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REVIEW

Standard protocols for antimicrobial susceptibility testing of Vibrionaceae isolated from aquatic animals

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Abstract
A review of the relevant literature has shown that it is safe to recommend that the antimicrobial susceptibility of the species of the *Vibrionaceae* most commonly isolated from aquatic animals can be established using internationally standardised protocols that specify the use of Mueller-Hinton media that has not been supplemented with additional NaCl.

Introduction

The OIE Aquatic Animal Health Code (http://www.oie.int/en/international-standard-setting/aquatic-code/access-online/) recommends that, if the data produced in programmes for monitoring and surveillance of antimicrobial susceptibility are to be comparable, it is essential that standardised and internationally harmonised susceptibility test protocols be used to the greatest extent possible (Smith et al., 2013).

The CLSI has published guidelines for the antimicrobial susceptibility testing of bacteria isolated from aquatic animals (CLSI, 2006; CLSI, 2014a). With respect to the testing of the *Vibrionaceae* these guidelines recommend that, for those that are facultative halophiles, disc diffusion tests can be performed using Mueller-Hinton agar (MHA) and MIC tests can be performed using cation adjusted Mueller-Hinton broth (CAMHB). For those *Vibrio* spp. that are obligate halophiles CLSI suggest that susceptibility tests should be performed on MHA or in CAMBB modified by the addition of 1% NaCl.

Quality control criteria are an essential component of any CLSI standardised testing protocol. The acceptable ranges of results for reference strains, which provide these criteria, have been developed for test protocols that specify the use of unmodified MHA and CAMHB and incubations at 35°C (CLSI, 2017), and at 28°C and 22°C (CLSI, 2014b). The acceptable ranges for tests using NaCl supplemented media have not yet been established. As the addition of NaCl has been shown to affect the data generated from susceptibility tests (Douglas et al., 2006) the acceptable ranges established for protocols using

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unmodified Mueller-Hinton media cannot be treated as applicable to data generated using these media supplemented with NaCl. Thus, at present, standardised susceptibility testing protocols exist for *Vibrio* species that can be treated as facultative halophiles but not for those species that are obligate halophiles.

It should be noted that Mueller-Hinton is not a defined medium and may contain some NaCl derived from the beef or casein used in its preparation. Therefore, in this paper, a species is categorised as a functional facultative halophile if its susceptibility can be adequately determined using unmodified MHA or CAMHB. If the susceptibility of a species can be tested only if these media are supplemented with additional NaCl that species must be categorised as a functional obligate halophile.

Literature search

A search of the literature was made for papers that had reported susceptibility testing of non-cholera *Vibrios* and/or *Photobacterium damselae*. A primary list of relevant papers was obtained using Google Scholar using as keywords either *Vibrio* or *Photobacterium*, with or without a species name, coupled with various combinations of the terms antibiotic, antimicrobial, susceptibility and resistance. This procedure generated a primary list that was then expanded by examining the papers that had been cited by those in the primary list. The final step in compiling the list of relevant papers was to examine those papers that had cited the papers identified in the expanded primary list. This process identified 199 papers and we were able to access full test copies of 193 of these. A full listing of the papers accessed is given in the supplementary files (available with the digital version of the manuscript in the EAFP Bulletin Archives; https://eafp.org/bulletin-archive/).

Although we do not believe that all relevant papers were accessed, we do believe that the number is adequate to provide a reasonably accurate picture of the susceptibility test conditions currently being used globally. The papers showed a reasonable global distribution. The majority (55%) were from laboratories in Asia and the remainder were from Europe (20%), America (15%), Africa (8%) and Australasia (2%). Although the earliest paper accessed had been published in 1974, the majority had been published in the present millennium with 62% being published between 2008 and 2017. The total number of citations that these 193 papers had received was 7112 and the median citations per paper was 16. The majority of the papers reported either disc diffusion or MIC studies but a few reported both. In total 158 studies were reported that used a disc diffusion method, 50 that used an MIC method and 7 that used the hybrid E-test gradient diffusion method. The papers were examined for the species investigated, the media and incubation temperature used.

Not all papers provided a species level classification of the bacteria they studied but, in those that did, studies of six species predominated. The most commonly studied was *V. parahaemolyticus* (91 studies) followed by *V. alginolyticus* (72) *V. vulnificus* (50), *V. harveyi* (49), *V. anguillarum* (20) and *P. damselae* (20).

Media used in susceptibility testing

With respect to the media used, approximately 90% of the studies stated that they used either unmodified or Mueller-Hinton media supplemented by the addition of various concentrations of NaCl. Table 1 shows the percentage of the studies for each of the dominant species that used MHA or CAMHB but that made no mention of supplementing their media with additional NaCl. These percentages varied from 40% for *V. anguillarum* to 76% for *V. vulnificus*. The data in this Table demonstrates that it would be reasonable to categorise these six species as functional facultative halophiles.

