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

Epidemiological cut-off values for *Yersina ruckeri* disc diffusion data generated by a standardised method

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ABSTRACT: In order to establish the meaning of data generated in antimicrobial agent susceptibility tests, it is necessary to develop internationally harmonised interpretive criteria. Currently, such criteria have not been developed for data generated in studies of the susceptibility of the fish pathogen *Yersinia ruckeri*. This work generated the data that would be required to set epidemiological cut-off values for the susceptibility data of this species that had been generated using a standardised disc diffusion method that specified the use of Mueller Hinton agar and incubation at 22°C for 24—28 h. Using this method, sets of inhibition zones data for 4 antimicrobial agents were generated by 3 independent laboratories. The data from these laboratories were aggregated and analysed using the statistically based normalised resistance interpretation. For ampicillin, florfenicol, oxytetracycline and trimethoprim-sulfamethoxazole the cut-off values calculated by this analysis were ≥16, ≥23, ≥24 and ≥30 mm, respectively. Evidence is presented demonstrating that the data for these 4 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 *Y. ruckeri*.

KEY WORDS: CLSI VET04 · Normalized resistance interpretation · Antimicrobial susceptibility · Disc diffusion · *Yersinia ruckeri*

1. INTRODUCTION

Infections of fish by *Yersinia ruckeri* result in serious disease and can cause significant economic losses to aquacultural enterprises. Although most frequently isolated from diseased salmonids (Tobback et al. 2007), infections of many other species have been reported (Pajdak-Czaus et al. 2019, Feng et al. 2020). The distribution of *Y. ruckeri* is world-wide. Infections of fish have, for example, been reported from North and South America, Europe, Australia, South Africa, the Middle East and China (Kumar et al. 2015).

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Vaccines against *Y. ruckeri* are commercially available (Deshmukh et al. 2012), and they have been licenced in some, but not all, countries where this species is a problem (Feng et al. 2022). Despite this Tobback et al. (2007) reported that control of *Y. ruckeri* infections in fish frequently relies on the use of antimicrobial compounds. Kumar et al. (2015) reported that the most frequently used agents were amoxicillin, oxolinic acid, oxytetracycline, sulphadiazine in combination with trimethoprim and florfenicol. However, such chemotherapy could be compromised by the emergence of strains resistant to these agents. A number of studies of antimicrobial agent susceptibility in *Y. ruckeri* isolates have been published in the last 10 yr (Calvez et al. 2014, Huang et al. 2014, Duman et al. 2017, Mesías Valle et al. 2020, Önalan & Çevik 2020, Feng et al. 2022). Unfortunately, these studies used a variety of susceptibility testing methods, which makes it difficult to combine or compare the data they generated. Even when 2 or more studies used a standardised Clinical and Laboratory Standards Institute (CLSI) method (CLSI 2020a), the lack of any internationally harmonised, consensus-interpretive criteria for susceptibility measures generated using those methods presents major difficulties for inter-study comparisons.

Baron et al. (2021) demonstrated that with respect to categorising *Y. ruckeri* isolates as wild type (WT) or non-wild type (NWT) minimum inhibitory concentration (MIC) and disc diffusion methods were equally valid. However, Smith (2007) reported that the majority of laboratories handling clinical isolates from diseased fish used the disc diffusion method. The work reported here was, therefore, undertaken to generate the data required to set internationally harmonised, consensus epidemiological cut-off values for data on the susceptibility of *Y. ruckeri* generated using a standardised disc diffusion method.

2. MATERIALS AND METHODS

2.1. Participating laboratories

Three laboratories performed the antimicrobial susceptibility tests of *Yersinia ruckeri* susceptibility. These were those of the Centre for Environment, Fisheries and Aquaculture Science Laboratory, Weymouth, UK (Cefas), the Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit of Ploufragan-Plouzané-Niort Laboratory of the French Agency for Food, Environmental and Occupational Health & Safety (MBA) and the National Reference Laboratory for fish, mollusc and crustacean diseases, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy (ISZVe).

