Vol. 155: 35–42, 2023
https://doi.org/10.3354/dao03740

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 3

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

Setting epidemiological cut-off values for *Vibrio harveyi* relevant to MIC data generated by a standardised microdilution method

Peter Smith[1,*], Luana Cortinovis[2], Tobia Pretto[2], Amedeo Manfrin[2], Daniela Florio[3], Marialetizia Fioravanti[3], Sandrine Baron[4], Laëtita Le Devendec[4], Eric Jouy[4], Alain Le Breton[5], Sara Picon-Camacho[5], Ivana Giovanna Zupičić[6], Dražen Oraić[6], Snježana Zrnčić[6]

[1]Department of Microbiology, University of Galway, Galway H91 TK33, Ireland
[2]Fish Pathology Unit, Istituto Zooprofilattico Sperimentale delle Venezie, 35020 Legnaro, Italy
[3]Department of Veterinary Medical Sciences, Alma Mater Studiorum Università di Bologna, 40064 Ozzano Emilia (BO), Italy
[4]Anses, Ploufragan-Plouzané-Niort Laboratory, Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit, 22440 Ploufragan, France
[5]Vet'eau-Selarl du Dr Alain Le Breton, 1989 Rue des Pyrénées, 31330 Grenade sur Garonne, France
[6]Laboratory for Fish and Molluscs Diseases, Croatian Veterinary Institute, 10000 Zagreb, Croatia

ABSTRACT: The lack of internationally harmonised criteria for interpreting the data generated by standardised susceptibility testing methods presents a serious obstacle for the development of prudent use of antimicrobials in aquaculture. The data required to set epidemiological cut-off values for minimum inhibitory concentrations for antibiotic agents against *Vibrio harveyi* was determined using a standard microdilution method that specified the use of cation-adjusted Mueller Hinton broth and incubation at 28°C for 24 to 28 h. In total, 120 observations were made in 4 independent laboratories from 109 unique isolates. The aggregated data from these laboratories were analysed by the normalised resistance method and by ECOFFinder to calculate epidemiological cut-off values. The data for chloramphenicol, meropenem and sulfamethoxazole were not considered as suitable for analysis. The data for ampicillin indicated that this species is innately resistant to this agent. No acceptable ranges for quality control strains have been set for ceftazidime and, therefore, only provisional cut-off values could be generated for this agent. The epidemiological cut-off values were, however, calculated for the other 6 agents. These values were ≤0.5 µg ml^{-1} for enrofloxacin, ≤1 µg ml^{-1} for florfenicol, oxolinic acid and oxytetracycline, ≤4 µg ml^{-1} for gentamicin and ≤0.5/9.5 µg ml^{-1} for trimethoprim/sulfamethoxazole. Evidence is presented demonstrating that the data for these 6 antimicrobial agents was of sufficient quantity and quality that they could be used by the relevant authorities to set internationally harmonised, consensus epidemiological cut-off values for *V. harveyi*.

KEY WORDS: *Vibrio harveyi* · Standardised MIC protocol · Epidemiological cut-off value · NRI · ECOFFinder

1. INTRODUCTION

Vibrio harveyi is a Gram-negative pathogenic bacteria that is associated with high mortalities in temperate and tropical areas worldwide in commercial marine aquaculture species (fish, crustaceans and shellfish) at all developmental stages (Pretto 2020, Zhang et al. 2020). In addition, cases of wound infections by *V. harveyi* have been recently reported in humans (Del Gigia-Aguirre et

