Short Communication

Broth microdilution protocol for minimum inhibitory concentration (MIC) determinations of the intracellular salmonid pathogen *Piscirickettsia salmonis* to florfenicol and oxytetracycline

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*Piscirickettsia salmonis* is a facultative intracellular Gram-negative bacterium (Mauel, Ware & Smith 2008; Mikalsen *et al.* 2008) originally isolated from fish in Chile and constitutes one of the main problems in farmed salmonids and marine fish worldwide (see reviews by Almendras & Fuentealba 1997; Mauel & Miller 2002; Fryer & Hedrick 2003). This fastidious pathogen was first observed in southern Chile in 1989 (Bravo & Campos 1989; Branson & Díaz-Muñoz 1991; Cvitanich, Garate & Smith 1991), then in western Canada (Brocklebank *et al.* 1992), Ireland (Rodger & Drinan 1993), Scotland (Grant *et al.* 1996), Norway (Olsen *et al.* 1997), eastern Canada (Cusack, Groman & Jones 2002) and southern USA (Arkush *et al.* 2005). Currently, an official report from the Chilean authorities indicates that piscirickettsiosis or salmonid rickettsial septicaemia (SRS) caused by *P. salmonis* has a high prevalence with 68% of fish diagnosed as positive (www.sernapesca.cl).

Treatments with antimicrobial drugs represent the only measure to control SRS in farmed fish. In fact, in 2010, its use in Chilean salmon farms added up to approximately 103 000 kg (www.sernapesca.cl). Chemotherapy with a fluorinated structural synthetic analogue to thiamphenicol and chloramphenicol, florfenicol (FLO) and oxytetracycline (OTC), two bacteriostatic agents with broad-spectrum activity, has often been the choice of drugs for treating the outbreaks. However, since their first use, the results of antimicrobial treatments have been inconsistent and have failed to effectively control SRS (Branson & Díaz-Muñoz 1991; Cvitanich *et al.* 1991). The irregular effects of the chemotherapy in controlling *P. salmonis* in salmonids indicate that the bacterium has developed drug resistance. The drug resistance mechanisms for some fish pathogens have been studied but have not been characterized for *P. salmonis* (http://www.who.int/topics/foodborne_diseases/aquaculture_rep_13_16June2006%20.pdf). Therefore, the appearance of potentially resistant isolates has raised efforts to provide data about the antimicrobial susceptibility of *P. salmonis* isolates to different antibiotics.
Susceptibility presented in the form of minimum inhibitory concentration (MIC) values is an alternative way to measure antibiotic resistance as proposed by Isachsen et al. (2012) for Francisella noatunensis subsp. noatunensis, also an intracellular pathogen. Smith et al. (1996) described the MIC values of five antimicrobial agents to four P. salmonis isolates grown in the Chinook salmon embryo (CHSE-214) cell line. On the other hand, although the Clinical Laboratory Standard Institute guideline M49-A (CLSI 2006a) reported the best framework for developing standard broth dilution susceptibility test protocols for different bacteria pathogenic to fish, P. salmonis is not included in the guidance document. Recently, a marine broth medium supplemented with L-cysteine, named AUSTRAL-SRS, which enhances the growth of P. salmonis isolates, was described (Yáñez et al. 2012). The aim of this research was to evaluate the use of AUSTRAL-SRS broth in the determination of MIC values and in the interpretation of antimicrobial susceptibility of P. salmonis. Moreover, in this study, the induction of drug resistance of the P. salmonis type strain to OTC and FLO under laboratory conditions was examined.

Two P. salmonis isolates, PPT-04 and PPT-05, recovered in 2008 from diseased farmed Atlantic salmon, Salmo salar L., and rainbow trout, Oncorhynchus mykiss (Walbaum), respectively, in Chile, were used to test the liquid culture media. The P. salmonis type strain ATCC VR-1361 (equivalent to LF-89) originally isolated from coho salmon, Oncorhynchus kisutch (Walbaum), was included for comparative purposes and was acquired from the American Type Culture Collection (ATCC). All bacteria were confirmed as P. salmonis using qPCR-based analysis described by Karatas et al. (2008) and also by an indirect fluorescent antibody test (IFAT, SRS—BiosChile), according to the manufacturer’s recommendation.

All P. salmonis were maintained frozen at −80 °C in Criobille tubes (AES Laboratory) and in 1 mL of L-15 Leibovitz medium containing 20% foetal bovine serum and 10% dimethyl sulphoxide.

