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WORKSHOP REPORT

Setting epidemiological cut-off values for bacteria isolated from aquatic animals: a toolbox for designing a 96-well plate for microdilution MIC assays

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Introduction

A workshop was held at the EAFP meeting in Porto to initiate discussion on the design of studies that could generate the data needed to set epidemiological cut-off values appropriate for application to antimicrobial susceptibility data for bacteria isolated from aquatic animals. To set such cut-off values, consortia of at least five laboratories are needed. The work required from individual laboratories, although not a relatively large number, was the susceptibility testing of approximately 30 isolates of each species. What is essential is, however, that the activities of the laboratories, and the experimental protocols that they use, are coordinated. A major aim of this workshop was to encourage the formation of informal groups of laboratories that could form consortia and coordinate their activities to produce the data needed to generate cut-off values for various species that were of interest to them. All laboratories with an interest in the possibility of joining such a consortia were, and are still, invited to contact Sandrine Baron (Sandrine.BARON@anses.fr) who has agreed to facilitate this activity.

In the hope that it could provide a template for studies appropriate to other species, the workshop presented a work schedule that had been developed by a multi-national consortium of laboratories for setting epidemiological cut-off values for minimal inhibitory concentration (MIC) and disc diffusion data for *Vibrio parahaemolyticus*, *V. vulnificus* and *V. anguillarum*. In this work schedule the MIC values were to be determined by the standard broth microdilution protocol provided in VET04-A2 (CLSI, 2014), that specifies the use of cation-adjusted Mueller-Hinton broth with no supplementary NaCl added. The disc diffusion zones were to be determined by the protocol provided inVET03-A (CLSI, 2006) that specifies the use of unmodified Mueller-

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Hinton agar. The incubation conditions for both methods were set as 28 ± 2 °C for 24-28h. The quantitative aspects of this work schedule were based on SOP 10.0 published by EUCAST (www.eucast.org >documents >sops) that presented the quantitative requirements for the data needed to set epidemiological cut-off values from MIC data. In essence this SOP requires that the observations (MIC values) must be made from at least five independent laboratories. The total number of observations must include >100 from independent isolates that were fully susceptible with respect to the agent being tested. No single laboratory should contribute > 50% of the observations and no laboratory should contribute < 15 observations from fully susceptible strains. Although these requirements had been set for MIC data they were used as guidelines in designing this work schedule for generating both the MIC and disc diffusion data.

This paper will not describe the details of this work schedule adopted for the *Vibrio* study, which can be obtained on application to the authors. However, in developing the work schedule it became clear that the design of the 96-well plates to be used in the microdilution MIC tests represented a critical step. Both logistical and financial considerations suggested the design had to be addressed before any experimental work could be commenced. This paper will, therefore, concentrate on illustrating the approach taken to designing the layout of the plates to be used in this work.

Designing a 96-well plate for microdilution MIC assays of bacteria isolated from aquatic animals.

Susceptibility testing of non-cholera *Vibrio* spp. isolated from aquatic animals or the aquatic environment may be undertaken for two main reasons. They may be performed to inform the selection of agents to be used in the therapy of aquatic animal disease or as part of a food safety study of the potential impact of aquatic animal products for human health. In this work schedule a plate layout that would facilitate the establishment of epidemiological cut-off values needed for both these aims was developed.

Background

In the performance of MIC tests, the use of commercial pre-loaded 96-well microdilution plates has the very considerable advantage, particularly for multi-laboratory studies, that most of the necessary quality controls will have been performed by the manufacturer. However, the microdilution plates that are commercially available have not been designed for studies of bacteria isolated from aquatic animals. As a consequence they often either include agents that are of little relevance and /or fail to include agents of importance to aquatic studies. In addition the ranges of agent concentrations are frequently not appropriate. An examination of the 50 papers that reported MIC studies of *Vibrio* spp. collected by Smith and Egan (2018) found a very significant number that reported susceptibilities to inappropriate agents, multiple agents of the same class and/or the use of ranges of agent concentrations that were incapable of capturing quantitative MIC for the isolates studied.

Thus, it was necessary to design custom-made microdilution plates that, with respect to the agents and their concentrations included, were appropriate.

Selection of agents

Three criteria were used in the selection of agents to be included in the plates.

Aquatic criteria (A) were applied to identify agents used in therapy of aquatic animals infected with *Vibrio* spp. The most frequently used agents were assumed to be amoxicillin, oxolinic acid, flumequine, enrofloxacin, oxytetracycline, florfenicol and trimethoprim/sulfamethoxazole. The use of these agents is allowed in most European countries.

Human criteria (H) were applied to identify agents used in therapy of humans infected with *Vibrio* spp. Wong et al. (2015) has reported that the most effective classes of antibiotics in the treatment of infections of humans by *Vibrio* spp. are the quinolones, the third generation cephalosporins and the tetracyclines. Treatment with trimethoprim/sulfamethoxazole and aminoglycosides (e.g. gentamicin) has also been reported. (Bier et al., 2015).

