

Efficacy of orally administered flumequine in the treatment of vibriosis caused by *Listonella anguillarum* in Atlantic cod *Gadus morhua*

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ABSTRACT: The efficacy of orally administered flumequine in the treatment of experimentally induced vibriosis in Atlantic cod *Gadus morhua* was investigated. Cod (mean \pm SD, 120 \pm 30 g) were randomly distributed to twelve tanks and bath challenged for 1 h with *Listonella anguillarum* serotype O2 α , strain HI-610, using a dose of 9.2×10^6 CFU ml⁻¹. At 3 d post-challenge, medication was introduced in 10 of the groups at doses of 2.5, 5, 10, 15 and 25 mg flumequine kg⁻¹ body weight d⁻¹ in duplicate. The medication was administered on Days 1, 2, 4, 6, 8, and 10 after the initiation of treatment. In challenged unmedicated fish, mortality started on Day 4 post-challenge, reaching a final cumulative mortality of 82% at Day 18. In the medicated groups, mortality started on Days 3 to 5 post-challenge, reaching final cumulative mortalities of 42, 49, 37, 37 and 23% respectively for the fish treated with 2.5, 5, 10, 15 and 25 mg flumequine kg⁻¹ body weight d⁻¹. Survival of medicated fish in all groups was significantly greater than in challenged unmedicated fish ($p < 0.001$). Twenty-four h following the final medication, HPLC analysis found a linear relationship between doses and mean concentrations of the drug in plasma, muscle and liver.

KEY WORDS: Efficacy · Flumequine · *Gadus morhua* · Vibriosis · *Listonella anguillarum*

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INTRODUCTION

Atlantic cod *Gadus morhua* is regarded as a valuable species for domestication in Norway and, during the past few years, both large-scale production of cod fry and production facilities have been established (Engelsen et al. 2004, Karlsen 2004). However, vibriosis caused by *Listonella anguillarum* (Colwell & Grimes 1984, Egidius 1987, Vadstein et al. 2004) is still responsible for serious losses in cod hatcheries (Johansen 2004). A clinical infection with vibriosis in fish is usually associated with a low rate of mortality over a long period of time (Torrissen et al. 1993). In cod, however, vibriosis may also occur as an acute onset condition with moderate to high mortality, often associated with seawater temperatures above 15°C (H. Bleie, National Veterinary Institute, Bergen, Norway, pers. comm.). Bacterial infections are usually treated with antibacterial agents and any potential treatment would

be particularly useful in cod hatcheries, as outbreaks usually occur prior to or in connection with vaccination (Bergh 2002).

The most common antibacterial agents in use in Norway for several years have been oxolinic acid and florfenicol (Grave et al. 2003). Oxolinic acid has been the agent of choice in treating vibriosis in cod but medication has been based on doses and dose regimes originally intended for Atlantic salmon *Salmo salar*. Recent studies have revealed that oxolinic acid, florfenicol and flumequine all possess excellent pharmacokinetic properties in cod (Hansen & Horsberg 2000, Samuelsen et al. 2003a,b), and have demonstrated high efficacy of oxolinic acid and florfenicol against experimentally induced vibriosis (Samuelsen & Bergh 2004). However, no study of the efficacy of flumequine in treating vibriosis in cod has been published.

The design of treatment regimes and prediction of their possible clinical outcomes represent practical