*Corresponding author: peter.smith@nuigalway.ie

Publisher: Inter-Research · www.int-res.com

al. 2017, Brehm et al. 2020). In Mediterranean aquaculture, *V. harveyi* outbreaks are seasonal, occurring mainly during warmer periods, from the end of spring to autumn (Pujalte et al. 2003). It is probable that the increase in water temperature resulting from climate changes will in turn increase the frequency of *V. harveyi* outbreaks in aquaculture systems (Amaro et al. 2020). Although there has been extensive research on the development of an effective vaccine to control *V. harveyi* outbreaks in aquaculture species (Nguyen et al. 2017, 2018, Mohd-Aris et al. 2019, Abu Nor et al. 2020, Gong et al. 2021), no commercial vaccines are currently available (Zhang et al. 2020). In the field, autogenous vaccines have been developed over the past 10 yr to control the disease in seabream and seabass production; however, their efficacy and application remain limited and effective control still relies on antimicrobial therapies (A. Le Breton pers. obs.). The isolation of strains showing reduced susceptibility to some of these antimicrobial agents has, however, been reported (Zhu et al. 2018, Deng et al. 2020), although it should be noted that both these studies manifested some of the shortcomings identified by Smith & Egan (2018). Importantly, neither applied internationally recognised interpretive criteria to establish the meaning of the data they obtained.

The Food and Agriculture Organisation of the United Nations (FAO 2022) has identified programmes to monitor the antimicrobial susceptibility of *Vibrio* species isolated from aquatic animals as a priority. The ubiquity and significance of *V. harveyi* infections in aquaculture strongly suggests that this species should be included in the *Vibrio* species to be studied in such programmes. The World Organization for Animal Health (WOAH) Aquatic Animal Health Code (www.woah.org/en/what-we-do/standards/codes-and-manuals/#ui-id-3) recommends that monitoring and surveillance of antimicrobial susceptibility of bacteria isolated from aquatic animals should be performed using standardised testing protocols. It is also recommended that the meaning of the data obtained through these standardised protocols should be generated by the application of relevant, internationally harmonised, consensus epidemiological cut-off values. With respect to the availability of standardised methods for antimicrobial susceptibility testing, those published by the Clinical Laboratory Standards Institute (CLSI 2020a) provide suitable conditions for the testing of the majority (84%) of the 44 bacterial species most frequently isolated from aquatic animals including the facultative halophilic *Vibrio* species such as *V. harveyi* (Smith 2019). However, international harmonised epidemiological cut-off values have been published for very few aquatic species so far (Smith 2020). With respect to susceptibility data generated using standard methods specifying incubation at temperatures <35°C, the latest edition of the CLSI guideline VET04 (CLSI 2020b) provides epidemiological cut-off values (ECVs) for only 4 species (*Aeromonas salmonicida*, *A. hydrophila*, *Flavobacterium columnare* and *F. psychrophilum*). Significantly, VET04 (CLSI 2020b) does not provide ECV values for any *Vibrio* species.

The work reported here was performed to obtain the data that would be required by CLSI (2018) to set ECVs for *V. harveyi* minimum inhibitory concentrations (MIC) data generated by a standardised microdilution method specifying incubation at 28°C for 24 to 28 h (CLSI 2020a).

2. MATERIALS AND METHODS

2.1. Participating laboratories

Five laboratories were involved in this study: the Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit of the Ploufragan-Plouzané-Niort Laboratory of the French Agency for Food, Environmental and Occupational Health & Safety (Anses), the Laboratory for Fish and Molluscs Diseases of the Croatian Veterinary Institute, Zagreb, Croatia (CVI), the Department of Veterinary Medical Sciences of Alma Mater Studiorum Università di Bologna, Italy (DIMEVET), the National Reference Laboratory for Fish, Mollusc and Crustacean Diseases of the Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy (IZSVe) and Vet'Eau, Grenade sur Garonne, France (VETEAU).

2.2. Isolate collections

The isolates studied in this work had been collected by the 5 participating laboratories. Using MALDI-TOF (Microflex LT, Bruker Daltonics) (Singhal et al. 2015, Florio et al. 2018, Culot et al. 2021), these isolates were classified as *Vibrio harveyi* clade members by those laboratories. For this work, their identification was further confirmed by *V. harveyi* species-specific amplification of the *toxR* gene sequence (382 bp) using the PCR

primers of Pang et al. (2006) developed on the hypervariable membrane-tether region of the *toxR* gene: toxRF1 5′-GAA GCA GCA CTC ACC GAT-3′ and toxRR1 5′-GGT GAA GAC TCA TCA GCA-3′ using the protocol recommended by Pretto (2020, 2018). As the extensive validation studies of Pang et al. (2006), Pretto (2018) and Triga et al. (2023) demonstrated that the these primers are species-specific, isolates from which they generated the expected 382 bp fragment were classified as *V. harveyi*.

