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Epidemiological cut-off values for non-O1/ non-O139 *Vibrio cholerae* disc diffusion data generated by standardised methods

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ABSTRACT: This work generates the data needed to set epidemiological cut-off values for disc-diffusion zone measurements of *Vibrio cholerae*. The susceptibility of 147 European isolates of non-O1/non-O139 *V. cholerae* to 19 antibiotics was established using a standardised disc diffusion method which specified incubation of Mueller Hinton agar plates at 35°C. Epidemiological cut-off values were calculated by analysis of the zone size data with the statistically based normalised resistance interpretation method. Cut-off values for 17 agents were calculated by analysis of the aggregated data from all 4 laboratories participating in this study. The cut-off values calculated were ≥18 mm for amoxicillin/clavulanate, ≥18 mm for amikacin, ≥19 mm for ampicillin, ≥27 mm for cefepime, ≥31 mm for cefotaxime, ≥24 mm for ceftazidime, ≥24 mm for chloramphenicol, ≥31 mm for ciprofloxacin, ≥16 mm for erythromycin, ≥27 mm for florfenicol, ≥16 mm for gentamicin, ≥23 mm for imipenem, ≥25 mm for meropenem, ≥29 mm for nalidixic acid, ≥28 mm for norfloxacin, ≥13 mm for streptomycin and ≥23 mm for tetracycline. For the other 2 agents the data from 1 laboratory was excluded from the censored aggregation because the data from that laboratory was considered excessively imprecise. The cut-off values for these 2 agents calculated for the aggregation of the data from 3 laboratories were ≥23 mm for trimethoprim and ≥24 mm for trimethoprim/sulfamethoxazole. These zone size data will be submitted to the Clinical Laboratory Standards Institute (CLSI) and European Committee for Antimicrobial Susceptibility Testing (EUCAST) for their consideration in setting international consensus epidemiological cut-off values for non O1/non-O139 *V. cholerae*.

KEY WORDS: *Vibrio cholerae* · Epidemiological cut-off values · Antimicrobial susceptibility · Disc diffusion · Normalized resistance interpretation

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1. INTRODUCTION

Vibrio cholerae are Gram-negative bacteria that are abundant in marine and brackish as well as in freshwater, subsaline and saline water environments and wastewater (Lesne et al. 1991, Vezzulli et al. 2020). The serogroups O1 and O139 of *V. cholerae* are associated with cholera in humans; the other serogroups are opportunistic pathogens for humans and numerous aquatic organisms (Senderovich et al. 2010). Studies of resistance to antimicrobial agents in *V. cholerae* have mainly concentrated on the 2 serotypes O1 and O139 (Loo et al. 2020). The frequency and spread of resistance determinants in these serotypes have been associated with their genomic plasticity and the presence of integrating conjugative elements, plasmids, superintegrations, transposable elements and insertion sequences (Kitaoka et al. 2011, Escudero & Mazel 2017, Verma et al. 2019, Das et al. 2020).

Resistance in non-O1/non-O139 *V. cholerae* isolates has, however, received less attention. Studies of resistance in these primarily environmental serotypes that used the susceptibility testing methods given for *Vibrio* spp. in the second and third editions of the Clinical Laboratory Standards Institute (CLSI) guideline M45 (CLSI 2010a, 2015a) have been reported from Austria (Lepuschitz et al. 2019), Germany (Bier et al. 2015, Fleischmann et al. 2022), France (Baron et al. 2017), Haiti (Baron et al. 2016) and the USA (Ceccarelli et al. 2015). The data obtained in these studies were interpreted using criteria for *Vibrio* spp. provided by these editions of M45 or the criteria for *Enterobacteriaceae* provided by various editions of the informational supplement M100 (CLSI 2010b, 2015b, 2016, 2018b). Although it should be noted that for the antimicrobial agents studied there were no differences in the criteria provided in these various editions, the specific editions of M45 and M100 used as sources for interpretive criteria in each of the 5 studies are given in Table S1 in the Supplement; www.int-res.com/articles/suppl/d156p115_supp.pdf.