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applications of microbiological and pharmacokinetic data. To prevent the development of *in vitro* resistance of pathogens, the clinical significance of pharmacokinetic data has been related to an assumption that the *in vitro* concentration of the antibacterial agent in use should exceed the pathogen's minimum inhibitory concentration (MIC) value by a factor of 4 (Stamm 1989). Recently, Shojaee AliAbadi & Lees (2000) studied dose regime optimisation for the treatment of animals with antibacterial agents. When the pharmacokinetic properties of antibacterial agents in relation to the MIC value of the pathogen involved are taken into account, they suggest an optimal dose regime for bactericidal drugs that acts via time- and concentration-dependent mechanisms. For such antibacterial agents, including flumequine, the AUC value (AUC:MIC ratio) (AUC = area under curve) should be at least 100 and the maximum plasma concentration (C_{\max}):MIC ratio at least 8 in order to obtain effective control of the pathogen. The C_{\max} values for the various doses therefore need to be determined. On the basis of the available data on the single-dose pharmacokinetics of flumequine in cod (Hansen & Horsberg 2000), presenting a T_{\max} (time to reach C_{\max}) of 24 h in plasma, it can be argued that C_{24} (the concentration measured 24 h following last medication) is a reasonable proxy for C_{\max} in cod.

This study was therefore performed with the aims of evaluating the efficacy of orally administered flumequine given in 5 different doses in controlling mortality in cod experimentally infected with *L. anguillarum*, and of determining the C_{24} concentrations of the drug in plasma and tissues following treatment.

MATERIALS AND METHODS

Experimental fish. Unvaccinated Atlantic cod *Gadus morhua* with a mean weight \pm SD of 120 ± 30 g were obtained from Parisvannet Research Station (Institute of Marine Research, Norway) and maintained in circular (1 m deep \times 2.5 m inside diameter) flow-through seawater storage tanks at the laboratory of the Institute of Marine Research in Bergen. The seawater had a salinity of 34.5‰, a flow rate of 12 l min^{-1} and a temperature of 9°C . The fish were fed an unmedicated ration of 1% body weight d^{-1} of dry pellets (Skretting AS).

Prior to challenge, fish from the storage tanks were randomly divided into 12 groups of 40 fish and transferred to 12 circular (0.8 m internal diameter) flow-through seawater tanks. The seawater had a salinity of 34.5‰, a flow rate of approximately 10 l min^{-1} and a temperature of 9°C . The fish were acclimatised for 3 wk, and at the end of the acclimatisation period they

were consuming the appointed amount of unmedicated feed of 1% of body weight d^{-1} . No signs of disease were observed prior to challenge.

Cultivation of bacteria. The strain of bacteria, *Listonella anguillarum* HI-610, serotype O2 α , was originally isolated from diseased cod from Parisvannet suffering from vibriosis. The MIC value for this strain had previously been determined to be $0.015 \mu\text{g ml}^{-1}$ for flumequine (Samuelsen & Lunestad 1996). Apparently identical strains with respect to API 20E and API 50CH (BioMérieux) profiles have frequently been isolated from moribund cod juveniles at this facility. The strain was used in a pre-challenge under the same conditions as the final challenge experiment and subsequently recultivated from kidney samples from moribund fish and cultivated on nutrient blood agar (Oxoid) supplemented with 5% sheep's blood and 1.5% (wt vol $^{-1}$) NaCl. After confirming that the isolated strain had a biochemical profile identical to the original strain using an API 20E kit (BioMérieux), the recultivated strain was used for the challenge experiment.

The bacteria were grown in a pre-culture of 200 ml Tryptone Soya Broth (TSB) (Difco Laboratories) with 2% (wt vol $^{-1}$) NaCl and incubated for 24 h at 20°C with moderate shaking (150 rpm). The main culture was made by inoculating 10 ml from the pre-culture into 1000 ml of TSB (Difco) with 2% (wt vol $^{-1}$) NaCl and incubated for 24 h at 20°C with moderate shaking (110 rpm). A total of 6 l of bacterial culture was prepared. The number of colony-forming units (CFU) was examined by making tenfold dilutions of the bacterial culture in 80% sterile seawater. Petri dishes with Tryptone Soya Agar (TSA) (Difco) were inoculated in triplicate with 100 μl from dilutions from 10^{-5} to 10^{-10} and incubated at 20°C . The colonies were counted after 3 d.

