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Epidemiological cut-off values for *Vibrio anguillarum* MIC and disc diffusion data generated by standardised methods

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ABSTRACT: This work aims to generate the data needed to set epidemiological cut-off values for minimum inhibitory concentration (MIC) and disc-diffusion zone measurements of *Vibrio anguillarum*. A total of 261 unique isolates were tested, applying standard methods specifying incubation at 28°C for 24–28 h. Aggregated MIC distributions for a total of 247 isolates were determined in 9 laboratories for 11 agents. Data aggregations of the disc zone for the 10 agents analysed contained between 157 and 218 observations made by 4 to 7 laboratories. Acceptable ranges for quality control (QC) reference strains were available for 7 agents and the related multi-laboratory aggregated data were censored, excluding the data of a laboratory that failed to meet QC requirements. Statistical methods were applied to calculate epidemiological cut-off values. Cut-off values for MIC data were calculated for florfenicol (≤1 µg ml^{-1}), gentamicin (≤4 µg ml^{-1}), oxytetracycline (≤0.25 µg ml^{-1}) and trimethoprim/sulfamethoxazole (≤0.125/2.38 µg ml^{-1}). The cut-off values for disc zone data were calculated for enrofloxacin (≥29 mm), florfenicol (≥27 mm), gentamicin (≥19 mm), oxolinic acid (≥24 mm), oxytetracycline (≥24 mm) and trimethoprim/sulfamethoxazole (≥26 mm). MIC and disc-diffusion zone data for the other agents where not supported by QC, thus yielding only provisional cut-off values (meropenem, ceftazidime). Regardless of whether QC is available, some of the aggregated MIC distributions (enrofloxacin, oxolinic acid), disc zone (sulfamethoxazole), and MIC and disc-diffusion distributions (ampicillin, chloramphenicol) did not meet the statistical requirements. The data produced will be submitted to the Clinical Laboratory Standards Institute for their consideration in setting international consensus epidemiological cut-off values.

KEY WORDS: CLSI VET04 · Normalized resistance interpretation · ECOFFinder · Antimicrobial susceptibility · Aquatic animals

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1. INTRODUCTION

Infections with *Vibrio anguillarum* can result in a typical haemorrhagic septicaemia in warm- and cold-water species of aquatic animals of economic importance (Austin & Austin 2007). Susceptible species include Pacific and Atlantic salmon (*Oncorhynchus* spp. and *Salmo salar*), rainbow trout *Oncorhynchus mykiss*, turbot *Scophthalmus maximus*, seabass *Dicentrarchus labrax*, seabream *Sparus aurata*, striped bass *Morone saxatilis*, cod *Gadus morhua*, Japanese eel *Anguilla japonica*, European eel *Anguilla anguilla* and ayu *Plecoglossu altivelis* (Toranzo et al. 2005, Haenen et al. 2014). Because of its high morbidity and mortality rates and wide host range, this disease has been responsible for severe economic losses worldwide (Frans et al. 2011).

Although commercial vaccines protecting against the main serotypes of *V. anguillarum* are available (Amaro et al. 2020), antimicrobial treatments are still extensively used to control losses to this disease (Frans et al. 2011, Lillehaug & Colquhoun 2020, Rigos et al. 2021). Therefore, it is argued that *V. anguillarum* should be included in programmes for the monitoring and surveillance of antimicrobial susceptibility of aquatic organisms that are recommended in the World Organization for Animal Health (WOAH) Aquatic Animal Health Code (WOAH 2019). This code recommends that such susceptibility studies should be performed using internationally harmonised and standardised testing protocols. It further states that the data generated by these protocols should be interpreted by the application of relevant international harmonised consensus epidemiological cut-off values.

Smith & Egan (2020) reported an extensive review of 203 published studies on the antimicrobial susceptibility of *Vibrio* species carried out to determine to what extent they followed the recommendations of the WOAH Aquatic Animal Health Code (WOAH 2019). This review revealed that >95% of the studies examined failed to meet the criteria set out in the code. The shortcomings frequently occurred with respect to the methods used and/or the details provided for those methods. Smith & Egan (2020) also noted that although all 203 studies reported frequencies of resistance, less than 10% of them reported the use of appropriate internationally harmonised consensus-based interpretive criteria. In the 18 studies involving *V. anguillarum* reviewed, only one (Uhland 2010) used an internationally standardised testing protocol. The others used a variety of temperatures and media to perform their susceptibility tests. Significantly, none of the 18 *V. anguillarum* studies applied internationally harmonised interpretive criteria. Smith (2020) has suggested a set of rules that, if implemented, would improve the quality of papers on the antimicrobial susceptibility of bacteria isolated from aquatic animals. These rules stressed the central importance of the use of standardised testing protocols and internationally harmonised, consensus-based interpretive criteria in susceptibility studies.

Baron et al. (2020a) addressed the issue of selecting a standardised testing protocol that would be appropriate for *V. anguillarum*. Their multi-laboratory study demonstrated that the susceptibility of this species could be established using the standard protocols given in the guideline VET03 (CLSI 2020a), specifying the use of Mueller–Hinton medium with no additional NaCl and incubation at 28°C for 24–28 h. They suggested that although other testing protocols could be used, there would be significant advantages if this testing protocol for *V. anguillarum* were to be adopted internationally.

Both the Clinical Laboratory Standards Institute (CLSI) guideline M23 (CLSI 2018) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) SOP 10.2 (EUCAST 2021) have provided some guidance concerning the quantity of the data they require for setting consensus epidemiological cut-off values for minimum inhibitory concentration (MIC) data. The guideline M23 (CLSI 2018) states that epidemiological cut-off values, for which they use the abbreviation ECV, can only be determined from aggregations of MIC data sets sourced from at least 3 separate laboratories and should include observations from greater than 100 unique isolates. Although it does not explicitly state that it refers to the number of unique isolates categorised as wild-type (WT), in the present study it has been assumed that this was the implied meaning of the guideline. EUCAST (2021) specify that setting an epidemiological cut-off value, for which they use the abbreviation ECOFF, requires aggregated MIC data from at least 5 laboratories, with each laboratory generating at least 15 observations from isolates categorised as WT. However, EUCAST would allow a tentative ECOFF value to be calculated when 3 or 4 laboratories contributed data. They also require that the aggregated data should include a minimum of 100 observations from isolates categorised as WT. As the quantitative requirements specified in EUCAST (2021) are more stringent, they were adopted for the present study. To the best of our knowledge, neither organisation has set quantitative requirements for disc-diffusion zone data. Given the absence of pub-

lished guidelines, it was decided to apply, where relevant, the EUCAST quantitative requirements for MIC data sets (EUCAST 2021) to the disc-diffusion data generated in this work.