In total, 109 *V. harveyi* isolates were studied: 93 were obtained from bony fish, 5 from crustaceans or crustacean farms, 10 from molluscs and 1 from an environmental sample. To minimise the inclusion of multiple isolates of individual clones, these 109 isolates were obtained from a variety of aquaculture facilities, in 11 different countries and over a 15 yr period (Table 1). Eighty-eight of the 109 isolates were from unique farms or environments. The other 21 were from 8 farms; however, they were collected from these farms in different years or, if from the same year, from different fish species.

Table 1. Isolates of *Vibrio harveyi* in the collections of the participating laboratories. Anses: Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit, Ploufragan-Plouzané-Niort Laboratory of the French Agency for Food, Environmental and Occupational Health & Safety, France; CVI: Laboratory for Fish and Molluscs Diseases, Croatian Veterinary Institute, Zagreb, Croatia; DIMEVET: Department of Veterinary Medical Sciences, Alma Mater Studiorum Università di Bologna, Italy; IZSVe: National Reference Laboratory for Fish, Mollusc and Crustacean Diseases, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy; VETEAU: Vet'Eau, Grenade sur Garonne, France

Laboratory	Isolate origin	Date of isolation	Number of isolates
Anses	Brazil	2018	2
CVI	Croatia	2016–2021	20
	Italy	2014–2019	8
	Spain	2019	1
	Turkey	2016	1
DIMEVET	Italy	2011–2018	22
	Albania	2016	2
IZSVe	Italy	2006–2021	24
VETEAU	France	2016–2020	22
	Turkey	2017–2018	2
	Tunisia	2018	1
	Portugal	2020	1
	Cyprus	2016	1
	Qatar	2021	1
	Madagascar	2018	1
Total			109

For some laboratories, the results of previous studies of susceptibility were available. Isolates that had been provisionally categorised as fully susceptible were preferentially selected for inclusion in the isolate sets for that laboratory used in this work. As a consequence of this bias, it would not be legitimate to use the frequencies of reduced susceptibilities in the isolates studied as measures of the frequency of such phenotypes in the areas or locations from which the isolates were made.

2.3. Analysis of isolates

With the exception of the 30 isolates collected by VETEAU, which were analysed by Anses, each laboratory performed MIC determinations on their own collections of isolates. In addition, the MIC values for 11 isolates were made in 2 laboratories. Ten of the isolates in the VETEAU collection were analysed in both the Anses and CVI laboratories and 1 of the isolates in the CVI collection was also analysed in the IZSVe laboratory. Thus, 40 MIC observations were reported by CVI, 31 by Anses, 25 by IZSVe and 24 by DIMEVET, giving a total of 120 observations from the 109 isolates studied in this work.

2.4. Susceptibility measurements

MIC values were determined by a microdilution method provided in the CLSI guideline VET03 (CLSI 2020a) that specified using cation-adjusted Mueller Hinton broth (CAMHB) that was not supplemented with NaCl and incubation at 28°C for 24 to 28 h. The custom made 96 well ECOFFVIB plates used in the MIC studies were manufactured by Thermo Fisher according to the layout developed by Baron et al. (2020b). The concentration ranges of the agents in these plates is shown in Table S1 in the Supplement (www.int-res.com/articles/suppl/d155p035_supp.pdf).

Each laboratory employed both the quality control (QC) reference strains *Escherichia coli* ATCC 2592 and *Aeromonas salmonicida* ATCC 33658 recommended by CLSI for this method (CLSI 2020a). The CLSI document VET04 (CLSI 2020b) provides acceptable ranges for these QC reference strains tested using the MIC protocols adopted in this work for ampicillin (AMP), enrofloxacin (ENR), florfenicol (FLO), gentamicin (GEN), oxytetracycline (OXY), oxolinic acid (OXO), and trimethoprim/sulfamethoxazole (TRS).

