne or contact-transmitted infections associated with aquacultural products.

Strain collection protocols

Strains for this type of investigation would be most efficiently collected from aquaculture products at the “point-of-sale”.

Statistically validated protocols governing the performance of microbiological sampling of animal products provided in the *OIE Terrestrial Animal Health Code* (OIE, 2018b) should be consulted.

Susceptibility testing protocols

Standardized protocols appropriate for use in these investigations have been published by CLSI (2015a, 2015b, 2016) and EUCAST³.

Interpretive criteria

Either epidemiological cut-off values or clinical breakpoints can be applied to the quantitative data generated in these investigations. If the aim of the study is limited to assessing the impact on the therapy of infections of humans by the bacterium being tested, then human clinical breakpoints may be the most appropriate. If, however, the study was interested in the contribution of the bacterium being studied to the total human resistome, then epidemiological cut-off values would be most appropriate.

³Available at www.eucast.org

4.4. Investigations of the public health implications of antimicrobial agent use in aquaculture mediated through aquacultural products

Rationale

Investigations of this type are similar in many ways to investigation outlined in Section 4.3. They differ in the fact that they are aimed at establishing only the additional risk to human health resulting from the use of antimicrobial agents in aquaculture.

In designing these studies it must be noted that bacteria with reduced susceptibility to antimicrobial agents in aquacultural products may have originated from three sources:

- a) They may have been present in the inputs (water, feed) of the aquaculture facility.
- b) They may have emerged as a response to the selective pressure exerted by antimicrobial agent use in the facility.
- c) They may have been present due to post-harvest contamination of the aquaculture product.

Investigations of this type are performed to gain information on the bacteria with reduced susceptibility that emerged as a consequence of the selective pressure exerted by antimicrobial use in aquaculture facilities (source b).

Relevant bacterial species

The bacterial species to be prioritized in studies of this type are the same as in the investigations discussed in Section 4.3.

Relevant antimicrobial agents

The antimicrobial agents to be prioritized in studies of this type are the same as in the investigations discussed in 4.3.

Strain collection protocols

These investigations aim to detect increases in the frequency of resistance or NWT phenotypes in specific human pathogens as they are exposed to any antimicrobial agents used in an aquaculture facility. Thus, two groups of strains need to be collected.

One group would be collected to allow the determination of resistance frequencies, in the species under examination, that are present in the water supply (or feed) to the aquaculture facility. This group could only be collected during sampling programmes specifically designed for this purpose.

The other group would be collected to allow the determination of resistance frequencies, in the species under examination, that are present in the aquaculture product as it left the facility where it was grown. It is possible that the most effective way to collect these strains would be by sampling the product as it arrives at the post-harvesting processing plant.

Susceptibility testing protocols and interpretive criteria

Standardized protocols and interpretive criteria appropriate for use in these investigations are the same as those discussed in Section 4.3.

4.5. Investigations of the public health implications of antimicrobial agent use in aquaculture mediated through the environmental resistome

Rationale

Antimicrobial agent use in aquaculture may result in an increase in the frequency of strains of reduced susceptibility in the microflora of the local environment (the local resistome). The major significance of any such increase is related to the ability of the genetic elements that code for these reductions in susceptibility to transfer to bacteria of other genera and ultimately to bacteria of importance to human health.

Relevant bacterial species

Approximately 90–99 percent of the bacteria in the local environment cannot be cultured in the laboratory. Therefore, phenotypic studies of reduced susceptibility in those species or group of species that can be cultured would provide only a very limited picture of the impacts that are the subject of these types of investigations.

However, changes in the frequencies of the genetic element that encoded “resistance” can be quantified by culture-independent methods. The molecular, culture-independent methods, which are most appropriate for the investigations of this type, will not, however, be discussed in this paper.

Relevant antimicrobial agents

The antimicrobial agents to be prioritized in studies of this type should include all those of concern to human therapeutic treatments.

Strain collection protocols

No strain sets are required for these types of investigation.

5. CONCLUSIONS

In 2016, FAO published *The FAO Action Plan on Antimicrobial Resistance 2016-2020* (FAO, 2016). One of the action plan objectives envisaged in this document involved the monitoring and surveillance of antimicrobial resistance. Consistent with the “One Health” concept, the action plan argues that AMRs occurring in all environments, including those of humans, terrestrial animals and plants and aquatic animals need to be considered in a coordinated and integrated manner. A necessary prerequisite for such integrated studies of AMR is the development, acceptance and application of internationally standardized methods of detecting AMR.