With respect to the application of protocol-specific epidemiological cut-off values to data for *V. anguillarum*, neither CLSI nor EUCAST have set any values for data from this species that have been generated by any testing protocol. Therefore, this work was undertaken to provide the data needed to set the relevant epidemiological cut-off values for *V. anguillarum* susceptibility data generated by the standard methods provided in VET03 (CLSI 2020a) that were recommended for this species by Baron et al. (2020a).

2. MATERIALS AND METHODS

2.1. Participating laboratories

Eleven laboratories were involved in the present study of *V. anguillarum* susceptibility. These were those of the Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit of Ploufragan-Plouzané-Niort Laboratory of the French Agency for Food, Environmental and Occupational Health & Safety (Anses), the Section for Fish and Shellfish Diseases at the Technical University of Denmark, National Institute of Aquatic Resources, Kgs Lyngby, Denmark (DTU), the National Reference Laboratories for Antimicrobial Resistance and for Fish Diseases of Wageningen Bioveterinary Research Lelystad, The Netherlands (WBVR), the Centre for Environment Fisheries and Aquaculture Science Laboratory, Weymouth, UK (Cefas), the Department of Contaminants and Biohazards at the Institute of Marine Research, Bergen Norway (IMR), the Laboratory for Fish and Molluscs Diseases, Croatian Veterinary Institute, Zagreb, Croatia (CVI), the Department of Veterinary Medical Sciences of Alma Mater Studiorum Università di Bologna, Ozzano Emilia (BO), Italy (DIMEVET), the National Reference Laboratory for Fish, Mollusc and Crustacean Diseases, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy (ISZVe), Vet'eau - Selarl du Dr Alain Le Breton, Grenade sur Garonne, France (VETEAU), the VISAVET Health Surveillance Centre of the Complutense University of Madrid, Spain (VISAVET), and Istanbul University, Faculty of Aquatic Sciences (IU-FAS).

2.2. Isolate collections

The 261 *V. anguillarum* isolates studied in this work were collected by the 11 participating laboratories. These isolates originated from 14 European countries and were collected over a period from 1981 to 2021. The identification of isolates was made using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Singhal et al. 2015, Florio et al. 2018). In a validation study involving 4 European veterinary institutes, Jansson et al. (2020) reported that MALDI-TOF-MS was capable of identifying *V. anguillarum* to the species level.

2.3. Susceptibility testing of isolates

Nine of the 11 participating laboratories performed susceptibility tests on the *V. anguillarum* isolates held in their own collections. The susceptibility testing of the isolates in the other 2 collections, those of VETEAU and IU-FAS, were performed in the Anses and CVI laboratories, respectively. All 9 laboratories performed MIC assays and, in total, they generated MIC values for 247 unique isolates for each of 11 agents (Table 1). Seven laboratories performed disc diffusion tests and, in total, they generated zone sizes for 218 unique isolates for each of 9 agents (Table 1). However, with respect to ampicillin, only 6 laboratories performed disc-diffusion tests and the total number of unique isolates tested for this agent was 187. Both

Table 1. Number of unique isolates tested by minimum inhibitory concentration (MIC) and disc-diffusion tests by the participating laboratories. The Anses numbers include the isolates in the VETEAU collection. The CVI numbers include the isolates in the IU-FAS collection (see Section 2.1 for a complete list of laboratory names)

Test	Number of unique isolates tested by each laboratory									
	Anses	Cefas	CVI	DIMEVET	DTU	ISZVe	IMR	VISAVET	WBVR	Total
MIC	51	18	30	27	20	25	40	15	21	247
Disc diffusion	63	20	30	27	20[a]	–	37	–	21[b]	218

[a]DTU did not perform disc zone tests for ampicillin
[b]For ampicillin, WBVR performed disc diffusion tests on only 10 isolates

MIC values and disc-diffusion zone sizes were determined for 204 unique isolates. For a further 43 isolates, only MIC values were determined, and for 14, only disc-diffusion zone sizes were determined. The numbers of unique isolates tested by each laboratory for both methods are shown in Table 1.

2.4. Susceptibility measurements

Both MIC and disc-diffusion tests against *V. anguillarum* were performed at 28°C with incubation for 24–28 h according to the protocols provided in the CLSI guideline VET03 (CLSI 2020a). For both tests, the inocula were prepared by the colony suspension method recommended by this guideline using cation-adjusted Muller–Hinton broth (CAMHB). The MIC values against *V. anguillarum* were determined by the microdilution method using CAMHB that was not supplemented with NaCl. The 96-well plates (ECOFFVIB) used in the MIC studies were manufactured by Thermo Fisher Scientific according to the layout developed by Baron et al. (2020b) (Table 2).

The MIC values were determined as the lowest concentration in which growth was not visible. When an isolate failed to grow in any of the wells in the ECOFFVIB plates that contained any concentration of the agent, but did grow in the control well without any agent, they were recorded as 'below scale'. When an isolate grew in all the concentrations of an agent, these were recorded as 'above scale'. In the EUCAST SOP 10.2, distributions that contain such 'off-scale' observations are termed 'truncated' (EUCAST 2021). Disc-diffusion zone sizes against *V. anguillarum* were determined on unmodified Muller–Hinton agar (MHA) using discs with contents recommended by CLSI (2017, 2020b). These were AMP 10 µg, CTZ 30 µg, CHL 30 µg, ENR 5 µg, FLO 30 µg, GEN 10 µg, MER 10 µg, OXO 2 µg, OXY 30 µg and TRS 1.25/23.75 µg ml^{-1}. The participating laboratories obtained their discs from different suppliers and this information is detailed in Table S1 (see the Supplement at www.int-res.com/articles/suppl/d155p109_supp.pdf). Each laboratory employed one or both of the quality control (QC) reference strains *Escherichia coli* ATCC 25922 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 and disc-diffusion protocols adopted in this work for AMP, ENR, FLO, GEN, OXO and TRS. However, the acceptable ranges for ENR disc data are provided for the *A. salmonicida* reference strain, but not for *E. coli* ATCC 25922.