2.5. Calculation of proposed epidemiological cut-off values

In this work, epidemiological cut-off values (CO_{WT}) were calculated from the aggregations of the MIC data from the participating laboratories using the 2 automatic spreadsheets, ECOFFinder (www.eucast.org/mic_distributions_and_ecoffs/) and NRI (www.bioscand.se/nri/), both available as free downloads. The ECOFFinder 99.9% 'exact' values were used in this work (European Committee for Antimicrobial Susceptibility Testing [EUCAST] 2021a). In generating cut-off values, the ECOFFinder spreadsheet calculates what it refers to as an 'exact' cut-off value and then rounds this value up to the next highest dilution actually used in the test. Similarly, the NRI spreadsheet calculates an exact value for the mean plus 2 standard deviations (mean + 2 SD) of the normalised distribution of MICs for putative wild-type (WT) isolates and then rounds this value up to generate a cut-off value. A single value for the epidemiological cut-off values proposed in this work was generated by averaging the 'exact' values generated by the 2 methods and rounding up to the next higher dilution actually used in the test.

The SD values of the best-fit curve generated by ECOFFinder and the normalised distribution of WT observations calculated by NRI were used as measures of the precision of the aggregated data sets for each agent (Smith et al. 2018). Data sets which exceeded the SD limits developed 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 were considered as too imprecise for reliable values of CO_{WT} to be calculated from them.

When an isolate failed to grow in any of the wells that contained an agent but did grow in the control well without any agent, the MIC could only be recorded as equal to or less that the lowest concentration of that agent in the ECOFFVIB plates. These observations were termed 'below scale'. Aggregations that contained 'below-scale' observations were considered suitable for analysis by ECOFFinder and NRI provided that the frequency of 'below-scale' observations was <10% of the putative WT observations and was not greater than the frequency recorded for the lowest MIC that could be quantified. For the purpose of analyses of such aggregations, the 'below-scale' observations were treated as having an MIC equal to the lowest concentration in the ECOFFVIB plate.

2.6. Terminology and abbreviations

With respect to the abbreviations used for epidemiological cut-off values, we followed the recommendations of Smith (2019). The abbreviation ECV was reserved for cut-off values published by CLSI. The abbreviation CO_{WT} was used for all epidemiological cut-off values not set by CLSI but calculated for data generated by laboratories that had demonstrated compliance with the QC requirement of the standard method they adopted. The abbreviation pCO_{WT} was used for the provisional epidemiological cut-off values generated for agents for which no QC requirements have been set by CLSI (2020b). Following the recommendations of Silley (2012) the abbreviation WT was used for isolates that manifested MIC values at or below the CO_{WT} and were, therefore, assumed to be fully susceptible members of their species. The abbreviation NWT was used for those that manifested MIC values greater than the CO_{WT} and were assumed to have acquired some mechanism conferring reduced susceptibility.

The abbreviations adopted for the antimicrobial agents (see Table 2) were those recommended in the EUCAST System for Antimicrobial Abbreviations (www.eucast.org/eucast/guidance_documents/).

3. RESULTS AND DISCUSSION

3.1. Quality control

The CLSI guideline VET04 (CLSI 2020b) provides acceptable ranges for the MIC values obtained with QC reference strains for AMP, ENR, FLO, GEN, OXO, OXY and TRS obtained using the testing protocol adopted in this work. All 4 laboratories recorded MIC values for these agents within the acceptable ranges, as presented in Table S2 in the Supplement.

3.2. *Vibrio harveyi* data sets

Table 2 shows the distributions of the MIC values in the aggregations of the data generated in the 4 laboratories for the 7 agents for which acceptable ranges have been set and for the 4 agents (CTZ, CHL, MER and SME) for which they have not yet been set. This table also presents the mean CO_{WT} or pCO_{WT} values calculated for the 7 data sets that were considered as suitable for analysis and the numbers of isolates categorised as WT by the application of

Table 2. The distributions of 120 MIC values determined by 4 laboratories from the study of 109 isolates of *Vibrio harveyi* using 11 antimicrobial agents. Unshaded boxes indicate that MIC values could be quantified using the ECOFFVIB plates. AMP: ampicillin; CTZ: ceftazidime; CHL: chloramphenicol; ENR: enrofloxacin; FLO: florfenicol; GEN: gentamicin; MER: meropenem; OXO: oxolinic acid; OXY: oxytetracycline; SME: sulfamethoxazole; TRS: trimethoprim/sulfamethoxazole; CO_{WT}: epidemiological cut-off value; pCO_{WT}: provisional epidemiological cut-off value; nc: values not calculated