There are, however 3 considerations that suggest that in surveys of the susceptibility of environmental *Vibrio* spp. the use of these interpretive criteria would not be appropriate. The first relates to the nature of the interpretive criteria. The criteria provided by M45 and M100 are clinical breakpoints that are designed to facilitate the prediction of the clinical outcomes of the administration of a predefined dose of an agent to human patients. Thus, their application is appropriate when a study is being performed to guide the choice

of agents to be used in the therapy of humans. Epidemiological cut-off values, in contrast, are interpretive criteria designed to facilitate the identification of isolates that possess any resistance mechanism at all (Silley 2012). Thus, as argued by the Aquatic Animal Health Code (www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access/), they represent the more appropriate criteria for studies designed to perform surveillance and monitoring of antimicrobial susceptibility in environmental isolates.

The second consideration relates to the taxonomic units to which the interpretive criteria are to be applied. The clinical breakpoints given in M45 (CLSI 2010a, 2015a) are given for the *Vibrio* spp., and those in M100 (CLSI 2010b, 2015b, 2016, 2018b) are given for the family *Enterobacteriaceae*, which includes multiple genera. A more detailed examination of the clinical breakpoints given for *Vibrio* spp. in M45 (CLSI 2010a, 2015a) shows that they are almost identical to those given for *Aeromonas* spp. in the same guideline and to those given in M100 (CLSI 2018b) for *Enterobacteriaceae*. However, both the guideline M23 (CLSI 2018a) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) SOP 10.2 (EUCAST 2021) state that epidemiological cut-off values should only be determined for a single species. Thus, the clinical breakpoint in the CLSI documents would appear to have been specified for wider taxonomic units than is currently recommended for epidemiological cut-off values.

The third consideration relates to studies of *V. cholerae*. Table 20 in the guideline M45 (CLSI 2010a, 2015a) provides clinical breakpoints for 24 antimicrobial agents against *Vibrio* spp. However, this table includes an unreferenced comment that the values for 17 of these agents should only be applied to data for *Vibrio* spp. other than *V. cholerae*. These 17 agents include 9 (amikacin, amoxicillin/clavulanate, cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, imipenem and meropenem) that were included in the present study.

These considerations suggest that to perform surveillance and monitoring of the susceptibility to antimicrobial agents of non-O1/non-O139 *V. cholerae* isolates epidemiological cut-off values will be required that can be applied to this species. The work presented in this study met the requirements of CLSI (2018a) and EUCAST (2021). It is, therefore, intended that these data will be submitted to these agencies in order to allow them to set the required internationally harmonised consensus epidemiological cut-off values.

2. MATERIALS AND METHODS

2.1. Participating laboratories

Four laboratories were involved in this study of *Vibrio cholerae* susceptibility. These were the Mycology-Bacteriology and Antimicrobial Resistance Unit of Ploufragan-Plouzané-Niort Laboratory of the French Agency for Food, Environmental and Occupational Health & Safety (Anses), the Inter-university Cooperation Centre Water & Health (ICC) at Karl Landsteiner University of Health Sciences, Division Water Quality & Health, Krems, Austria, the Institute for Medical Microbiology and Hygiene at the Austrian Federal Agency for Health and Food Safety, Vienna, Austria (AGES) and the Consultant Laboratory for *Vibrio* spp. in Food hosted at the German Federal Institute for Risk Assessment, Berlin, Germany (BfR).

2.2. Source and classification of isolates

One hundred and fifteen unique isolates analysed in this work were obtained from waters of various salinity in France and Austria. In addition, 32 were isolated from cockles *Cerastoderma edule* collected in France. The French isolates were obtained using the methods of Baron et al. (2007, 2017) and the Austrian isolates using the methods of Pretzer et al. (2017). The identification of isolates was performed with the species-specific PCR primers for the *ompW* gene (Nandi et al. 2000). The genes coding for the O1 and O139 surface antigens were assessed with PCR using O1- and O139-specific primers (Hoshino et al. 1998). The cholera toxin gene *ctxA* was screened using PCR (Hoshino et al. 1998, Nandi et al. 2000). All 147 isolates included in this study did not belong to either the O1 or the O139 serogroups and did not harbour *ctxA* gene.