This technical paper provides a review of the current state, as of 2018, of standard susceptibility testing protocols, their quality control requirements and their respective interpretive criteria for bacteria isolated from aquatic animals. This review demonstrates that internationally standardized susceptibility testing protocols have been developed and published for the majority of the bacterial species encountered in aquaculture. There would appear to be no reason why, when they are available, these standard protocols are not adopted in all future monitoring and surveillance programmes. However, a serious lack of harmonized consensus epidemiological cut-off values was identified. The work needed to set these values was discussed and it is suggested that such studies should be given some priority.

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Annex 1.
CLSI documents cited in this Circular

Code	Document title
M02-A12	Performance standards for antimicrobial disc susceptibility tests
M07-A10	Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically
M24-A2	Susceptibility testing of <i>Mycobacteria</i> , <i>Nocardiae</i> , and other aerobic <i>Actinomycetes</i>
M37-A3	Development of <i>in-vitro</i> susceptibility testing criteria and quality control parameters for veterinary antimicrobial agents
M45-A3	Methods for antimicrobial dilution and disc susceptibility testing in infrequently isolated or fastidious bacteria
M100-S27	Performance standards for antimicrobial susceptibility testing
VET01-S5	Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals
VET03-A	A Method for antimicrobial disk susceptibility testing of bacteria isolated from aquatic animals
VET04-A2	Methods for broth dilution susceptibility testing of bacteria isolated from aquatic animals

Notes:

An updated version of the material in VET03-A and VET04-A2 is expected to be published as VET03-A2 in 2019 or 2020.

An updated version of the material in VET03/ VET04-S2 is expected to be published as VET04-S3 in 2019 or 2020.

Annex 2.
Antimicrobial agents used in aquaculture

There is no comprehensive list of all the agents used in global aquaculture. The following provisional list, however, probably includes those agents that are used most frequently.

Class	Agent	Disc content
Anti-folates		
	Trimethoprim/Sulfamethoxazole	1.25/23.75 µg
	Trimethoprim	5 µg
	Sulfonamides	250 or 300 µg
β-lactams		
	Amoxicillin	
	Ampicillin ¹	10 µg
Macrolides		
	Erythromycin	15 µg
	Josamycin	
	Neomycin	
Nitrofurans		
	Nitrofurantoin ²	300 µg
Phenicols		
	Chloramphenicol ²	30 µg
	Florfenicol	30 µg
	Thiamphenicol	
Quinolones		
	Oxolinic acid	2 µg
	Flumequine	30 µg
	Enrofloxacin	5 µg
Tetracyclines		
	Oxytetracycline	30 µg
	Doxycycline	30 µg

¹ Ampicillin is not frequently used in aquaculture but can be used as a reporter for amoxicillin.

² The use of these agents in food-producing animals is banned in most countries.

Notes:

Cross-resistance: The frequency of cross-resistance between some agents is sufficiently high that it is not necessary to test the susceptibility to both of them. Categorization of isolates with respect to one of the following pairs can be taken as a valid indication of the appropriate categorization with respect to the other. Examples of agents that show high or complete cross-resistance:

- Ampicillin and amoxicillin
- Oxolinic acid and flumequine
- Oxytetracycline and doxycycline
- Trimethoprim/sulfamethoxazole and ormetoprim/sulfamonomethoxine
- Most sulfonamides

Innate resistances: Some bacterial species are innately resistant to particular antimicrobial agents or antimicrobial agent classes. Care should be taken in reporting these innate resistances. They must not be treated as having emerged due to any selective pressure resulting from antimicrobial agent use. In this context it should be noted that mesophilic *Aeromonas* spp. and many species of *Vibrio* are innately resistant to the aminopenicillins and other β -lactamase sensitive agents.

Anti-folates: There is evidence that the use of combined trimethoprim and sulfamethoxazole in susceptibility test may fail to detect as non-wild type (NWT) some isolates that possess genes encoding reduced susceptibility to sulfamethoxazole. Preference should be given to tests of these antimicrobial agents as separate and individual agents.

Annex 3.

