Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from the United Kingdom

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Abstract

Routine application of antimicrobials is the current treatment of choice for rainbow trout fry syndrome (RTFS) or bacterial coldwater disease (BCWD) caused by *Flavobacterium psychrophilum*. In this study, the antimicrobial susceptibilities of 133 *F. psychrophilum* isolates, 118 of which were from the UK, were evaluated by broth microdilution and disc diffusion methods following VET04-A2 and VET03-A guidelines of Clinical and Laboratory Standards Institute (CLSI), respectively. Isolates were categorised as wild type (fully susceptible, WT) or non-wild type (NWT) using normalised resistance interpretation (NRI) determined cut-off values (CO_{WT}). Broth microdilution testing showed that only 12% of UK isolates were WT to oxolinic acid (MIC CO_{WT} ≤0.25 mg L^{-1}) and 42% were WT for oxytetracycline (MIC CO_{WT} ≤0.25 mg L^{-1}). In contrast, all the isolates tested were WT (MIC CO_{WT} ≤2 mg L^{-1}) for florfenicol, the main antimicrobial for RTFS control in the UK. Disc diffusion-based CO_{WT} values were ≥51 mm for 10 µg amoxicillin, ≥44 mm for 30 µg florfenicol, ≥30 mm for 2 µg oxolinic acid and ≥51 mm for 30 µg oxytetracycline. There was a high categorical agreement between the classifications of the isolates by two testing methods for florfenicol (100%), oxytetracycline (93%), and oxolinic acid (99%).

Keywords: *Flavobacterium psychrophilum*, antimicrobial susceptibility, epidemiological cut-off values, disc diffusion, broth microdilution, rainbow trout fry syndrome.

Introduction

Flavobacterium psychrophilum, a Gram-negative, filamentous, psychrotrophic bacterium, is the aetiological agent of rainbow trout fry syndrome (RTFS) and bacterial coldwater disease (BCWD), which was first described in USA in 1946 (Borg 1948). *F. psychrophilum* infection has been found throughout North America, Europe and elsewhere in Turkey, Australia, Peru, Japan and Korea (Barnes & Brown 2011). A commercial vaccine against RTFS/BCWD is still not available (Gómez *et al.* 2014). Alhtough phage therapy (Stenholm *et al.* 2008; Kim *et al.* 2010a; Castillo *et al.* 2012) and the use of probiotic bacteria (StrömBesto & Wiklund 2011; Korkea-aho *et al.* 2011; Boutin *et al.* 2012; Burbank *et al.* 2012) have been suggested to be a promising alternative to the use of antibiotics in aquaculture, further studies are needed to prove the consistent effect of these green/blue technologies on preventing the infection of *F. psychrophilum*. Therefore, the use of antibiotics is currently the treatment of choice for controlling RTFS/BCWD outbreaks, resulting in a concern about the development of antimicrobial resistance by *F. psychrophilum* (Gómez *et al.* 2014). In the UK, three antibiotics (florfenicol, oxytetracycline and amoxicillin) are licensed for use in aquaculture by the UK Veterinary Medicines Directorate (VMD) (http://www.vmd.defra.gov.uk/).

Several studies have examined the antimicrobial susceptibility of *F. psychrophilum* isolated from the USA (Pacha 1968, Soule *et al.* 2005; Van Vliet *et al.* 2017), the UK (Rangdale *et al.* 1997; Verner-Jeffreys & Taylor 2015; Smith *et al.* 2016), Denmark (Lorenzen *et al.* 1997; Bruun *et al.* 2000; Dalsgaard & Madsen 2000; Schmidt *et al.*

2000; Bruun *et al.* 2003; Smith *et al.* 2016), France (Michel *et al.* 2003), Japan (Izumi & Aranishi 2004), Turkey (Kum *et al.* 2008; Durmaz *et al.* 2012; Boyacioğlu & Akar 2012; Boyacioğlu *et al.* 2015), Canada (Hesami *et al.* 2010), Spain (Del Cerro *et al.* 2010), Norway (Nilsen *et al.* 2011), Chile (Henríquez-Núñez *et al.* 2012; Miranda *et al.* 2016) and Finland (Sundell *et al.* 2013). However, differences in the medium and growth conditions used in these studies and variations in the interpretive criteria used make comparisons difficult. In addition, some of these studies included only a small number of isolates, while others produced susceptibility data that was too diverse to allow any estimate of cut-off values to interpret their meaning.

Smith *et al.* (2013) addressed the need for standardised and internationally recognized protocols for laboratory *in vitro* susceptibility testing in monitoring and surveillance programmes and the use of standardised methods to calculate epidemiological cut-off values for interpretation of the meaning of the data collected in such surveys.

The aim of the present study was to evaluate the antimicrobial susceptibility of 140 *F. psychrophilum* isolates, 125 of which were obtained within the UK, by the disc diffusion and standardised broth microdilution methods following VET03-A (CLSI 2006) and VET04-A2 (CLSI 2014a) guidelines respectively, as recommended by the Clinical and Laboratory Standards Institute (CLSI) for aquatic bacteria with an optimal growth temperature below 35ºC.

Materials and Methods

Bacterial isolates and growth conditions

A total of 140 *F. psychrophilum* isolates, previously described by Ngo *et al.* (2017) for genetic and serological diversity, were examined in this study. This collection comprised 125 isolates obtained within the UK during 2005-2015 and 15 isolates from other countries (France, Denmark, Finland, Ireland, Chile and the USA) (Table 1 and 2); 123 *F. psychrophilum* isolates were obtained from rainbow trout (*Oncorhynchus mykiss*), 16 from Atlantic salmon (*Salmo salar*) and one from coho salmon (*O. kisutch*). *F. psychrophilum* type strain NCIMB 1947^T (ATCC 49418^T) was included for comparative purposes. For all the experiments, the *F. psychrophilum* isolates were grown in Modified Veggietone (MV) medium [veggitones GMO-free soya peptone (Oxoid, UK), 5 g L^{-1}; yeast extract (Oxoid, UK), 0.5 g L^{-1}; magnesium sulphate heptahydrate (Fisher chemicals, UK), 0.5 g L^{-1}; anhydrous calcium chloride (BHD), 0.2 g L^{-1}; dextrose (Oxoid, UK), 2 g L^{-1}; agar (solid medium; Oxoid, UK), 15 g L^{-1}; pH 7.3] at 18^{o}C for 72 – 96 h. Broth cultures were shaken at 140 rpm. Stock cultures were maintained at -70^{o}C in tryptone–yeast extract–salts medium supplemented with glucose [FLP – tryptone (Oxoid, UK), 4.0 g L^{-1}; yeast extract, 0.4 g L^{-1}; anhydrous calcium chloride, 0.2 g L^{-1}; magnesium sulphate heptahydrate, 0.5 g L^{-1}; D(+)-glucose (Sigma, UK), 0.5 g L^{-1}; Cepeda *et al*. 2004] with 10% glycerol and on Protect-Multi-purpose cryobeads (Technical Service Consultants Ltd, UK).

