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Contribution to the Virtual DAO Special 'Epidemiological cut off values for aquatic bacteria'

Setting epidemiological cut-off values relevant to MIC and disc diffusion data for *Aeromonas salmonicida* generated by a standard method

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ABSTRACT: The Clinical and Laboratory Standards Institute has published epidemiological cut-off values for susceptibility data generated at 22°C and read after 44–48 h for florfenicol, oxolinic acid and oxytetracycline against *Aeromonas salmonicida.* The cut-off values for the minimum inhibitory concentration (MIC) and disc diffusion were derived from data obtained by 1 laboratory and 2 laboratories respectively. The present work reports the generation of susceptibility data from additional laboratories and the calculation of provisional cut-off values from aggregations of these data with previously published data. With respect to MIC data, the provisional cut-off values, derived from aggregations of the data from 4 laboratories, were $\leqslant 4$ µg ml^{-1} for florfenicol, $\leqslant 0.0625$ µg ml^{-1} for oxolinic acid and $\leqslant 1$ µg ml^{-1} for oxytetracycline. For disc diffusion data, the provisional cut-off values derived from aggregations of the data from 5 laboratories were $\geqslant 30$ mm for florfenicol, $\geqslant 32$ mm for oxolinic acid and $\geqslant 25$ mm for oxytetracycline. In addition, a cut-off value of $\geqslant 29$ mm for ampicillin was derived from the aggregation of data from 4 laboratories.

KEY WORDS: *Aeromonas salmonicida* · Epidemiological cut-off values · Antimicrobial susceptibility · MIC · Minimum inhibitory concentrations · Disc diffusion · Normalised resistance interpretation · ECOFFinder

1. INTRODUCTION

The application of epidemiological cut-off values to antimicrobial agent susceptibility data enables isolates to be categorised as either fully susceptible wild-type (WT) members of their species or as non-wild-type (NWT) isolates that manifest a susceptibility significantly lower than that of the WT isolates (Silley

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2012). The Clinical and Laboratory Standards Institute (CLSI) supplement VET04 (CLSI 2020a) details the internationally harmonised, consensus epidemiological cut-off values (for which they use the acronym ECV) that are applicable to data on the susceptibility to a number of antimicrobial agents for various bacterial species isolated from aquatic animals. VET04 (CLSI 2020a, their Table 2B) lists the ECVs that can be applied to minimum inhibitory concentration (MIC) and inhibition zone (IZ) diameter values for both typical and atypical *Aeromonas salmonicida* when these data have been obtained in tests performed at 22 ± 2°C with incubation for 44–48 h as specified in the guideline VET03 (CLSI 2020b). The empirical data used to develop ECVs for florfenicol (FLO), oxolinic acid (OXO) and oxytetracycline (OXY) MIC data were those published by Miller & Reimschuessel (2006). Those authors reported the MIC values for 163 typical isolates and 54 atypical isolates of *A. salmonicida* generated in a single laboratory. The empirical data used to develop ECVs for IZ data with respect to FLO, OXO and OXY were those published by Miller & Reimschuessel (2006), Ruane et al. (2007) and Smith et al. (2007). Both Ruane et al. (2007) and Smith et al. (2007) published IZ data for 2 laboratories. However, as both those laboratories had studied the same set of 106 isolates, the data from only one of them was used by CLSI in developing the ECVs presented in VET04 (CLSI 2020a).