MIC (µg ml^{-1})	Antimicrobial agent										
	AMP	ENR	FLO	GEN	OXO	OXY	TRS[a]	CTZ	CHL	MER	SME
Below scale									86	79	6
0.004											
0.008											
0.015										35	
0.03		1					2			5	
0.06		6				1	50				
0.125		48			19	17	31	1			
0.25		57	12		70	76	34	40			
0.5		7	101		29	23	2	66			
1			5	32	2		1	12	33		
2				80				1			
4			1	8							
8											11
16			1								27
32									1		31
64											24
128											4
256											2
512											7
Above scale	120	1				3				1	8
SD (ECOFFinder)[b]	nc	0.65	0.33	0.47	0.60	0.53	1.00	0.59	nc	nc	1.28
SD (NRI)[c]	nc	0.80	0.50	0.58	0.85	0.62	0.76	0.72	nc	nc	1.54
CO_{WT}[d]	nc	≤0.5	≤1	≤4	≤1	≤1	≤0.5				
$_pCO_{WT}$[d]								≤1	nc	nc	nc
WT[e]	nc	108	107	109	109	106	109	108	nc	nc	nc

[a]The MIC values for TRS are recorded in this table as the trimethoprim concentrations in the wells; [b]The SD of the best-fit curves calculated by ECOFFinder; [c]The SD of the normalised distribution of WT observations calculated by NRI; [d]These values are the mean CO_{WT} or $_pCO_{Wt}$ values calculated by ECOFFinder and NRI; [e]The number of isolates categorised as wild-type by application of CO_{WT} or $_pCO_{Wt}$ values

those values. Table S2 details the MIC values generated by each laboratory and the MIC values obtained by those laboratories for the QC reference strains.

3.3. Interpretation of the off-scale observations

The CLSI guideline M23 (CLSI 2018) suggests that, in so far as possible, the MIC values to be used in setting ECVs should be on scale. In aggregations for 7 agents, all observations were on scale. However, the concentration range in the ECOFFVIB plates resulted in off scale observations being recorded for 4 agents. With respect to the aggregation of the data for SME, 6 of the 120 observations were 'below scale' and were reported as ≤4 µg ml^{-1}. For the purposes of ECOFFinder and NRI analyses, the MICs of these 8 observations were treated as being 4 µg ml^{-1}. In the aggregations of MIC data for AMP, all 120 observations were above scale. These data were assumed to indicate that all the *V. harveyi* isolates studied in this work manifested a reduced susceptibility to AMP and, therefore, a CO_{WT} value was not calculated for this agent. Resistance to this agent is considered an innate property of some, but not all species of *Vibrio* (CLSI 2020a), and reduced susceptibility to AMP has also been reported for all isolates of *V. anguillarum* studied by Baron et al. (2020a).

In the aggregations for CHL and MER, 72 and 66% respectively of the observations were below scale. As the data for these 2 agents failed to capture the quantitative MIC values for the majority of the putative WT isolates, pCO_{WT} could not be calculated from them. A recent EUCAST document (EUCAST 2021b) presented the distributions of MICs for MER that were generated at 35°C for 5 *Vibrio* species. For 1 group, *V. cholerae* and *V. fluvialis*, the modes of the MIC distributions were be-

tween 0.125 and 0.25 µg ml^{-1}. For a second group, *V. alginolyticus, V. parahaemolyticus* and *V. vulnificus*, the modes were in the range of 0.008 to 0.016 µg ml^{-1}. In this work, although the data for *V. harveyi* were generated at 28°C and were, therefore, not directly comparable to the data for these 5 species, they suggest that, with respect to MER susceptibility, it is probably related to the second group. Analyses of whole genome sequences have also suggested a close relationship of *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* (Urbanczyk et al. 2013).

