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OPINION PIECE

Eight rules for improving the quality of papers on the antimicrobial susceptibility of bacteria isolated from aquatic animals

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ABSTRACT: The methods for antimicrobial susceptibility testing of bacteria, although relatively simple, are not robust. As a consequence, if the data generated in such tests are to be commensurate, all susceptibility tests must be performed using standard protocols. A review of the published literature of antimicrobial susceptibility testing of bacteria from aquatic animals revealed a frequent occurrence of significant errors, particularly with regard to testing methodology, quality control and the use of appropriate interpretive criteria in the performance and reporting of susceptibility tests. This opinion piece provides a set of rules that, if followed, would help authors to avoid these frequently detected shortcomings.

KEY WORDS: Antimicrobial susceptibility testing · Standard methods · Interpretive criteria · Aquatic animals

INTRODUCTION

Smith & Egan (2018) collected 186 published papers that reported on 203 studies of antimicrobial resistance in non-cholera *Vibrio* spp. A review of these papers revealed some serious shortcomings in the methods used and/or the descriptions of those methods in these studies (Smith & Egan 2020). For example, only 72 of the 200 studies examined claimed to have used a standardised testing protocol. However, 34 of these stated that they made modifications to the standard protocol, with the result that only 38 (19%) had actually used the testing conditions specified in the standard protocol. Compliance with the quality control (QC) requirements of a protocol is an essential requirement, if a laboratory wishes to claim that their data were generated by that protocol. Many of the 38 studies mentioned using QC reference strains, but only 4 provided a reference to an appropriate source of the acceptable ranges for the reference strains they used. Only one provided a comment that the results they obtained were actually within the acceptable range. The dramatic conclusion of the review was that 99.5% of the 200 studies failed to provide explicit evidence that the data they reported had been obtained using standard testing protocols.

Problems with the way in which susceptibility data have been reported are not confined to studies of bacteria isolated from aquatic animals. Turner & Ashley (2019) and Schwarz et al. (2010) commented that non-compliance with stated laboratory methods also appears to be relatively common in the published literature on the susceptibility of isolates from humans and veterinary animals. In a joint editorial published in the *Journal of Antimicrobial Chemotherapy and Veterinary Microbiology*, Schwarz et al. (2010) identified a number of shortcomings that regularly occur in papers reporting antimicrobial susceptibility. They provided a very valuable and detailed set of recom-

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mendations as to how such shortcomings could be avoided in reporting on susceptibility testing of isolates from land-based agriculture. However, the susceptibility testing of isolates from aquaculture faces some unique problems, particularly in the availability of interpretive criteria. For this reason, this opinion piece addresses the improvement of the presentation of data on the susceptibility of isolates from aquatic animals. Eight rules are suggested that, if followed, would result in a significant improvement in the future quality and utility of papers published in this area.

RULE 1. USING STANDARD SUSCEPTIBILITY TESTING PROTOCOLS

All papers on the susceptibility of bacteria should present only data that have been obtained using internationally recognised and standardised testing protocols. They should provide a reference to the source of the protocol they used and should provide explicit evidence of compliance with the QC requirements of that protocol.

Standardised susceptibility testing protocols have been published for tests performed at 35°C by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org/ast_of_bacteria/) and the Clinical and Laboratory Standards Institute (CLSI 2007, 2015, 2016) and for tests performed at <35°C by CLSI (2020a). Between them, these protocols provide test conditions that are suitable for testing 37 of 44 (85%) of the species encountered most frequently in aquatic studies (www.fao.org/3/ca6028en/ca6028en.pdf). Except for the very few, and rarely occurring, species for which suitable standard protocols are not yet available, there would appear to be no valid reasons why all studies of the susceptibility of isolates from aquatic animals should not use one of these standardised protocols.

As noted by Schwarz et al. (2010), the detailed instructions provided in standard protocols are not optional but are strict rules that must be adhered to. When using a standard protocol, it is never acceptable to alter parameters, such as the incubation temperature or times or the media composition. If such changes are made, the data must be presented as having been obtained using a new and non-standardised protocol. In this context, it should be noted that it is never legitimate to apply QC requirements or interpretive criteria developed for a standard testing protocol to data generated using a non-standard protocol, even if the 2 protocols show many similarities.

