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Epidemiological cut-off values for *Vibrio parahaemolyticus* calculated from minimal inhibitory concentration data generated at 35 and 28°C

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ABSTRACT: This work was performed to generate the data needed to set epidemiological cut-off values for minimal inhibitory concentrations (MICs) of 10 antimicrobial agents against *Vibrio parahaemolyticus* determined using standardised broth microdilution protocols. Eight laboratories performed broth microdilution tests with incubation at 35°C for 16 to 20 h, and 7 also performed tests on the same isolates with incubation at 28°C for 24 to 28 h. Data were analysed by the ECOFFinder and normalised resistance interpretation algorithms. The cut-off values calculated for ceftazidime, florfenicol and trimethoprim/sulfamethoxazole, 1, 1 and 0.25/4.75 µg ml^{-1}, respectively, were the same when calculated from data obtained at both temperatures. The cut-off values calculated from data obtained at 35°C and from data obtained at 28°C were 0.25 and 0.5 µg ml^{-1} for enrofloxacin, 2 and 4 µg ml^{-1} for gentamicin, 0.5 and 1 µg ml^{-1} for oxolinic acid and 2 and 1 µg ml^{-1} for oxytetracycline, respectively. The influence of incubation temperature on MIC values was investigated by comparing MICs obtained at 35 and 28°C for a specific antimicrobial agent with a particular isolate by an individual laboratory. Results showed that 56% of 1473 of these paired MIC values were identical, while 38% differed from one another by not more than 1 dilution step. The data generated in this work will be submitted to the Clinical and Laboratory Standards Institute for consideration in their setting of internationally agreed epidemiological cut-off values for *V. parahaemolyticus* that are essential for interpreting antimicrobial susceptibility testing data of this species.

KEY WORDS: *Vibrio parahaemolyticus* · Epidemiological cut-off values · Minimal inhibitory concentration · Incubation temperature · MIC · CO^{WT} · Antimicrobial

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1. INTRODUCTION

Vibrio parahaemolyticus is a gram-negative bacterium that is frequently isolated from marine and estuarine waters (Baker-Austin et al. 2010) and aquaculture products (Yang et al. 2020). Infections of humans with *V. parahaemolyticus*, which normally result in self-limiting gastroenteritis (Onohuean et al. 2022, Baker-Austin et al. 2010), are most frequently associated with the consumption of raw or undercooked aquatic animals (Daniels et al. 2000). *V. parahaemolyticus* has also been reported as the causative agent of diseases in a wide variety of aquatic animals including prawns, tilapia and catfish and a wide variety of shellfish (Ina-Salwany et al. 2019). Of these, acute hepatopancreatic necrosis disease, which primarily affects penaeid shrimp, is probably the most economically significant (Kumar et al. 2020).

The 2023 edition of the Aquatic Animal Health Code published by the World Organisation for Animal Health (WOAH) recommends that appropriate authorities should initiate monitoring and surveillance programmes of the antimicrobial susceptibilities of bacteria isolated from aquatic animals (WOAH 2023). It also states that such programmes should include the investigation of the susceptibility to antimicrobial agents used to treat diseases in aquatic animals. These investigations should be performed using standardised antimicrobial susceptibility testing methods, with the resulting susceptibility data interpreted by the application of internationally agreed epidemiological cut-off values whenever available. In the aforementioned code, *V. parahaemolyticus* was listed as a species that should be studied in routine monitoring and surveillance programmes (WOAH 2023).

V. parahaemolyticus is not an obligate halophile. Therefore, the standardised methods for testing non-fastidious bacterial species published in the Clinical and Laboratory Standards Institute (CLSI) guideline VET03 (CLSI 2020a), which specify media not supplemented with salt, are suitable for testing the antimicrobial susceptibility of this species. However, as yet, no internationally agreed epidemiological cut-off values have been set that would facilitate the interpretation of *V. parahaemolyticus* susceptibility data. The work reported in this manuscript was undertaken to produce the data needed to set internationally agreed epidemiological cut-off values for *V. parahaemolyticus*.