The predictive ability of any epidemiological cut-off value is, in part, a function of the number of laboratories and the number of observations they contribute to the aggregated data sets used to set the cut-off value. It can always be enhanced by increasing the numbers and sources that contribute to the aggregation. The aggregated data used to generate the cut-off for MIC data for ampicillin (AMP) against *Escherichia coli* given by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (https://www.eucast.org/mic_and_zone_distributions_and_ecoffs) was, for example, calculated from 105 483 observations made by 53 laboratories. It is unlikely that anything approaching these numbers could ever be realised in setting cut-off values for bacteria isolated from aquatic animals. It has, however, been possible to suggest minimum numbers of laboratories and observations necessary to set reliable cut-off values. The CLSI guideline M23 (CLSI 2018) states that to set reliable ECVs for MIC, data must be sourced from at least 3 laboratories. EUCAST (2021) states that values from at least 5 laboratories are required to set epidemiological cut-off values (ECOFFs) for MIC data, although they will set tentative cut-offs (TECOFFs) when values are derived from at least 3 laboratories. Both CLSI (2018) and EUCAST (2021) would also require a minimum of 100 observations from WT isolates. EUCAST apply similar rules to the setting of ECOFF values for IZ data (https://www.eucast.org/mic_and_zone_distributions_and_ecoffs).

The ECVs with respect to FLO, OXO and OXY against *A. salmonicida* given in the current edition of VET04 (CLSI 2020a) for MIC data were generated after consideration of data from a single laboratory, and those for IZ data from the aggregated data from 2 laboratories. Thus, they do not meet the current quantitative requirements of CLSI (2018). The present study was undertaken to provide data from an additional 3 laboratories to facilitate the setting of ECVs for both MIC and IZ data in accordance with CLSI requirements.

2. MATERIALS AND METHODS

2.1. Laboratories

This work presents the data generated in 3 laboratories, namely the Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit of Ploufragan-Plouzané-Niort Laboratory of the French Agency for Food, Environmental and Occupational Health & Safety (MBA); the Centre for Environment, Fisheries and Aquaculture Science Laboratory, Weymouth, UK (Cefas); and the Institute of Aquaculture, University of Stirling, Scotland (UoS).

In addition, 3 data sets that had been previously published were analysed: data generated by the Division of Animal Research at the Office of Research, Center for Veterinary Medicine, Food and Drug Administration, Laurel, Maryland, USA (FDA) that were accessed from Miller & Reimschuessel (2006); and data generated by the Fish Health Unit, Marine Institute, Oranmore, Galway, Ireland (MI) and the Department of Microbiology, University of Galway, Ireland (UG), who studied the same 106 isolates, which were accessed from Ruane et al. (2007) and Smith et al. (2007).

2.2. Isolate identification

Miller & Reimschuessel (2006) reported that their isolates were classified as *Aeromonas salmonicida* using the PCR primers of Gustafson et al. (1992). Using the PCR primers specific for typical *A. salmonicida* subsp. *salmonicida* (Miyata et al. 1996), 163 of these isolates were classified as typical isolates, while the other 54 were classified as atypical isolates. The UoS

isolates were derived from samples collected in the UK and Norway (Buba 2021) and classified as *A. salmonicida* by serology (Mono-AS, Bionor). Using the PCR method of Gulla et al. (2016), 82 of these isolates were classified as atypical, with 68 classified as *vapA* type V, 12 as *vapA* type VI and 1 each as *vapA* types I and II.

The methods used by the other 4 laboratories (Cefas, MBA, MI and UG) to identify their isolates were not capable of separating typical and atypical *A. salmonicida* isolates. MBA isolates collected in France were identified as *A. salmonicida* by the PCR methods of Kupfer et al. (2006) and Byers et al. (2002). The Cefas laboratory isolates collected in the UK were identified as *A. salmonicida* by API20E (bioMérieux) and serology (Mono-AS, Bionor). The isolates for which Ruane et al. (2007) and Smith et al. (2007) reported the data generated by UG and MI originated from Denmark, France, Ireland, Norway and Scotland and were identified as *A. salmonicida* by serology (Mono-AS, Bionor).

2.3. Susceptibility testing

The MIC values for FLO, OXO and OXY generated against their isolates by Cefas, MBA and UoS were obtained using the standardised microdilution method that specified the use of cation-adjusted Mueller-Hinton broth and incubation at 22 ± 2°C for 44–48 h (CLSI 2020b). Details of the 96-well microplates used by each laboratory are given in Table S1 in the Supplement at www.int-res.com/articles/suppl/d159p029_supp.pdf. The Cefas and UoS laboratories generated MIC values for all 3 agents from 50 and 82 of their isolates respectively. The MBA laboratory generated MIC values for FLO from 154 of their isolates. The numbers of isolates from which MIC values for OXO and OXY were obtained by the MBA laboratory were 144 and 68 isolates respectively. The same MIC testing method had been used by the FDA laboratory to generate the MIC data for the same 3 agents against 217 isolates (Miller & Reimschuessel 2006).

