

# Antimicrobial susceptibility of *Francisella noatunensis* subsp. *noatunensis* strains isolated from Atlantic cod *Gadus morhua* in Norway

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**ABSTRACT:** A total of 30 isolates of *Francisella noatunensis* subsp. *noatunensis* isolated from Atlantic cod *Gadus morhua* L. were tested for susceptibility, in the form of minimal inhibitory concentration (MIC) values, against the following antibacterial agents: flumequine, oxolinic acid, ciprofloxacin, florfenicol, oxytetracycline, erythromycin, streptomycin sulphate, trimetoprim/sulphadiazine and rifampin. All the isolates had a low susceptibility to oxytetracycline, trimetoprim/sulphadiazine (Tribrissen®), erythromycin, ciprofloxacin and streptomycin with MIC values of 64, 64 to 128, 16, 8 and 32 to 128 µg ml<sup>-1</sup>, respectively. The strains were, on the other hand, susceptible to florfenicol, oxolinic acid, flumequine and rifampin with MIC values of 0.5, 0.25, 0.25 and 0.25 to 1 µg ml<sup>-1</sup>, respectively.

**KEY WORDS:** *Francisella noatunensis* subsp. *noatunensis* · Susceptibility · Atlantic cod

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## INTRODUCTION

The bacterium *Francisella noatunensis* subsp. *noatunensis* is a facultative intracellular bacterium causing the systemic granulomatous inflammatory disease francisellosis in cod *Gadus morhua* L. Since 2004, francisellosis has been found to increase in prevalence and severity, and *Francisella* sp. has been diagnosed in clinically infected cod from numerous grow-out facilities spanning most of the Norwegian coastline from Rogaland to Nordland (Olsen et al. 2006, Ottem et al. 2008, Hjeltnes 2009). This disease is now regarded as a major problem in the cultivation of cod in Norway (Hjeltnes 2009). Internationally, various *Francisella* spp. are known to cause disease epidemics in farmed and wild fish from fresh, brackish and marine water (Mikalsen 2008, Birkbeck et al. 2011). Treatment of francisellosis in cod with antibacterial agents has not yet been described in the literature.

In order to obtain a successful treatment, a bacterium must be susceptible to the antibacterial agent in use and the concentration of the drug achieved in the infected organ or organs must be high enough over a long enough period to have an effect. Pharmacokinetic data are available for the antibacterial agents flumequine, oxolinic acid, florfenicol and Romet®30, and a combination of ormetoprim and sulphadimetoxine, in cod (Hansen & Horsberg 2000, Samuelsen et al. 2003a,b, Samuelsen 2006a,b). Basic knowledge of susceptibility, presented in the form of minimum inhibitory concentration (MIC) values of *Francisella noatunensis* subsp. *noatunensis* against antibacterial agents is, however, presently not available. The antibacterial agents oxytetracycline, florfenicol, flumequine, oxolinic acid and Tribrissen, and a combination of trimethoprim and sulfadiazine in a ratio of 1:5 are at present, or have been, licensed and used in Norwegian fish farming, whereas ciprofloxacin is a bactericidal drug that was found to be

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markedly more active against oxolinic-acid-resistant isolates of *Aeromonas salmonicida* (Lewin & Hastings 1990). *F. noatunensis* subsp. *noatunensis* is a slow-growing bacterium, and rifampin, erythromycin and streptomycin sulphate are all antibacterial agents that are used in human medicine in the treatment of *Mycobacterium tuberculosis*, another slow-growing bacterium. Erythromycin is also occasionally used against bacterial kidney disease (BKD) in salmonids.

The aim of the present study was to test the susceptibility, in the form of MIC values, to a number of isolates of *Francisella noatunensis* subsp. *noatunensis* against the selected group of antibacterial agents.

## MATERIALS AND METHODS

### Bacterial strains

A total of 30 isolates of *Francisella noatunensis* subsp. *noatunensis* from Atlantic cod *Gadus morhua* L. were analysed. Of these, 28 isolates were provided by the Norwegian Veterinary Institute (Norway) and 2 isolates, GM2212/LMG 24256 (Nylund et al. 2006, Ottem et al. 2007) and EK-4b, were provided by the Institute of Marine Research (Norway). All isolates originate from farmed cod, except EK-4b, which was isolated from a wild cod. The strains were previously identified as *F. noatunensis* subsp. *noatunensis* using the methods described by Ottem et al. (2008).