Essential QC components of all standard susceptibility test protocols are the acceptable ranges of susceptibility measures for a specific agent that must be achieved for QC reference strains when using that protocol. If a paper claims to have used a standard susceptibility test protocol, it must cite the source of the QC requirements relating to that protocol. It must also include quantitative data demonstrating that these QC requirements have been met and that the reference strains were tested with the required frequency.

RULE 2. INTERPRETIVE CRITERIA

Any paper that presents the meaning of the raw, observational data obtained must present the interpretive criteria that were used to establish that meaning. When internationally harmonised consensus criteria are available, these must be used, and their source must be referenced. When consensus criteria are not available, full details of the methods by which the meanings of the observational data were established must be given.

Either clinical breakpoints or epidemiological cutoff values may be used to provide interpretive criteria. It should be noted that both are protocol-specific and, therefore, limited in the data to which they can be applied. They can be applied only to data for the specified agent against the members of a specified bacterial group, usually a defined species, which had been generated by the standard protocol for which they were developed. It is never legitimate to apply criteria developed for a protocol specifying one set of test conditions to data generated using a different protocol or different test conditions. Equally, it is never legitimate to apply criteria developed for one bacterial group to data generated in studies of a different bacterial group.

In contrast to the wide-spread availability of interpretive criteria that can be applied to susceptibility data for bacteria that are isolated from humans and other terrestrial animals, there have been very few such criteria published for data isolated from aquatic animals.

Clinical breakpoints aim to give information on the probable clinical outcome of the treatment with a specified dose regimen of an antimicrobial agent of an infection of a specified host by the bacterium being tested. Clinical breakpoints have been published by CLSI for *Aeromonas* spp., *Vibrio* spp. and *Streptococcus* spp. (CLSI 2016, 2017) and by EUCAST for various streptococci (www.eucast.org/

ast_of_bacteria/). These were all developed for data generated by protocols that specify incubation at 35°C and are relevant to predicting the clinical outcomes of human infections. They cannot be validly applied to data produced at temperatures <35°C or to predict the clinical outcomes of treatments of other animals. It should also be noted that CLSI (2016) have stated that the empirical evidence for their breakpoints is considerably weaker than is normally required. The values for *Aeromonas* spp. and *Vibrio* spp. are in fact just copies of the values developed for *Enterobacteriaceae* (CLSI 2017). CLSI (2020b) have also published clinical breakpoints for data obtained at 22°C/48 h for oxytetracycline and oxolinic acid against *A. salmonicida*. The breakpoints relate to treatments of salmonid fish at low temperatures in fresh water and again the empirical evidence for them is extremely limited.

Epidemiological cut-off values on the other hand aim to categorise isolates on the basis of whether or not they possess mechanisms which reduce their susceptibility (Silley 2012). With respect to consensus epidemiological cut-off values, CLSI (2020b) published these for a limited number of species (*A. salmonicida, A. hydrophila, Flavobacterium columnare* and *F. psychrophilum*).

In summary, for application to data generated in studies of bacteria isolated from aquatic animals, very few internationally harmonised consensus interpretive criteria are currently available. Thus, many studies will have to generate their own criteria to establish the meaning of their data. Generating clinical breakpoints is an extremely difficult and complex task. In contrast, setting epidemiological cut-off values is relatively simple. Excel spreadsheets that automatically calculate these values from a statistical analysis of *in vitro* susceptibility data are available. The normalised resistance interpretation (NRI) method (www.bioscand.se/nri/) can be applied to either minimum inhibitory concentration (MIC) or disc diffusion data, and ECOFFinder (www.clsi.org/standards/micro/ecoffinder/) can be applied to MIC data. The precision with which cut-off values can be calculated by these methods increases as the number of susceptibility observations increases. However, our current understanding is that values can be calculated with reasonable precision from a data set obtained in a single laboratory that contains as few as 20–30 observations from fully susceptible isolates. When cut-off values are calculated from observations made in a single laboratory, they cannot take account of inter-laboratory variation and must, therefore, be treated as 'local' and should be applied only to data obtained in the laboratory that generated them. In setting internationally harmonised consensus values, it is generally accepted that, to allow for inter-laboratory variation, over 100 observations from at least 5 laboratories would be required. Consensus epidemiological cut-off values set from such data are not fixed but, as new data become available, are capable of continuing evolution. For example, the current epidemiological cut-off value published by EUCAST for gentamicin against *Escherichia coli* was set from a consideration of 78 138 observations made in 98 laboratories (www.eucast.org/mic_distributions_and_ecoffs/).