Challenge. A bath challenge protocol, used in previous studies with cod (Samuelsen & Bergh 2004) was utilised. The fish were starved for 48 h before the challenge. On the day of challenge, the water supply to the tanks was turned off and the water volume reduced to 100 l. A 0.5 l suspension of the *Listonella anguillarum* strain was added to each tank and the fish were maintained in the bath for 1 h, followed by restoration of the water supply. During challenge the tanks were oxygenated. Following challenge the water temperature in the tanks was raised from 8 to 12°C over a period of 6 h. Bacterial concentrations in the *L. anguillarum* suspension were measured by plate counts, corresponding to final concentration in the tanks of $9.2 \times 10^6 \text{ CFU ml}^{-1}$.

Medicated feeds. Medicated feed was made by adding 0.14, 0.28, 0.54, 0.84 or 1.4 g of flumequine to 280 g pellets, corresponding to a daily dose of 2.5, 5, 10, 15 and 25 mg flumequine kg^{-1} body weight d^{-1} .

using a feed ration of 0.5 % of body weight d^{-1} . In order to obtain a more uniform distribution on the pellets, the drug was mixed with glucose to a 50 % premix before coating. The drug was coated on the surface of the pellets using capelin oil.

Treatment. Medication started 3 d after challenge. Two parallel groups of fish were treated with 2.5, 5, 10, 15 and 25 mg flumequine kg^{-1} body weight d^{-1} , while 2 groups were given unmedicated feed and served as positive controls. Medicated feeds were administered on Days 1, 2, 4, 6, 8 and 10 following the start of medication. In the days between medications the fish were offered unmedicated feed. The fish were fed once a day; approximately 1 h after feeding, excess food was collected from each tank and the amount of feed consumed was estimated. Dead fish were removed from the tanks once a day and mortality was recorded. The amount of feed provided was adjusted every day to take the fish that had died during the previous 24 h into account. Twenty-four h after the final medication, 6 fish from each dose regime were killed by a blow to the head and samples of plasma, muscle and liver were collected for drug analysis.

In the post-medication period, the feeding regime was as described for the acclimatisation period. The fish were observed for 19 d post-challenge.

Microbiological analysis. Kidney samples were taken from the first 3 dead fish in each group post-challenge, and from 3 survivors from each group 24 h following the final medication. Kidney samples were inoculated on Petri dishes containing TSA (Difco) with 2% NaCl and incubated for 48 h at 20°C. Cultures were re-streaked until pure cultures were obtained. Pure cultures were tentatively identified by an agglutination test (Mono-Va, Bio-Nor), followed by verification using a commercial API 20 E biochemical test kit (BioMérieux) used according to the manufacturer's instructions.

High-performance liquid chromatography (HPLC). The plasma and tissue samples were analysed for flumequine according to previously described methods (Samuelsen & Ervik 2001). These methods have quantitation limits of 0.01, 0.025 and 0.1 $\mu g ml^{-1}$ (g^{-1}) for flumequine in plasma, muscle and liver respectively.

Statistical analysis. A *G*-test of independence was used to compare the observed mortality between replicate groups (Sokal & Rohlf 1981). A pairwise *G*-test of independence was used to compare patterns of mortality among groups (rows \times columns of independence) and a pairwise comparison between the groups with a Bonferroni-corrected *p*-value (Sokal & Rohlf 1981). The relative percentage survival (RPS) value, quantifying the effect of the treatment against the severity of the challenge, was calculated as described by Inglis et al. (1991).

Tissue and plasma levels of flumequine within treatment groups were compared using a *t*-test for independence. Between the different treatment groups, ANOVA was used to determine the overall significance of the treatment. A Newman-Keuls post hoc test was used to make pairwise comparisons where significant overall differences were detected.