2. MATERIALS AND METHODS

2.1. Participating laboratories

Eight laboratories were involved in the determination of minimal inhibitory concentrations (MICs) for *Vibrio parahaemolyticus.* These were the Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit, Ploufragan-Plouzané-Niort Laboratory, French Agency for Food, Environmental and Occupational Health & Safety; the Centre for Environment Fisheries and Aquaculture Science Laboratory, Weymouth, UK; the Consultant Laboratory for *Vibrio* spp. in Food, Department Biological Safety, German Federal Institute for Risk Assessment, Berlin, Germany (BfR); the Animal and Plant Health Agency, Addlestone, UK; the Institute of Microbiology and Epizootics, Freie Universität Berlin, Germany; the Center for Veterinary Medicine, US Food and Drug Administration; the Washington Animal Disease Diagnostic Laboratory (WADDL); and the Centre for Animal and Veterinary Science, National Parks Board, Singapore (NParks).

2.2. Isolate collections

The 235 *V. parahaemolyticus* isolates studied in this work were collected by participating laboratories from 1993 to 2021. Of these isolates, 146 originated from European countries, 64 from the USA, 17 from Asia and 8 from South America. In addition, 194 isolates were obtained from aquatic animals, 22 from humans and 19 from water samples. The isolates were identified using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Singhal et al. 2015, Florio et al. 2018, Liu et al. 2022) or specific PCR assays for the *tox*R (Kim et al. 1999), *tlh* (Bej et al. 1999, Nordstrom et al. 2007) and collagenase genes (Di Pinto et al. 2005).

2.3. Terminology and abbreviations

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) developed the concept of epidemiological cut-off values 21 yr ago (Kahlmeter et al. 2003). For a given microbial species and antimicrobial agent, these cut-off values were defined as the highest MIC for wild-type (WT) organisms devoid of phenotypically detectable acquired resistance mechanisms (EUCAST 2003). Since then, internationally agreed epidemiological cut-off values have

been published by CLSI, which uses the abbreviation ECV (CLSI 2020b, 2024), and EUCAST, which uses the abbreviation ECOFF (https://www.eucast.org/mic_distributions_and_ecoffs/). In this work, the abbreviation CO_{WT} is used for all epidemiological cut-off values not set by EUCAST or CLSI but that had been calculated from data generated by laboratories that were in compliance with the quality control (QC) requirements of these agencies. The abbreviations adopted for each antimicrobial agent were those recommended in the EUCAST system for antimicrobial abbreviations (EUCAST 2022).

2.4. Antimicrobial susceptibility testing

The MIC values for 10 antimicrobial agents against *V. parahaemolyticus* were determined by the broth microdilution method using unmodified cation-adjusted Mueller Hinton broth according to the protocol provided in CLSI guideline VET03 (CLSI 2020a) for non-fastidious (Group 1) species. Tests were performed using 2 of the incubation conditions specified in this guideline. Seven of the participating laboratories performed tests at 35°C with incubation for 16 to 20 h, and 6 of them also performed tests on the same isolates at 28°C with incubation for 24 to 28 h. The 96-well plates (AQGNECV) used for testing were manufactured by Thermo Fisher. The antimicrobial agents and concentration ranges in these plates were ampicillin (AMP), 0.015–16 μg ml^{-1}; ceftazidime (CTZ), 0.03–8 μg ml^{-1}; enrofloxacin (ENR), 0.0005–0.25 μg ml^{-1}; florfenicol (FLO), 0.03–16 μg ml^{-1}; gentamicin (GEN), 0.06–8 μg ml^{-1}; meropenem (MER), 0.008–1 μg ml^{-1}; oxolinic acid (OXO), 0.002–1 μg ml^{-1}; oxytetracycline (OXY), 0.015–8 μg ml^{-1}; sulfamethoxazole (SME), 1–512 μg ml^{-1}; and trimethoprim/sulfamethoxazole (TRS), 0.008/0.15–1/19 μg ml^{-1}.