RULE 3. TERMINOLOGY

All papers that present the categorisation of isolates, based on the interpretation of antimicrobial susceptibility data, must ensure that they use appropriate terminology when referring to those categories.

Much confusion in the field of antimicrobial susceptibility has resulted from the use of the word 'resistant' in situations where the meaning of this word has not been defined. To reduce this confusion, it is essential that all papers follow the recommendations of Silley (2012) with respect to the terminology used. He argued that the word 'resistant' should only be used when it refers to clinical resistance. Thus, the categorisation of an isolate as 'resistant' should always be taken to mean that, because of its reduced susceptibility to an agent, the treatment of infections it causes in a specified host by application of standard therapy is unlikely to be successful. It follows that an isolate can be categorised as 'resistant' only when the meaning of its *in vitro* susceptibility has been interpreted by the application of a relevant clinical breakpoint. As clinical breakpoints are established from data derived from treatments of infections of a specified host, with a specified dose of a specified agent, the meaning that can be given to the term 'resistant' is significantly limited. They are, of course, antimicrobial agent-specific but always also dose regimen-specific and host-specific. The pharmacokinetics of an agent in an aquatic animal may be significantly different from its pharmacokinetics in humans. Thus, clinical breakpoints established to predict the probable outcome of a treatment of an infection of humans should not be used to predict the probable outcome of the treatment of aquatic animals. It should also be noted that the pharmacokinetics in any aquatic animal will vary depending on the species of the treated animal and the environmental

conditions (temperature and salinity) (Rigos & Smith 2015) under which it is treated and the method of administration (oral or immersion) used (O'Grady et al. 1988). As a consequence, these parameters will impose further limitations on the application of the term 'resistant' in the context of bacteria isolated from aquatic animals.

Epidemiological cut-off values are set from a consideration of the distribution of *in vitro* susceptibility data only and take no account of any clinical parameters. Thus, following Silley (2012), the categories identified by their application must never be termed as 'resistant' or 'sensitive'. These cut-off values are calculated statistically as the limit values for the susceptibility measures for fully susceptible members of a bacterial species or group of species. For isolates whose susceptibility is not distinguishable from those manifested by the fully susceptible members of their group, the correct terminology is wild-type (WT). For those whose susceptibility is different from that of WT isolates, the correct terminology is non-wild-type (NWT). As with all interpretive criteria, the numerical values that define the categories WT and NWT are protocol- and agent-specific. However, the terms WT and NWT refer to properties of the isolates themselves and, as opposed to the terms 'resistant' and 'sensitive', their meanings are not affected by the aims of the study in which the bacteria were isolated or the parameters of any therapeutic use.

RULE 4. TAXONOMY

All papers should provide adequate details of the methods by which the taxonomic status of the isolates studied was established.

It is essential that, in any paper reporting the susceptibility of isolates from aquatic animals, the taxonomic status of the isolates studied is established. The most appropriate taxonomic method will vary according to the species or group being investigated, and it is not possible to make overall recommendations as to those that are appropriate for all groups. Authors should, however, ensure that they use an up-to-date method whose use has been validated for the group they are studying.

Current best practice is that species-specific interpretive criteria should be developed. However, modern taxonomic methods have led to a rapid increase in the number of species in genera that contain members that infect aquatic animals. For example, over 100 species of *Vibrio* are currently recognised (Romalde et al. 2014). It may well prove necessary and possible to develop interpretive criteria that can be applied to multi-species groups. The decision to develop multi-species interpretive criteria will not reduce the requirement for accurate taxonomic identification of isolates; rather, it would make it even more essential.

RULE 5. RAW DATA

All papers should provide quantitative measurements of the *in vitro* susceptibility of the isolates studied in an unprocessed form.