2.3. Antimicrobial susceptibility testing

The susceptibility of the *V. cholerae* isolates were determined using a disc diffusion method recommended for non-fastidious organisms that specifies incubation on Mueller-Hinton (MH) agar at 35°C for 16–18 h (CLSI 2015a). This method is functionally similar to that recommended for *Vibrio* spp. in the EUCAST disc diffusion method for antimicrobial susceptibility testing version 11.0 (www.eucast.org). The antibiotic discs used contained

amoxicillin/clavulanate (20/10 µg), amikacin (30 µg), ampicillin (10 µg), cefepime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), gentamicin (10 µg) imipenem (10 µg), meropenem (10 µg), nalidixic acid (30 µg), norfloxacin (10 µg), streptomycin (10 µg), tetracycline (30 µg), trimethoprim (5 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg). All these antimicrobial agents have been categorised as critical, highly important or important for human medicine (WHO 2019). Details of the manufacturers of the discs used by the 4 laboratories are shown in Table S2. For assays with erythromycin discs, the quality control reference strain *Staphylococcus aureus* ATCC 25923 was used, for assays with imipenem and meropenem discs, the strain *Pseudomonas aeruginosa* ATCC 27853 was used, for amoxicillin/clavulanate, *Escherichia coli* ATCC 35218 and for the remaining 15 discs *E. coli* ATCC 25922 was used. The acceptable ranges for the reference stains relevant to the test protocols used in this work were accessed from the CLSI guidelines VET04 (CLSI 2020), M45 (CLSI 2015a) and M100 (CLSI 2021).

2.4. Additional data sets for various species of terrestrial bacteria

Twenty-one disc zone data sets for terrestrial species that had been generated using the standard method specifying incubation at 35°C and the use of MH agar (www.eucast.org), were accessed from the EUCAST website (www.eucast.org/mic_distributions_and_ecoffs/). The sets selected were those that had been generated from more than 3 and less than 9 laboratories and consisted of less than 700 observations and had been considered as of sufficient quality by EUCAST to allow them to set epidemiological cut-off values or, for those with less than 5 laboratories, tentative cut-off values.

2.5. Analysis of zone data sets

Epidemiological cut-off values (CO_{WT}) were calculated from zone size data sets by the normalised resistance (NRI) method (www.bioscand.se/nri/). This analysis also calculates the mean and the standard deviation (SD) of the normalised distribution of the zones obtained from isolates categorised as wild type (WT). The SD values calculated by NRI analysis can

provide a measure of the precision of zone data sets (Smith et al. 2018). They suggested that, for zone size data sets obtained at 35°C by a single laboratory, an upper limit for these SD values could be set at 3.38 mm. For each agent, NRI analyses were performed on the zone data obtained from each laboratory individually and also on the aggregated zone data from all 4 laboratories. In preparing the multiple laboratory aggregations, single laboratory zone size data sets from which NRI analysis generated an SD value in excess of the suggested limit (>3.38 mm) were considered excessively imprecise and were omitted from any aggregation.

3. RESULTS AND DISCUSSION

3.1. Setting acceptable precision limits for multiple laboratory aggregations

The SD values of the 21 sets of multiple laboratory aggregations of inhibition zone size data for terrestrial organisms accessed from EUCAST (www.eucast.org/mic_distributions_and_ecoffs/) were calculated by NRI analysis. The mean and SD of the SD values for the 21 data sets were 2.1 and 0.8 mm respectively. Using the approach of Smith et al. (2018) these data, therefore, suggested that a provisional upper precision limit that would be acceptable for the SD of a multiple laboratory aggregation to be used in setting CO_{WT} values would be 3.7 mm (mean + 2 SDs). The number of laboratories contributing to the 21 data sets accessed from EUCAST ranged from 3 to 8 with a mean of 4.5, and the numbers of observations in the data sets ranged from 111 to 695 with a mean of 309. The data sets for these terrestrial species had, therefore, been generated under similar test conditions, contained similar numbers of observations and had been generated by a similar number of laboratories as the aggregated data sets for the *Vibrio cholerae* isolates generated in this work. It was, therefore, considered that the acceptable limit value of 3.7 mm could be applied to assessing the precision of the aggregated *V. cholerae* data sets.