The 125 isolates from 27 sites within the UK in this study had been isolated between 2005 and 2015 with the majority (110 strains, 88%) being retrieved between 2011 and 2013. Among these isolates, 51 genotypes and 7 plasmid profiles were detected

(Ngo *et al.* 2017) (Table 1). However, within this set of 125 isolates, there were five groups of two or three isolates that were recorded as having the same site, sampling time point, genetic profiles and susceptibilities. In order to avoid the over-representation, 7 potential replicates were eliminated from the analysis and only 118 UK isolates were included in the analyses. The epidemiological cut-off values were calculated from the data obtained from 118 UK isolates and 15 isolates from other countries. The frequencies of NWT phenotypes circulating the UK during 2005 – 2015 were estimated from the analysis of 118 isolates.

Minimum inhibitory concentration (MIC) testing

The MICs for *F. psychrophilum* isolates were performed using Sensititre CMP1MSP plates (Trek Diagnostic Systems; ThermoScientific.com/microbiology). These test plates were 96-well, dry-form plates that contained twofold serial dilutions of the following antimicrobial agents: ampicillin (AMP) 0.03–16 mg L^{-1}, amoxicillin (AMOX) 0.25–16 mg L^{-1}, erythromycin (ERY) 0.25–128 mg L^{-1}, enrofloxacin (ENRO) 0.002–1 mg L^{-1}, florfenicol (FFN) 0.03–16 mg L^{-1}, flumequine (FLUQ) 0.008–4 mg L^{-1}, ormetoprim/sulphadimethoxine (PRI) 0.008/0.15–4/76 mg L^{-1}, oxolinic acid (OXO) 0.004–2 mg L^{-1}, oxytetracycline (OTC) 0.015–8 mg L^{-1} and trimethoprim/sulphamethoxazole (SXT) 0.015/0.3–1/19 mg L^{-1}.

The MIC assays were determined using the broth microdilution protocol recommended for *F. psychrophilum* in the CLSI guideline VET04-A2 (CLSI 2014a).

Colony counts on inoculum suspensions were performed to ensure that the final inoculum density was close to 5.0×10^5 colony-forming units (CFU) per millilitre.

Disc diffusion testing

Disc diffusion susceptibility of the *F. psychrophilum* strains was determined by the protocol suggested in the guideline VET03-A (CLSI 2006) with modification on the agar percentage of the culture medium. It should be noted, however, that this protocol has not been formally accepted as a standard by CLSI. The test was performed on diluted Mueller-Hinton medium (Sigma-Aldrich, UK; 3 g L^{-1}) containing 1.5% agar (Agar No. 1, LP0011, Oxoid, UK) (MHA) and 5% foetal calf serum (FCS; Gibco, Fisher chemicals, UK) and plates were incubated at 15°C for 68 – 72 h. Antimicrobial agent discs (Oxoid, Basingstoke, UK) containing 10 µg amoxicillin ($AMOX_{10}$), 5 µg enrofloxacin ($ENRO_5$), 30 µg florfenicol (FFN_{30}), 2 µg oxolinic acid (OXO_2), 30 µg oxytetracycline (OTC_{30}) and 25 µg trimethoprim/sulphamethoxazole (SXT_{25}) were employed.

Quality control

As specified in VET04-A2 (CLSI 2014a) the quality control strain *Escherichia coli* NCIMB 12210 (ATCC® 25922) was included in every MIC test run and was assayed on diluted CAMHB at 18°C as described above. However, no quality control ranges have been established for any disc diffusion protocol specifying these incubation conditions

144 (CLSI 2006). Therefore, the *F. psychrophilum* type strain NCIMB 1947^T was also
145 included in every test run to monitor the performance of the method.
146
147 **Statistical analysis**
148 The antimicrobial susceptibility patterns of 133 *F. psychrophilum* isolates used in this
149 study were analysed by application of protocol and species-specific epidemiological cut-
150 off values (CO_{WT}). These values allow isolates to be categorised as fully susceptible
151 (wild type, WT) or manifesting reduced susceptibility (non-wild type, NWT). In this
152 work, CO_{WT} values were calculated for both the MIC and disc diffusion data by the
153 normalised resistance interpretation (NRI) method (Kronvall 2003; 2010). This NRI
154 method was used with permission from the patent holder, Bioscand AB, TÄY, Sweden
155 (European patent No 1383913, US patent No 7,465,559).
156 MIC distributions were analysed using the NRI method of Kronvall (2010). A fully
157 automatic Excel spreadsheet for performing these NRI analyses is available on-line
158 (http://www.bioscand.se/nri/). In data sets where a small percentage (<5 %) of the WT
159 observations were "below-scale", these observations were treated as having the MIC
160 value immediately below the limit of the plate quantitation. When the percentage of the
161 WT observations "below-scale" was >5%, the data set was considered as unsuitable for
162 NRI analysis (Smith *et al.* 2016).
163 The NRI analyses for zone histograms were performed using a modification of the
164 standardised protocol developed by Kronvall & Smith (2016). In this modification, the

peak values of the zone sizes for the putative WT isolates were established using 8-point rather than 4-point rolling means.

Terminology

The acronyms ECV and ECOFF have been used by the CLSI and European Committee on antimicrobial susceptibility testing (EUCAST) respectively for epidemiological cut-off values set from data generated in multiple laboratories. In the present study, the term CO_{WT}, as previously employed by Smith *et al.* (2016), was used to indicate epidemiological cut-off values that have not been set by either of these international agencies. It has been suggested that the terms resistant and sensitive should not be used to refer to the categories identified by epidemiological cut-off values (Silley 2012). Following this suggestion, when isolates are categorised by epidemiological cut-off values, the terms wild type (WT) and non-wild type (NWT) should be used for fully susceptible isolates and isolates exhibiting reduced susceptibility respectively.

Results

Quality control

The MIC values obtained with the quality control reference strain *E. coli* NCIMB 12210, grown at 18^{o}C for 72-96 h in diluted CAMHB, were within the acceptable range published by CLSI in VET03/04-S2 guideline (CLSI 2014b). All the inoculum suspensions used in MIC tests were confirmed to have the density ranging from 4.8 x 10^5 to 5.3 x 10^5 CFU mL^{-1} by colony counts.

F. psychrophilum type strain NCIMB 1947^T was included in all disc diffusion tests and the inhibition zone data of this strain were 56 – 72 mm for $AMOX_{10}$, 60 – 75 mm for $ENRO_5$, 57 – 64 mm for FFN_{30}, 64 – 86 mm for OTC_{30}, 45 – 56 mm for OXO_2 and 16 – 44 mm for SXT_{25}. The mean of the ranges of these zone sizes for these six agents against the *F. psychrophilum* type strain was 16.5 ± 7.6 mm.