RESULTS

The values of cumulative mortalities presented here are the means of parallel treatment. Among challenged unmedicated fish, mortality started on Day 5 post-challenge, plateaued at Day 10 and reached a final cumulative mortality of 82 % on Day 18. In the medicated groups, mortality started on Days 3 to 5 post-challenge, reaching final cumulative mortalities of 42, 49, 37, 37 and 23 % respectively for the fish treated with 2.5, 5, 10, 15 and 25 mg flumequine kg^{-1} body weight d^{-1} (Fig. 1). The RPS (Inglis et al. 1991) was calculated to be 49, 40, 54, 55, and 72 % respectively for treatment with 2.5, 5, 10, 15 and 25 mg flumequine kg^{-1} body weight d^{-1} .

Microbiological analysis of the posterior kidney from the first 3 dead fish in each group indicated the presence of bacteria with API 20E and agglutination profiles identical to the HI-610 strain. However, the pathogen was not isolated from any of the medicated or unmedicated survivors sampled 24 h after termination of the medication.

No significant differences in survival were observed between replicate tanks within any of the treatments

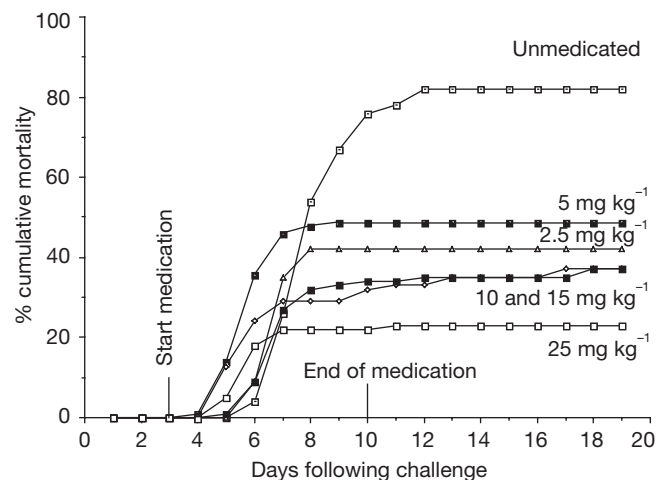


Fig. 1. *Gadus morhua* challenged with *Listonella anguillarum*. Cumulative mortality over time in 5 groups of fish treated with flumequine and an unmedicated control. Medication started 3 d after challenge and medicated pellets were administered on Days 1, 2, 4, 6, 8 and 10 following start of medication. Each curve is the mean value of 2 parallel groups ($n = 40$ in each group)

Table 1. Mean concentrations (n = 6) of flumequine in muscle, plasma and liver of Atlantic cod *Gadus morhua* following multiple dose administration. The samples were collected 24 h following the last drug administration. The minimum inhibitory concentration (MIC) value for the pathogen was determined to be 0.015 $\mu\text{g ml}^{-1}$ (Samuelsen & Lunestad 1996). An area under curve (AUC) value of 533 mg h l^{-1} from Hansen & Horsberg (2000) was used

Treatment dose of flumequine (mg kg^{-1} body weight d^{-1})	Muscle ($\mu\text{g g}^{-1}$)	Liver ($\mu\text{g g}^{-1}$)	Plasma ($\mu\text{g ml}^{-1}$)	MIC $\times 4^a$ > pl. cons.	$C_{\text{max}}/\text{MIC}^b$ > 8	AUC/MIC ^b > 100
2.5	1.0 \pm 0.4	0.4 \pm 0.3	0.5 \pm 0.2	0.06	33	–
5	1.4 \pm 0.8	0.5 \pm 0.3	0.9 \pm 0.5	0.06	60	–
10	1.9 \pm 1.4	1.1 \pm 1.0	1.3 \pm 0.7	0.06	86	35533
15	4.3 \pm 1.2	2.1 \pm 0.8	2.7 \pm 0.9	0.06	180	–
25	7.8 \pm 2.9	3.1 \pm 2.5	4.5 \pm 1.5	0.06	300	–

^aCriteria suggested by Stamm (1989), pl. cons. = *in vivo* plasma concentration
^bCriteria suggested by Shojaee AliAbadi & Lees (2000), C_{max} (maximum plasma concentration) = C_{24} (plasma concentration 24 h after last medication)