3.2. Setting epidemiological cut-off values

To comply with the quality control requirements of the testing protocol used in this work, all 4 laboratories recorded zone sizes for the appropriate quality control (QC) reference strains for each of

the 19 agents (Table S3.1–S3.19). All these zone sizes were within the acceptable ranges set for the testing protocol used in this work (CLSI 2018b, 2020, 2021). With respect to assessing the quality of the 76 zone data sets generated by the individual laboratories, their precision was assessed by examining the SD values calculated from them by NRI analysis (Table S3). For 74 of them the SD values ranged between 0.2 and 2.9 mm. These SD values were smaller than the suggested upper limit for data from individual laboratories of 3.38 mm (Smith et al. 2018), indicating that these data sets manifest acceptable precision. These data sets were, therefore, included in the multi-laboratory aggregations. The SD values calculated for the remaining 2 data sets from individual laboratories, one for trimethoprim and the other for trimethoprim/sulfamethoxazole were both 4.1 mm (Table S3.18, S3.19). These data were, therefore, excluded from the censored aggregations used to calculate CO_{WT} .

The SD values calculated for the 17 aggregations and the 2 censored aggregations of *V. cholerae* zone size data sets generated in this work (Table 1) ranged from 0.8 to 2.5 mm with a mean and SD of 1.7 and 0.5 mm, respectively. As all these SD values were less than the provisional upper limit (3.7 mm) calculated in this work from the EUCAST multiple laboratory aggregations of zone size data sets, it was assumed that these 19 aggregations of *V. cholerae* data had sufficient precision to be used in calculating cut-off values.

With respect to the minimum quantity of data required for setting CO_{WT} values for disc zone data, we are unaware of any recommendations by CLSI or EUCAST. Therefore, we decided to follow Smith et al. (2023), who adapted the requirements presented in the guideline M23 (CLSI 2018b) and the EUCAST SOP 10.2 (EUCAST 2021) for MIC data sets to be used in setting CO_{WT} values. The minimum quantitative requirements adopted in this work were that aggregated or censored aggregations must contain at least 100 observations from unique isolates categorised as WT that were generated in at least 3 laboratories.

Table 1 presents a summary of the results of the analysis, by NRI, of the 19 multi-laboratory aggregations of the *V. cholerae* zone data. For 17 of the agents the data sets were aggregations of the data from 4 laboratories and contained between 138 and 147 observations from isolates categorised as WT. For the other 2 agents, the censored aggregations were made from the data generated by 3 laboratories and contained 107 and 118 observations from isolates

Table 1. Epidemiological cut-off values (CO_{WT}) calculated by normalised resistance (NRI) analysis of the aggregations and censored aggregations of the zone size data generated by the participating laboratories. Total: total number of isolates in the aggregation or censored aggregation. WT: number of isolates categorized as wild type by the application of CO_{WT} calculated for them in this work

Agent (disc contents)	NRI analysis of zones (mm)			Isolates	
	Mean	SD	CO_{WT}	Total	WT
Amoxicillin/clavulanate (20/10 µg)	22	1.5	≥18	147	147
Amikacin (30 µg)	21	1.2	≥18	147	147
Ampicillin (10 µg)	24	1.6	≥19	147	138
Cefepime (30 µg)	32	1.7	≥27	147	146
Cefotaxime (30 µg)	38	2.4	≥31	147	147
Ceftazidime (30 µg)	30	2.3	≥24	147	147
Chloramphenicol (30 µg)	31	2.5	≥24	147	147
Ciprofloxacin (5 µg)	37	2.3	≥31	147	145
Erythromycin (15 µg)	21	1.7	≥16	147	147
Florfenicol (30 µg)	32	1.6	≥27	147	146
Gentamicin (10 µg)	22	2.2	≥16	147	147
Imipenem (10 µg)	26	1.3	≥23	147	146
Meropenem (10 µg)	28	0.8	≥25	147	146
Nalidixic acid (30 µg)	33	1.4	≥29	147	146
Norfloxacin (10 µg)	35	2.4	≥28	147	147
Streptomycin (10 µg)	16	1.0	≥13	147	147
Tetracycline (30 µg)	29	1.9	≥23	147	147
Trimethoprim (5 µg) ^a	27	1.3	≥23	108	107
Trimethoprim/sulfamethoxazole (1.25/23.75 µg) ^a	29	1.9	≥24	118	118

^aCensored aggregation of data from only 3 of the 4 laboratories involved in this study

categorised as WT. The CO_{WT} calculated for all 19 agents (Table 1) were made from data sets that met the quantitative and qualitative criteria that were applied. It is, therefore, intended that the data used to generate these cut-off values will be submitted to CLSI and EUCAST for their consideration in setting international consensus epidemiological cut-off values.