The disc diffusion IZ data for AMP, FLO, OXO and OXY generated against their isolates by Cefas and MBA were obtained using the standardised method that specified the use of Mueller-Hinton agar (MHA) and incubation at 22°C for 44–48 h (CLSI 2020b). The Cefas laboratory generated IZ data from 58 of their isolates for all 4 agents. The MBA laboratory generated IZ data for AMP and FLO from 75 of their isolates, and OXO and OXY data from 90 and 28 isolates, respectively. The same disc diffusion testing method had been used by the MI and UG laboratories (Ruane et al. 2007, Smith et al. 2007) to generate IZ data for AMP, FLO, OXO and OXY. Both these laboratories generated IZ data from the same 106 isolates. The FDA laboratory also used the same disc diffusion testing method to generate IZ data for FLO, OXO and OXY discs against the 217 isolates they examined (Miller & Reimschuessel 2006). Full details of the number of isolates on which susceptibility tests were performed is given in Table S2.

2.4. Data analysis

The analyses of susceptibility data were performed on aggregations of the available data of Cefas, MBA and UoS obtained in the present work and those previously obtained by FDA, MI and UG. For the aggregated MIC data, analyses were performed using 2 automatic methods, namely normalised resistance interpretation (NRI) (www.bioscand.se/nri/) and ECOFFinder (https://clsi.org/meetings/susceptibility-testing-subcommittees/ecoffinder/). To generate their proposed epidemiological cut-off values, both of these methods calculate exact measures for the cut-off values and then round them up to the next highest dilution in the test series. The ECOFFinder spreadsheet provides a variety of exact values of the cut-off calculated by that method and, as specified in the EUCAST standard operating procedure 10.2 (EUCAST 2021), the 99.9% values were used in the analyses reported here. The NRI spreadsheet provides a similar exact value which is presented as the mean + 2 SD of the normalised WT distribution. In the present work, the proposed cut-off values were derived by rounding up the mean of these 2 exact values. As these exact values are on a logarithmic scale, the mean of these values, termed the 'exact average', was calculated as the antilog of the means of their $\log_2$-transformed values. The analysis of the aggregated IZ data sets was performed using the NRI spreadsheets (www.bioscand.se/nri/).

2.5. Terminology and abbreviations

With respect to the abbreviations used for epidemiological cut-off values, we follow the recommendations of Smith (2019). The abbreviation ECV is reserved for cut-off values set by CLSI, and the abbreviation CO_{WT} is used for all epidemiological cut-off values not set by CLSI but were calculated for data generated by laboratories that have demonstrated compliance with the quality control (QC) require-

ments of the standard method adopted. The abbreviations adopted for the antimicrobial agents were those recommended in the EUCAST system for antimicrobial abbreviations (EUCAST 2018).

3. RESULTS AND DISCUSSION

3.1. QC

Four laboratories contributed to the aggregated MIC data sets analysed. All determinations of the MIC values made by Cefas, MBA and UoS for the reference strain *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 were within the acceptable ranges described in VET04 (CLSI 2020a) for the 3 antimicrobial agents tested. For the fourth laboratory (FDA), no MIC values for the reference strains they used were provided in Miller & Reimschuessel (2006). Details of these QC data are presented in Table S3.

Five laboratories contributed to the aggregated IZ sets analysed. Four of them (Cefas, MBA, MI and UG) recorded IZ data for one or both of the QC reference strains, *Escherichia coli* ATCC 25922 and *A. salmonicida* subsp. *salmonicida* ATCC 33658, that were within the acceptable ranges set in VET04 (CLSI 2020a) for the 4 agents tested. For the fifth laboratory (FDA), no IZ values for the reference strains they used were provided in Miller & Reimschuessel (2006). Details of these QC data are presented in Table S4.