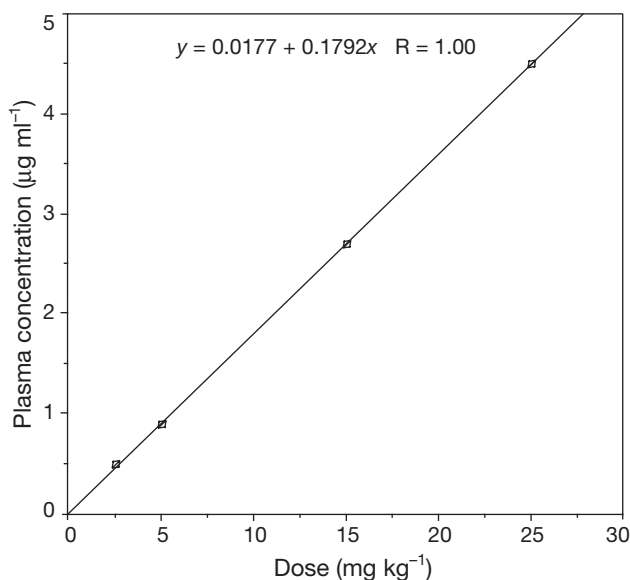


Fig. 2. *Gadus morhua*. Plasma concentration versus dose of flumequine in cod following multiple dose administrations. The samples were collected 24 h following the last drug administration

(independent *G*-test, all $p > 0.05$, $df = 1$), whereas between the various treatments, including controls, significant differences in survival were found ($R \times C$ test of independence using *G*-test, $df = 5$, $G = 78.5$, $p < 0.001$). A pairwise *G*-test of independence, with a Bonferroni-corrected p -value, showed that all treatments had significantly lower mortalities than the unmedicated group ($p < 0.001$). Furthermore, the tests showed that the groups that had received the 5 mg dose had a significantly higher mortality than the groups receiving the 25 mg dose ($p < 0.001$).

In all tanks throughout the experiment, 100% of the ration of medicated feed was consumed. The HPLC analysis showed the highest concentrations of flumequine in the fish that received the highest doses

(Table 1) and using the mean concentrations, a clear dose/concentration relationship for plasma, muscle and liver was found. This is shown for plasma in Fig. 2. Statistical treatment of the data (*t*-test) showed no differences in the mean flumequine concentration within replicate groups, while ANOVA identified significant differences among the different treatment groups for all samples ($df = 5$, $p < 0.0001$). A post hoc analysis showed that the 15 and 25 mg treatments had a significantly higher concentration of flumequine than the other groups (Newman-Keuls test, $df = 5$, $p < 0.002$).

DISCUSSION

The culture and identification of *Listonella anguillarum* from the dead fish in all groups indicate the pathogen as the probable cause of death.

The unmedicated groups suffered a final mean cumulative mortality of 82%. This is similar to the 87.5% obtained by Samuelsen & Bergh (2004) using a similar experimental set-up and confirms the validity of the challenge model and the dramatic effect of vibriosis on unprotected juvenile cod. Furthermore, the value obtained is within or close to the recommended mortality range for unmedicated controls suggested for drug dose titration trials: 50 to 90% (Amend 1981) and 30 to 70% (Elston et al. 1995). It should be emphasised that the pre-challenge is an integrated part of the model, and higher variation in mortality has been observed in challenges where pre-challenge has not been used (O. B. Samuelsen & Ø. Bergh unpubl. data).