Each laboratory employed the QC reference strain *Escherichia coli* ATCC® 25922 recommended by CLSI for this method (CLSI 2020a). The acceptable QC ranges are provided in CLSI documents VET04 (CLSI 2020b) and M100 (CLSI 2024) for 9 of the 10 antimicrobial agents when tested at 35°C and for 7 when tested at 28°C. Acceptable QC ranges have not yet been established by CLSI for CTZ or MER at 28°C or for SME at either temperature.

2.5. Calculation of CO_{WT}

In this study, CO_{WT} values for *V. parahaemolyticus* were calculated using MIC distributions generated by the participating laboratories, supplemented with additional data accessed from EUCAST (www.eucast.org/mic_distributions_and_ecoffs/). These EUCAST data consisted of MIC distributions of CTZ and TRS against isolates of *V. parahaemolyticus* at 35°C with incubation for 16 to 20 h. Although 2 unnamed independent laboratories generated these data for the purpose of calculating CO_{WT} values, they were combined into a single distribution.

Two automatic algorithms, ECOFFinder (https://www.eucast.org/mic_distributions_and_ecoffs/) and normalised resistance interpretation (NRI) (www.bioscand.se/nri/), both of which can be downloaded for free, were used to calculate CO_{WT} values. EUCAST standard operating procedure (SOP) 10.2 (EUCAST 2021) details the method to be used in determining epidemiological cut-off values from MIC distributions obtained from multiple laboratories when the ECOFFinder algorithm is used. The first step is to estimate the exact 99.9% CO_{WT} values of each contributing distribution. Then, the mean of these exact values is calculated (using their $\log_2$ values). The ECOFF is set as the antilog of this $\log_2$ mean rounded up to the next 2-fold dilution. A similar method was applied when the data were analysed using NRI. Using the NRI algorithm, the mean plus 2 SDs (using the $\log_2$ values) were determined for each of the contributing distributions, and the mean of these values was calculated. The CO_{WT} values were then set as the antilog of this $\log_2$ mean rounded up to the next 2-fold dilution.

2.6. Precision of individual laboratory MIC distributions

The ECOFFinder algorithm calculates the SD of the best-fit curve for each individual MIC distribution. The NRI algorithm also provides SD values for the normalised WT distributions. Both SD values can serve as a measure of the precision of the distribution from which they were derived (Smith et al. 2018). Smith (2022) recommended that any individual distribution which generated an SD value in excess of the acceptable precision limit of 1.11 $\log_2$ μg ml^{-1} when analysed by ECOFFinder or 1.18 $\log_2$ μg ml^{-1} when analysed by NRI should be considered excessively imprecise and would not be suitable for inclusion in an effort to establish a reliable epidemiological cut-off value. Therefore, in calculating CO_{WT} values in this work, individual distributions categorised as excessively imprecise were excluded.

3. RESULTS AND DISCUSSION

3.1. QCs

With respect to tests performed at 35°C, CLSI guideline VET04 (CLSI 2020b) and standard M100-S34 (CLSI 2024) provide acceptable QC ranges for the MICs obtained with the QC reference strain *Escherichia coli* ATCC® 25922 for AMP, CTZ, ENR, FLO, GEN, MER, OXO, OXY and TRS but not for SME. The ranges of the MIC values determined for these 9 antimicrobial agents against this reference strain by all 8 laboratories that reported test results at 35°C were all within the acceptable QC ranges (Table S1 in the Supplement at www.int-res.com/articles/suppl/d160p127_supp.pdf). With respect to tests performed at 28°C, CLSI guideline VET04 (CLSI, 2020b) provides acceptable ranges for the MICs obtained with the QC reference strain *E. coli* ATCC® 25922 for AMP, ENR, FLO, GEN, OXO, OXY and TRS but not for CTZ, MER or SME. The range of MIC values for these 7 agents against this reference strain reported by all 7 laboratories that performed tests at 28°C were also all within the acceptable ranges (Table S2).