### Chemicals

Flumequine, oxolinic acid, oxytetracycline, florfenicol, Tribriksen (trimethoprim/sulfadiazine), erythromycin, ciprofloxacin, streptomycin sulphate and rifampin were all obtained from Norwegian Medical Depot (Bergen, Norway). Stock solutions of antibacterial agents were prepared at a concentration of 1.0 mg ml<sup>-1</sup> in methanol (florfenicol, Tribriksen, rifampin), in water (oxytetracycline, erythromycin, streptomycin sulphate), 0.03 M NaOH (flumequine, oxolic acid) and 0.03 M NaOH/methanol (1:1) (ciprofloxacin).

### MIC determination

In order to meet the demands of *Francisella noatunensis* subsp. *noatunensis* for specific growth factors, the method of choice in the present work was a modified agar dilution test for the determinations of

MIC values (Alderman & Smith 2001). Briefly, *F. noatunensis* subsp. *noatunensis* isolates were grown on modified Mueller-Hinton agar (MMHA) supplemented with 3% foetal bovine serum (FBS) (PAA Laboratories), 0.8% glucose (Merck), 0.4% L-cystein (Sigma) and 4% Yeastolate Ultrafiltrate (Gibco) and containing 2-fold dilutions of the antibacterial agents tested. All supplements and antibacterial agents were aseptically treated and added when the temperature of the agar (after sterilization 121°C, 15 min) had fallen to 50°C (Andrews 2001), except FBS, which was added at 60°C. Finally, pH was adjusted to 7.3 ± 0.1. Agar plates were inoculated with standardized inoculums of the different *F. noatunensis* subsp. *noatunensis* isolates and incubated for 72 h at 22°C. Three controls included the solvents methanol, 0.03 M sodium hydroxide, or water in amounts corresponding to the highest quantity present in the agar dilution assay. Inoculated agar plates without antibiotics served as positive controls. The type strain LMG 24256 was included on all test plates as control of the culture medium, as none of the recommended control strains were suitable for the specific medium required by *F. noatunensis* subsp. *noatunensis* (Alderman & Smith 2001, Colquhoun & Duodu 2011). Tests were performed in triplicate, and the MIC values were defined as the lowest concentration exhibiting no visible bacterial growth.

## RESULTS

The MIC values for the various antibacterial agents are shown in Table 1. No variations between parallels and between isolates were found for most of the antibacterial agents. All the isolates had a low susceptibility to oxytetracycline, Tribriksen, erythromycin, ciprofloxacin and streptomycin sulphate. The isolates were susceptible to florfenicol, oxolinic acid, flumequine and rifampin. The results show that the MICs for all antibacterial agents and strains fell within a narrow range, apart from streptomycin, which showed greater variation.

The MIC values for the type strain LMG 24256 were similar to the clinical strains for all antibacterial agents, with the exception of streptomycin, where the MIC value for LMG 24256 was higher (128 µg ml<sup>-1</sup>).

## DISCUSSION

In order to establish correct dosage regimes and thereby promote optimal use of a drug, data derived

Table 1. Minimum inhibitory concentration (MIC) of antibacterial agents against *Francisella noatunensis* isolates and *F. noatunensis* reference strain (LMG 24256)

Antibacterial agents	Test range ( $\mu\text{g ml}^{-1}$ )	MIC value ( $\mu\text{g ml}^{-1}$ )		
		<i>F. noatunensis</i> (n = 28)	EK-4b	LMG 24256
Florfenicol	0.03–2	0.5 <sup>a</sup>	0.5 <sup>a</sup>	0.5 <sup>a</sup>
Oxytetracycline	4–128	64	64	64
Erythromycin	2–128	16	16	16
Trimethoprim/ Sulfadiazine	4–128	64–128 <sup>a</sup>	64 <sup>a</sup>	64–128 <sup>a</sup>
Flumequine	0.03–4	0.25	0.25	0.25
Oxolinic acid	0.015–16	0.25	0.25	0.25
Ciprofloxacin	0.12–16	8	8	8
Streptomycin sulphate	4–128	>32	32–64	>128
Rifampicin	0.25–128	0.25–1 <sup>a</sup>	0.25 1 <sup>a</sup>	0.25–1 <sup>a</sup>

<sup>a</sup>No growth in solvent control plate

from pharmacokinetic investigations and the pathogens' susceptibility to the drug in use are essential. However, pharmacokinetic data in fish are known to vary, e.g. with dose, temperature, salinity, species and size.

In cod, pharmacokinetic data are available for the antibacterial agents flumequine, oxolinic acid, florfenicol and Romet<sup>30</sup> and where the studies were performed at ambient temperature, salinity and size and applying doses and regimes as recommended by the producers (Hansen & Horsberg 2000, Samuelsen et al. 2003a,b, Samuelsen 2006a,b). For the remaining drugs, pharmacokinetic data from other fish species were used, introducing more inaccuracy in the concluding arguments.