NRI analysis of susceptibility data

The distribution of MIC values of 133 *F. psychrophilum* isolates for ten antimicrobial agents is shown in Table 3 and 4. MIC-based CO_{WT} values of antimicrobial agents are presented in Table 5. The distribution of disc diffusion zones of the isolates for six antimicrobials is presented in Figure 1 and the zone data-based CO_{WT} values of antimicrobial agents are shown in Table 6.

Oxytetracycline

MIC data for OTC showed a clear bimodal distribution (Table 3). The modal group with lower MICs was assumed to represent the WT group. NRI analysis calculated the standard deviation of the log_2 normalised WT distribution as 0.68 and a CO_{WT} value of $\leq$0.25 mg L^{-1} (Table 5). Applying this cut-off, fifty-six (42%) of the 133 isolates analysed were categorised as WT.

The disc diffusion zone sizes for OTC_{30} showed considerable diversity at the high zone end (Figure 1A). However, NRI analysis of these data identified a high zone modal group with a standard deviation of 7.44 mm. If this modal group was assumed to

represent zones obtained from WT isolates, a provisional CO_{WT} value of ≥51 mm could be calculated (Table 6). Applying this cut-off, sixty-five (49%) of the 133 isolates analysed were categorised as WT.

The categorisation of isolates resulting from applying the cut-off of ≤0.25 mg L^{-1} to the MIC data agreed with the categorisation resulting from applying the disc zone cut-off of ≥51 mm to the zone data for 93% of the 133 isolates studied (Figure 2A).

Amoxicillin and ampicillin

For AMOX, 98 observations (100% of the lower MIC modal observations) and for AMP, 24 observations (24% of the lower MIC modal observations) were recorded as "below-scale" (Table 3). On this basis, neither of these data sets was considered suitable for NRI analysis.

As in MIC data set for AMP there was a clear separation of the low MIC and high MIC modal groups, this data set was considered suitable for estimating CO_{WT} by visual examination. The estimated value generated by this subjective method was ≤0.125 mg L^{-1} for AMP. A scatterplot of the paired MIC data for these two beta-lactam agents (Figure 3A) suggested a high correlation between them and also demonstrated that AMOX might have the same distribution as AMP.

The disc diffusion zone sizes for $AMOX_{10}$ were also bimodal (Figure 1B). NRI analysis of these data calculated a standard deviation of the normalised WT distribution of 5.2 mm and a CO_{WT} value of ≥56 mm (Table 6). A scatterplot of the paired MIC

values vernus inhibition zone sizes for amoxicillin suggested a high correlation between them (Figure 2B).

Florfenicol

MIC data for FFN showed a clear unimodal distribution (Table 3). This modal group was assumed to represent the WT isolates. NRI analysis calculated a standard deviation of the log_2 normalised WT distribution of 0.68 and a CO_{WT} value of $\leq$2 mg L^{-1} (Table 5).

The disc diffusion zone sizes for FFN_{30} were also unimodal (Figure 1C). NRI analysis of these data calculated a standard deviation of the normalised WT distribution of 5.6 mm and a CO_{WT} value of $\geq$45 mm (Table 6).

Applying the cut-off of $\leq$2 mg L^{-1} to the MIC data and the disc zone cut-off of $\geq$41 mm to the zone data categorised 100% of the 133 isolates studied as WT (Figure 2C).

Oxolinic acid, Flumequine and Enrofloxacin

The MIC values of OXO, FLUQ and ENRO were bimodally distributed (Table 3). NRI analysis calculated the standard deviation of the log_2 normalised WT distribution as 0.67, 0.57 and 0.74 for OXO, FLUQ and ENRO respectively. The MIC CO_{WT} values calculated from these data were $\leq$0.25 mg L^{-1} for OXO, $\leq$0.125 mg L^{-1} for FLUQ and $\leq$0.032 mg L^{-1} for ENRO (Table 5). When these CO_{WT} values were applied, 21 (16%), 20 (15%) and 20 (15%) of the 133 isolates were categorised as WT with respect to OXO, FLUQ and ENRO respectively.

Scatterplots of the MIC data for OXO against those for FLUQ and ENRO (Figure 3B and 3C) demonstrated a high (>97.7%) categorical agreement in both cases. This suggests that it would be safe to accept MIC data for OXO as a predictor of reduced susceptibility to the FLUQ and ENRO (Smith *et al.* 2016). Adoption of this proposal would reduce the cost of routine susceptibility testing.

The disc diffusion zone sizes for OXO_2 were bimodal (Figure 1D). NRI analysis of these data calculated a standard deviation of the normalised WT distribution of 8.5 mm. This high standard deviation is probably a result of the fact that high zone modal group was diverse and composed of only a few observations. This suggests that the disc CO_{WT} value calculated by NRI analysis of ≥30 mm (Table 6) should only be treated as a provisional value. Applying the cut-off of ≤0.25 mg L^{-1} to the MIC data for OXO and the disc zone cut-off of ≥30 mm to the zone data resulted in 99% agreement in the categorisation of the 133 isolates studied (Figure 2D).

The disc diffusion zone sizes for FLUQ were not determined and those for ENRO did not show any visually obvious high zone modal group and were not subject to NRI analysis (Figure 1E).

Erythromycin

MIC values of ERY had a unimodal distribution. NRI analysis calculated a standard deviation of the log_2 normalised WT distribution of 0.98 and the CO_{WT} value was calculated as ≤8 mg L^{-1} (Table 3 and 5). This value determined that all 133 *F. psychrophilum* isolates analysed were WT for ERY.

Ormetoprim/Sulphadimethoxine and Trimethoprim/Sulphamethoxazole

The distributions of the MIC values for these two potentiated sulfonamide agents were diverse but appeared to be unimodal (Table 3). NRI analysis generated provisional CO_{WT} values for PRI and SXT of ≤320 mg L^{-1} and ≤160 mg L^{-1}, respectively. However, the standard deviations calculated for the normalized distribution of these putative WT observations, 1.39 log_2 mg L^{-1} and 1.67 log_2 mg L^{-1} for PRI and SXT respectively, were higher than those recorded for all other agents in this work (Table 5). Therefore, the validity of these CO_{WT} values was questionable.

The disc diffusion zone sizes for SXT did not show any visually obvious high zone modal group and were not subject to NRI analysis (Figure 1F).

Discussion

Data precision

Precision of MIC data sets

The precision of any CO_{WT} value is a function of the precision of the observational data used to calculate it. Smith *et al.* (2012) demonstrated that the standard deviations of the normalised distributions of the log_2 WT observation calculated by the NRI analysis could provide a proxy measurement of precision. The median of the standard deviation calculated for 22 *F. psychrophilum* data sets published by Michel *et al.* (2003), Smith *et al.* (2016) and Van Vliet *et al.* (2017) was 0.70 log_2 mg L^{-1}. In this work, the median value of standard deviations calculated for ENRO, ERY, FFN, FLUQ, OTC and OXO

(Table 5) was 0.72 $\log_2$ mg L^{-1}. This suggests that the MIC data sets obtained in this work for these agents were of an acceptable level of precision and were of sufficient quality that they could be used to calculate CO_{WT} values.