Table 2. Range of concentrations (µg ml^{-1}) of the antimicrobial agents used in ECOFFVIB 96-well plates during the present study, and for the improved AQGNECV plate layout described in Section 4.4 (all manufactured by Thermo Fisher)

Agent		ECOFFVIB	AQGNECV
Ampicillin	AMP	0.03–16	0.015–16
Ceftazidime	CTZ	0.03–16	0.002–16
Chloramphenicol	CHL	0.5–64	
Enrofloxacin	ENR	0.002–0.5	0.0005–0.25
Florfenicol	FLO	0.06–16	0.03–16
Gentamicin	GEN	0.06–8	0.06–8
Meropenem	MER	0.008–1	0.008–1
Oxolinic acid	OXO	0.008–1	0.002–1
Oxytetracycline	OXY	0.015–4	0.015–1
Sulfamethoxazole	SME	4–512	1–512
Trimethoprim/ sulfamethoxazole	TRS	0.015/0.30–2/28	0.008/0.15–1/18

2.5. Aggregation of MIC and disc zone data

For the majority of the agents, the available data from all participating laboratories were combined to construct aggregated data sets for the calculation of epidemiological cut-off values. However, in a few cases, censored aggregations were used. Censored aggregations were those from which data from one or more individual laboratories for a specific agent was excluded. When a laboratory recorded a result for one or both of the QC reference strains that was outside the acceptable range for that agent, the MIC or disc zone data from that laboratory was not included in the censored aggregations for that agent. As VET04 (CLSI 2020b) provides acceptable limits for ENR disc data for *A. salmonicida* ATCC 33658 and not for *E. coli* ATCC 25922, the ENR disc data from the 2 laboratories that used only the *E. coli* and not the *A. salmonicida* reference strain were also excluded. With respect to the agents for which VET04 (CLSI 2020b) does not provide acceptable ranges (CTZ, CHL, MER and SME), all available data were included in the agent-specific aggregations.

Three additional criteria for the exclusion of MIC data generated by individual laboratories from multi-laboratory aggregations, that were recommended in EUCAST SOP 10.2 (EUCAST 2021), were applied to the data analysed in this work. MIC distributions from an individual laboratory for an agent were excluded if their mode differed by more than one dilution from the most common mode in the data from the rest of the participating laboratories. The

data from an individual laboratory for an agent were also excluded if they contained <15 WT observations. The EUCAST SOP 10.2 (EUCAST 2021) also states that 'distributions where truncation is unacceptable and at the lower end of the distribution' should be excluded. However, they do not provide any specific criteria for establishing the unacceptability of any truncations. In this study, truncated distributions from a laboratory where the frequencies of below-scale observations were <10% of the WT observations they made and were not greater than the frequency recorded for the lowest MIC that could be quantified, were treated as acceptable. The below-scale observations in distributions with acceptable truncations were, for the purpose of ECOFFinder and NRI analyses, assumed to have an MIC equal to the lowest concentration in the ECOFFVIB plate.

2.6. Calculation of proposed epidemiological cut-off values

Epidemiological cut-off values (CO_{WT}) were used to categorise isolates as either fully susceptible 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 2012). For the disc zone data against *V. anguillarum*, the CO_{WT} were calculated from the aggregation or the censored aggregations of the data from the participating laboratories using the automatic normalised resistance interpretation (NRI) spreadsheet (www.bioscand.se/nri/). For the MIC data, CO_{WT} were calculated against *V. anguillarum* from the aggregations of the data from the participating laboratories using the 2 automatic spreadsheets ECOFFinder (https://www.eucast.org/mic_distributions_and_ecoffs/) and NRI (www.bioscand.se/nri/). Both of these methods can be downloaded for free. As specified in the EUCAST SOP 10.2 (EUCAST 2021) with respect to the ECOFFinder method, the 99.9% values were used in calculating CO_{WT}.

In generating cut-off values, the ECOFFinder spreadsheet calculates what it refers to as an 'exact' value and then rounds this value up to the next higher dilution 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 WT isolates and then rounds this value up to generate a cut-off value. In the present study, a mean value for the proposed CO_{WT} was calculated by averaging the 'exact' values generated by the 2 methods and then rounding up this average in the normal way.

The ECOFFinder spreadsheet provides values for the SD of the best-fit curve it calculates and the NRI spreadsheet provides SD values for the normalised WT distributions it calculates. Both of these values were used as a measure of the precision of the data sets from which they were derived (Smith et al. 2018). Following the recommendations of Smith (2022), any multi-laboratory aggregated data set that 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 was considered to be excessively imprecise and not suitable for calculating a reliable CO_{WT} value.

2.7. 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 set by CLSI, and ECOFF for those set by EUCAST. In the present study, the abbreviation CO_{WT} is used for all epidemiological cut-off values not set by these agencies but calculated for data generated by laboratories that have demonstrated compliance with the QC requirements of the standard method adopted. The abbreviation pCO_{WT} was used for the provisional epidemiological cut-off values generated for agents for which no QC requirement had been set. The abbreviations adopted for the antimicrobial agents were those recommended in the EUCAST System for Antimicrobial Abbreviations (EUCAST 2018).

3. RESULTS

3.1. Quality control

The guideline VET04 (CLSI 2020b) provides acceptable ranges for the MIC and the disc zones obtained with QC reference strains for AMP, ENR, FLO, GEN, OXO, OXY and TRS. All 9 laboratories that recorded *V. anguillarum* MIC values for these 7 agents also reported MIC values for the reference strains they tested that were within these acceptable ranges (Table S2 in the Supplement). Seven laboratories reported disc zones for ENR, FLO, GEN, OXO, OXY and TRS and 6 reported zones for AMP. All of these laboratories reported the zones they obtained either with one or both of the relevant QC reference strains (CLSI 2020b). These data are reported in Table S3 in the Supplement. On 8 occasions these zones were outside the acceptable range. Two labo-

ratories recorded reference strain data outside the acceptable range for GEN. Of these laboratories, one also recorded a zone outside the acceptable range for FLO. Zones for reference strains that were outside the acceptable range were also recorded for AMP and TRS by a third laboratory and for OXY by a fourth. In addition, 2 laboratories recorded QC data for ENR using a reference strain (*E. coli* ATCC 25922) for which no acceptable ranges have been set. The composition of the censored multi-laboratory aggregations of the zone size data considered as valid for NRI is shown in Table 3.

