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Short communication

Setting epidemiological cut-off values for *Aeromonas salmonicida* disc diffusion data capable of discriminating between strains on the basis of their possession of *sul1* genes

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ABSTRACT

This study evaluated the ability of the disc diffusion protocols and epidemiological cut-off values published by the Clinical and Laboratory Standards Institute (CLSI) to detect *sul1* containing strains of *Aeromonas salmonicida*. Molecular analysis of sixteen strains demonstrated the presence of *sul1* in seven of them. Two sets of disc diffusion data for these sixteen strains, produced in two studies preformed five years, apart, were used in the evaluation. Data sets produced using a disc containing both sulfamethoxazole and trimethoprim, as recommended by CLSI, were analysed using the recommended epidemiological cut-off value. When this cut-off value was applied to the data generated in the first study, fifteen of the sixteen strains were categorised as wild-type. When it was applied to the data generated in the second study, all sixteen strains were categorised as wild-type.

When the strain susceptibilities were investigated using discs that contained 100 μg sulfamethoxazole only, strains that were *sul1* negative manifested zones ≥18 mm in one study and ≥24 mm in the other. None of the *sul1* containing strains manifested any zones of inhibition in either study. A provisional epidemiological cut-off value (≥9 mm) has been suggested for sulfamethoxazole disc data generated under the test conditions specified by CLSI. Evidence is presented that it is highly likely that application of this cut-off value to such data would lead to the categorisation of all *sul1* negative strains as wild type and all *sul1* containing strains as non-wild type.

It is argued that, in order to maximise their ability to detect the presence of *sul1* containing strains, standardised monitoring and surveillance programmes should adopt protocols that specify the use of discs containing only sulfamethoxazole.

1. Introduction

The new edition of the Aquatic Animal Code issued by the World Organisation for Animal Health (OIE) recommends that relevant competent authorities should initiate surveillance and monitoring programmes of antimicrobial agent resistance in bacteria isolated from aquatic animals (OIE, 2012). Trans-national comparisons of the data collected in these programmes will be possible only if there is international harmonisation of the standardised test protocols that are employed by various authorities to produce quantitative data on *in-vitro* susceptibility. The standardised susceptibility test protocols published in the Clinical and Laboratory Standards Institute (CLSI) guidelines M42-A (CLSI, 2006a) and M49-A (CLSI, 2006b) are much more developed than any other possible alternatives (Smith, 2012). It, therefore, seems obvious that the requisite harmony will be most efficiently achieved if there is agreement that these protocols should be employed in all surveillance and monitoring of the susceptibility of bacteria isolated from aquatic animals.

There also needs to be agreement with respect to the criteria to be used to interpret the meaning of the *in-vitro* data generated by these protocols. As the aim of monitoring and surveillance programmes is to gain information on the prevalence and emergence of antimicrobial resistant microorganisms and antimicrobial resistance determinants, Silley et al. (2011) and OIE (2012) have argued that epidemiological cut-off values (ECVs) represent the most appropriate type of interpretive criteria for use in these programmes. Application of ECVs to *in-vitro* measures of susceptibility allows the categorisation of strains as either wild-type (WT) or non wild-type (NWT) members of a species. According to the European Committee on Antimicrobial Susceptibility Testing, a microorganism is defined as WT for a species by the absence of acquired and mutational resistance mechanisms to the drug in question (EUCAST, 2000).

Although sulfamethoxazole is normally administered to aquatic animals in a synergistic combination with trimethoprim (Smith et al.,

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2008), bacteria acquire resistance to these two agents independently (Huovinen et al., 1995; Sköld, 2001). Three genes, *sul1*, *sul11* and *sul111* have been reported as conferring resistance to sulfonamides (Grape et al., 2005) and all three have been detected in the aquaculture environment (Gao et al., 2012). In a study of *Aeromonas salmonicida* from diverse geographical regions, *sul1* was shown to be present in all sulfonamide resistant isolates tested (Schmidt et al., 2001) and Kadlec et al. (2011) reported the presence of this gene in all sulfonamide resistant Aeromonads isolated from German sources. However, L'Abée-Lund and Sørum (2001) reported that although *sul1* was present in all European isolates of *A. salmonicida* they tested, *sul11* was present in some Japanese isolates. These data suggest that a minimum requirement of the susceptibility test conditions and interpretive criteria to be used in phenotype-based monitoring or surveillance programmes of bacteria isolated from aquatic animals should be that strains possessing the *sul1* gene could be detected and placed in a different category than those that did not.

In disc diffusion investigations of bacterial susceptibility to sulfamethoxazole/trimethoprim CLSI (2006a) recommends the use of a single disc (SXT_{25}) containing a combination of 1.25 μg trimethoprim and 23.75 μg sulfamethoxazole (CLSI, 2006a). It has also published an ECV (≥20 mm) that is recommended for the interpretation of diffusion zone data obtained with *A. salmonicida* using this combined disc (CLSI, 2010). This ECV of ≥20 mm was based on data obtained with SXT_{25} discs by Douglas et al (2007). However, these authors also investigated the distributions of zone sizes when discs containing single agents, either 100 μg sulfamethoxazole (SFM_{100}) or 5 μg trimethoprim (TMP_5), were used. They demonstrated that the categorisation of their strains using the SXT_{25} disc data and the ECV of ≥20 mm lacked the sensitivity needed to detect strains manifesting reductions in phenotypic susceptibility to the sulfamethoxazole component of the synergistic mixture. Using the single drug discs, SFM_{100} and TMP_5, they detected 21 strains that manifested a sulfamethoxazole NWT and trimethoprim WT phenotype. When these 21 strains were examined using the SXT_{25} discs containing both drugs and the data was interpreted using the ECV of ≥20 mm recommended by CLSI (2010) only 4 were categorised as NWT and 17 (81%) were classified as WT.