3.2. Analysis of the aggregated MIC data sets

The individual data sets from the Cefas, FDA, MBA and UoS laboratories against FLO, OXO and OXY contained between 23 and 217 isolates that were categorised as putative WT (Table S5). The multi-laboratory aggregations for these 3 agents contained between 276 and 449 such isolates (Table 1). Thus, all data sets met the quantitative requirements of >15 WT isolates from individual laboratories and >100 WT isolates in multi-laboratory aggregates, as set by CLSI (2018) and EUCAST (2021).

Analysis with both ECOFFinder and NRI allowed the calculation of the respective SD values for the multi-laboratory aggregations of the data for FLO, OXO and OXY (Table 1). It should be noted that, as these methods employ different statistical approaches, the numerical SD values they calculate are not directly comparable. Similarly, SD values were also calculated for each of the individual laboratory data sets (Table S5). In all cases, these SD values were below the upper limit suggested by Smith (2022) of 1.18 $\log_2$ µg ml^{-1} when calculated by NRI analysis, or 1.11 $\log_2$ µg ml^{-1} when calculated by ECOFFinder. Thus, these data sets were considered to be sufficiently precise for reliable CO_{WT} to be calculated from them.

EUCAST (2021) requires that the MIC data from any individual laboratory should be excluded from any multi-laboratory aggregation if the modal value of its putative WT distribution is >1 dilution different from the most common mode of the distributions for the other laboratories. None of the modal values of the putative WT distributions in the data sets generated by the individual laboratories in the present work differed by >1 dilution from those recorded for the same agent by the other 3 laboratories (Table S5). Thus, the application of this EUCAST rule did not require the exclusion of any data set. On the basis of these considerations of their quantitative and qualitative properties, it was therefore concluded that it would be legitimate, for all 3 agents (FLO, OXO and OXY), to use aggregations of all the data generated by the Cefas, MBA and UoS laboratories, and those previously reported by Miller & Reimschuessel (2006), to calculate proposed CO_{WT} values. The distributions of the MIC values in these aggregated data sets are given in Table 2. A summary of the results of the an-

Table 1. Summary of the analysis of aggregated minimum inhibitory concentration (MIC) data from 4 laboratories by ECOFFinder and normalised resistance interpretation (NRI). For ECOFFinder, the SD and exact cut-off values are for the best-fit line calculated by ECOFFinder. For NRI, the SD and mean + 2 SD are for the normalised wild-type (WT) distribution calculated by NRI. Exact average is the antilog of the means of the $\log_2$-transformed exact cut-off values generated by ECOFFinder and the $\log_2$-transformed mean + 2 SD calculated by NRI. FLO: florfenicol; OXO: oxolinic acid; OXY: oxytetracycline; CO_{WT}: WT epidemiological cut-off value

		Agent		
		FLO	OXO	OXY
Unique isolates tested		493	503	417
ECOFFinder	SD ($\log_2$ µg ml^{-1})	0.84	0.48	0.62
	Exact cut-off (µg ml^{-1})	2.73	0.047	0.74
NRI	SD ($\log_2$ µg ml^{-1})	0.93	0.77	0.85
	Mean + 2 SD (µg ml^{-1})	1.85	0.077	0.85
Exact average (µg ml^{-1})		2.25	0.060	0.79
Proposed CO_{WT} (µg ml^{-1})		⩽4	⩽0.0625	⩽1
Unique WT isolates tested		449	313	276

Table 2. Distribution of MIC values for the aggregated *Aeromonas salmonicida* data sets. Shaded areas: concentrations at which, as a result of the design of the 96-well plates used by the participating laboratories, the frequency of isolates in the aggregations could not be quantified. Off scale: number of isolates for which quantitative determinations of their MIC values could not be made. Abbreviations as in Table 1