Notes on selection of test protocols for selected Gram-positive Cocci

Species under consideration:

Mesophilic species

<i>Aerococcus viridans</i>	<i>Streptococcus iniae</i>
<i>Lactococcus garvieae</i>	<i>Streptococcus phocae</i>
<i>Streptococcus agalactiae</i>	<i>Weissella</i> spp.
<i>Streptococcus dysgalactiae</i>	

Psychrophilic species

<i>Lactococcus piscium</i>	<i>Vagococcus salmoninarum</i>
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This annex does not attempt to provide a comprehensive review of the literature with respect to the protocols that have been reported as being used in susceptibility testing of the Gram-positive (G +ve) species listed above. Rather it attempts to establish the answers to three questions that are relevant to the selection of susceptibility testing protocols for this group of species.

- *Have any of these species been reported as able to infect warm-blooded animals?*

It would seem reasonable to assume that any bacterium capable of infecting humans or other warm-blooded animal would be capable of sufficient growth on MHA or in CAMHB at 35 °C to allow their susceptibility to be established using protocols that specify these conditions. The ability to infect warm-blooded animals has been reported for *A. viridans* (Rasmussen, 2013), *L. garvieae* (Chan *et al.*, 2011), *S. agalactiae* (Garland *et al.*, 2011), *S. dysgalactiae* (Bengtsson *et al.*, 2009), *S. iniae* (Agnew and Barnes, 2007), *S. phocae* (Skaar *et al.*, 1994) and *Weissella* spp. (Ayeni *et al.*, 2011). Thus, all mesophilic species under consideration have been reported as capable of infecting warm-blooded animals.

- *Are there reports that the susceptibility of any of the species under consideration has been tested at 35 °C?*

The susceptibility of *A. viridans* (Christensen *et al.*, 1996; Pitkälä *et al.*, 2004), *L. garvieae* (Elliott and Facklam, 1996; Chang, Li and Lee, 2002), *S. agalactiae* (Bengtsson *et al.*, 2009; Garland *et al.*, 2011), *S. dysgalactiae* (Pitkälä *et al.*, 2004; Bengtsson *et al.*, 2009), *S. iniae* (Weinstein *et al.*, 1997), *S. phocae* (Skaar *et al.*, 1994) and *Weissella* spp. (Ayeni *et al.*, 2011) had been reported in studies that employed incubation at 35 °C. Thus, the susceptibilities of all of the seven mesophilic species under consideration have been reported in studies that used 35 °C as the incubation temperature.

With respect to *V. salmoninarum*, Didinen *et al.* (2011) reported susceptibility test performed at 37 °C but Ruiz-Zarzuela *et al.* (2005) reported that some strains of this species failed to grow at this temperature. No reports of susceptibility testing of *L. piscium* have been accessed, but Leroi *et al.* (2012) have reported that the maximum growth temperature of this species was 27 °C.

- *Are there reports that the susceptibility of any of the species under consideration has been tested on MHA or in CAMHB without their supplementation with blood products?*

The susceptibility of *L. garvieae* (Diler *et al.*, 2002; Baeck *et al.*, 2006; Didinen *et al.*, 2014), *S. agalactiae* (Amal *et al.*, 2012), *S. dysgalactiae* (Bengtsson *et al.*, 2009), *S. iniae* (Suanyuk *et al.*, 2010; Barnes pers. comm.), *V. salmoninarum* (Michel *et al.*, 1997; Didinen *et al.*, 2011) and *Weissella* spp. (Ayeni *et al.*, 2011; Jeong and Lee, 2015) has been reported in studies that employed MHA or CAMHB without blood product supplementation. Thus, there are reports of the susceptibility testing of six of the nine species under consideration using Mueller-Hinton media without blood product supplementation. Romalde *et al.* (2008) reported susceptibility testing of *S. phocae* using Mueller-Hinton media without blood product supplementation but with an additional 1 percent NaCl.

No studies reporting the determination of the susceptibility of *A. viridans* that were performed using MH media without blood product supplementation have been accessed. Although no reports of susceptibility testing of *L. piscium* have been accessed, Pothakos *et al.* (2014) have reported the growth of this species on tryptone soya agar without the addition of any blood products, indicating that it may be possible to perform susceptibility tests for this species using unmodified MHA or CAMHB.