3.2. Elimination of distributions with excessive truncation

EUCAST (2021) refers to MIC distributions which include observations from isolates for which MIC values cannot be quantified, because they are either below or above the concentrations tested, as truncated. In this work, the data sets for 2 agents, AMP and MER, were extensively truncated. In the 8 distributions for AMP obtained at 35°C, the average frequency of observations that were above scale (>16 µg ml^{-1}) was 38%, and in the 6 distributions obtained at 28°C, this frequency was 71% (Table S3.1). These distributions were therefore not considered suitable for calculating a CO_{WT} value. One hypothesis for these off-scale observations is that members of *Vibrio parahaemolyticus* are intrinsically resistant to this agent. Intrinsic resistance to AMP has also been reported for *V. anguillarum* (Smith et al. 2023a) and *V. harveyi* (Smith et al. 2023b).

In the 8 MER MIC distributions obtained at 35°C, the average frequency of observations that were below scale (⩽0.008 µg ml^{-1}) was 84%, and in the 7 distributions obtained at 28°C, this average value was 83% (Table S3.6). These results for MER are consistent with those reported by Karatuna et al. (2024), who, in a study of 119 *V. parahaemolyticus* isolates at 35°C, reported that the modal MIC value for this agent was 0.008 µg ml^{-1}. Smith et al. (2023b) also reported similar results for *V. harveyi*, with 84% of the MIC values for MER being <0.008 µg ml^{-1}. In contrast, the MIC values recorded for *V. anguillarum* had a modal value of 0.25 µg ml^{-1} (Smith et al. 2023a). Thus, it must be concluded that the concentrations of MER in the AQGNECV plates were not appropriate for determining the distribution of MICs for WT *V. parahaemolyticus*.

3.3. Determination of precision of MIC distributions from individual laboratories

The precision of MIC distributions generated by individual laboratories was investigated by calculating the SD of their WT distributions produced by the NRI and ECOFFinder analyses (Smith et al. 2018). As their distributions were excessively truncated, these analyses were not performed for the AMP and MER data sets. For 22 of the 122 MIC distributions (18%), the SD values for the remaining 8 agents determined by 1 or both of the methods were in excess of the limits suggested by Smith (2022) (Table 1). These individual laboratory distributions were considered as insufficiently precise and, therefore, were excluded from the distributions used to calculate CO_{WT} values. A further distribution for SME obtained at 28°C, provided by an individual laboratory, was also identified as aberrant. This distribution was so dispersed that the ECOFFinder spreadsheet could not process the data, and analysis by NRI suggested the distribution was bimodal. The putative WT group contained only 8 observations. As this number of WT observations was less than the 15 required by EUCAST (2021), this data set was also excluded.

3.4. Determination of interlaboratory variation among MIC distributions from individual laboratories

To limit the degree of interlaboratory variation between individual MIC distributions, EUCAST (2017) requires that observations from an individual laboratory should be included in an analysis only if the mode of its WT distribution is equal to or within one 2-fold dilution of the most common mode observed in the collection of valid distribu-

Table 1. Number of individual laboratory distributions for which the SDs calculated by normalised resistance interpretation (NRI) and/or ECOFFinder exceeded the precision limits suggested by Smith (2022)

Agent	Abbrev.	Temp. (°C)	— No. of distributions — NRI[a]	ECOFFinder[b]	Both[c]
Ceftazidime	CTZ	35	2		1
		28	1	1	
Enrofloxacin	ENR	35	2		
		28	1		
Florfenicol	FLO	35			
		28			
Gentamicin	GEN	35			
		28			
Oxolinic acid	OXO	35	3		
		28	2		
Oxytetracycline	OXY	35			
		28			
Sulfamethoxazole	SME	35	3		1
		28	1		2
Trimethoprim/ sulfamethoxazole	TRS	35	1		
		28			1