The MIC values were determined using a standardized broth microdilution method recommended by CLSI (2006a), with some modifications in the medium composition, incubation time and temperature as required for P. salmonis (Yáñez et al. 2012). The strains were inoculated in AUSTRAL-SRS broth medium, which contained twofold dilutions of the antibacterial agents tested, ranging from 0.016–256 µg mL⁻¹. OTC and FLO used in the MIC testing were all obtained from Sigma-Aldrich. Standard stock solutions were prepared by dissolving 10 mg of each antibacterial agent with 500 µL of methanol 100% (OTC) and ethanol 95%, and the final volume was adjusted with distilled water to 10 mL. All stock solutions were stored at 4 °C and were used within 24 h. Each microdilution tray included a growth control well (without antibiotics) and a negative (uninoculated) well as well as three controls including the solvents (methanol and ethanol) in amounts corresponding to the highest quantity present in the assay. Each test was carried out ten times in separate cultures for each strain, and the MIC value was defined as the lowest concentration exhibiting no visible bacterial growth at 18 °C for 92–96 h and/or 116–120 h. In addition, the microtitre plates were read with a spectrophotometer Synergy™ 2 (Biotek) to compare any possible difference in sensitivity between reading methods. As recommended in the CLSI M49-A, the Escherichia coli reference strain ATCC 25922 was included in this study to check the performance of the MIC test, as the MIC values of this strain are well known. It was grown in cation-adjusted Mueller–Hinton (CAMH) broth and incubated at 18 °C for 92–96 h and 35 °C for 16–20 h.

The MIC values for the antibacterial agents are shown in Table 1. No variation between replicates performed with each P. salmonis isolate was

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>MIC µg mL⁻¹</th>
<th>Florfenicol</th>
<th>Oxytetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPT-04</td>
<td>Atlantic salmon</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>PPT-05</td>
<td>Rainbow trout</td>
<td>0.5</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>ATCC VR-1361</td>
<td>Coho salmon</td>
<td>0.25</td>
<td>0.25</td>
<td>2.5*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quality control</th>
<th>Florfenicol</th>
<th>Oxytetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 ± 2 °C after</td>
<td>35 ± 2 °C</td>
<td>18 ± 2 °C after</td>
</tr>
<tr>
<td>92–96 h</td>
<td>16–20 h</td>
<td>92–96 h</td>
</tr>
</tbody>
</table>

*MIC value obtained by Smith et al. (1996) using chloramphenicol and oxytetracycline.
found, regardless of the antibacterial agents tested. All isolates tested had a high susceptibility and fell within a narrow range. The isolates showed MIC values ranging from 0.5 to 1 for FLO and from 0.125 to 0.5 μg mL⁻¹ for OTC. In addition, P. salmonis ATCC VR-1361 showed an MIC value of 0.25 μg mL⁻¹ for OTC and FLO. Smith et al. (1996) classified a type strain and two isolates (EM-90 and SLGO-90) as susceptible to OTC having MIC values ranging from 0.5 to 3 μg mL⁻¹ (Table 1).

In the case of FLO, the mechanism of action is similar to that of chloramphenicol; in fact, resistance to FLO is most commonly mediated by mono- and diacetylation via chloramphenicol acetyltransferase enzymes, which prevents the binding of FLO to the 50S ribosomal subunit (Shaw 1984). In general, MIC values found by Smith et al. (1996) for chloramphenicol tested against the type strain ATCC VR-1361 (equivalent to LF-89) were similar to that obtained in our study for FLO. In the case of the isolates PPT-04 and PPT-05, they exhibited a high susceptibility level, which was different from the other three P. salmonis isolates studied by Smith et al. (1996).

Interestingly, there are dramatic inconsistencies with the SRS susceptibility results observed in field treatments when compared with the biological results obtained in vitro, probably because during natural outbreaks of the disease, P. salmonis invades the fish cells, growing within cytoplasmic vacuoles of hepatocytes and liver-associated macrophages, as well as in macrophages from the kidney, spleen and peripheral blood (Branson & Díaz-Muñoz 1991; Cvitanich et al. 1991; McCarthy et al. 2008), thus allowing the antibiotic to reach the site of action, that is, inside the fish cells. In addition, it can be speculated that a possible origin of the variations is also due to the repeated application of FLO and OTC in Chilean farms during the last years, in doses that are much higher than that recommended by the pharmaceutical companies (San Martín et al. 2010). In fact, from 2010 to 2011, its use in Chilean salmon farms added up to 177 597 and 160 266 kg, respectively, for FLO and OTC (www.sernapesca.cl).