3.4. Precision of data sets

For 7 agents (CTZ, ENR, FLO, GEN, OXO, OXY and TRS) of the 8 that were analysed by ECOFFinder and NRI, the observed MICs were tightly grouped. Between 92 and 100% of the observations for each of these agents were within a 3 dilution range. The SD values calculated for these 7 multi-laboratory aggregates by ECOFFinder and NRI (Table 2) were all below the upper limits suggested by Smith (2022) of >1.11 $\log_2$ and >1.18 $\log_2$ µg ml^{-1}, respectively. These data were, therefore, considered as sufficiently precise to allow the calculation of reliable cut-off values. The distribution of the MIC values in the aggregation for SME was more diverse than those for the other 7 agents for which CO_{WT} or pCO_{WT} values were calculated. Analysis of the SME data by ECOFFinder generated an SD of 1.28 $\log_2$ µg ml^{-1} and analysis by NRI generated an SD of 1.54 $\log_2$ µg ml^{-1}. As both these SD values were in excess of the suggested limits (Smith 2022), the SME data was considered too imprecise for a reliable CO_{WT} value to be calculated for this agent.

3.5. Epidemiological cut-off values

The aggregated MIC data for 7 agents were considered suitable for analysis by ECOFFinder and NRI. All 4 laboratories had demonstrated compliance with the QC requirements of the test protocol (CLSI 2020b) for 6 of these agents (Table S2). It was, therefore, possible to calculate mean CO_{WT} values from their MIC distributions. The results of these analyses are shown in Table 3. The calculated mean CO_{WT} values were ≤0.5 µg ml^{-1} for ENR, ≤1 µg ml^{-1} for FLO, OXO and OXY, ≤4 µg ml^{-1} for GEN and ≤0.5/9.5 µg ml^{-1} for TRS (Table 3). For the seventh agent, CTZ, acceptable ranges for QC reference strains tested under the standard method used in this work have not yet been set (CLSI 2020b) and, therefore, only a mean pCO_{WT} of ≤1 µg ml^{-1} could be calculated for this agent.

4. CONCLUSIONS

The CLSI guideline M23 (CLSI 2018) states that ECVs for MIC data can be determined only if that data is obtained for a single species and if they were generated by a recognised reference (standardised) method. This guideline also states that ECVs should account for both strain to strain variation and inter-laboratory variation in the performance of MIC assays. To achieve this, the data used to set ECVs should be sourced from at least 3 laboratories and the aggregated data sets should include MIC observations from at least 100 unique strains. As ECVs are based on the assumption that the distribution of MIC values of WT isolates do not vary geographically or over time (CLSI 2018), the guideline does not specify any requirements with respect to these parameters.

Table 3. The exact cut-off values and the proposed epidemiological cut-off values (CO_{WT}) or provisional epidemiological cut-off values (pCO_{WT}) calculated from the analysis of 120 observations made in 4 laboratories for *Vibrio harveyi*. See Table 2 for antimicrobial abbreviations

Agent	Exact cut-off values (µg ml^{-1})			Proposed epidemiological cut-off values (µg ml^{-1})	
	ECOFFinder[a]	NRI[b]	Mean	CO_{WT}	pCO_{WT}
CTZ	1.042	0.931	0.987		≤1
ENR	0.518	0.450	0.484	≤0.5	
FLO	0.686	0.840	0.763	≤1	
GEN	3.356	2.931	3.143	≤4	
OXO	0.694	0.857	0.776	≤1	
OXY	0.573	0.497	0.535	≤1	
TRS	0.672	0.216	0.444	≤0.5/9.5	

[a]The exact cut-off values reported in this column were those calculated for the 99.9% ECOFF by ECOFFinder; [b]The exact cut-off values reported in this column were the mean +2 SD for the normalised WT distributions calculated by NRI analyses

The aggregated MIC data sets for *Vibrio harveyi* with respect to 6 agents, ENR, FLO, GEN, OXO, OXY and TRS, were obtained in 4 independent laboratories. All MIC assays were performed using the standard test method specifying the use of unmodified CAMHB and incubation at 28°C for 24 to 28h (CLSI 2020a), and all the participating laboratories reported meeting the QC requirements set for this method (CLSI 2020b) (Table S2). The mean CO_{WT} values for these agents were calculated by analysis of aggregated data sets containing 120 observations made from 109 isolates. Although the guideline M23 (CLSI 2018) specifies a requirement for >100 unique isolates, it can be argued that the number of observations from unique isolates categorised as WT is a more relevant parameter. In this work the aggregated data sets for the 7 agents contained between 106 and 109 observations from isolates categorised as WT (Table 2). The aggregated data sets obtained for the 6 agents ENR, FLO, GEN, OXO, OXY and TRS met all the requirements specified in M23 (CLSI 2018). It is, therefore, intended that the CO_{WT} calculated for these agents (Table 3) will be submitted to CLSI for consideration as ECV values.