The standard deviations calculated for potentiated sulphonamide MIC data**,** 1.39 $\log_2$ mg L^{-1} and 1.61 $\log_2$ mg L^{-1} for PRI and SXT respectively in this work, were, however, considerably larger and were taken to indicate significant imprecision. Smith *et al.* (2016) and Van Vliet *et al.* (2017), who used the same testing protocol as was used in this work also reported very low precision in the MIC data they obtained for these agents (mean 1.43 $\log_2$ mg L^{-1}). Due to their low precision, it was considered that valid CO_{WT} could not be established for PRI and SXT data obtained in this work.

Precision of disc diffusion data sets

Smith & Kronvall (2015) analysed zone data for reference control strains *E. coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658 and demonstrated a reduction in precision as the incubation temperature decreased and time increased. Analysis of the data obtained from the reference strain *F. psychrophilum* NCIMB 1947^T and from the test isolates in this work suggest a similar effect of temperature and time on precision of zone size data.

The mean of the ranges of zone sizes for the control reference strain *E. coli* NCIMB 12210 provided in the guideline VET03-A (CLSI 2006) for tests performed at 35°C, 28°C and 22°C were 7.7 ± 0.8 mm, 8.0 ± 1.5 mm and 11.8 ± 2.0 mm respectively (Smith

316 and Kronvall 2015). In this work, the mean range obtained at 15ºC for six agents against
317 the control strain *F. psychrophilum* NCIMB 1947^{T} was 16.5 mm ± 7.6 mm

318 The mean of standard deviations of the 19 zone data sets obtained at 28ºC in studies
319 of *Edwardsiella tarda* and *Vibrio harveyi* was 2.53 mm (Lim *et al.* 2016). For 13 data
320 sets of *A. salmonicida* obtained at 22ºC, the mean was 3.9 mm (Miller & Reimschuessel
321 2006; Smith *et al.* 2007). In this work, the disc diffusion assays were performed at 15ºC
322 and the mean standard deviation of the normalised distributions of the four disc data sets
323 was 6.7 mm.

324 These comparisons suggest that the low precision of the zone data sets obtained in
325 this work was most probably a function of the inherent property of this type of assay
326 rather than any laboratory specific errors in the performance of the assays. However, the
327 low level of precision suggests that any CO_{WT} calculated from these zone data should be
328 treated as only provisional estimates.

329

330 **Categorical agreements**

331 With the calculated MIC CO_{WT} and provisional disc diffusion-based CO_{WT} of FFN, OXO
332 and OTC, it is possible to calculate the percentage agreement between the categorisation
333 of the 133 isolates obtained by analysing the observed MIC measures and the zone size
334 data. The values of these categorical agreements were 100% for FFN, 99% for OXO and
335 93% for OTC. This high level of categorical agreement raise the possibility that, although
336 the disc diffusion protocol used in this work generated data of low precision, the

provisional CO_{WT} calculated from them may have some value in detecting isolates of reduced susceptibility.

It should, however be noted that Smith & Kronvall (2015) demonstrated that reduced temperatures and prolonged incubation time increased not only the level of intra-laboratory variation but also the level of inter-laboratory variation in the data generated. High inter-laboratory variation of the data will have the consequence that although any provisional disc CO_{WT} calculated in one laboratory may have some value in interpreting zone data produced in that laboratory, it may be misleading if applied to zone data obtained in another laboratory. In other words, the CO_{WT} values for MIC data calculated in this work are probably laboratory-independent and of general or 'universal' applicability. However, it is probably safer to treat the CO_{WT} values for inhibition zone data generated in this work as only of local applicability. As a consequence, each laboratory using this protocol to perform disc diffusion assays would have to generate their own CO_{WT} values. The disc diffusion test protocol used in this work has not been accepted as a standard by CLSI. It is possible that further optimisation such as using a higher incubation temperature (18^{o}C) may lead to a protocol with increased precision.

Comparison of CO_{WT} values calculated for MIC measures determined by standardised broth microdilution protocols of CLSI for *F. psychrophilum*

The values for any CO_{WT} are protocol-specific. It is, therefore, legitimate to compare the CO_{WT} calculated in this work with those published by Smith *et al.* (2016) and Van Vliet *et al.* (2017), who also used the standardised broth microdilution protocol (CLSI 2014a)

and NRI method to calculate CO_{WT} for *F. psychrophilum* from MIC data. This comparison can be made with respect to three agents (FFN, OXO and OTC). For FFN and OXO, the same CO_{WT} values ($\leq$2 mg L^{-1} and 0.25 mg L^{-1} respectively) were calculated from all three studies. For OTC, Smith *et al.* (2016) and Van Vliet *et al.* (2017) calculated a CO_{WT} of $\leq$0.125 mg L^{-1} compared to the 0.25 mg L^{-1} calculated in this work. It should, however, be noted that in this work, no isolates were recorded as manifesting an MIC of 0.25 mg L^{-1} for OTC (Table 3) and the categorisation of the 133 isolates studied here would be the same if either CO_{WT} value was applied to them. This agreement in the CO_{WT} values calculated illustrates the value of the use of standardised test protocols and statistically based interpretive criteria and suggests that it should be possible for CLSI to set internationally applicable, laboratory-independent ECVs for this species.

Frequencies of UK *F. psychrophilum* isolates with reduced susceptibility

Applying the CO_{WT} values calculated or, in the case of AMP, estimated in this work (Table 5) to the MIC data from these 118 UK *F. psychrophilum* isolates, the frequencies of those with reduced susceptibility were 92% for FLUQ, 90% for ENRO, 88% for OXO, 58% for OTC, 32% for AMP and no isolates were recorded with reduced susceptibility for FFN and ERY. However, as noted by Smith *et al.* (2016), ERY, a drug whose primary value is in treating infections by gram-positive bacteria, has never been recommended for the control of *F. psychrophilum* infections of aquatic animal. There have been two earlier studies of NWT frequencies in UK *F. psychrophilum* isolates. Rangdale *et al.* (1997)

investigated the susceptibility of 47 *F. psychrophilum* isolates, 36 of which were collected in the UK. However, their MIC data sets were of very low precision (mean log2 standard deviation for FFN, OTC and OXO of 3.24 $\log_2$ mg L^{-1}) and therefore, reliable estimates of NWT frequencies could not be assessed. In a smaller study (27 UK *F. psychrophilum* isolates) that used the same testing protocol and statistically based interpretive criteria as used in this work, Smith *et al.* (2016) reported NWT frequencies similar to those reported here. When the isolates studied here are combined those studied by Smith *et al.* (2016), the frequency of NWT phenotypes in the 145 UK isolates obtained during 2005 – 2015 were 85%, 59% for OXO and OTC respectively, and no NWT phenotypes were reported for FFN.