3.2. Data sets

Table 4 shows the distributions of the MIC values in the aggregations, and in the case of TRS the censored aggregation, generated by 9 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. The data for TRS generated by one laboratory had a modal value that differed by 2 dilutions from the most common mode in the distributions from the other 8 laboratories. The distribution for this agent from another laboratory showed unacceptable truncation. Therefore, the data from these 2 laboratories were excluded from the censored aggregation for TRS presented in Table 4. This table also presents a summary of the analyses of these aggregations and censored aggregations using ECOFFinder and NRI. Details of the MIC values generated by each laboratory and the MIC values obtained by those laboratories for the QC reference strains are provided in Table S2.

Seven laboratories participated in the disc-diffusion analyses. However, some laboratories did not generate any or sufficient (≤15 observations) data for all 10 agents. Also a number of laboratories did not demonstrate compliance with the QC requirements for some of the agents for which they reported zone data. As a result, the aggregations or censored aggregations of the disc zone data for the 10 agents analysed in this work contained between 157 and 218 observations made by 4 to 7 laboratories (Table 3). Table 5 shows the distributions of the disc zone values against *V. anguillarum* in these aggregations or censored aggregations. These tables also present the CO_{WT} or pCO_{WT} values and the SD values calculated by NRI analysis of those data sets that were considered as suitable for analysis. Details of the disc zone sizes generated by each laboratory and the zone sizes obtained by those laboratories for the QC reference strains are provided in Table S3.

3.3. Ampicillin (AMP)

Eighty-seven percent of the aggregated MIC data against *V. anguillarum* for AMP were above scale (the MIC values were higher than the highest test concentration). With respect to the disc zone data for this agent, one laboratory reported no data. Another reported zone data for only 10 isolates and was excluded from the aggregation. A third recorded a zone outside the acceptable range for a QC reference strain and the data from this laboratory were also excluded. Ninety-six percent of the 157 zones recorded for this agent by the other 4 laboratories were ≤13 mm. These MIC and zone data were interpreted as indicating that the vast majority, if not all, of the *V. anguillarum* isolates tested manifested a NWT phenotype with respect to this agent. Therefore, the susceptibility data obtained from them were not suitable for calculating any CO_{WT} values.

Table 3. Composition of the aggregations and censored aggregations of the disc-diffusion zone data generated by the 7 participating laboratories. Antimicrobial agent abbreviations as in Table 2. 'na' indicates that acceptable ranges for the QC reference strains were not available for these agents

Agent	Number of laboratories reporting		Composition of aggregations	
	No data or <15 observations	QC non-compliance	Number of laboratories	Number of unique isolates
AMP	2	1	4	157
ENR		2	5	161
FLO		1	6	198
GEN		2	5	160
OXO			7	217
OXY		1	6	188
TRS		1	6	198
CAZ		na	7	218
CHL		na	7	218
MER		na	7	218

3.4. Enrofloxacin (ENR)

The MIC data against *V. anguillarum* for ENR contained 247 observations from 9 laboratories, 35 of which (14%) were below scale (the MIC values were lower or equal to the lowest test concentration) (Table 4). However, these data showed excessive variation in the modes of the WT distributions from

Table 4. Distributions of MIC values for *Vibrio anguillarum* in the aggregations data from the participating laboratories and the results of their analysis by ECOFFinder and NRI. The distribution shown for TRS was that of the censored aggregation. Unshaded boxes indicate the MIC values for each agent (abbreviations as in Table 2) could be quantified using the ECOFFVIB plates. 'na' indicates that the data set was not considered suitable for ECOFFinder or NRI analysis and that epidemiological cut-off values (CO_{WT}), provisional epidemiological cut-off values (pCO_{WT}) and SD values were not generated

MIC (µg ml^{-1})	AMP	ENR	FLO	GEN	OXO	OXY	TRS[c]	CTZ	CHL	MER	SME
Below scale[a]		35	1	1	1		2		190		130
0.004		41									
0.008		14									
0.015		8			68						
0.03		53			53	2	119				
0.06		78			102	57	51			1	
0.125		8	1	3	13	149	3	1		65	
0.25		3	52	11	1	26		3		172	
0.5			187	63	2	5	1	25		8	
1			6	117		1		164	54	1	
2				46				47	1		
4				6				5			
8	6										41
16	28										17
32									1		17
64											26
128											7
256											1
512											2
Above scale[b]	213	1			7	7	1	2			6
CO_{WT}/pCO_{WT}[d]	na	na	≤1	≤4	≤0.5	≤0.25	≤0.125	≤2	na	≤0.5	na
SD (ECOFFinder)[e]			0.36	0.81	1.02	0.55	0.36	0.50		0.41	
SD (NRI)[f]			0.74	0.87	1.35	0.59	0.64	0.53		0.57	
WT[g]			247	247	240	234	175	240		247	

[a]MIC was lower or equal to the lowest test concentration
[b]MIC value was higher than the highest test concentration
[c]MIC values for TRS are recorded in this table as the trimethoprim concentrations in the plates
[d]Mean CO_{WT}/pCO_{WT} values calculated from ECOFFinder and NRI analysis
[e]The SD of the best-fit curves calculated by ECOFFinder
[f]The SD of the of the normalised distribution of wild-type (WT) observations calculated by NRI
[g]The number of isolates categorised as WT by the mean CO_{WT}/pCO_{WT} values calculated in this work

individual laboratories (Table S2). Six laboratories, with modes in the range 0.06 to 0.03 µg ml^{-1}, showed reasonable agreement. Two other laboratories reported distributions with modal values of 0.004 µg ml^{-1} and one reported that 60% of their isolates had MICs ≤0.002 µg ml^{-1}. As the extent of the inter-laboratory variation in modal values was greater than that allowed for by EUCAST (2021), the aggregated MIC data for ENR were not considered as suitable for calculating a reliable CO_{WT} value. With respect to the disc zone data for this agent, 2 laboratories did not report any zones for the appropriate QC reference strain. The censored aggregation of the disc zone data for ENR from the other 5 laboratories contained 161 observations. Analysis of these data with NRI gave a CO_{WT} of ≥29 mm with an SD value of 3.26 mm. Application of this cut-off value categorised 156 observations (97%) as being from WT isolates.