Acknowledgements. This study received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 727315 (MedAID) and grant agreement No. 727610 (PerformFISH). This publication solely reflects the views of the authors and the European Union cannot be held responsible for any use which may be made of the information contained therein. Authors are grateful to Süheyla Steinum Karataş from Istanbul University, Turkey and Daniel Gijón from Skretting Espana, SA, Spain for providing isolates of *Vibrio harveyi*.

LITERATURE CITED

Abu Nor N, Zamri-Saad M, Md Yasin IS, Salleh A, Mustaffa-Kamal F, Matori MF, Azmai MN (2020) Efficacy of whole cell inactivated *Vibrio harveyi* vaccine against vibriosis in a marine red hybrid Tilapia (*Oreochromis niloticus* × *O. mossambicus*) model. Vaccines 8:734

Amaro C, Fouz B, Sanjuan E, Romalde JL (2020) Vibriosis. In: Woo PTK, Leong JA, Buchmann K (eds) Climate change and infectious fish diseases. CAB International, Wallingford, p 182–210

Baron S, Ceccarelli D, Dalsgaard I, Granier SA and others (2020a) Influence of incubation time on antimicrobial susceptibility testing of pathogenic *Vibrio anguillarum* and *Vibrio vulnificus* isolated from fish. Aquaculture 524: 735258

Baron S, Smith P, Verner-Jeffreys DW (2020b) Setting epidemiological cut-off values for bacteria isolated from aquatic animals: a toolbox for designing a 96-well plate for microdilution MIC assays. Bull Eur Assoc Fish Pathol 40:55–61

Brehm TT, Berneking L, Rohde H, Chistner M, Schlickewe C, Martins MS, Schmiedel S (2020) Wound infection with *Vibrio harveyi* following a traumatic leg amputation after a motorboat propeller injury in Mallorca, Spain: a case report and review of literature. BMC Infect Dis 20:104

CLSI (Clinical Laboratory Standards Institute) (2018) Development of in vitro susceptibility testing criteria and quality control parameters. CLSI guideline M23, 5th edn. Clinical Laboratory Standards Institute, Wayne, PA

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

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

Culot A, Grosset N, Bruey Q, Auzou M and others (2021) Isolation of *Harveyi* clade *Vibrio* spp. collected in aquaculture farms: How can the identification issue be addressed? J Microbiol Methods 180:106106

Del Gigia-Aguirre L, Sánchez-Yebra-Romera W, García-Muñoz S, Rodríguez-Maresca M (2017) First description of wound infection with *Vibrio harveyi* in Spain. New Microbes New Infect 19:15–16

Deng Y, Xu L, Liu S, Wang Q, Guo Z, Chen C, Feng J (2020) What drives changes in the virulence and antibiotic resistance of *Vibrio harveyi* in the South China Sea? J Fish Dis 43:853–862

EUCAST (European Committee for Antimicrobial Susceptibility Testing) (2021a) SOP 10.2 MIC distributions and the setting of epidemiological cut-off (ECOFF) values. www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/EUCAST_SOPs/2021/EUCAST_SOP_10.2_MIC_distributions_and_epidemiological_cut-off_value__ECOFF__setting_20211202.pdf

EUCAST (2021b) EUCAST proposed breakpoints for *Vibrio* species. www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/2021/Vibrio_breakpoints_consultation_1OCT2021.pdf

FAO (Food and Agriculture Organisation of the United Nations) (2022) Monitoring and surveillance of antimicrobial resistance in bacterial pathogens from aquaculture. Regional guidelines on monitoring and surveillance of antimicrobial resistance, use and residues in food and agriculture, Vol 3. FAO, Bangkok