Antibiotic use in UK rainbow trout farming

Verner-Jeffreys & Taylor (2015) reported the use of four agents (FFN, OXY, AMOX and OXO) in attempts to control RTFS in the UK. The survey revealed that FFN was the treatment of choice in the industry. These FFN treatments were generally considered very effective. Where other antimicrobials (OTC, OXO or AMOX) were used, the therapeutic response was reported as either mixed or poor.

These anecdotal reports of comparative treatment efficacies reflect closely the frequencies with which isolates of reduced susceptibility were detected in this work. This in turn suggests that routine susceptibility testing, associated with appropriate interpretation of these data obtained, would be cost-effective and an essential element in the prudent use of antibiotics in aquaculture.

Verner-Jeffreys & Taylor (2015) reported that within the UK most batches of rainbow trout were treated with FFN at least once during every production cycle. Thus, given the relatively high frequency of NWT phenotypes detected with respect to the alternative agents available (OXO, OTC and AMOX), it would appear that, as it currently operates, the UK rainbow trout industry is critically dependent on the continued clinical efficacy of FFN. Some concern must be expressed about the long-term sustainability of an industry that would be affected by the emergence of strains of *F. psychrophilum* that were clinically resistant to this agent.

As FFN is the agent of choice to treat *F. psychrophilum* infection in many countries, it is reasonable to postulate that this critical dependence of the continued clinical efficacy of FFN is not unique to the UK but is wide-spread in the global trout farming industry. The global situation with respect to FFN susceptibility of *F. psychrophilum* can be assessed from a number of studies that have been published. Studies that have employed standard MIC testing protocols and that generated data of adequate precision have been reported from Denmark and the UK (Smith *et al.* 2016), Chile (Miranda *et al.* 2016) and USA (Van Vliet *et al.* 2017). Studies of the antimicrobial susceptibility of Danish (Bruun *et al.* 2000) and French (Michel *et al.* 2003) *F. psychrophilum* isolates that used non-standardised agar dilution protocols have also been published Combining the data presented in these studies with the data generated in this study provides a total of 829 measurements of FFN susceptibility, of which only two (0.2%), both collected in Chile, were categorised as NWT with respect to FFN. Recently the presence of a region containing resistance genes to florfenicol (*floR*), tetracycline (*tet*X), streptothricin and

chloramphenicol acetyltransferase gene was detected in *Chryseobacterium* spp. from rainbow trout (Verner-Jeffreys *et al.* 2017). However, this resistance gene cassette was not widely distributed in Flavobacteriaceae isolates (Verner-Jeffreys *et al.* 2017).

These data would indicate, rather surprisingly, that the selective pressure that must have resulted from the use of this agent during the 25 or more years since the introduction of the antibiotic to aquaculture (Smith 2008) has not yet resulted in any significant emergence of strains of *F. psychrophilum* strains with reduced susceptibility to this agent. However, it cannot be automatically assumed that this situation will continue. Given the significance of FFN to the global trout farming industry, and as recommended by the World Animal Health Organisations (http://www.oie.int/international-standard-setting/aquatic-code/access-online), it is essential that programmes designed to detect any emergence of isolates of *F. psychrophilum* with reduced susceptibility to this agent are implemented as a matter of urgency by all relevant authorities.

Conclusions

Interpretation of MIC data by NRI analysis for *F. psychrophilum* generated by the standardised microdilution protocol (CLSI 2014a) provided an overview of the frequencies of isolates manifesting reduced susceptibility in 118 UK isolates. There was a general agreement between the frequencies of isolates manifesting WT phenotypes for the agents FFN, OXO and OTC, observed in this work, and the reports of their clinical success when used in commercial farms in the UK. On the basis of this, it is strongly recommended that, in order to ensure rational and prudent use of antibiotics to control *F.*

psychrophilum infections, susceptibility testing using standardised methods should be performed in association with all on-farm administrations of the antibiotics.

The Aquatic Animal Health Code of the World Animal Health Organisation recommends that all relevant authorities should implement programmes for the monitoring and surveillance of the susceptibility of aquatic animal pathogens to antibiotics used in their areas. As the UK trout farming industry is critically dependent on the continued efficacy of FFN in the control of RTFS, the implementation of such a programme with respect to *F. psychrophilum* would appear to be a priority for the UK. The standardised MIC susceptibility testing protocol of CLSI and the epidemiological cut-off values developed in this work would provide the analytical methods for such a programme.

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Chryseobacterium isolates from rainbow trout. Exception to the general rule? *FEMS Microbiol Ecol*. Doi: 10.1093/femsec/fix015.

664 **Table 1.** Summary of 118 UK *F. psychrophilum* isolates analysed in this study

Location	Host source	No. of sites	Site	Year of isolation	No. of sampling times	No. of strains	No. of genotypes (*)	No. of plasmid profiles (*)
Scotland	***RT(93)/ AS(14)***	***20***		***2005-2015***	***46***	***102***	***47***	***7***
			Scot I	2005-2014	16	25	11	4
			Scot II	2013	1	1	1	1
			Scot III	2011-2015	4	13	9	4
			Scot IV	2013	1	5	2	2
			Scot V	2013-2015	4	27	16	5
			Scot VI	2009	1	1	1	1
			Scot VII	2007	1	1	1	1
			Scot VIII	2005	1	1	1	1
			Scot IX	2006	1	1	1	1
			Scot X	2011-2013	2	3	2	1
			Scot XI	2015	1	4	3	1
			Scot XII	2010	1	1	1	1
			Scot XIII	2005	1	1	1	1
			Scot XIV	2013	1	2	1	1
			Scot XV	2013	2	3	2	1
			Scot XVI	2014-2015	4	9	4	3
			Scot XVII	2007	1	1	1	1
			Scot XVIII	2009	1	1	1	1
			Unknown (2)	2009-2012	2	2	2	2
England	***RT***	***6***		***2007-2015***	***8***	***13***	***5***	***2***
			Eng I	2013	3	8	4	2
			Eng II	2015	1	1	1	1
			Eng III	2015	1	1	1	1
			Eng IV	2015	1	1	1	1
			Eng V	2007	1	1	1	1
			Eng VI	2007	1	1	1	1
Northern Ireland	***RT***	***1***	***N Ire I***	***2013***	***2***	***3***	***2***	***2***
Total	**RT(111)/ AS(14)**			**2005-2015**	**56**	**118**	**51**	**7**

665 RT, rainbow trout; AS, Atlantic salmon

666 (*) Genotypes and plasmid profiles of *F. psychrophilum* isolates determined by Ngo *et al.* (2017).