[a]SDs are >1.11 $\log_2$ µg ml^{-1} when analysed by ECOFFinder and <1.18 $\log_2$ µg ml^{-1} when analysed by NRI

[b]SDs are <1.11 $\log_2$ µg ml^{-1} when analysed by ECOFFinder and >1.18 $\log_2$ µg ml^{-1} when analysed by NRI

[c]SDs are >1.11 $\log_2$ µg ml^{-1} when analysed by ECOFFinder and >1.18 $\log_2$ µg ml^{-1} when analysed by NRI

tions prior to aggregation. All the individual data sets for CTZ, ENR, FLO, GEN, OXO, OXY and TRS generated at both temperatures met this requirement. Furthermore, in all the aggregations of the MIC distributions for each agent at each temperature, >91% of the MICs were within 3 dilutions of each other (Table 2). These calculations indicate that the degrees of interlaboratory variation between all these distributions were within acceptable limits. It was therefore considered that it would be valid to include them in the ECOFFinder and NRI analyses.

The distributions for SME showed a large degree of interlaboratory variation (Table S3.9). Four individual distributions obtained for SME at 35°C were of sufficient precision to be included in the aggregation. However, the modes of the WT distributions for 2 of them were 64 µg m l^{-1} and for the other 2 were 4 and 16 µg ml^{-1}. The 3 individual SME distributions of sufficient precision obtained at 28°C had modes of 16, 32 and 128 µg ml^{-1}. The degree of interlaboratory variation at both temperatures was considered unacceptable; therefore, no attempt was made to calculate CO_{WT} values for SME.

3.5. Calculations of CO_{WT} values

The MIC values obtained by the participating laboratories for all 10 antimicrobial agents at 35 and 28°C are shown in Table S3. To calculate CO_{WT} values for 3 agents (FLO, GEN and OXY), ECOFFinder and NRI algorithms were applied to all the distributions of MIC values generated by the participating laboratories at both temperatures. For 4 agents (CTZ, ENR, OXO and TRS), these algorithms were applied to the distributions censored by the exclusion of those individual distributions for which the SD values calculated by 1 or both of the algorithms exceeded the limits suggested by Smith (2022) (Table 1). All 14 of the aggregated distributions that were analysed contained >100 observations from WT isolates, and all 92 of the individual distributions contained >15 observations from WT isolates. Thus, these data met the quantitative requirement of EUCAST SOP 10.2 (EUCAST 2021). The results of these analyses are shown in (Table 3).

For CTZ at 35°C, the MIC distributions generated by 5 of the participating laboratories together with 1 distribution accessed from the EUCAST website (www.eucast.org/mic_distributions_and_ecoffs/) were analysed, and a CO_{WT} value of 1 µg ml^{-1} was calculated. For CTZ at 28°C, as acceptable QC ranges have not been set, only a provisional CO_{WT} of 1 µg ml^{-1} could be calculated from the analysis of the 5 individual distributions available. For ENR at 35°C, analysis of the MIC distributions generated by 6 of the participating laboratories gave a CO_{WT} value of 0.25 µg ml^{-1}, and at 28°C, analysis of 6 individual distributions gave a CO_{WT} value of 0.5 µg ml^{-1}. For FLO at 35°C, analysis of the MIC distributions generated by 8 of the participating laboratories gave a CO_{WT} value of 1 µg ml^{-1}, and at 28°C, analysis of 7 distributions gave a CO_{WT} value of 1 µg ml^{-1}. For GEN at 35°C, analysis of the MIC distributions generated by 8 of the participating laboratories gave a CO_{WT} value of 2 µg ml^{-1}, and at 28°C, analysis of 7 individual distributions gave a CO_{WT} value of 4 µg ml^{-1}. For OXO at 35°C, analysis of 5 individual distributions gave a CO_{WT} value of 0.5 µg ml^{-1}, and at 28°C, analysis gave a CO_{WT} value of 1 µg ml^{-1}. For OXY at 35°C, analysis of the MIC distributions from 8 of the participating laboratories gave a proposed CO_{WT} value of 2 µg ml^{-1}, and at 28°C, analysis of 7 individual distributions gave a proposed CO_{WT} value of 1 µg ml^{-1}. For