Summary of suggestions regarding testing conditions and protocols

	35 °C		28 °C		22 °C	
	MH	MH + blood	MH	MH + blood	MH	MH + blood
<i>A. viridans</i>	D	B	D	C		
<i>L. garvieae</i>	A	B	A	C		
<i>L. piscium</i>	D	D	D	D	A	C
<i>S. agalactiae</i>	A	B	A	C		
<i>S. dysgalactiae</i>	A	B	A	C		
<i>S. iniae</i>	A	B	A	C		
<i>S. phocae</i>	?	B	?	C		
<i>V. salmoninarum</i>	D	D	D	D	A	C
<i>Weissella</i> spp.	A	B	A	C		

A: Conditions for which standard testing protocols exist with quality control (QC) data for some agents.

B: Conditions for which standard testing protocols exist with QC data for some agents and some clinical breakpoints for *Streptococcus* spp. relevant to human therapies.

C: Conditions for which standard testing protocols have been suggested but no QC data have been developed.

D: Conditions probably inappropriate for species.

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Annex 4.

On the possible use of epidemiological cut-off values in a clinical context

One of the reasons for determining the susceptibility to a particular antimicrobial agent of a bacterium isolated from an infected population of aquatic animals is to assess whether the administration of that agent to that population is likely to be therapeutically beneficial. In these situations, at least in theory, clinical breakpoints would appear to provide the most appropriate interpretive criteria for understanding the clinical significance of the *in-vitro* susceptibility data obtained. Few, if any, however, clinical breakpoints relevant to the metaphylactic treatments encountered in aquaculture have even been proposed and none have been validated. It has also been argued that it will prove extremely difficult, expensive and time consuming to generate even one of the large number of clinical breakpoints required (Smith, 2008).

Thus, for the foreseeable future, epidemiological cut-off values will be the only interpretive criteria available to guide health care professionals in attempting to understand the clinical significance of *in-vitro* susceptibility data. The central problem with this use of epidemiological cut-off values is that because they are set from a consideration of susceptibility data alone, the categories WT and NWT that they generate can have no inherent meaning in a clinical context.

Although the categories WT and NWT do not have any inherent clinical meaning, testing laboratories can use them to generate reports that may provide useful guidance to those faced with making choices concerning therapeutic options. If an isolate has been categorized as WT to a specific agent, it would be legitimate for a laboratory to report:

“Laboratory susceptibility tests have not detected any evidence of reduced susceptibility in the isolate tested. These tests provide no reason why therapy should not be initiated”

If, on the other hand, an isolate has been categorized as NWT to a specific agent, it would be legitimate for a laboratory to report:

“Laboratory susceptibility tests have detected reduced susceptibility. The isolate may contain resistance mechanisms. Initiation, or continuation with therapy would be imprudent and is likely to result in clinical failure”

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The Performance of Antimicrobial Susceptibility Testing Programmes Relevant to Aquaculture and Aquaculture Products technical paper provides good guidance to countries with respect to standard susceptibility testing protocols, the quality control requirements and the respective interpretive criteria for bacteria isolated from aquatic animals. An initial draft of this paper was produced as a discussion document for a series of regional workshops organized by FAO under the project FAO FMM/RAS/298: Strengthening capacities, policies and national action plans on prudent and responsible use of antimicrobials in fisheries. This was prepared under the auspices of FAO's Strategic Programme 4: Enable more inclusive and efficient agricultural and food systems and specifically 4.1.1: Public sector institutions are supported to improve their capacity to design and implement better policies and regulatory frameworks, and to provide public services related to plant and animal health, food safety and quality. The document: (1) provides a general background to the principles of antimicrobial susceptibility testing and stresses the absolute need for the use of internationally agreed standardized test protocols and the adherence to the quality control requirement of those protocols; (2) discusses the current status of the standard protocols that can be recommended for use in antimicrobial susceptibility testing of bacteria isolated from aquatic animals; (3) discusses the importance of the design of programmes aimed at monitoring or surveillance of antimicrobial resistance associated with the use of antimicrobial agents in the rearing of aquatic animals and outlines four designs, each of which will provide data for programmes aimed at answering different questions; and (4) examines the aims and design of studies to investigate the antimicrobial agent susceptibility of bacteria isolated from aquatic animals.

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