In this investigation the susceptibility of various *Francisella noatunensis* subsp. *noatunensis* strains to both bacteriostatic drugs (florfenicol, oxytetracycline, erythromycin, Tribissen) and bactericidal drugs (flumequine, oxolinic acid, ciprofloxacin, streptomycin sulphate, rifampin) were studied (Table 1). Few studies have been conducted to evaluate the antibacterial susceptibility to *Francisella* sp. strains isolated from fish. Bohle et al. (2009) reported MIC values of 0.25  $\mu\text{g ml}^{-1}$  for flumequine and oxolinic acid and 1.0  $\mu\text{g ml}^{-1}$  for florfenicol for *F. philomiragia* cultures isolated from infected Atlantic salmon *Salmo salar*. These values are similar to the values found in the present study (Table 1). However, Bohle et al. (2009) also reported a MIC value of 0.5  $\mu\text{g ml}^{-1}$  for oxytetracycline, which is much lower than the value of 64  $\mu\text{g ml}^{-1}$  found in our investigation (Table 1). In a recent study, Soto et al. (2012) present MIC values for 10 isolates of *F. noatunensis* subsp. *orientalis* against a large number of antibacterial agents. Similar to our investiga-

tion, they found their isolates to be less susceptible to streptomycin, erythromycin and potentiated sulphonamides and susceptible to florfenicol and fluoroquinolones (enrofloxacin and ciprofloxacin). However, unlike our results, they found their isolates to be susceptible to oxytetracycline (MIC value of 0.25  $\mu\text{g ml}^{-1}$ ) and ciprofloxacin (MIC value of 0.5  $\mu\text{g ml}^{-1}$ ).

According to Shojaee AliAbadi & Lees (2000), the length of the period for which the concentration in plasma exceeds the MIC value is an important determinant for the outcome of therapy for bacteriostatic drugs. For the predominantly concentration-dependant bactericidal drugs with

significant post-antibiotic effect, which include the bactericidal drugs tested here, the outcome of therapy requires the attainment of high concentrations and is correlated to the  $\text{AUC}_{0-24}/\text{MIC}$  ratio, whereas prevention of the development of resistance is correlated with the  $C_{\text{max}}/\text{MIC}$  ratio (Shojaee AliAbadi & Lees 2000).  $\text{AUC}_{0-24}$  is defined as the area under the concentration (time curve) from 0 to 24 h following a single administration of a drug, whereas  $C_{\text{max}}$  refers to the peak plasma concentration of the drug. For the quinolones, rifampin and streptomycin a  $C_{\text{max}}/\text{MIC}$  ratio of 8 should be obtained and an  $\text{AUC}_{0-24}/\text{MIC}$  ratio of 100 and, in some cases, 250 is desirable (Shojaee AliAbadi & Lees 2000).

The MIC value for both flumequine and oxolinic acid for all the *Francisella noatunensis* subsp. *noatunensis* strains tested in this investigation was 0.25  $\mu\text{g ml}^{-1}$ . A single oral administration of 25 mg  $\text{kg}^{-1}$  of oxolinic acid to cod resulted in a plasma  $C_{\text{max}}$  value of 1.2  $\mu\text{g ml}^{-1}$  and an  $\text{AUC}_{0-24}$  value of 140 h  $\mu\text{g ml}^{-1}$  (Samuelsen et al. 2003a). From these data, an  $\text{AUC}_{0-24}$  value of approximately 23 h  $\mu\text{g ml}^{-1}$  could be calculated giving an  $\text{AUC}_{0-24}/\text{MIC}$  ratio of 92 and a  $C_{\text{max}}/\text{MIC}$  ratio of 4.8. These values are slightly lower than recommended, and an increase in the daily dosage should be considered when using oxolinic acid. For flumequine, following administration of 10, 15 and 25 mg  $\text{kg}^{-1}$  on Days 1, 2, 3, 5, 7 and 9,  $C_{\text{max}}$  values of 1.3, 2.7 and 4.5  $\mu\text{g ml}^{-1}$ , respectively, could be determined (Vik-Mo et al. 2005). The 2 highest doses resulted in  $C_{\text{max}}/\text{MIC}$  values of >8, while the 10 mg  $\text{kg}^{-1}$  dose failed (Vik-Mo et al. 2005). Unfortunately, no AUC values could be determined from their study. However, based on data presented by Hansen & Horsberg (2000), following a single oral

dose of 10 mg kg<sup>-1</sup> of flumequine, an AUC<sub>0–24</sub> of approximately 60 to 70 h µg ml<sup>-1</sup> could be determined giving an AUC<sub>0–24</sub>/MIC ratio of 240 to 280, indicating efficacy.