The Aquatic Animal Health Code (www.oie.int/en/international-standard-setting/aquatic-code/access-online/) recommends that all reports of studies of susceptibility in isolates from aquatic animals should present the raw, quantitative and unprocessed laboratory data obtained in the study (Smith et al. 2013). There are 2 major reasons for this recommendation. The first is that if the raw data are published and subsequently developments in the relevant interpretive criteria occur, the meaning of those raw data can be reinterpreted. The second is that if they are available, the data can be combined with other data produced for the same bacterial group and using the same testing protocol either to produce new epidemiological cut-off values or to facilitate the continued evolution of existing ones.

The prevalence of journals that now allow the publication of raw data in supplementary files greatly facilitates the publishing of the raw susceptibility data. When this option is not provided by the journal, and authors provide their raw data within the main text, preference should be given to the use of tables rather than histograms. In situations where it proves impossible to present raw data in a publication, authors must provide details of how or from whom those data could be obtained.

RULE 6. ISOLATES WITH REDUCED SUSCEPTIBILITY TO MULTIPLE AGENTS

In calculating frequencies of isolates with reduced susceptibility to multiple agents, papers should not use data on the susceptibility to multiple members of the same antimicrobial agent class or to an agent to which the species under consideration is innately resistant.

The term multiple drug resistance (MDR) occurs frequently in the literature. However, following Rule 3, this term should only be used when the isolates

have been categorised as resistant by the application of clinical breakpoints to interpret the *in vitro* susceptibility data obtained from them. When these data have been interpreted using epidemiological cut-off values, a more appropriate term would be multiple drug reduced susceptibility (MDRS).

Schwarz et al. (2010) provided a detailed discussion on this issue with respect to isolates from terrestrial animals. Their recommendations are equally relevant to studies of isolates from aquatic animals and will not be repeated here. Anybody planning to report on the frequency of multiresistance in isolates from aquatic animals should consult this paper.

RULE 7. ABBREVIATIONS

All papers should as far as is possible use standard acronyms or abbreviations.

In referring to the epidemiological cut-off values, CLSI uses the acronym 'ECV' and EUCAST uses 'ECOFF'. It is strongly suggested that these acronyms are only used to refer to cut-off values that have been set by these bodies. In order to reduce confusion, the acronym CO_{WT} (wild-type cut-off) should be used for epidemiological cut-off values other than those set by these agencies.

In the published literature, there have also been many variations in the acronyms or abbreviations used to refer to antimicrobial agents. EUCAST published a System for Antimicrobial Abbreviations which provides rules for generating these acronyms (www.eucast.org/ast_of_bacteria/guidance_documents/). It is strongly suggested that these EUCAST rules should be followed in all future publications.

RULE 8. PRECISION

All papers should provide quantitative estimates of the precision of any *in vitro* data they present.

Although it is addressed here in Rule 8, the precision of any *in vitro* susceptibility data set is in many ways the most critical issue. If the primary data are imprecise, any categorisation of isolates based on them will be inaccurate.

Smith et al. (2018) demonstrated that the standard deviations in data generated by NRI and ECOFFinder analyses of the distributions of WT zone sizes and the $\log_2$ transformed MIC values provide a measure of precision of any susceptibility data set. These standard deviations should always be presented for each data set analysed.

Smith et al. (2018) also calculated provisional limit values for these standard deviations. From an analysis of 137 MIC data sets, they calculated that 97.7% of the standard deviations calculated by NRI were >1.19 $\log_2$ µg ml^{-1}. They, therefore, suggested that MIC data sets for which the standard deviations calculated by NRI were ≤ 1.2 $\log_2$ µg ml^{-1} should be considered as excessively imprecise. A similar approach to the available disc zone data was made. For zone data obtained at 35°C, a precision limit of ≤3.38 mm was calculated from 40 data sets, and for zone data obtained at 28°C a precision limit of ≤3.95 mm was calculated from 43 data sets. For zone data obtained at 22°C, a precision limit of ≤6.49 mm was calculated, but as this was based on the analysis of only 26 data sets, the authors suggested that this limit value should be treated as a provisional estimate. As these suggested limit values are based on analyses of published data, they will be modified as more data become available. However, it is argued that if the standard deviations for any data set presented in a paper exceed these limit values, this evidence of excessive imprecision must be mentioned in the text.