The efficacy of flumequine in treating vibriosis in cod is clearly demonstrated in this study. The cumulative mortalities at all doses are significantly lower than the mortality of unmedicated controls. Among the treated groups the difference in mortality was statistically significant only between the 25 and 5 mg treatments, so that the doses of 2.5, 10, 15 and 25 mg

were statistically equivalent in efficacy. However, the data set suggests that a daily dose of 25 mg is needed to obtain the desired effect. The RPS values were calculated to be 49, 40, 54, 55, and 72% respectively for the 2.5, 5, 10, 15 and 25 mg treatments. In a similar study that examined the efficacy of florfenicol and oxolinic acid in the treatment of vibriosis in cod, RPS values of 61 and 68% (oxolinic acid) and 64 and 77% (florfenicol) were found using doses of 10 and 20 mg kg⁻¹ body weight d⁻¹, respectively (Samuelsen & Bergh 2004). By recalculating data from Nordmo et al. (1998), a RPS value of 64% was estimated when furunculosis-infected Atlantic salmon were treated with a dose of 25 mg flumequine kg⁻¹ body weight d⁻¹ for 10 d consecutively. On the basis of RPS values, therefore, the greatest efficacy was clearly obtained with the 25 mg dose in this study. Since no lower recommended RPS value from efficacy studies is available from the literature, a less expensive and more environmentally friendly medication regime than suggested in this study would be possible by lowering the standard of efficacy.

Tissue and plasma analysis showed that flumequine was readily absorbed and distributed from plasma to tissues (Table 1). This confirmed the results obtained by Hansen & Horsberg (2000), who calculated the volume of distribution (V_d) of flumequine in cod to be 2.4 l kg⁻¹ following a single bolus intravenous administration. In the same study, a plasma C_{max} of 3.5 µg ml⁻¹ was found 24 h after a single oral administration of 10 mg kg⁻¹ body weight. In the present study a plasma concentration of 1.3 µg ml⁻¹ was found 24 h (C_{24}) after the final medication at a dose of 10 mg kg⁻¹ d⁻¹. There is thus a difference in concentration of more than 60% between single-dose and multiple-dose studies. However, a similar effect has also been described for the quinolone oxolinic acid in cod. Using oxolinic acid, and accepting C_{24} as a proxy for C_{max} , it was shown that repeated doses of the drug generated lower maximum concentrations for plasma, muscle and liver in cod than a single-dose (Samuelsen et al. 2003b, O. B. Samuelsen unpubl.). The authors suggested that enzyme induction and increased metabolic activity might account for this effect. It should be noted, however, that the studies involving oxolinic acid involved a temperature difference of 2°C (6 and 8°C) while there is a 4°C difference (8 and 12°C) between the flumequine studies. This might have influenced the results to a certain extent by increasing the difference in C_{max} values.

An important application of pharmacokinetic data is the establishment of appropriate treatment regimes. In that respect several authors, including the corresponding author of this paper, related pharmacokinetic data of antibacterial agents in fish to the assumptions by Stamm (1989) and Shojaee AliAbadi & Lees (2000)

with the aim of predicting clinical outcome of the treatment. Taking the plasma C_{24} concentrations found in this study, a MIC value of 0.015 µg ml⁻¹ determined by Samuelsen & Lunestad (1996) and a calculated AUC value of 533 mg h l⁻¹ for flumequine in cod (Hansen & Horsberg 2000) in combination with the statements by Stamm (1989) and Shojaee AliAbadi & Lees (2000), it can be shown that all the doses of flumequine used in the present study fulfil the criteria (Table 1). A plot of the mean plasma concentrations against doses, excluding the 10 mg dose, shows a linear relationship (Fig. 2). Assuming a linear relationship in the lower part of the graph, we can predict that a dose of 0.5 mg flumequine kg⁻¹ d⁻¹ would meet the criteria of Stamm (1989) and Shojaee AliAbadi & Lees (2000) with a good margin. However, on the basis of the data presented in Fig. 1 it is unlikely that a dose of 0.5 mg kg⁻¹ d⁻¹ would provide an acceptable level of efficacy. Our data thus suggest that the assumptions by Stamm (1989) and Shojaee AliAbadi & Lees (2000) cannot be used rationally to predict clinical outcomes, at least in fish, and other criteria should be considered.

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