Agent	Off scale	MIC (μg ml^{-1}) 0.0008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	Off scale
FLO						10	55	181	151	35	17	10	17	17
OXO	3	5	64	213	29	6	5	14	50	55				59
OXY					3	36	147	70	20	5				136

alyses of the aggregations of the data using the ECOFFinder and NRI spreadsheets is presented in Table 1. The proposed CO_{WT} values calculated by rounding the relevant 'exact average' values were ⩽4 μg ml^{-1} for FLO, ⩽0.0625 μg ml^{-1} for OXO and ⩽1 μg ml^{-1} for OXY (Table 1). For FLO and OXY, these proposed CO_{WT} are identical to the ECVs published in VET04 (CLSI 2020a), but for OXO, the proposed CO_{WT} is 1 dilution lower (Table 3). Although it would be interesting to test whether there are significant differences in the CO_{WT} values calculated from the MIC data for FLO, OXO and OXY determined for typical and atypical *A. salmonicida* strains, this was not possible, as the methodologies for identification used by some of the laboratories did not permit accurate differentiation of typical and atypical isolates.

3.3. Analysis of the aggregated disc diffusion data sets

With respect to their acceptability for setting epidemiological cut-off values, neither CLSI or EUCAST have published minimum quantitative requirements for IZ data sets. For the purposes of the present work, we decided to follow Smith et al. (2023), who set the minimum requirement as ⩾15 observations from isolates categorised as WT from each laboratory and for ⩾100 such observations in any aggregation of data from a minimum of 3 laboratories. The rationale for the requirement that an aggregation should include ⩾100 WT isolates observations is to ensure that any cut-off value calculated from it would adequately capture the variations in IZ recorded for WT isolates which will inevitably arise in the studies of various laboratories that might wish to apply such cut-off values. The 2 main sources of such variation would be the minor differences in the susceptibilities of isolates categorised as WT and the inter-laboratory variations in the performance of the disc diffusion tests. To ensure the capture of the variations arising from any differences in the susceptibilities of WT isolates, it is necessary that aggregated data contain a minimum number of observations from unique isolates determined after the elimination of duplicates. However, it is argued that the inclusion of observations of the IZ of the same isolates made in >1 laboratory in the aggregates used to calculate CO_{WT} would increase the extent that such a value would capture inter-laboratory variations in the performance of disc diffusion tests. Therefore, in the present work, CO_{WT} values were calculated from all IZ data, including any duplicates, always provided that the aggregated data contained observations for ⩾100 unique WT isolates.

The individual IZ data sets from the Cefas, FDA, MBA, MI and UG laboratories for AMP, FLO, OXO and OXY each contained observations for between 20 and 217 isolates that were categorised as WT and, when the duplications resulting from the analysis of the same isolates by both MI and UG were eliminated, the aggregates of these data contained between 227 and 436 observations for unique WT isolates (Table S6). Thus, the quantity of IZ data considered in the present work is sufficient to allow their use in setting CO_{WT} values.

The SD values calculated by NRI analysis can provide a measure of the precision of IZ data sets. An

Table 3. Comparison of the epidemiological cut-off values for *Aeromonas salmonicida* for susceptibility tests performed on unmodified Mueller-Hinton media with incubation at 22 ± 2°C for 44–48h published in VET04Ed3 (CLSI 2020a) with those calculated in the present work. AMP: ampicillin; IZ: inhibition zone; na: not available. Other abbreviations as in Table 1

	MIC data (μg ml^{-1}) FLO	OXO	OXY	IZ data (mm) AMP	FLO	OXO	OXY
VET04	⩽4	⩽0.125	⩽1	na	⩾27	⩾30	⩾28
This work	⩽4	⩽0.0625	⩽1	⩾29	⩾30	⩾32	⩾25

acceptable upper limit of ⩾6.49 mm has been suggested for SD values calculated from IZ data sets obtained at 22°C by a single laboratory (Smith 2019). All the SD values calculated from the 19 individual laboratory data sets (Table S6) were within this limit. As yet, acceptable upper limits have not been developed for multi-laboratory aggregations obtained at this temperature. Nevertheless, the SD values for the 4 aggregations analysed in the present work ranged from 3.4 to 4.6 mm (Table 4).