The mean of the normalised WT distributions of the aggregated disc zone data for 5 of the laboratories that recorded the modes of their MIC data in the range 0.03–0.06 µg ml^{-1} was 38.3 mm. The equivalent mean zone size for the laboratory that generated a mode for their MIC distributions of ≤0.002 µg ml^{-1} was 39.7 mm. Thus, the extensive inter-laboratory variation apparent in the MIC data was not apparent in the disc zone data. However, the validity of this comparison of the MIC and disc zone data is limited by the observation that the laboratory that recorded the lowest MIC values reported ENR zone QC data only for the *E. coli* ATCC 25922 reference strain. The guideline VET04 (CLSI 2020b) does not provide any acceptable ranges for this strain and, therefore, it is not legitimate to treat the disc zone data generated by this laboratory as having been obtained using the standard protocol adopted in this work.

3.5. Florfenicol (FLO)

The aggregated MIC data against *V. anguillarum* for FLO contained 247 observations from 9 laboratories and only one was below scale. As this below-scale observation represented 3.3% of the WT observations made by that laboratory (Table S2), the degree of truncation was considered as acceptable.

Table 5. Frequency of disc-diffusion zones for *Vibrio anguillarum* isolates. The zone frequencies shown are those in the aggregations or censored aggregations of the data generated for these agents (abbreviations as in Table 2) by the participating laboratories. 'na' indicates that the data set was not considered suitable for NRI analysis and that CO_{WT}, pCO_{WT} and SD values were not generated. CO_{WT}, pCO_{WT} and SD values were calculated by NRI analysis. WT indicates the number of isolates categorised as wild type by the mean CO_{WT}/pCO_{WT} values calculated in this work

	AMP	ENR	FLO	GEN	OXO	OXY	TRS	CTZ	CHL	MER
Labs	4	5	6	5	7	6	6	7	7	7
Zone (mm)										
6	69				4	6	1			
7	7				1					
8	9									
9	9									
10	18									
11	14									
12	17								1	
13	8									
14	2									
15	3				1	1				
16										
17	1							1		
18								1		
19				1				4	1	
20				3				12	1	
21		1	1	21				8		
22				26				10		
23		1		29	1		2	19		3
24		1		23	1			32		4
25		2		27	2		1	34		24
26				21	2	5	2	38		28
27				6	2		1	28		40
28			1	2	2	16	4	20		24
29			1	1	5	14	9	4		33
30		1	3		21	34	16	5	3	21
31		8	4		21	26	11	1	6	24
32		12	8		47	25	29	1	12	2
33		10	7		28	21	16		8	5
34		25	17		37	11	40		33	3
35		24	23		16	18	25		19	4
36		11	30		6	6	14		28	1
37		15	20		6	2	10		10	2
38		8	33		4	3	8		33	
39		8	6		3		6		13	
40		14	18		6		1		32	
41		12	6				2		5	
42		3	12		1				5	
43		2	4						3	
44			2						1	
45		1	2						1	
46		1							3	
47										
48										
49		1								
No result		1		1	1					
CO_{WT}/pCO_{WT}	na	≥29	≥27	≥19	≥24	≥24	≥26	≥21	≥32	≥19
SD	na	3.26	3.68	1.90	3.23	2.97	2.90	1.91	2.47	3.02
WT		156	197	160	211	181	194	200	206	218

The modes of the distributions for individual laboratories were all within one dilution of the most common modal value in the aggregation. The SD values calculated for this aggregated data set by ECOFFinder and NRI, 0.36 and 0.74 $\log_2$ µg ml^{-1}, respectively, indicated that the data set was of adequate precision. Analysis of these data by ECOFFinder and NRI gave the 'exact' cut-off values of 0.664 and 1.167 µg ml^{-1}, respectively, giving a mean value of 0.916 µg ml^{-1} and a CO_{WT} of ≤1 µg ml^{-1}. Application of this cut-off value categorised all 247 observations (100%) as being from WT isolates. With respect to the disc zone data for this agent, one laboratory reported zones outside the acceptable range for the QC reference strain they used. The censored aggregation of the disc data for this agent from the other 6 laboratories contained 198 observations. The zone frequencies showed a unimodal distribution and NRI analysis calculated a CO_{WT} of ≥27 mm with an SD of 3.68 mm. Application of this cut-off value categorised 197 observations (>99%) as being from WT isolates.

3.6. Gentamicin (GEN)

The aggregated MIC data against *V. anguillarum* for GEN contained 247 observations from 9 laboratories. Only one of the MIC values was off scale. As this below-scale observation represented 2.5% of the WT observations made by that laboratory (Table S2), the degree of truncation was considered as acceptable. The modes of the distributions for individual laboratories were all within one dilution of the most common modal value in the aggregation. The SD values calculated for this aggregated data set by ECOFFinder and NRI, 0.81 and 0.87 $\log_2$ µg ml^{-1}, respectively, indicated that data set was of adequate precision. Analysis of these data by ECOFFinder and NRI gave the 'exact' cut-off values of 3.654 and 2.260 µg ml^{-1}, respectively, giving a mean value of 2.96 µg ml^{-1} and a CO_{WT} of ≤4 µg ml^{-1}. Application of this cut-off value categorised all 247 observations (100%) as being from WT isolates. With respect to the disc zone data for this agent, 2 laboratories reported zones outside the acceptable range for the QC reference strain they used. The censored aggregation of the disc zone data for this agent from the other 5 laboratories contained 160 observations. The zone frequencies showed a uni-modal distribution and NRI analysis calculated a CO_{WT} of ≥19 mm with an SD of 1.90 mm. Application of this cut-off value categorised all of the 160 observations (100%) as being from WT isolates.