3.3. Comparison of clinical breakpoints and CO_{WT} values

CO_{WT} values represent the lowest limit of the zone size for isolates that possess no resistance mechanisms. The susceptible (S) cut-off values, provided by clinical breakpoints, represent the lowest zone size for isolates that either possess no resistance mechanisms or possess resistance mechanisms that code only for a reduction in susceptibility that is not sufficient to predict the failure of a predetermined antimicrobial treatment of a specified host. Table 2 presents the difference between

the CO_{WT} values calculated in this work and the S values given in the published clinical breakpoints (CLSI 2010a,b, 2015a,b, 2016, 2018b, 2021). Overall, as would be expected, the CO_{WT} were either equal to or larger than the S values. However, agents in the same class tended to show similar degrees of difference. For 7 agents, 3 aminoglycosides, the 2 aminopenicillins and the 2 carbapenems, the S values and the CO_{WT} were within 2 mm of each other. In contrast, for 6 agents, the CO_{WT} were considerably larger than the S values. For the 3 quinolones, the differences were 10–11 mm, for the 2 folate inhibitors they were 6–8 mm and for tetracycline it was 8 mm. It is not possible from the data available to determine whether the differences, when they occur, between the S values and the CO_{WT} derive from the fact that the S values were established from a wider taxonomic group than the CO_{WT} or because their aim was to detect only those isolates with clinically significant resistance.

3.4. Application of clinical breakpoints and CO_{WT} values

When both clinical breakpoints and CO_{WT} values are available, the question is which should be applied to any susceptibility data set. Essentially the factor influencing this decision is the primary aim of the study which generated the data. Bier et al. (2015), Ceccarelli et al. (2015), Baron et al. (2016, 2017), Lepuschitz et al. (2019) and Fleischmann et al. (2022) studied the susceptibility of non-O1/non-O139 *V. cholerae* isolated from aquatic environments. However, the studies of Bier et al. (2015) and Fleischmann et al. (2022) were designed to investigate the risks to human health resulting from reduced susceptibility to any antimicrobial of non-O1/non-O139 *V. cholerae* isolates. In these studies, and others that share similar aims, it is clear that the application of clinical breakpoints to the susceptibility data would be the most appropriate. In contrast, the studies of Ceccarelli et al. (2015), Baron et al. (2016, 2017) and Lepuschitz et al. (2019) were primarily focused on investigating *V. cholerae* as a potential reservoir of en-

Table 2. Comparison of epidemiological cut-off values (CO_{WT}) calculated for 19 antimicrobial agents in the present work with the clinical breakpoint (CB) values for susceptible isolates (S) provided by M45 (CLSI 2010b, 2015a) or M100 (CLSI 2010b, 2015b, 2016, 2018b). na: no clinical breakpoints given in either document

Agent	CO _{WT} (mm)	S (mm)		Difference (CO _{WT} – CB)
		M45	M100	
Aminoglycosides				
Amikacin	≥ 18	≥ 17 ^a		1
Gentamicin	≥ 16	≥ 15 ^a		1
Streptomycin	≥ 13		≥ 15	-2
Aminopenicillins				
Ampicillin	≥ 19	≥ 17		2
Amoxicillin/ clavulanate	≥ 18	≥ 18 ^a		0
Carbapenems				
Meropenem	≥ 25	≥ 23 ^a		2
Imipenem	≥ 23	≥ 23 ^a		0
Cephems				
Cefotaxime	≥ 31	≥ 26 ^a		5
Ceftazidime	≥ 24	≥ 21 ^a		3
Cefepime	≥ 27	≥ 25 ^a		2
Folate pathway inhibitors				
Trimethoprim/sulfamethoxazole	≥ 24	≥ 16		8
Trimethoprim	≥ 23		≥ 16	7
Macrolides				
Erythromycin	≥ 16	na	na	
Phenicols				
Chloramphenicol	≥ 24	≥ 18		6
Florfenicol	≥ 27	na	na	
Quinolones				
Norfloxacin	≥ 28		≥ 17	11
Ciprofloxacin	≥ 31	≥ 21		10
Nalidixic acid	≥ 29		≥ 19	10
Tetracyclines				
Tetracycline	≥ 23		≥ 15	8

^aValues indicated as relevant for *Vibrio* spp. other than *V. cholerae*

vironmental antimicrobial resistance mechanisms. In these studies, and other which share similar aims, the application of CO_{WT} values developed in the present work would be the most appropriate.

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