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Use of normalised resistance analyses to set interpretive criteria for antibiotic disc diffusion data produce by *Aeromonas* spp

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ABSTRACT

Normalised resistance interpretive (NRI) analysis was applied to the development of interpretive criteria for antibiotic disc diffusion zones of mesophilic *Aeromonas* spp. Minimum quantitative and qualitative properties of data sets from which normalised resistance interpretation could generate acceptable cut-off values were established by examining published data that had been used to set the epidemiological cut-off values for bacteria of human and aquatic interest. Applying these criteria to disc diffusion data generated in a previous study of 129 mesophilic *Aeromonas* spp. isolated from pet fish, demonstrated that normalised resistance interpretation was capable of generating acceptable cut-off values for seven (erythromycin ≥ 8 mm, gentamicin ≥ 18 mm, moxalactam ≥ 32 mm, oxytetracycline ≥ 21 mm, streptomycin, ≥ 20 mm tetracycline ≥ 28 mm, and trimethoprim/sulfamethoxazole ≥ 26 mm) of the agents tested. This analysis demonstrated, with respect to these agents, that a single set of interpretive criteria could be developed for application to data generated from all the Aeromonads studied.

Provisional cut-off values were generated for three other agents (chloramphenicol ≥ 35 mm, florfenicol ≥ 35 mm and furazolidone ≥ 23 mm). The distribution of zone sizes for these agents showed an apparent bimodality in the high-zone end. As a result, the question of whether, for these three agents, a single set of interpretive criteria could be validly set for all Aeromonads could not be resolved. With respect to the five quinolone agents studied, a cut-off value could only be estimated for oxolinic acid. Because of the high frequency of resistance to quinolones in the strain set, this value (≥ 32 mm) should be treated as a provisional estimate.

With respect to some agents, strains manifesting a low-level resistant phenotype, zones ≤ 8 mm smaller than the wild-type cut-off, represented a significant proportion of the strains classified as non wild-type. The difficulties that these frequencies of low-level resistance would present for attempts to set and apply universal, laboratory-independent interpretive criteria are discussed.

1. Introduction

The publication of the guidelines M42-A by the Clinical and Laboratory Standards Institute (CLSI, 2006) marked a major milestone in the development of rational antibiotic susceptibility testing of bacteria associated with aquatic animal disease. This guideline provided the test protocols and their associated quality control requirements for disc diffusion testing of any bacterium that was capable of yielding reliable results when tested on unmodified Mueller–Hinton agar within 48 h incubation at 22 °C or 28 °C. Importantly, however, M42-A did not provide any criteria for interpreting the data produced by the protocol. Recently an informational supplement (CLSI, 2010) published criteria for interpretation of *Aeromonas salmonicida* data.

Interpretive criteria can be of two types (CLSI, 2010). Clinical breakpoints aim to provide a clinically relevant interpretation. Setting them requires extensive clinical outcome data or pharmacokinetic/pharmacodynamic data (CLSI, 2007; Turnidge and Paterson, 2007). However, these data are not currently available for the majority of bacteria of interest to aquaculture and Smith (2008a, 2008b) has argued that they will difficult and very expensive to produce. The alternative set of criteria, epidemiological cut-off values (ECO), can be set from a consideration of laboratory generated susceptibility data (CLSI, 2007; Kronvall et al., 2011), which, in contrast, are relatively simple to produce. These ECO values can be applied to classifying strains, on the basis of their in-vitro phenotypes, as fully susceptible or wild type (WT) or not fully susceptible or non-wild type (NWT). Although they are of less direct clinical relevance than clinical breakpoints (Smith, 2008a,c), ECO are, at least in theory, easier to set. In this paper, in order to limit ambiguities in the terminology used in association with cut-off values the term epidemiological cut-off values (ECO) will be reserved for values that have been accepted and published by either CLSI or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The term wild-type cut-off (CO_{WT}) will be used for values proposed by individual laboratories.

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Kronvall (2003, 2010) and Kronvall et al. (2006) developed a statistically based method, normalised resistance interpretation (NRI), for estimating CO_{WT} from laboratory generated susceptibility data. This method has been applied to disc diffusion data obtained for bacteria associated with fish disease by Smith et al. (2007), Douglas et al. (2007), Ruane et al. (2007) and Avendaño-Herrera et al. (2011). A particular advantage of NRI analysis in the setting of CO_{WT} is that it provides a formal method of dealing with situations where the distribution of zones obtained for NWT strains manifesting low-level resistance (LLR) overlaps those obtained for WT strains. NRI analysis was developed to assist in the interpretation of data on bacteria of human relevance obtained in hospital laboratories (Kronvall, 2003). Characteristically these laboratories generate very large data sets (see http://www.eucast.org/mic_distributions/). In aquaculture it would be unlikely that such large data sets would be available. Smith et al. (2009) demonstrated that the precision associated with any CO_{WT}, set by NRI analysis, is a function of the log of the number of strains used in the analysis. They suggested that strain sets that contain a minimum of 20–40 strains would be capable of generating a reasonably precise CO_{WT}.