667

668

669 **Table 2.** Fifteen *F. psychrophilum* isolates from outside the UK used in this study
670

Countries	Host source	Year of isolation	No. of strains	No. of genotypes[a]	No. of plasmid profiles[a]
Chile	RT	1995-1997	2	2	2
Denmark	RT	1990-1994	3	3	1
Finland	RT	1996	2	2	1
France	RT	unknown-2013	3	2	1
Ireland	AS	2006	1	1	1
USA[b]	RT(3)/CS(1)	unknown - 2004	4	4	2
Total	**RT(13)/AS(1)/CS(1)**	**unknown - 2013**	**15**	**13**	**3**

671 RT, rainbow trout; AS, Atlantic salmon; CS, coho salmon
672 [a] Pulsotypes and plasmid profiles of *F. psychrophilum* isolates determined by Ngo *et al.* (2017).
673 [b] including the *F. psychrophilum* type strain NCIMB 1947^T
674
675

Table 3. MIC values (mg L^{-1}) determined for 133 *F. psychrophilum* isolates

	Off scale	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	Off scale
β lactams																
AMOX	93										19	13	8			
AMP	24						66	3			15	17	8			
Macrolides																
ERY	4									4	24	93	8			
Phenicols																
FFN								1	9	69	54					
Quinolones																
ENRO				7	10	3	3	51	17	14	28					
FLUQ						3	16	1	1	3	41	13	13			42
OXO							7	13	1	1	6	47				58
Tetracyclines																
OTC						12	40	4		2		7	33	32		3
	Off scale	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	Off scale

Shaded areas indicate MIC values could not be determined using Sensititre CMP1MSP plates.

Off scale indicates the number of strains whose MIC lay outside of the range that could be determined using these plates.

681 **Table 4.** MIC values (mg L^{-1}) determined for potentiated sulphonamide drugs against 133 *F. psychrophilum* isolates

682

	Off scale	0.008/0.15	0.015/0.30	0.03/0.59	0.06/1.19	0.12/2.38	0.25/4.75	0.5/9.5	1/19	2/38	4/76	Off scale
PRI						1	2	15	28	43	29	15
SXT					1	11	37	30	48			6

683

684 Shaded areas indicate MIC values could not be determined using Sensititre CMP1MSP plates.

685 Table 5. Cut-off values (CO_{WT}) calculated using NRI from 133 MIC observations

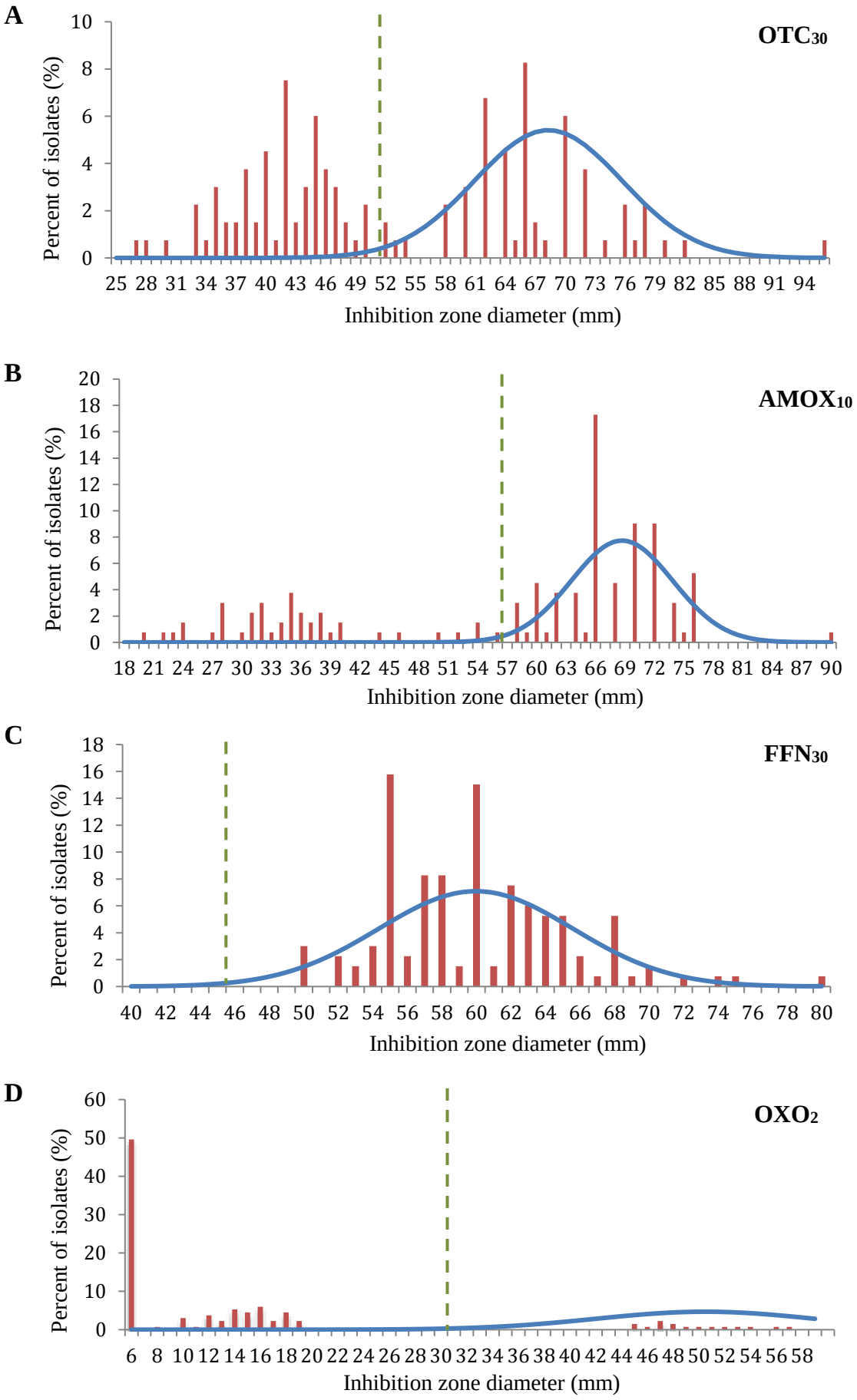
Agent	Number of WT observations	Standard deviation [a] (log_2 mg L^{-1})	CO_{WT} (mg L^{-1})
AMP[b]	93 (70%)	ND[c]	≤ 0.125
ENRO	20 (15%)	0.74	≤ 0.032
ERY	133 (100%)	0.98	≤ 8
FFN	133 (100%)	0.68	≤ 2
FLUQ	20 (15%)	0.57	≤ 0.125
OTC	56 (42%)	0.68	≤ 0.25
OXO	21 (16%)	0.67	≤ 0.25
PRI	133 (100%)	1.39	≤ 16/304
SXT	133 (100%)	1.61	≤ 8/152

686 [a] Standard deviation of the normalised distribution of MIC values for WT strains.
687 [b] CO_{WT} value of AMP was estimated by visual examination.
688 [c] ND: not determined
689
690