2.2. Source and classification of isolates

The 111 Cefas isolates were sourced from their culture collection of isolates recovered from farmed salmonids in the UK since the disease first emerged in the 1980s. These were identified via primary biochemical identification tests (Wheeler et al. 2009) and use of the Bionor Mono Yr agglutination test kit (BIONOR). The MBA collection was composed of 100 isolates provided by several French veterinarians and laboratories (Baron et al. 2021). They were all isolated from clinical samples and identified by Maldi-ToF MS (V7.0.0.0). The IZSVe collection derived from routine diagnostic activity (2021–2023) and included isolates obtained from diseased rainbow trout *Oncorhynchus mykiss* (n = 58) and manila clam *Ruditapes philippinarum* (n = 1). All the isolates were identified by MALDI-ToF MS (Microflex Biotyper LT, Bruker Daltonics), and for 47 isolates the identification was further confirmed by the amplification and Sanger sequencing of the ribosomal 16S gene (Davidovich et al. 2022).

2.3. Susceptibility measurements

Disc diffusion tests against *Y. ruckeri* were performed using unmodified Mueller Hinton agar with incubation at 22°C for 24–28 h according to the protocol provided in the CLSI guideline VET03 (CLSI 2020a). As recommended by CLSI (2017, 2020b), the discs used contained 10 µg ampicillin (AMP), 30 µg florfenicol (FLO), 30 µg oxytetracycline (OXY) and 1.25/23.75 µg trimethoprim/sulfamethoxazole (TRS). The abbreviations used for antimicrobial agents were generated using the system advocated by EUCAST (2022). The participating laboratories obtained their discs from different suppliers and this information is detailed in Table S1 in the Supplement (www.int-res.com/articles/suppl/d158p021_supp.pdf). The numbers of inhibition zone (IZ) observations in the data sets from the individual laboratories participating in this study are shown in Table 1. Each laboratory employed one or both of the quality control (QC) reference strains *Escherichia coli* American Type Culture Collection (ATCC) 25922 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 recommended by CLSI for this method (CLSI 2020a).

Table 1. Number of unique isolates for which inhibition zone data was obtained for each antimicrobial agent by the participating laboratories. Cefas: Centre for Environment, Fisheries and Aquaculture Science Laboratory. MBA: Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit of Ploufragan-Plouzané-Niort Laboratory of the French Anses. IZSVe: Istituto Zooprofilattico Sperimentale delle Venezie

Antimicrobial agent	Laboratory		
	Cefas	MBA	IZSVe
Ampicillin	110	100	30
Florfenicol	111	100	41
Oxytetracycline	70	32	58
Trimethoprim-sulfamethoxazole	110	100	41[b]

The CLSI document VET04 (CLSI 2020b) provides acceptable ranges for these QC reference strains tested using the standardised disc diffusion method adopted in this work for all 4 antimicrobial agents.

2.4. Calculation of proposed epidemiological cut-off values

Epidemiological cut-off values categorise isolates as either fully susceptible WT members of their species or as NWT isolates that manifest a susceptibility significantly lower than that of the WT isolates (Silley 2012). In this work epidemiological cut-off values (CO_{WT}) for each antimicrobial agent were calculated by the automatic normalized resistance interpretation (NRI) method (www.bioscand.se/nri/) from aggregations of the IZ data generated by the individual participating laboratories.

3. RESULTS AND DISCUSSION

For all 4 antimicrobial agents (AMP, FLO, OXT and TRS) the IZ for the QC reference strains *Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658 recorded by the participating laboratories were within the acceptable ranges set by the CLSI document VET04 (2020b) (Table S2). The distributions of the IZ observations for *Yersinia ruckeri* for each laboratory and for the 3 laboratory aggregations are shown in Table S3. A graphical presentation of the distributions for the aggregations are shown in Fig. 1 together with the curves of the normalised distributions of the WT observations and the CO_{WT} calculated by NRI. A summary of the results of the NRI analysis of the aggregations is shown in Table 2. For AMP, FLO, OXT and TRS the cut-off values calculated by this analysis were ≥16, ≥23, ≥24 and ≥30 mm, respectively.