Florio W, Tavanti A, Barnini S, Ghelardi E, Lupetti A (2018) Recent advances and ongoing challenges in the diagnosis of microbial infections by MALDI-TOF mass spectrometry. Front Microbiol 9:1097

Gong H, Wang Q, Lai Y, Zhao C and others (2021) Study on immune response of organs of *Epinephelus coioides* and *Carassius auratus* after immersion vaccination with inactivated *Vibrio harveyi* vaccine. Front Immunol 11: 622387

Mohd-Aris A, Saad MZ, Daud HM, Yusof MT, Ina-Salwany MY (2019) *Vibrio harveyi* protease deletion mutant as a live attenuated vaccine candidate against vibriosis and transcriptome profiling following vaccination for *Epinephelus fuscoguttatus.* Aquacult Int 27:125–140

Nguyen HT, Thu Nguyen TT, Tsai MA, Ya-Zhen E, Wang PC, Chen SC (2017) A formalin-inactivated vaccine provides good protection against *Vibrio harveyi* infection in orange-spotted grouper (*Epinephelus coioides*). Fish Shellfish Immunol 65:118–126

Nguyen HT, Nguyen TTT, Chen YC, Vu-Khac H, Wang PC, Chen SC (2018) Enhanced immune responses and effectiveness of refined outer membrane protein vaccines against *Vibrio harveyi* in orange-spotted grouper (*Epinephelus coioides*). J Fish Dis 41:1349–1358

Pang L, Zhang XH, Zhong Y, Chen J, Li Y, Austin B (2006) Identification of *Vibrio harveyi* using PCR amplification of the toxR gene. Lett Appl Microbiol 43:249–255

Pretto T (2018) Vibriosi da *Vibrio harveyi*: studi di eziopatogenesi e di efficacia vaccinale nel branzino (*Dicentrarchus labrax*). PhD thesis, University of Bologna

Pretto T (2020) *Vibrio harveyi* group. In: Zrnčić S (ed) Diagnostic manual for the main pathogens in European seabass and gilthead seabream aquaculture. Options Méditerranéennes, Series B, No. 75, CIHEAM, Zaragoza, p 75–82

Pujalte MJ, Sitjà-Bobadilla A, Macián MC, Belloch C and others (2003) Virulence and molecular typing of *Vibrio harveyi* strains isolated from cultured dentex, gilthead sea bream and European sea bass. Syst Appl Microbiol 26:284–292

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

Singhal N, Kumar M, Kanaujia PK, Virdi JS (2015) MALDI-TOF mass spectrometry: An emerging technology for microbial identification and diagnosis. Front Microbiol 6: 791

Smith P (2019) The performance of antimicrobial susceptibility testing programmes relevant to aquaculture and aquaculture products. FAO Fisheries and Aquaculture Circular No. 1191. FAO, Rome

Smith P (2020) Eight rules for improving the quality of papers on the antimicrobial susceptibility of bacteria isolated from aquatic animals. Dis Aquat Org 139:87–92

Smith P (2022) Precision limits for MIC data required to set epidemiological cut-off values. Bull Eur Assoc Fish Pathol 42:34–38

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, 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

Urbanczyk H, Ogura Y, Hayashi IT (2013) Taxonomic revision of Harveyi clade bacteria (family *Vibrionaceae*) based on analysis of whole genome sequences. Int J Syst Evol Microbiol 63:2742–2751

Zhang XH, He X, Austin B (2020) *Vibrio harveyi:* a serious pathogen of fish and invertebrates in mariculture. Mar Life Sci Technol 2:231–245

Zhu ZM, Dong CF, Weng SP, He JG (2018) The high prevalence of pathogenic *Vibrio harveyi* with multiple antibiotic resistance in scale drop and muscle necrosis disease of the hybrid grouper, *Epinephelus fuscoguttatus* (♀) × *E. lanceolatus* (♂), in China. J Fish Dis 41:589–601

Editorial responsibility: Andrew Barnes, Brisbane, Queensland, Australia
Reviewed by: C. Miranda 2 anonymous referees

Submitted: January 31, 2023
Accepted: June 1, 2023
Proofs received from author(s): July 31, 2023