This study used NRI analysis to re-analyse disc diffusion data, produced by 16 antibiotics agents against 129 strain of *Aeromonas* spp. isolated from pet fish, that had been generated by Verner-Jeffreys et al. (2009). The major aim was to generate agent-specific CO_{WT} values, which could be applied as criteria for the interpretation of these data. To facilitate this aim, qualitative and quantitative data set requirements were developed that should be met before the CO_{WT} generated from that data could be considered as reasonably valid.

In developing CO_{WT} or ECO, a key question is whether such values need to be species-specific (Kronvall et al., 2011) or whether, without excessive loss of precision, they can be applied to multi-species groups such as the *Aeromonas* spp. (CLSI, 2010). The qualitative and quantitative data set requirements developed in this work were used to make an evidence-based evaluation of this issue.

2. Materials and methods

2.1. Data sets

Three sets of inhibition zone data were analysed in this work.

2.1.1. Aeromonas spp. data sets

The detail of the sources, isolation and identification methods for 129 bacteria, classified as mesophilic *Aeromonas* spp., have been described by Verner-Jeffreys et al. (2009). The protocol used by these workers to perform the disc diffusion assays was essentially that described in the CLSI guideline M42-A (CLSI, 2006). However, the disc contents used were not, in all cases, those recommended by the guideline. These discs and their contents used by them are described in Table 1. The disc contents recommended by the CLSI are also shown in this Table. The assays were performed at 22 ± 2 °C and *Escherichia coli* ATCC 25922 was used as the control strain.

2.1.2. Human pathogen data sets

Data on distribution of agent-specific zone sizes for bacterial species relevant to human medicine were obtained from the EUCAST web site http://www.eucast.org/mic_distributions/. The twenty-two data sets selected from this site were all sets from which EUCAST had set ECO.

2.1.3. Aquatic bacteria data sets

Data on zone size distribution for aquatic bacteria were obtained from Miller and Reimschuessel (2006), Smith et al. (2007) and Avendaño-Herrera et al. (2011). These authors all applied the protocols recommended in the guideline M42-A (CLSI, 2006) and performed their disc diffusion tests at 22 °C.

Table 1
Discs used by Verner-Jeffreys et al. (2009).

Class	Agent (content)	Symbol	CLSI (2006) recommendation
Aminoglycosides	Streptomycin (10 μg)	STP_{10}	–
	Gentamicin (10 μg)	GEN_{10}	10
Cephalosporin	Moxalactam (30 μg)	MOX_{30}	–
Foliate pathway inhibitor	Trimethoprim/sulfamethoxazole (1:19) (25 μg)	SXT_{25}	25
Macrolide	Erythromycin (10 μg)	ERY_{10}	15
Nitrofuran	Furazolidone (20 μg)	FUR_{20}	–
Phenicols	Chloramphenicol (25 μg)	CAM_{25}	30
	florfenicol (30 μg)	FLO_{30}	30
Penicillin	Amoxicillin (10 μg)	AMX_{10}	–
Quinolones	Ciprofloxacin (5 μg)	CIP_5	–
	Enrofloxacin (5 μg)	ENO_5	5
	Flumequine (4 μg)	FLU_4	–
	Ofloxacin (5 μg)	OFL_5	–
	Oxolinic acid (4 μg)	OXA_4	2
Tetracyclines	Tetracycline (30 μg)	TET_{30}	30
	Oxytetracycline (20 μg)	OTC_{20}	30

2.2. Data analysis

The various data sets were analysed using the NRI method developed by Kronvall (2003). This method involved plotting a frequency histogram of the zone size data. Outliers in the data sets were eliminated (Smith et al., 2009) and 5 point rolling mean analysis was used to identify the 'peak value' in the high zone end of the distribution of this data. In NRI analysis, this 'peak value' is assumed to be the modal value of the zones obtained from WT strains. To avoid interference by NWT strains manifesting low-level resistance (LLR), the normalised distribution of the WT strains, defined by this 'peak value', were generated using only the high-zone half of the data. Cut-off values were set at the mean minus 2.5 times the standard deviation (SD) of the calculated normalised distributions (Smith et al., 2007).

Statistical analyses were performed using InStat 3.1a (GraphPad Software Inc.).

3. Results and discussion

3.1. Quantitative and qualitative data set requirements

A central step in performing NRI analysis is the generation of a normalised distribution of putative WT zones. As WT strains are assumed to be homogeneous with respect to their susceptibility, the SD of these normalised distributions should reflect the random experimental error in determining the WT zone sizes. Excessive SD values for these distributions would indicate either an unacceptable imprecision in the determinations of zone sizes or that the putative WT strain set was not in fact homogeneous. Lack of homogeneity might arise from an excessive taxonomic diversity in the strain set. This is particularly possible when strain sets include members of more than one species. Lack of homogeneity may also be manifest in situations where significant numbers of NWT strains manifesting LLR and, therefore, only a slight decrease in zone size, are present in the strain set. In such a situation it may prove difficult or impossible to identify the zone sizes of a group of WT strains that is uncontaminated with NWT strains.

3.1.1. Standard deviations of normalised distributions calculated by NRI analysis of previously published data

The arguments above suggest that CO_{WT} calculated from a data set that during NRI analysis yielded a normalised distribution with a SD larger than would be expected from experimental error should be treated with a degree of suspicion, or should be rejected. The published literature was consulted to provide an estimate of the SD