With respect to the quantity of the data, Smith et al. (2023) have argued that to provide adequate data for the setting of epidemiological cut-off values the aggregated inhibition zone diameter sets should contain at least 100 observations from unique isolates, categorised as WT, that had been generated in at least 3 laboratories. The numbers of IZ observations that were categorised as being from WT isolates in the 3 laboratory aggregated data sets for the 4 agents ranged from 153 to 246 (Table 2).

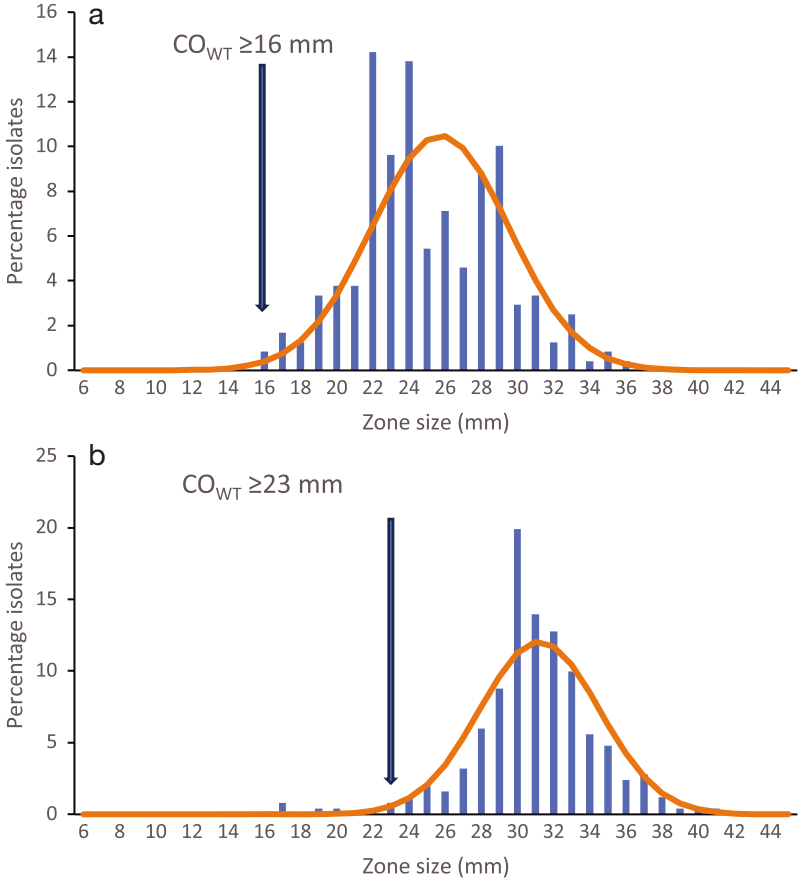

Fig. 1. Distributions of inhibition zone frequencies (vertical bars) in the aggregated data sets from the 3 participating laboratories for 4 antimicrobial agents. Curved line: normalised distribution of wild-type observations calculated by normalised resistance interpretation (NRI) analysis. Arrow: wild-type cut-off values (CO_{WT}) calculated by NRI analysis

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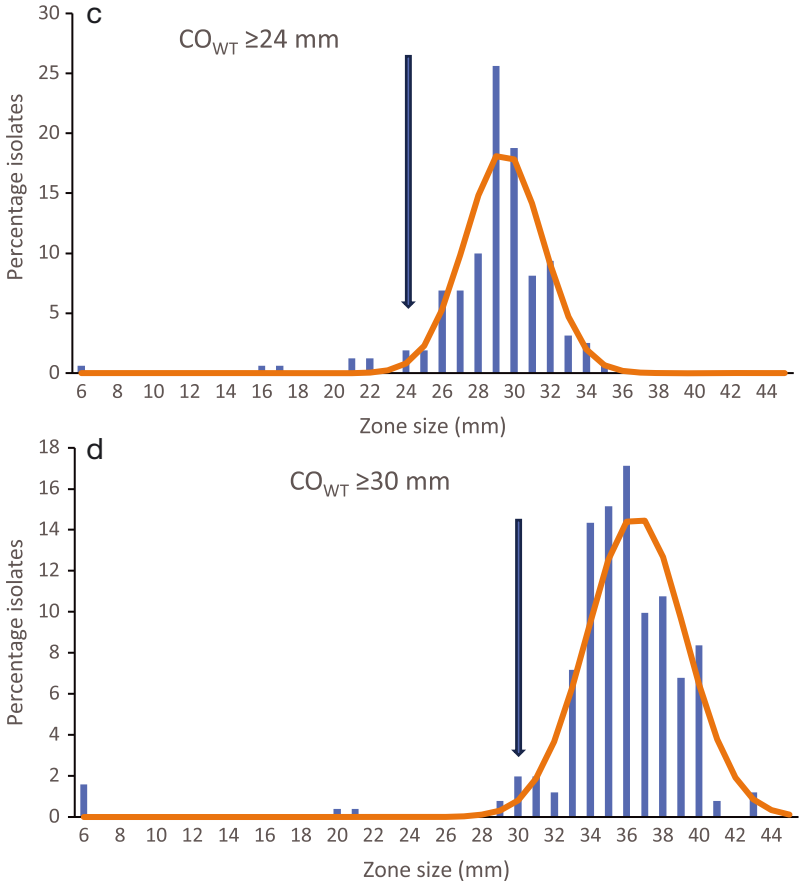