The distributions of IZ data generated by the individual laboratories are shown in Table S6. Those of the multi-laboratory aggregates are shown for each of the agents in Table 5. The NRI analysis of the aggregated data sets generated CO_{WT} values of ⩾29 mm for AMP, ⩾30 mm for FLO, ⩾32 mm for OXO and ⩾25 mm for OXY (Table 4). The CO_{WT} values for FLO, OXO and OXY, calculated by NRI analysis of aggregated data from 5 laboratories, were only slightly different from the ECVs published in VET04 (CLSI 2020a) that had been estimated from the aggregated data derived from 2 laboratories (Table 3). For FLO and OXO, the CO_{WT} values calculated herein (Table 4) were 3 and 2 mm larger respectively than the published ECV, whilst for OXY, the CO_{WT} value was 3 mm smaller.

3.4. Setting internationally harmonised, consensus epidemiological cut-off values

The process for setting internationally harmonised, consensus ECVs by CLSI is that susceptibility data are first submitted to the Aquatic Working Group of CLSI, who then, following a consideration of these data, prepare a report for the CLSI subcommittee on

Table 4. Summary of the NRI analysis of the aggregated *Aeromonas salmonicida* IZ data sets for 4 antimicrobial agents. Values for SD and CO_{WT} are the SD of the normalised distribution observations for WT isolates and the epidemiological cut-off values calculated from those distributions respectively. WT observations: number of observations from isolates categorised as WT by application of the CO_{WT} shown in this table. Unique observations: number of observations determined after the elimination of duplicate observations made from any isolate. Abbreviations as in Tables 1 & 3

Agent	Laboratories	Total observations	NRI analysis SD (mm)	NRI analysis CO_{WT} (mm)	WT observations Total	WT observations Unique
AMP	4	345	3.4	⩾29	322	227
FLO	5	562	4.2	⩾30	539	436
OXO	5	577	3.4	⩾32	346	303
OXY	5	515	4.6	⩾25	338	283

Table 5. Distribution of IZ diameters for the aggregated *Aeromonas salmonicida* data sets. Shaded areas: zone sizes for isolates that would be categorised as WT by application of the epidemiological cut-off values calculated by NRI analysis of the aggregated data from multiple laboratories. Abbreviations as in Tables 1 & 3

IZ (mm)	AMP	FLO	OXO	OXY
6	5	11	42	66
7		3	2	32
8			13	43
9		1	4	19
10	2		10	8
11	1		7	6
12	1		12	1
13	1		11	
14	1		12	
15			18	
16	1		12	
17	2		9	
18			8	
19			9	
20	1		13	
21			12	
22		1	6	
23			3	
24	1		1	2
25	1		2	
26			4	
27		3	5	
28	5	2	6	2
29	6	2	2	1
30	3	6	5	4
31	3	7	3	5
32	17	11	1	11
33	16	7	3	15
34	18	15	6	40
35	26	22	15	29
36	23	18	10	52
37	21	38	18	32
38	29	40	34	35
39	29	44	39	35
40	55	43	61	18
41	29	34	49	15
42	17	70	40	10
43	18	55	22	9
44	5	39	15	10
45	3	17	10	8
46	3	31	14	3
47		8	5	1
48	1	8	1	2
49		3		1
50		2	1	
51	1	6		
52		3	1	
53		1		
54			1	
55		2		
56		4		
57		1		
58		1		
59				
60				
61		2		

Veterinary Antimicrobial Susceptibility Testing (VAST) to make the final decision. The data generated in the present work has been submitted to CLSI, and the ECVs for *A. salmonicida* susceptibility data generated at 22 ± 2°C with incubation for 44–48 h, to be published in the next edition of the CLSI supplement VET04, will be those set following a consideration of these data by the VAST subcommittee.

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