This work was undertaken to investigate the role of *sul1* genes in determining the phenotypes of the strains that were categorised by Douglas et al. (2007) as WT when the ECV of ≥20 mm was applied to the zones that manifested with SXT_{25} discs but NWT when the zones manifested with SFM_{100} were examined. The aim was to generate a data set that would allow the evaluation of the suitability of the disc diffusion protocols and interpretive criteria recommended by CLSI (2006a; 2010) for adoption in programmes for monitoring and surveillance of antibiotic resistance.

2. Materials and methods

2.1. Bacterial strains

A. salmonicida NCIMB 1102 (ATCC 33658), used as a control strain in this work, was obtained from NCIMB (Aberdeen, UK). The other sixteen strains of *A. salmonicida* analysed in this work were members of the strain set used and described by Douglas et al. (2007). The 16 strains were selected on the basis that, using discs containing 5 μg of that agent (TMP_5), they had been categorised as WT with respect to trimethoprim by Douglas et al. (2007). They were also selected to represent the two major classes (WT and NWT) identified by the application of disc diffusion assays using discs containing 100 μg sulfamethoxazole (SFM_{100}). Group A comprised seven strains that were categorised as NWT with respect to sulfamethoxazole by Douglas et al. (2007). Group B comprised 9 strains categorised as WT with respect to this agent (Table 1).

Table 1
Detection of *sul1* and inhibition zones (mm) recorded for strains of *A. salmonicida*.

Strain[a]	SFM (100 μg)[b]		SXT (25 μg)[c]		*sul1*
	This work	Douglas et al. (2007)	This work	Douglas et al. (2007)	
Group A					
MT 1123	<6	<6	31	17	+
MT 1712	<6	<6	29	21	+
MT 684	<6	<6	32	21	+
MT 1114	<6	<6	31	22	+
MT 545	<6	<6	26	23	+
FR 2705/03	<6	<6	34	25	+
FR 2147/04	<6	<6	38	25	+
Mean (sd)			31.6 (3.8)	22.0 (2.8)	
Group B					
MT 1413	24	18	37	28	−
MT 809	26	20	39	28	−
FR 2294/02	26	20	43	35	−
MT 1020	27	22	43	32	−
N 1528/91	30	16	42	29	−
MT 1163	30	16	44	31	−
FR 2436/05	31	19	42	35	−
N 3719/90	32	18	39	29	−
MT 783	32	15	44	31	−
Mean (sd)	28.7 (2.9)	18.2 (2.3)	41.4 (2.5)	30.9 (2.7)	
Control					
NCIMB 1102	19	13	36	26-28	−

[a] Strains with the prefix MT were isolated from Scotland and the FR and N strains were isolated from France and Norway respectively.
[b] Zone recorded using discs containing 100 μg sulfamethoxazole.
[c] Zone recorded using discs containing 1.25 μg trimethoprim and 23.75 μg sulfamethoxazole.

2.2. Antibiotic susceptibility testing

The antibiotic susceptibilities of strains were determined using the disc diffusion protocol described in the guideline M42-A (CLSI, 2006a) using discs containing both 1.25 μg trimethoprim and 23.75 μg sulfamethoxazole (SXT_{25}). The same test conditions were used in assays using discs containing 100 μg sulfamethoxazole (SFM_{100}). Discs were obtained from Oxoid (Basingstoke, UK). Assays were performed at 22 ± 2 °C and read after 44–48 h. The strain *A. salmonicida* NCIMB 1102 was used as the quality control strain.

2.3. Detection of sul1 genes

To prepare DNA template for conventional PCR, colonies of approximately 2 mm in diameter were picked with a sterilized toothpick and directly transferred to a 1.5 ml microfuge tube containing 50 μl of sterile nuclease free water and mixed thoroughly. 1 μl of this suspension was added to the PCR tube as DNA template.

Conventional PCR was performed on the 16 strains of *A. salmonicida* and the control strain NCIMB 1102, using primers targeting a 437 bp region of the *sul1* gene; Sul1F 5′-CTTCGATGAGAGCCGGCGGC-3′ and Sul1R 5′-GCAAGGCGGAAACCCGCGCC-3′ (L'Abée-Lund and Sørum, 2001; Sundström et al., 1988) on the iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., CA, USA). All reactions were carried out in 0.2 ml PCR tubes, containing 2.5 μl 10X buffer (15 mM $MgCl_2$), forward and reverse primers (0.2 μM final conc.), 0.5 μl dNTP mix (10 mM: deoxynucleoside triphosphate set, Roche Diagnostics, Basel, Switzerland), 5% dimethyl sulfoxide (DMSO), 0.5 U Taq DNA polymerase (Roche Diagnostics), 1 μl of DNA template and the final volume adjusted to 25 μl with the addition of nuclease free water (Applied Biosystems, CA, USA).

The thermal cycling parameters consisted of an initial denaturation step at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C (30 s), amplification at 50 °C (30 s), and extension at 72 °C (30 s), followed by a final elongation at 72 °C for 7 min.