Fig. 1 (continued)

Table 2. Summary of the normalised resistance interpretation (NRI) analysis of the aggregated inhibition zone sizes (mm) generated in 3 laboratories. SD: standard deviation of the normalised distribution of wild-type (WT) isolates calculated by NRI analysis. CO_{WT}: WT cut-off values calculated by NRI analysis. Total: total number of observations in the aggregation. WT: number of observations from isolates categorised as WT by the CO_{WT} calculated in this work

Antimicrobial agent	NRI analysis		Observations	
	SD	CO_{WT}	Total	WT
Ampicillin	3.8	≥16	240	240
Florfenicol	3.3	≥23	251	246
Oxytetracycline	2.1	≥24	160	153
Trimethoprim-sulfamethoxazole	2.7	≥30	251	243

The European Committee on Antimicrobial Susceptibility Testing (EUCAST 2021) has stated that, in order to set reliable epidemiological cut-off values, the data from individual laboratories should include >15 observations that were categorised as being from WT isolates. Although this minimum limit was proposed for MIC data, Smith et al. (2023) suggested that it was reasonable to also apply it to IZ data. The numbers of IZ observations in the data sets from the individual laboratories participating in this study ranged from 30 to 111 (Table 1).

Precision is an important qualitative parameter of any IZ data set (Smith 2020). In this context the standard deviation (SD) of the normalised distribution of WT observations calculated by NRI analysis of a data set can provide a measure of the precision of that data set (Smith et al. 2018). Smith (2019) suggested that any IZ data set generated by a single laboratory at 22°C for which the SD was ≥6.5 mm should be treated as excessively imprecise. The SD values from the 12 IZ data sets from individual laboratories calculated in this work were all within this suggested limit. They ranged from 1.9 to 3.5 mm with a median value of 2.5 mm (Table S3). These calculations suggest that the data sets from individual laboratories were adequately precise. A limit has not yet been suggested for SD values calculated for multiple laboratory aggregated data sets. However, for the 4 agents the SD values for the 4 aggregated data sets ranged from 2.0 to 3.8 mm with a median of 3.0 mm (Table S3). This suggests that any inter-laboratory variation there was in the data for any of the 4 agents was not sufficient to result in a major decline in the precision of the aggregated data sets for that agent.

On the basis of the arguments above, the data sets generated in this work were considered of sufficient quantity and quality for them to be used in setting reliable epidemiological cut-off values. These data will, therefore, be presented to the Aquatic Working Group of CLSI. It is hoped that these data, taken together with data of adequate quantity and quality from any other sources, will support CLSI in publishing internationally harmonised, consensus epidemiological cut-off values in future editions of VET04.

It should be noted that many of the isolate collections studied in this work had been deliberately constructed to include as many isolates as possible that initial susceptibility testing had suggested might be fully susceptible. For this reason it would not be in any way legitimate to treat the frequencies of NWT

phenotypes in the *Y. ruckeri* isolates studied in this work as indications of the frequencies circulating in the European countries from which the isolates were made.

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