Miscellaneous criteria (M). An additional three agents were selected under this criteria. Chloramphenicol was included to facilitate the detection of illegal use. The use of this agent in food animals is prohibited in most countries. Ceftazidime was included as it has been reported to be an effective pre-screen for isolates containing extended-spectrum β-lactamase-/AmpC β-lactamase resistance mechanisms (Livermore and Brown, 2001; Aerts et al., 2019). The spread of these determinants in human pathogens is of major concern. Further meropenem is also included to screen for isolates producing carbapenems (Aerts et al., 2019). Sulfamethoxazole was included to facilitate the detection of isolates possessing *sul* genes. These genes have been reported as occurring with a high frequency in some aquatic environments (Shimizu et al., 2013) and cannot be reliably detected using trimethoprim/sulfamethoxazole (Kim et al., 2018).

In selecting agents to be included the recommendation of CLSI (2014) that routine testing should include only one representative agent from a class of antimicrobial agents that has activity against a spectrum of bacteria was followed. Because of the high frequencies of cross resistance that have been reported between the quinolones (flumequine, nalidixic acid, oxolinic acid), the fluroquinolones (ciprofloxacin, enrofloxacin and levofloxacin), the tetracyclines (oxytetracycline, tetracycline and doxycycline) and the aminopenicillins (amoxycillin and ampicillin) it was decided that only one agent of each of these classes should be included. The final agent selection is shown in Table 1.

Note on the significance of quality control (QC) criteria

Epidemiological cut-off values are protocol-specific. They can be set only from a consideration of data produced by a specific standard protocol. Laboratories can claim to have used a specific protocol if they have demonstrated compliance with the QC requirements that are an essential component of that protocol. It follows that protocol-specific epidemiological cut-off values can be set with respect to a specific agent only if the protocol-specific QC requirements for that agent have been published.

The decision to perform the susceptibility tests at 28 °C in this study was taken because of the risk that some fish pathogenic *Vibrio* species, *V. anguilarum* for example, may not grow suffi-

Table 1. Selection of agents

Class	Agent[a]	Cross resistance	Animal group[b]	QC requirements	Importance[d]	
				@28°C	OIE	WHO
Aminopenicillin	AMP	amoxicillin, 1st gen cephalosporins	A, H	yes	C	C
3rd generation cephalosporin	CTA	3rd generation cephalosporins	H	no		C
Carbapenem	MER	3rd generation cephalosporins	H	no		C
Quinolone	OXO	flumequine	A	yes	H	
Fluroquinolone	ENR	ciprofloxacin	A, H	yes	C	C
Phenicol	FLO		A	yes	C	
	CHL		M	no		H
Aminoglycoside	GEN		H	yes		C
Tetracycline	OXY	doxycycline, tetracycline,	A, H	yes	C	H
Anti-folate	SME	sulphonamides	M	no	C	H
	TRS		A, H	yes	C	H

a Abbreviations for agents follow the EUCAST system. ampicillin AMP, ceftazidime CTA, chloramphenicol CHL, enrofloxacin ENR, florfenicol FLO, gentamicin GEN. meropenem MER, oxolinic acid OXO, oxytetracycline OXY, sulfamethoxazole SME, trimethoprim / sulfamethoxazole TRS.
b A indicates important in treatment of aquatic animals, H indicates important in treatment of humans and M indicates miscellaneous importance.
c Acceptable ranges for reference strains tested at 28°C provided in VET04-A2 (CLSI, 2014).
d Categorisation of agents in the OIE list of important antimicrobial agents for fish (www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_OIE_List_antimicrobials_July2019.pdf /) and the WHO Critically Important Antimicrobials for Human Medicine (https://www.who.int/foodsafety/publications/antimicrobials-sixth/en/). C indicates critically important and H indicate highly important.

ciently at 35 °C to allow precise determination of their MIC (Smith and Egan, 2018). This decision has, however, consequences for the availability of QC requirements. For seven of the eleven of the agents selected (Table 1.) QC requirements for data generated at 28°C are available and, therefore, epidemiological cut-off values for them can be calculated using data generated by the suggested plate layout. However, for four agents selected (ceftazidime, chloramphenicol, meropenem and sulfamethoxazole) no QC requirements for data generated at 28°C have yet been published and, therefore, data generated for these four agents using the suggested plate layout cannot be used in setting protocol-specific epidemiological cut-off values. They can provide only evidence suggesting a reduced susceptibility of isolates to any of them. It is argued that evidence of a reduced susceptibility can be used as an important prescreen that indicates the need for further tests or investigations.