3.7. Oxolinic acid (OXO)

The aggregated MIC data against *V. anguillarum* for OXO contained 247 observations from 9 laboratories and only one of the MIC values was off scale. As this below-scale observation represented 4.8% of the WT observations made by that laboratory (Table S2), the degree of truncation was considered as acceptable. The mode of the distributions of the MIC values for 4 laboratories was 0.06 µg ml^{-1}, for another it was 0.03 µg ml^{-1} but for another 4 laboratories it was 0.015 µg ml^{-1}. This degree of inter-laboratory variation was greater than that allowed for by EUCAST (2021), and its extent meant that it could not be resolved by excluding the data from 1 or 2 aberrant laboratories. Despite this degree of inter-laboratory variation, these aggregated data were analysed by ECOFFinder and NRI. These analyses gave the 'exact' cut-off values of 0.259 and 0.268 µg ml^{-1}, respectively, giving a mean value of 0.264 µg ml^{-1} and a CO_{WT} of ≤0.5 µg ml^{-1}. Application of this cut-off value categorised 240 (96%) of the 247 isolates as WT. However, the SD value calculated for this aggregated data set by ECOFFinder was 1.05 $\log_2$ µg ml^{-1}, which was very close to the suggested upper limit for acceptable precision suggested for this method of 1.11 $\log_2$ µg ml^{-1}. The SD generated by NRI analysis of 1.35 $\log_2$ µg ml^{-1} was significantly above the upper acceptable limit of 1.18 $\log_2$ µg ml^{-1} suggested for this method. The extent of inter-laboratory variation and the consequent low precision of the aggregated data were taken to indicate that the CO_{WT} calculated from these MIC data could not be considered reliable. The aggregation of the disc zone data for OXO contained 217 observations from 7 laboratories. The zone frequencies showed a unimodal distribution. NRI analysis calculated a CO_{WT} of ≥24 mm with an SD of 3.74 mm. Application of this cut-off value categorised 210 of the 217 observations (97%) as being from WT isolates.

The mean of the normalised distribution of the 36 WT observations for OXO disc zones generated by the laboratory that recorded the mode of its MIC distribution as 0.015 µg ml^{-1} was calculated using NRI analysis as 33.4 mm. This was almost identical to the mean of 33.0 mm calculated for the 145 WT isolates studied in 4 laboratories that reported MIC distributions with the larger modes of 0.06 µg ml^{-1}. Thus, the extensive inter-laboratory variation in the MIC data was not apparent in the disc zone data. This analysis does not provide any evidence that would support a hypothesis that differences in the susceptibilities in the isolates tested in the different labora-

tories can explain the differences in MIC values they reported.

3.8. Oxytetracycline (OXY)

The aggregated MIC data against *V. anguillarum* for OXY contained 247 observations from 9 laboratories. None of the MIC values were below scale, and the modes of the distributions for individual laboratories were all within one dilution of the most common modal value in the aggregation. The SD values calculated for this aggregated data set by ECOFFinder and NRI, 0.55 and 0.59 $\log_2$ µg ml^{-1}, respectively, indicated that the data set was of adequate precision. Analysis of these data by ECOFFinder and NRI gave the 'exact' cut-off values of 0.264 and 0.184 µg ml^{-1}, respectively, giving a mean value of 0.224 µg ml^{-1} and a CO_{WT} of ≤0.25 µg ml^{-1}. Application of this cut-off value categorised 234 of the 247 (95%) isolates as WT. With respect to the disc zone data for this agent, one laboratory reported zones outside the acceptable range for one of the QC reference strains. The censored aggregation of the disc data for this agent from the other 6 laboratories contained 188 observations. The zone frequencies showed a unimodal distribution and NRI analysis calculated a CO_{WT} of ≥24 mm with an SD of 2.97 mm. Application of this cut-off value categorised 181 observations (96%) as being from WT isolates.

3.9. Trimethoprim/sulfamethoxazole (TRS)

Nine laboratories reported MIC values for TRS against *V. anguillarum*. For simplicity, in Tables 3 and 4 and in ECOFFinder and NRI analyses, the concentration in each dilution was expressed as the concentration of the trimethoprim component. The mode of the distribution of MIC values from one laboratory was 2 dilutions higher than the most common modal value in the other 8 laboratories. The data from this laboratory were, therefore, excluded from the censored aggregation of MIC data for TRS. The distributions from 2 other laboratories were truncated (Table S2). For one, the below-scale observations represented 10% of the WT observations and were considered acceptable. For the other, the below-scale observations represented 18% of the WT observations and the data from this laboratory were excluded from the aggregation. The SD values calculated for the censored data from 7 laboratories set by ECOFFinder and NRI, 0.36 and 0.64 $\log_2$ µg ml^{-1}, respectively, indicated that this data set was of adequate precision. For the censored TRS aggregated data, ECOFFinder and NRI analyses calculated 'exact' cut-off values of 0.060 and 0.080 µg ml^{-1}, respectively, giving a mean value of 0.070 µg ml^{-1}. This gave a CO_{WT} for TRS of ≤0.125/2.38 µg ml^{-1} and application of this cut-off value categorised 175 of the 177 observations (99%) as being from WT isolates.

With respect to the disc zone data for this agent, one laboratory reported zones outside the acceptable range for one of the QC reference strains. The censored aggregation of the disc data for this agent from the other 6 laboratories contained 198 observations. The zone frequencies showed a unimodal distribution and NRI analysis calculated a CO_{WT} of ≥26 mm with an SD of 2.90 mm. Application of this cut-off value categorised 194 observations (98%) as being from WT isolates.

3.10. Ceftazidime (CTZ)

The aggregated MIC data against *V. anguillarum* for CTZ contained 247 observations from 9 laboratories. None of the MIC values were off scale, and the modes of the distributions for individual laboratories were all within one dilution of the most common modal value in the aggregation. The SD values calculated for this aggregated data set by ECOFFinder and NRI, 0.50 and 0.53 $\log_2$ µg ml^{-1}, respectively, indicated that the data set was of adequate precision. Analysis of these data by ECOFFinder and NRI gave the 'exact' cut-off values of 2.157 and 1.745 µg ml^{-1}, respectively, giving a mean value of 1.951 µg ml^{-1} and a pCO_{WT} of ≤2 µg ml^{-1}. Application of this cut-off value categorised 240 of 247 observations (97%) as being from WT isolates. The aggregation of the disc zone data for this agent contained 218 observations from 7 laboratories. The zone frequencies showed a unimodal distribution and NRI analysis calculated a pCO_{WT} of ≥21 mm with an SD of 1.91 mm. Application of this cut-off value categorised 200 observations (92%) as being from WT isolates.