Cohen et al. (1989) have shown that sublethal antibiotic treatment can result in multidrug resistance induction, which has been associated with mutations in multidrug efflux pumps (Ma et al. 1993). There is evidence suggesting that antibiotic resistance mechanisms would be via radical-induced mutagenesis or induction of the expression of multidrug resistance genes (Kohanski, DePristo & Collins 2010). Therefore, our further objective was to study the influence of sublethal concentrations of OTC and FLO in the susceptibility of P. salmonis ATCC VR-1361 in laboratory conditions.

One hundred microlitres of bacterial cultures with 0.064 μg mL⁻¹ of OTC and FLO was transferred into 100-ml flasks containing 50 mL of AUSTRAL-SRS broth supplemented with each antibiotic at a concentration of 0.064 μg mL⁻¹. Each bacterial culture was incubated for 6 days at 18 °C and then the P. salmonis culture was washed and adjusted with fresh medium and used as an inoculum in the MIC assays against the two antibiotics, ranging from 0.016 to 256 μg mL⁻¹. Each microdilution tray was determined under the same culture conditions as described above. The same experiments were repeated five times.

The results obtained from the MIC analysis for ATCC VR-1361, which was previously grown in a sublethal concentration of OTC (0.064 μg mL⁻¹), showed an increase of twofold dilutions when compared with the initial MIC value (Fig. 1). For FLO, natural variation of the broth microdilution was of onefold dilution, equivalent to

![Figure 1](https://example.com/figure1.png)

**Figure 1** Minimum inhibitory concentration using the P. salmonis type strain previously exposed to 0.064 μg mL⁻¹ of florfenicol and oxytetracycline under in vitro conditions.
0.5 μg mL⁻¹. However, when we observed the growth kinetics of the bacterium, some variations in the distribution of the MIC data were detected, suggesting that the type strain was able to evolve and adapt to each drug. Microscopic observations under phase contrast of P. salmonis cultured into AUSTRAL-SRS broth did not reveal changes in the morphology and size of the bacteria, which were always observed as Gram-negative cocci. In addition, all samples analysed were positive by the IFAT test and qPCR analysis, confirming the cultured organism’s identity as P. salmonis. As expected, liquid culture without the addition of P. salmonis did not yield any growth.

Although the findings obtained with the type strain shed light on the increase in resistance, it needs to be confirmed by testing other isolates recovered from outbreaks and using molecular tools. Further studies are being performed to support this hypothesis. Interestingly, the rapid and combined appearance of resistance caused by the use of these antibiotics in Chilean fish farms has also been described for three other fish pathogens: Streptococcus phocae (Avendaño-Herrera et al. 2012), Flavobacterium psychrophilum (Henriquez-Núñez et al. 2012) and Vibrio ordalii (Poblete-Morales et al. 2013), but the mechanism of resistance acquisition by P. salmonis remains unknown.

Our study was performed using AUSTRAL-SRS broth as the base medium, which showed a good overall correspondence with the MICs obtained by Smith et al. (1996). Considering that P. salmonis replicates by binary fission within membrane-bound cytoplasmic vacuoles in cells of susceptible fish hosts or fish cell lines, inducing a characteristic cytopathic effect (Fryer & Hedrick 2003), the data obtained in our work indicate that this medium can be successfully used in susceptibility tests of P. salmonis isolates and that CLSI should consider accepting it in the next edition of the guideline.

According to Smith (1998), the composition of the medium used for the susceptibility tests should provide enough growth conditions for the strains to be tested and should not contain materials interfering with the test itself or react with any antimicrobial tested. Although CLSI (2006a,b) suggests that the susceptibility testing (disc diffusion and MIC assays) should be performed using Mueller–Hinton medium or that it should be based on some other version, until now P. salmonis has not been included in the guidelines because it does not grow in this medium.

Despite the fact that the study included a small number of bacterial strains (n = 3), no differences were observed among the MIC values obtained for each strain in the independent experiments performed (n = 10), which included separate preparation of the inoculum and/or the test itself. In the same way, MIC values of E. coli ATCC 25922 grown in CAMH broth showed acceptable values for all drugs (Table 1), that is, within the approved quality control ranges. As expected, the liquid culture without the addition of P. salmonis did not yield any growth.

In summary, the results suggest that the AUSTRAL-SRS broth is a suitable medium to determine MIC values of P. salmonis strains. Moreover, it facilitates the in vitro drug susceptibility tests of this fastidious pathogen and can contribute to an understanding of the pharmacokinetic data in fish as well as evaluate the drug resistance of this pathogen and help salmon producers to take treatment decisions to control the disease.

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