No pharmacokinetic studies are presented for streptomycin sulphate, ciprofloxacin, or rifampin in fish. However, MIC values above 32 µg ml<sup>-1</sup> for streptomycin sulphate and 8 µg ml<sup>-1</sup> for ciprofloxacin show that the susceptibilities of the isolates towards these drugs are low, and it is not likely that adequate concentrations can be achieved in the fish unless the administered dose is very high. For rifampin the high susceptibility may indicate potential efficacy, but this is an important drug in human medicine, and it is therefore unlikely that it will be licensed for use in aquaculture in Norway.

For the bacteriostatic drugs (florfenicol, oxytetracycline, erythromycin, Tribriksen) and time-dependant bactericidal drugs, the time for which concentration exceeds the MIC value is an important determinant for the outcome of therapy. In periods when concentrations decrease below MIC, re-growth of microbes occurs.

The MIC value for florfenicol for all the *Francisella noatunensis* subsp. *noatunensis* strains tested in this investigation was 0.5 µg ml<sup>-1</sup>. Pharmacokinetic data following a single oral dose of 10 mg kg<sup>-1</sup> show that florfenicol is well absorbed in cod with C<sub>max</sub> values of 10.8 µg ml<sup>-1</sup> in plasma, 13.0 µg g<sup>-1</sup> in muscle and 12.1 µg g<sup>-1</sup> in liver and is eliminated slowly with an elimination half-life (t<sub>1/2β</sub>) value of 39 h in plasma, 21 h in muscle and 23 h in liver (Samuelsen et al. 2003b). Following 10 d of medication with 10 and 20 mg florfenicol kg<sup>-1</sup> fish d<sup>-1</sup>, produced plasma and muscle concentrations of 5.0 µg ml<sup>-1</sup> and 4.6 µg g<sup>-1</sup>, respectively, for the 10 mg kg<sup>-1</sup> dose and 6.5 µg ml<sup>-1</sup> and 7.0 µg g<sup>-1</sup>, respectively, for the 20 mg kg<sup>-1</sup> dose in cod, 24 h following the last application (Samuelsen & Bergh 2004). These values therefore show that, following recommended medication with florfenicol (10 mg kg<sup>-1</sup> daily for 10 consecutive days), the mean concentrations in plasma will exceed the MIC value of 0.5 µg ml<sup>-1</sup> by a good margin for the entire medication period and have the potential of providing a therapeutic effect (Samuelsen et al. 2003b). In comparison, a single dose of 10 mg kg<sup>-1</sup> florfenicol to tilapia (*Oreochromis niloticus* × *O. aureus*) gave C<sub>max</sub> concentrations of 4.46 µg ml<sup>-1</sup> in plasma, 6.88 µg g<sup>-1</sup> in muscle and 5.87 µg g<sup>-1</sup> in liver and faster elimination with t<sub>1/2β</sub> values of 10 h in plasma, 11 h in muscle and 14 h in liver (Feng & Jia 2009), which is considerably lower than the values found in cod. However, according to Bowser et al. (2009) and Kosoff et al.

(2009), a 10 d administration of 10 and 15 mg kg<sup>-1</sup> d<sup>-1</sup> resulted in plasma and tissue concentrations in tilapia similar to or higher than those found in cod by Samuelsen & Bergh (2004). Furthermore, a medication regime of 15 mg kg<sup>-1</sup> daily for 10 consecutive days showed high efficacy in the treatment of a *Francisella asiatica* infection in tilapia if the drug was administered during the early stages of infection (1 to 3 d post challenge) (Soto et al. 2010). The MIC value for the bacterial isolate was 2.0 µg ml<sup>-1</sup>.