The most common source of imprecision in susceptibility data derives from the performance of the susceptibility tests themselves. Although these tests are technically relatively simple to perform, they are not robust. Minor changes in their performance can produce significant changes in the data obtained. Thus, as with all tests that lack robustness, the quality of the data they generate is a function of the experience of the operator who performs them. An operator with good bench skills and significant experience must be seen as the most valuable member of any group wishing to investigate antimicrobial susceptibility phenotypes.

CONCLUSIONS

It is argued that the adoption of these 8 rules would be highly cost-effective. As, in the main, they relate to the design of studies and the manner in which data are presented, their adoption would not require a laboratory to perform extra work. Thus, the cost of their implementation would be small, whereas the benefits would be significant. Their adoption would result in an increase in the meaning that could be given to the data presented in any individual paper. Possibly more significantly, it would also facilitate the correlation of the data produced in different papers.

The literature on antimicrobial susceptibility of bacteria isolated from aquatic animals has generated

large amounts of data. However, only when we know what they mean can data become useable information. Currently, because of difficulties in establishing the meaning of the data presented, reading the literature provides limited information. The rules presented here are designed to increase and standardize the information content of papers.

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LITERATURE CITED

CLSI (2007) Method for dilution antimicrobial susceptibility testing for bacteria that grow aerobically. CLSI document M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA

CLSI (2015) Performance standards for disk susceptibility tests. CLSI document M02-A12. Clinical and Laboratory Standards Institute, Wayne, PA

CLSI (2016) Methods for antimicrobial dilution and disc susceptibility testing of infrequently isolated or fastidious bacteria. CLSI guideline M45-A3. Clinical and Laboratory Standards Institute, Wayne, PA

CLSI (2017) Performance standards for antimicrobial susceptibility testing; twenty-seventh informational supplement. CLSI Document M100-S27. Clinical and Laboratory Standards Institute, Wayne, PA

CLSI (2020a) Methods for antimicrobial broth dilution and disk diffusion susceptibility. Testing of bacteria isolated from aquatic animals, 2nd edn. CLSI guideline VET03-A2. Clinical and Laboratory Standards Institute, Wayne, PA

CLSI (2020b) Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals, 3rd edn. CLSI supplement VET04-S. Clinical and Laboratory Standards Institute, Wayne, PA

O'Grady P, Moloney M, Smith PR (1988) Bath administration of the quinoline antibiotic flumequine to brown trout *Salmon trutta* and Atlantic salmon *S. salar*. Dis Aquat Org 4:27–33

Rigos G, Smith P (2015) A critical approach on pharmacokinetics, pharmacodynamics, dose optimization and withdrawal times of oxytetracycline in aquaculture. Rev Aquacult 7:77–106

Romalde JL, Diéguez AL, Lasa A, Balboa S (2014) New *Vibrio* species associated to molluscan microbiota: a review. Front Microbiol 4:413

Schwarz S, Silley P, Simjee S, Woodford N, van Duijkeren E, Johnson AP, Gaastra W (2010) Assessing the antimicrobial susceptibility of bacteria obtained from animals. J Antimicrob Chemother 65:601–604

Silley P (2012) Susceptibility testing methods, resistance and breakpoints: What do these terms really mean? Rev Sci Tech 31:33–41

Smith P, Egan S (2018) Standard protocols for antimicrobial susceptibility testing of Vibrionaceae isolated from aquatic animals. Bull Eur Assoc Fish Pathol 38:160–164

Smith P, Egan S (2020) A review of the shortcomings in the testing methods used and reporting of data in the literature on antimicrobial susceptibility. Bull Eur Assoc Fish Pathol (in press)

Smith P, Alday-Sanz V, Matysczak J, Moulin G, Lavilla-Pitogo CR, Prater D (2013) Monitoring and surveillance of antimicrobial resistance in microorganisms associated with aquatic animals. Rev Sci Tech 32:583–593

Smith P, Finnegan W, Ngo T, Kronvall G (2018) Influence of incubation temperature and time on the precision of MIC and disc diffusion antimicrobial susceptibility test data. Aquaculture 490:19–24

Turner P, Ashley EA (2019) Standardising the reporting of microbiology and antimicrobial susceptibility data. Lancet Infect Dis 19:1163–1164

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