3.11. Chloramphenicol (CHL)

The aggregated MIC data against *V. anguillarum* for CHL contained 247 observations from 9 laboratories. However, as the MIC values for 190 of these (77%) were below scale, these data were not suitable for calculating any CO_{WT} values. The aggregation of the disc zone data for this agent contained 218 obser-

vations from 7 laboratories. The zone frequencies showed a unimodal distribution and NRI analysis calculated a pCO_{WT} of ≥32 mm with an SD of 2.47 mm. Application of this cut-off value categorised 206 observations (94%) as being from WT isolates.

3.12. Meropenem (MER)

The aggregated MIC data against *V. anguillarum* for MER contained 247 observations from 9 laboratories. None of the MIC values were off scale, and the modes of the distributions for individual laboratories were all within one dilution of the most common modal value in the aggregation. The SD values calculated for this aggregated data set by ECOFFinder and NRI, 0.41 and 0.57 $\log_2$ µg ml^{-1}, respectively, indicated that data set was of adequate precision. Analysis of these data by ECOFFinder and NRI gave the 'exact' cut-off values of 0.335 and 0.360 µg ml^{-1}, respectively, giving a mean value of 0.358 µg ml^{-1} and a pCO_{WT} of ≤0.5 µg ml^{-1}. Application of this cut-off value categorised 246 of 247 observations (99%) as being from WT isolates. The aggregation of the disc zone data for this agent contained 218 observations from 7 laboratories. The zone frequencies showed a unimodal distribution and NRI analysis calculated a pCO_{WT} of ≥19 mm with an SD of 3.02 mm. Application of this cut-off value categorised all observations (100%) as being from WT isolates.

3.13. Sulfamethoxazole (SME)

Fifty-three percent of the 247 MIC observations for SME against *V. anguillarum* from 9 laboratories were below scale; therefore, these data were not suitable for calculating any CO_{WT} values.

4. DISCUSSION

4.1. Setting proposed ECV values for agents with acceptable QC ranges

The primary aim of the work reported here was to generate data that would allow the setting of ECV values for *V. anguillarum* susceptibility data generated by the standard methods for micro-dilution MIC and disc-diffusion tests specifying incubation at 28°C for 24–28 h provided in the guideline VET03 (CLSI 2020a). The minimal criteria that data must meet to fulfil this function are that they must have been generated by laboratories that strictly adhered to the CLSI standard and that they should also have met the quantitative requirements set out in the guideline M23 (CLSI 2018). Compliance with the QC requirements specified in a standard method is an essential component of that method. With respect to the standard methods performed at 28°C for 24–28 h used in this study, CLSI (2020b) have set acceptable ranges for QC reference strains for 7 (AMP, ENR, FLO, GEN, OXO, OXY and TRS) of the 11 agents tested in this study. Consequently, only the data obtained for these 7 agents could be considered as having been generated using a standard method. With respect to the MIC data sets for these 7 agents, all 9 laboratories recorded values for the QC reference strains that were within the published acceptable ranges. The MIC aggregations for 5 of these agents (ENR, FLO, GEN, OXO and OXY) were generated in 9 laboratories and contained between 234 and 247 observations from unique isolates (Table 4). The aggregation of the data for TRS was censored and was compiled using the data from 7 laboratories and contained only 175 observations from unique isolates categorised as WT. Thus, the data for these 6 agents met the quantitative requirements of both CLSI (2018) and EUCAST (2021a). The aggregation of the MIC data for AMP contained 247 MIC observations, but as these were all considered as having been obtained from NWT isolates, a CO_{WT} was not calculated from this aggregation. Although they met the quantitative requirements, the uncensored aggregations of the MIC data for ENR and OXO showed evidence of low precision and an excessive variation in the modes of the data sets from individual laboratories. Therefore, it was not considered advisable to propose a CO_{WT} from the MIC data for these 2 quinolones.

In the absence of specific guidance, we decided to adopt the requirement that aggregations should contain a minimum of 100 observations from unique isolates categorised as WT that had been generated in at least 5 laboratories. The aggregations and censored aggregations against *V. anguillarum* analysed for 6 (ENR, FLO, GEN, OXO, OXY and TRS) of the 7 agents were obtained from 5 to 7 laboratories and contained from 160 to 217 observations from unique isolates categorised as WT (Table 3) and, therefore, were considered to have met these requirements. The disc-diffusion data for AMP were considered as having been obtained from NWT isolates and, therefore, were not analysed.

In summary, the MIC and disc zone data sets against *V. anguillarum* for FLO, GEN, OXY and TRS and the disc zone data for ENR and OXO generated

in this work were considered to have met the criteria proposed in the CLSI guideline M23 (CLSI 2018). They also met the more stringent criteria published by EUCAST (2021). It is intended that the CO_{WT} values calculated from these MIC and disc zone data (Table 6) and the data used to calculate them will be submitted to CLSI for adoption as ECVs.

4.2. Agents with MIC data showing excessive inter-laboratory variation

For both ENR and OXO, 2 quinolones for which a high degree of cross-resistance would be expected, the differences in the modes of the distributions of MIC values for *V. anguillarum* isolates by individual laboratories exceeded the limits recommended by EUCAST (2021). Similar inter-laboratory differences were not observed in the data obtained by the individual laboratories for these agents when the QC reference stains were tested (Table S2). Nor, with the exception of the TRS data set from one laboratory, were excessive inter-laboratory variations observed in the modes of the MIC data with respect to the *V. anguillarum* isolates or the QC reference strains for any of the other agents studied (Table S2). The examination of the disc zones recorded for these quinolones, particularly those for OXO, did not reveal any extensive inter-laboratory variation in these data. Thus, it would appear that this phenomenon is confined to the data obtained in the testing of the MIC values of *V. anguillarum* isolates to these 2 quinolones. Investigations of this large inter-laboratory variation in the quinolone MIC data against *V. anguillarum* are planned, but at this stage, it must be considered imprudent or unwise to offer any CO_{WT} based on these data.