The MIC values for the combination of trimethoprim and sulphadiazin (Tribriksen) were from 64 to 128 µg ml<sup>-1</sup>. No pharmacokinetic data following administration of this combination were available in cod, but a single oral administration of the comparable compounds ormethoprim (5 mg kg<sup>-1</sup>) and sulphadimethoxine (25 mg kg<sup>-1</sup>) in combination (Romet®30) showed plasma C<sub>max</sub> values of 22.1 µg ml<sup>-1</sup> (sulphadimethoxine) and 1.6 µg ml<sup>-1</sup> (ormethoprim) after 72 and 9 h, respectively. Corresponding values for muscle were 11.1 µg g<sup>-1</sup> at 72 h and 2.0 µg g<sup>-1</sup> at 9 h, respectively, and for liver 9.7 µg g<sup>-1</sup> at 48 h and 14.7 µg g<sup>-1</sup> at 6 h, respectively (Samuelsen 2006b). Since a comparison of the pharmacokinetic properties of Tribriksen and Romet®30 revealed only minor differences in Atlantic salmon (Samuelsen et al. 1995, Horsberg et al. 1997), it is likely that this is valid also for cod. Our result therefore indicates that the concentrations of both drugs in plasma and tissues do not exceed the MIC values, and it is therefore unlikely that Tribriksen will have a therapeutic effect on cod.

The MIC value for oxytetracycline for all the *Francisella noatunensis* subsp. *noatunensis* strains tested in this investigation was found to be 64 µg ml<sup>-1</sup>. The pharmacokinetic profile for this drug has been studied in a number of marine fish species but not in cod (Elema et al. 1996, Rigos et al. 2003, 2004a,b, Wang et al. 2004). The plasma C<sub>max</sub> for oxytetracycline varies considerably in marine fish, and the absorption of oxytetracycline from the intestine is, due to complexation with di- and tri-valent cations, normally rather low. The highest plasma concentration measured in marine fish species was in black seabream *Sparus macrocephalus* with 8.48 µg ml<sup>-1</sup> (Wang et al. 2004) following administration of 100 mg kg<sup>-1</sup> daily for 5 d. If one can assume that the pharmacokinetic profile of oxytetracycline in cod is similar to the profiles in other marine fish species, the plasma concentrations will most certainly not reach a concentration high enough to exceed the MIC value of 64 µg ml<sup>-1</sup>. It is therefore unlikely that oxytetracycline will have a therapeutic effect in the treatment of a francisellosis



infection in cod. In comparison, Chern & Chao (1994) considered 10 to 14 d treatment with 30 to 50 mg kg<sup>-1</sup> oxytetracycline as a most-likely effective treatment of infection with rickettsia-like organisms in tilapia. The MIC value for tetracycline was determined to be 1 µg ml<sup>-1</sup> (Chern & Chao 1994). Furthermore, Mauel et al. (2005) and Ostland et al. (2006) reported successful treatment of francisellosis in tilapia and striped bass *Morone chrysops* × *M. saxatilis*, respectively, using tetracycline. The dosage used by Ostland et al. (2006) was approximately 250 mg kg<sup>-1</sup> daily for 10 d.

A rather intensive and extended treatment of juvenile Chinook salmon *Oncorhynchus tshawytscha* with erythromycin (100 mg kg<sup>-1</sup>) daily for 3 × 28 d during the period of February to June led to a maximum tissue concentration of 10.4 µg g<sup>-1</sup> (Fairgrieve et al. 2005). No pharmacokinetic data for erythromycin exist for cod, but, with a MIC value of 16 µg ml<sup>-1</sup> against all strains of *Francisella noatunensis* subsp. *noatunensis* tested, this drug will most probably not obtain concentrations high enough in cod to have a therapeutic effect.

Except for florfenicol, flumequine, oxolinic acid and rifampin, the antibacterial susceptibilities of the *Francisella noatunensis* subsp. *noatunensis* isolates tested in the present study were low (Table 1). In comparison, a number of studies testing the susceptibility of these antibacterial agents to strains of the bacterium *Francisella tularensis*, in general, showed higher susceptibility (Maurin et al. 2000, Johansson et al. 2002, Tomaso et al. 2005, Urich & Petersen 2008). The MIC values varied from 0.125 to 4 µg ml<sup>-1</sup> for tetracycline, 0.032 to 8 µg ml<sup>-1</sup> for streptomycin sulphate, 0.12 to 4 µg ml<sup>-1</sup> for erythromycin, 0.015 to 2 µg ml<sup>-1</sup> for rifampin and 0.004 to 0.25 µg ml<sup>-1</sup> for ciprofloxacin. One study, however, showed lower susceptibility, with MIC values of 4 to 64 µg ml<sup>-1</sup> for tetracycline, 4 to 32 µg ml<sup>-1</sup> for streptomycin sulphate, and 4 to 16 and 0.06 to 0.25 µg ml<sup>-1</sup>, respectively, for the quinolones nalidixic acid and ciprofloxacin (García del Blanco et al. 2004).

In conclusion, florfenicol and the quinolones flumequine and oxolinic acid seem to be the best alternatives for treatment based on MIC values and available pharmacokinetic data.

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