691 Table 6. Provisional cut-off values (CO_{WT}) calculated using NRI from 133 inhibition zone
692 observations

Agent	Number of WT observations	Standard deviation* (mm)	CO_{WT} (mm)
$AMOX_{10}$	90 (68%)	5.20	≥56
FFN_{30}	133 (100%)	5.61	≥45
OTC_{30}	65 (49%)	7.44	≥51
OXO_2	20 (15%)	8.50	≥30

693 * Standard deviation of the normalised distribution of MIC values for WT strains.
694
695
696
697
698


A
OTC30
Percent of isolates (%)
0 2 4 6 8 10
25 28 31 34 37 40 43 46 49 52 55 58 61 64 67 70 73 76 79 82 85 88 91 94
Inhibition zone diameter (mm)
B
AMOX10
Percent of isolates (%)
0 2 4 6 8 10 12 14 16 18 20
18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66 69 72 75 78 81 84 87 90
Inhibition zone diameter (mm)
C
FFN30
Percent of isolates (%)
0 2 4 6 8 10 12 14 16 18
40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80
Inhibition zone diameter (mm)
D
OXO2
Percent of isolates (%)
0 10 20 30 40 50 60
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58
Inhibition zone diameter (mm)

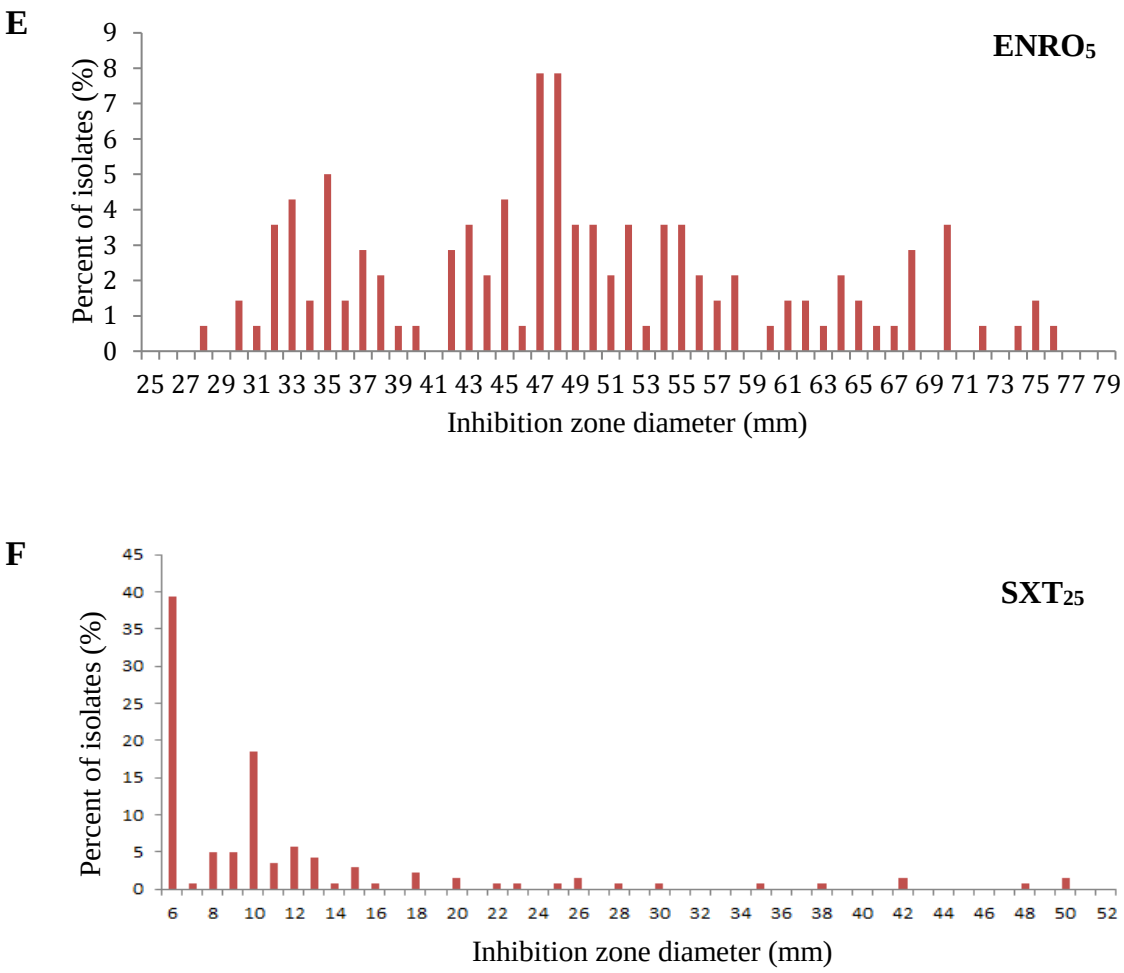


Figure 1. Distribution of 133 *F. psychrophilum* strains according to inhibition zone diameters generated by disc diffusion method for 30 µg oxytetracycline (A, OTC_{30}), 10 µg amoxicillin (B, $AMOX_{10}$), 30 µg florfenicol (C, FFN_{30}), 2 µg oxolinic acid (D, OXO_2), 5 µg enrofloxacin (E, $ENRO_5$) and 25 µg trimethoprim/sulphamethoxazole (F, SXT_{25}). The continuous line represents the 8 point rolling means, the vertical dashed line represents the calculated disc diffusion-based cut-off value.