4.3. Agents without acceptable ranges for QC reference strains

With respect to the agents (CTZ, CHL, MER and SME) for which there are no relevant acceptable ranges for any QC reference strains, CLSI would not be able to set ECVs against *V. anguillarum.* In this work, MIC values and zone sizes were determined for the QC reference strains for these agents (Table S2) in order that if and when CLSI set acceptable ranges for these agents, it may become possible to use the data generated in this work to set relevant ECVs. However, Baron et al. (2020b) argued that even in the absence of the necessary acceptable ranges, the pCO_{WT} calculated in this study could perform a useful function in a pre-screening role.

Table 6. Proposed epidemiological cut-off values for *V. anguillarum* MIC and disc zone data obtained using standard methods specifying incubation at 28°C for 24–28 h (CLSI 2020a)

Agent	MIC (μg ml^{-1})	Disc zone (mm)
Enrofloxacin		≥29
Florfenicol	≤1	≥27
Gentamicin	≤4	≥19
Oxolinic acid		≥24
Oxytetracycline	≤0.25	≥25
Trimethoprim/sulfamethoxazole	≤0.125/2.38	≥26

The pCO_{WT} values for *V. anguillarum* with respect to CTZ and MER could have a role in increasing our understanding of the spread of genetic elements encoding extended spectrum β-lactamases and carbapenemases. Resistances of this type are of major concern in human medicine (Laxminarayan et al. 2013, Aerts et al. 2019). Although the use of third-generation cephalosporins and carbapenems in the treatment of aquatic animal infections is thought to be very rare, the 'One Health' approach (FAO 2016) suggests that it would be prudent to monitor any emergence of resistance to these agents in the aquatic microbiome. With respect to *V. anguillarum* it is suggested that this might be most effectively achieved if isolates that were detected as manifesting reduced susceptibility to CTZ or MER by the application of the pCO_{WT} values, calculated in this work, were investigated with further, more specific tests to determine the mechanisms underlying these phenotypes. A report by the European Food Safety Authority (Aerts et al. 2019) provides a review of the methods that might be appropriate for this purpose.

EUCAST (2021b) published data on the distributions of MICs generated at 35°C for 5 *Vibrio* species. With respect to MER, the species were demonstrated to be in 2 distinct groups. For one group (*V. cholerae* and *V. fluvialis*), the modes of the MIC distributions were in the range 0.125–0.25 μg ml^{-1}. For the other group (*V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus*), the modes were in the range 0.008–0.016 μg ml^{-1}. Although they were generated at 28°C, and therefore are not directly comparable, the data obtained in this work suggest that with respect to MER susceptibility, *V. anguillarum* is probably related to the first group.

Bacteria showing resistance to sulfonamides mediated by *sul* genes have been reported to be present in

the aquatic environment (Gao et al. 2012, Suzuki et al. 2013). The rationale for the inclusion of SME as a single agent in the ECOFFVIB plates used in this work was to provide data that would facilitate the selection of isolates to be examined by molecular techniques for the possession of *sul* genes (Kadlec et al. 2011). However, the range of concentrations of SME in these plates was not sufficient to capture quantitative MIC values for over 50% of the 247 isolates tested, and therefore pCO_{WT} values could not be set.

4.4. Plate layout

Epidemiological cut-off values are calculated from the distribution of the values obtained from WT isolates. In performing MIC determinations to calculate these cut-off values, both CLSI (2018) and EUCAST (2021) stress the importance of using concentration ranges that capture quantitative MIC values for all WT isolates, including the most susceptible. The ECOFFVIB plates used in this work were not capable of capturing the MIC values for a large percentage of the most susceptible isolates of *V. anguillarum* with respect to 3 agents: ENR, CHL and SME (Table 4). This suggested that redesign of this plate layout would be desirable. An international group of scientists from Asia, Europe and the USA worked with FAO to formulate a guide to an improved 96-well plate layout that might be suitable for adoption globally in all studies of Gram-negative bacteria isolated from aquatic animals (FAO 2023). In order to facilitate maximal compatibility of the data generated, this guide suggests that all plates should include a fixed portion of 67 wells containing the same concentrations of seven agents AMP, ENR, FLO, GEN, OXO, OXY and TRS. The remaining 28 wells (the free portion) would include concentrations of 3 additional agents specifically chosen to reflect the interest of any particular study. A group of European laboratories led by Cefas and Anses are currently using a new plate (AQGNECV) in studies of Gram negative bacteria. In the fixed portion these plates incorporate the 7 agents and their concentrations recommended by FAO (2023) and in the free portion they also include CTZ, MER and SME. The details of the agents and their concentrations in the AQGNECV plate are shown in Table 2.

4.5. Frequencies of NWT phenotypes

As identified in the WOAH Aquatic Animal Health Code (WOAH 2019), one of the main uses of internationally harmonised epidemiological cut-off vales is to interpret the data collected in studies performed to monitor and survey the frequencies of isolates manifesting reduced susceptibility. Smith (2019) has stressed that in such studies, care should be taken to ensure that the isolate sets tested should be unbiased and, as far as is possible, represent those circulating in the region under investigation. However, the isolate collections of *V. anguillarum* used in this work were not unbiased. As epidemiological cut-off values are set from the distribution of observations from WT isolates, many of the isolate collections studied in this work had been deliberately constructed to exclude as many isolates as possible that initial susceptibility testing had suggested might possess any resistance mechanism. For this reason, it would not be in any way legitimate to treat the frequencies of NWT *V. anguillarum* isolates calculated in this work as indications of the frequencies circulating in the European countries from which the isolates were made.

5. CONCLUSIONS

This study is the first to generate the data necessary to set ECV values for *V. anguillarum* for the agents FLO, GEN, OXY, TRS, ENR and OXO, based on susceptibility data obtained by stringent standard protocols with acceptable ranges for the QC strains. Furthermore, provisional cut-off values were set for CTZ, CHL and MER, providing data important for the future development of standardised protocols for these agents. This comprehensive study demonstrates the importance of assessing inter-laboratory variations, as this was observed for quinolones.

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