Isolates of *Vibrio* spp. manifesting reduced susceptibility to ceftazidime or meropenem have been reported (Briet et al., 2018) but would be

expected only at a very low frequency. Reduced susceptibility to these agents in human pathogens is, however, an important issue. Evidence generated at 28°C of a possible reduced susceptibility to either of these agents in *Vibrio* spp. would indicate the need for confirmation either by molecular methods (Bier et al., 2015) or additional phenotypic tests at 35°C.

Sulfamethoxazole was included to facilitate the detection of isolates that possessed *sul* genes. The presumptive evidence of a reduced susceptibility in an isolate would be sufficient grounds for initiating the molecular studies needed to confirm the presence of these genes. Chloramphenicol was included to detect any possible illegal use of this agent. Again evidence of a possible reduced susceptibility would be sufficient to trigger a more detailed analysis.

Selection of concentrations

Both methods that are available for setting epidemiological cut-off values for MIC data, NRI (www.bioscand.se/nri/) and ECOFFinder, (www.clsi.org/standards/micro/ecoffinder/) calculate these values from statistical analyses of the distribution of MIC observations for fully susceptible (WT) isolates. To generate epidemiological cut-off values the range of concentrations to be included in a plate layout must, therefore, be capable of capturing the full range of quantitative MIC values of all WT isolates of the species under consideration including the most susceptible. Clearly, in situations where the aim is to generate epidemiological cut-off for a species, the full range of MIC values for all WT isolates of that species cannot be established in advance with any certainty. In this work three sources of data were used to provide estimates of the most suitable ranges and all have limitations. Source A was any MIC data sets that were available, from published and unpublished sources, for bacteria isolated from aquatic animals that had been generated using any standard CLSI protocol. The major limitation of these data was that they came from studies of a variety of different species and a variety of test conditions.

The second source was the published data sets used by EUCAST to set ECOFF values (www.eucast.org/mic_distributions_and_ecoffs/). These had the advantage that for each species/agent combination they were comprised of a very large number of observations by multiple laboratories. However, their value was limited by the fact that they were all generated in tests carried out at 35°C. This source presented data for multiple species but particular emphasis was placed on those for the Gram-negative enteric bacteria *Escherichia coli* and *Salmonella* spp. (Table 2).

The third source was the clinical breakpoints for *Vibrio* spp. published in M45-A3 (CLSI, 2016). One limitation of these data is that they were human clinical breakpoints, not epidemiological cut-off values, and, importantly, the distributions of MIC values used to calculate them have not been published. A second limitation of these breakpoints was that they were not based on any susceptibility measures made on *Vibrio* spp but rather were simply copied from breakpoints originally published for *Enterobacteriaceae* (CLSI, 2017).

Table 2 presents the quantitative data obtained from these three sources. Possibly surprisingly, given the various species and

Table 2. Selection of agent concentrations. The shaded areas indicate the concentrations included in the plate layout. The table presents the percentage distributions of MIC values.

Agent[a]	AMP		ENR		GEN		TRS		FLO	OXO	OXY	CHL	CTA	SUL	MER
Source[b]	A	H	A	H	A	H	A	H	A	A	A	H	H	H	H
Observations[c]	625	58816	921	2979	1347	64934	849	7192	1832	1176	1266	44172	19416	1109	7870
0.002			1												
0.004			5												
0.008			30							10					13
0.016			50				2	1		28					52
0.031			13	28			18	7		25	7		1		31
0.063	5		1	67			37	34		14	24		9		4
0.125	30			5	1	1	33	24	2	19	31		29		
0.250	40				4	13	10	20	9	5	29		29		
0.5	3				23	49		11	44		9		17		
1	18	15			42	31		3	34			5	9		
2	4	44			24	5			8			50	6	2	
4		36			7				3			43		6	
8		5										2		23	
16														43	
32														23	
64														4	
128															
256															
512															
M45-A3[d]	≥8				≥4		≥2							≥256	≥1

Abbreviations for agents as in Table 1
[b] A indicates data from bacteria isolated from aquatic animals. H indicates data for *E.coli* and *Salmonella* spp. published by EUCAST
[c] Number of individual MIC observations
[d] Clinical breakpoints for *Vibrio* spp. isolates categorised as Susceptible given in Table 20 of M45-A3 (CLSI, 2016).

test conditions used to generate the data sets, a reasonably simple and consistent pattern emerged. Setting the concentrations of the various agents to be incorporated in the 96-well plates proved to be a relatively simple task (Table 2).

Potential variations in plate layout

It is important to note that this plate layout has not, as yet been validated by application to any species or group of species isolated from aquatic animals and that field testing of the layout might suggest minor modifications. It is also possible that studies involv-

ing bacterial species other than *Vibrios* may require minor alterations. However, it is argued that the approach to plate layout design developed for this work schedule presents a useful template. Its use should go a long way to eliminating many of the errors that have limited the value of so many studies in this area.

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