Table 2. Aggregations of minimal inhibitory concentration (MIC) values (µg ml^{-1}) for *Vibrio parahaemolyticus* after the exclusion of those individual distributions for which the SDs were categorised as excessive (Table 1). The distributions for ampicillin and meropenem are not shown, as they were excessively truncated and it was not possible to determine their SDs. Unshaded boxes indicate concentrations included in the AQGNECV plates. Abbreviations as in Table 1

MIC (µg ml^{-1})	CTZ		ENR		FLO		GEN		OXO		OXY		SME		TRS[a]	
	35°C	28°C[b]	35°C	28°C	35°C	28°C	35°C	28°C	35°C	28°C	35°C	28°C	35°C	28°C	35°C	28°C
Below[c]													2			1
0.0005																
0.001																
0.002			1													
0.004			1	2												
0.008			2													
0.015			3	1					2	1					3	3
0.03			17	1					6	3					15	3
0.06	2		67	10					31	3		1			83	47
0.125	30	12	75	106		1			53	20	1	10			180	102
0.25	117	56	4	45	28	35	6		53	90	36	70			41	16
0.5	119	71			151	136	59	16	5	20	140	96			1	
1	1	5			55	22	116	106		1	56	17			2	1
2					1	2	46	65			1		5			
4							8	9				1	29			
8													16	17		
16													18	25		
32													9	14		
64													35	20		
128													10	24		
256													3	5		
512													1	7		
1024																
Above[d]	1	1	2	1							1	1		11	1	
Distributions[e]	7	5	6	6	8	7	8	7	5	5	8	7	4	4	8	6
Isolates[f]	270	145	232	166	235	196	235	196	150	138	235	196	128	123	326	173

[a]MIC values are given as the trimethoprim concentration; [b]No acceptable quality control ranges have been set for CTZ at 28°C; thus, the data in this column have not been generated using a standard method; [c]MICs could not be quantified, as they were lower than or equal to the lowest test concentration; [d]MICs could not be quantified, as they were higher than the highest test concentration; [e]Number of distributions contributing data to the aggregation; [f]Number of isolates in each aggregation

Table 3. Mean of the exact cut-off values (µg ml^{-1}) generated by ECOFFinder and NRI analyses of the individual distributions for *Vibrio parahaemolyticus* for each agent at each temperature and the wild-type (WT) rounded-up cut-off values (CO_{WT}) (µg ml^{-1}) calculated from them. Abbreviations as in Table 1

Agent	Temp. (°C)	No. of distributions[a] (WT isolates[b])	Mean exact cut-off value		Rounded-up cut-off value (CO_{WT})	
			ECOFFinder	NRI	ECOFFinder	NRI
CTZ	35	6 (269)	0.606	0.837	1	1
	28[c]	5 (144)	0.529	0.973	1	1
ENR	35	6 (172)	0.158	0.222	0.25	0.25
	28	6 (166)	0.280	0.318	0.5	0.5
FLO	35	8 (234)	0.755	0.996	1	1
	28	7 (194)	0.704	0.790	1	1
GEN	35	8 (235)	1.881	1.965	2	2
	28	7 (195)	2.366	2.571	4	4
OXO	35	5 (150)	0.373	0.447	0.5	0.5
	28	5 (138)	0.501	0.670	1	1
OXY	35	8 (234)	1.052	1.169	2	2
	28	7 (194)	0.797	0.726	1	1
TRS[d]	35	8 (301)	0.176	0.239	0.25	0.25
	28	6 (172)	0.169	0.247	0.25	0.25

[a]Individual distributions for which the SD values exceeded the precision limits suggested by Smith (2022) were excluded from these analyses; [b]Number of isolates categorised as WT in each aggregation; [c]No acceptable quality control ranges have been set for CTZ at 28°C; thus, the cut-off values in this table cannot be used to set internationally agreed epidemiological cut-off values; [d]MIC values are given as the trimethoprim concentration