Temperature of susceptibility testing

Not all the studies reported the temperatures at which they performed their susceptibility tests. Table 2 shows an analysis of the temperatures used in the studies that specifically mentioned this parameter. The very high frequencies of studies performed at ≥28°C strongly suggest that susceptibility testing protocols which specify incubation at 28°C could be used for all the six species. It should be noted, however, that with respect to *P. damselae* CLSI have suggested that prolonged incubation time (48h) may be required at 28°C (CLSI, 2006; CLSI 2014a) and 80% of the papers reporting studies of this species also reported using prolonged incubation times. Four of the six species (*V. alginolyticus, V. harveyi, V. parahaemolyticus* and *V. vulnificus*) have been reported to be capable of infecting humans (Austin, 2010). It is, therefore, not surprising that studies in which their susceptibility testing was performed at ≥35°C were frequently reported (Table 2). For these species it would appear reasonable to recommend that their susceptibility could also be tested using protocols that specify incubation at 35°C using Mueller-Hinton media without NaCl supplementation (CLSI, 2015a; CLSI, 2015b). Evidence that *V. anguillarum* or *P. damselae* could be tested at 35°C is, however, weak or non-existent. Testing of these two species at 35°C could not be recommended.

Interpretive criteria

The interpretation of the meaning of MIC or disc zone data can be made by applying protocol-specific clinical breakpoints or epidemiological cut-off values to observational data generated by a standard protocol (Smith et al., 2013).

Clinical breakpoints for application to MIC and disc zone data for *Vibrionaceae* have been published in the CLSI guideline M45-A3 (CLSI, 2016). Three issues should, however, be considered before applying these breakpoints to the

Table 1. Frequency of the use of NaCl supplementation in susceptibility studies of six species of the *Vibrionaceae*.

Species	Number[a]	Number (%) not mentioning added NaCl
V. alginolyticus	63	35 (56%)
V. anguillarum	15	6 (40%)
V. harveyi	48	25 (52%)
V. parahaemolyticus	81	55 (68%)
V. vulnificus	42	32 (76%)
P. damselae	19	13 (68%)

[a] This Table is limited to studies that used modified or unmodified Mueller-Hinton media

Table 2. Incubation temperature, when stated, of susceptibility studies for six species of the *Vibrionaceae*.

Species	Total[a]	≥35°C	≥28°C
V. alginolyticus	50	44%	80%
V. anguillarum	17	0%	76%
V. harveyi	37	22%	84%
V. parahaemolyticus	43	65%	95%
V. vulnificus	26	35%	85%
P. damselae	15	13%	73%

[a] This Table is limited to studies that specifically mentioned their incubation temperature.

data generated in any study. The first is that they are protocol-specific and their application is valid only to data generated by those protocols (CLSI, 2015a; CLSI 2015b) that specify testing at 35°C without NaCl supplementation. The second arises from the fact that clinical breakpoints are host specific. Thus, the clinical breakpoints in M45-A3 are only relevant if the aim of the study is to establish the significance of the susceptibility data for prediction of the probable clinical outcomes of antimicrobial therapies of humans. The third is that the foreward to M45-A3 states that "the very extensive microbiological, clinical, and pharmacodynamic databases normally used for setting breakpoints by CLSI do not exist" for the *Vibrionaceae*. Thus, with the possible exception of *V. cholera*, the empirical evidence supporting the application of these clinical breakpoints to the *Vibrionaceae* must be considered as weak.

Currently no consensus epidemiological cut-off values have been published for the interpretation of *Vibrio* susceptibility data generated by any of the appropriate CLSI test protocols. However, fully automated statistically based methods for calculating these cut-off values are available. For MIC data either ECOFFinder (clsi.org/standards/micro/ecoffinder/) or normalised resistance interpretation (NRI) (http://www.bioscand.se/nri/) are available. In addition a version of NRI (http://www.bioscand.se/nri/) is available for application to disc zone data. The generation of the data to establish valid epidemiological cut-off values is, therefore a relatively simple task that should be given urgent priority.

Conclusions

This review of the literature suggests that it would be safe to recommend that the antimicrobial susceptibility of *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* could be assessed using the existing standardised protocols published in VET03-A (CLSI, 2006) and VET04-A2 (CLSI, 2014a) that specify the use of unmodified MHA or CAMHB without additional NaCl and incubation at 28±2°C for 24-28h. For the testing of *P. damselae* it may be necessary to modify these protocols by increasing the incubation time. The testing of *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* could also be performed using protocols published in M02-A12 (CLSI, 2015b) and M07-A10 (CLSI. 2015a) that specify the use of unmodified MHA and CAMHB but with incubation at 35±2°C for 16-18h.

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