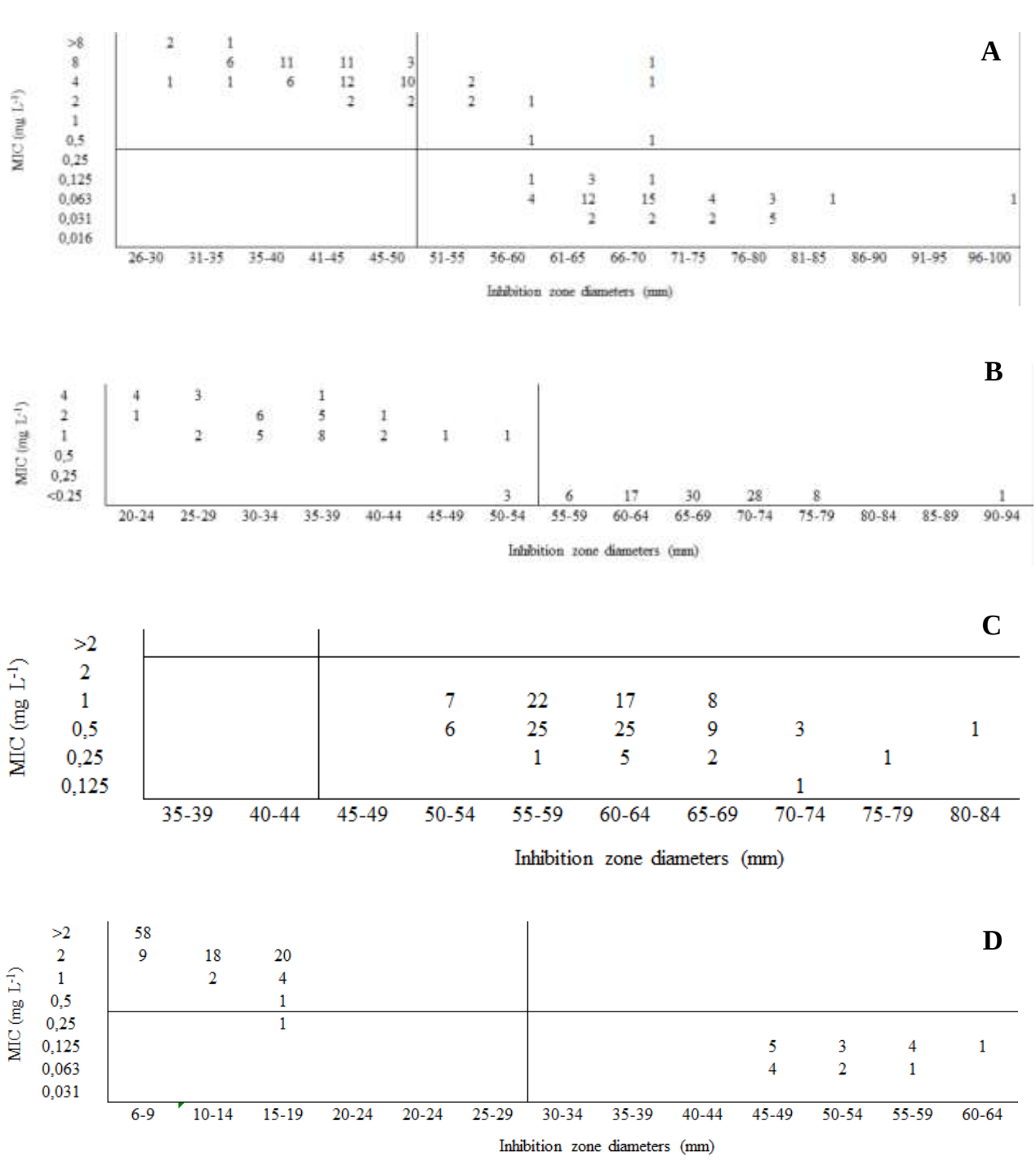


Figure 2. Plot of 133 paired MIC values versus disc diffusion zone diameters for oxytetracycline (A), florfenicol (B) and oxolinic acid (C). A continuous thick line presents the calculated cut-off line of the microbial agent.

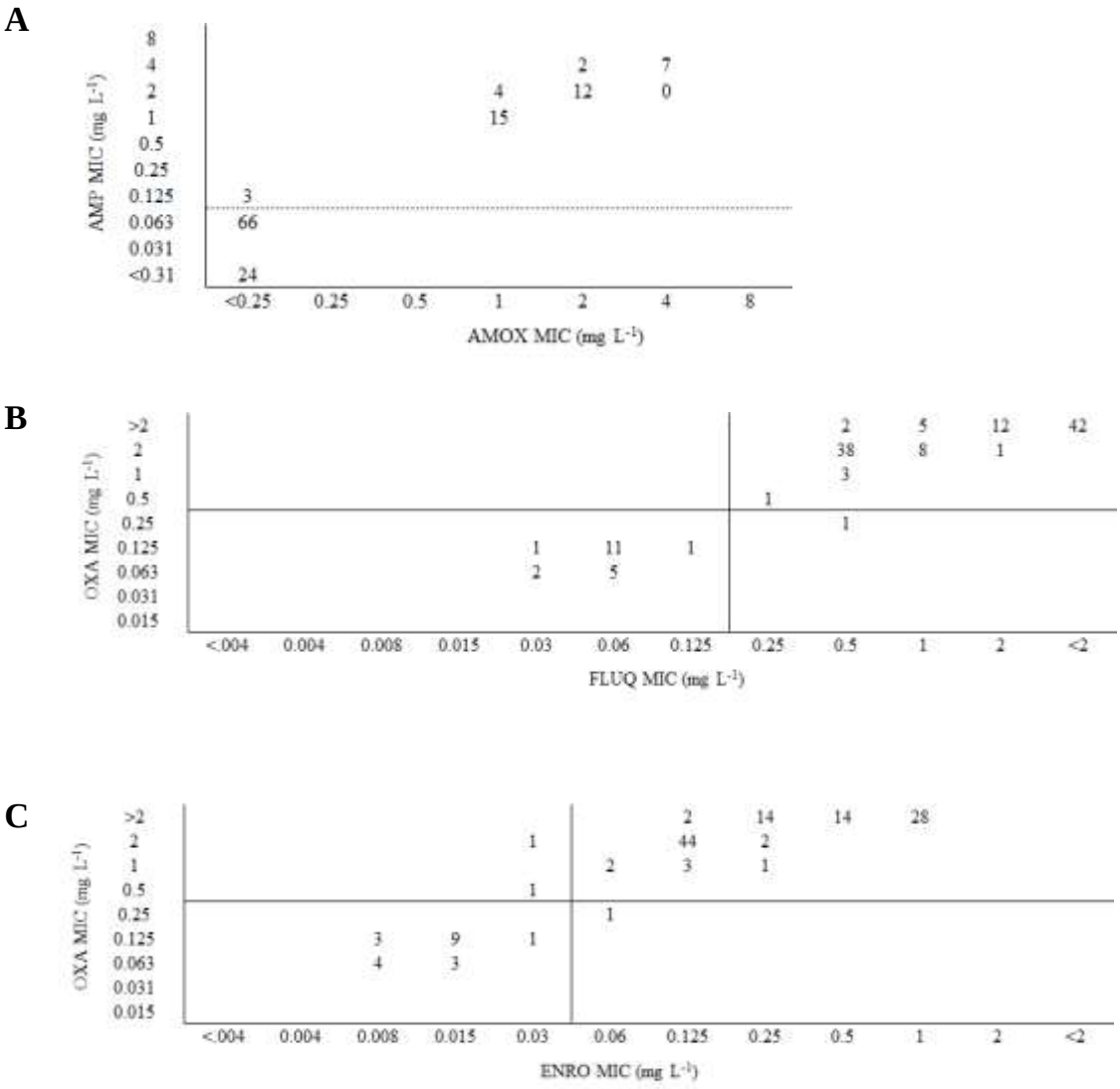


Figure 3. Plot of 133 paired MIC values between antimicrobial agents within beta-lactam group (A: ampicillin and amoxicillin) and quinolone group (B: oxolinic acid and flumequine; C: oxolinic acid and enrofloxacin). A continuous thick line presents the calculated cut-off line of the microbial agent. A dashed line presents an estimated cut-off value.