TRS at 35°C, the MIC distributions generated by 7 of the participating laboratories together with 1 accessed from the EUCAST website (www.eucast.org/mic_distributions_and_ecoffs/) were analysed, and a CO_{WT} value of 0.25/4.8 µg ml^{-1} was calculated. For TRS at 28°C, analysis of the MIC distributions generated by 6 of the participating laboratories gave a CO_{WT} value of 0.25/4.8 µg ml^{-1}.

3.6. Effect of temperature on MIC and calculated CO_{WT} values

Examination of the CO_{WT} values calculated from the distributions obtained at 35°C and 28°C (Table 3) suggests that the temperature at which these distributions were obtained does not have major and consistent influence on the calculations. For 3 of the 7 antimicrobial agents (CTZ, FLO and TRS), these values were the same. For 3 of the remaining 4 antimicrobial agents (ENR, GEN and OXO), the CO_{WT} values calculated from the 35°C distributions were 1 dilution step lower than those calculated from the 28°C distributions, and for OXY, the value at 35°C was 1 dilution step higher than the value at 28°C.

A more detailed examination of the extent of the effect of temperature on the observed MICs, however, can be obtained by comparing the values obtained at the 2 temperatures for a specific antimicrobial agent against a particular isolate by each individual laboratory. For a total of 1473 such paired observations, the difference in the MICs was calculated as the $\log_2$ of MIC values determined at 35°C minus the $\log_2$ of MIC values determined at 28°C (Table 4). The average difference in the paired observations was 0.04 dilution. In 55.9% of the paired observations, the MICs were identical at the 2 temperatures; in 20.9%, the MICs determined at 35°C were 1 dilution lower than those determined at 28°C, and in 17.5%, they were 1 dilution higher. Thus, in 1390 (94.4%) of 1473 paired observations, the MICs determined at the 2 temperatures were within 1 dilution of each other. In a decision document, EUCAST (2003) stated that it is generally accepted that broth microdilution tests are reproducible to within 1 doubling dilution of the real end point. Thus, the data analysed in this work would suggest that, at least for *V. parahaemolyticus,* the variation between MICs determined at 35 or 28°C is no greater than the inherent variation in the MIC determinations at a single temperature.

3.7. Limitations to application of data generated in this work

The setting of internationally harmonised epidemiological cut-off values is the prerogative of either EUCAST or CLSI, which use the acronyms ECOFFs and ECVs, respectively. Thus, it is important to note that the CO_{WT} values calculated in this work should not be treated as internationally harmonised epidemiological cut-off values; rather, a goal of this work is to submit the data generated in this study to these agencies to facilitate their setting of the ECOFFs/ECVs that are needed to perform monitoring and surveillance of antimicrobial susceptibility of *V. parahaemolyticus.*

One of the aims of any investigation into the antimicrobial susceptibility of *V. parahaemolyticus* is to establish the frequency of the incidence of isolates with reduced susceptibility in a given region. Inherent in this is that such studies require the collection of isolates that accurately reflect those occurring in that region (Smith et al. 2013). In this work, many of the isolates were included after previous disc diffusion tests, the results of which had suggested that they were WT. As a result of this deliberate bias, the data presented in this work cannot be used to estimate any frequency of isolates with reduced susceptibility in any of the regions from which the isolates were obtained.

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Table 4. Distribution of the difference between 1473 paired observations of MICs determined at 35°C and at 28°C. The differences were calculated as the $\log_2$ MIC determined at 35°C minus the $\log_2$ MIC determined at 28°C obtained for the same isolates and the same antimicrobial agent in the same laboratory

Difference	−4	−3	−2	−1	0	1	2	3	4
No. of isolates	2	5	40	308	824	258	33	5	2
Percentage (%)	<1	<1	3	21	56	18	2	<1	<1

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