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On the validity of setting breakpoint minimum inhibition concentrations at one quarter of the plasma concentration achieved following oral administration of oxytetracycline

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Abstract

Plasma concentrations of oxytetracycline (OTC) were established in two Atlantic salmon (*Salmo salar*) pre-smolts populations after they had received OTC medicated feed at a rate of 75 mg OTC/kg over 10 days. One population was experiencing an epizootic of furunculosis in a commercial freshwater farm and the other was held in a laboratory. Both populations were maintained at approximately 13 °C. The mean plasma concentration in 26 health farm fish was 0.25 ± 0.06 and the 80th percentile was 0.21 mg/l. The mean concentration for 26 laboratory fish was 0.21 ± 0.06 mg/l with an 80th percentile of 0.15 mg/l.

The validity of setting a breakpoint minimum inhibitory concentration (MIC) at a quarter of these plasma concentrations was investigated. The MIC of the *Aeromonas salmonicida* isolated from the farmed fish ($n = 7$) was 0.5 mg/l and the breakpoints generated by application of the 4:1 ratio were in the range $0.03125–0.0625$ mg/l. These breakpoint values would, therefore, predict that the therapy should have had no beneficial effect and that any strain of *A. salmonicida* with MIC>0.0625 mg/l must be considered as resistant. A consideration of the pattern of the mortalities before and during the period of therapy suggests that the therapy was probably beneficial. Thus, the data obtained in this work suggest that the application of the 4:1 ratio is not a valid method of generating meaningful breakpoint MIC values.

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Published values for the MIC of OTC against *A. salmonicida* and the plasma concentrations achieved after oral administration of OTC medicated feed were applied to investigate the validity of the application of the 4:1 ratio. Breakpoints generated by the application of this ratio to these data would suggest that OTC could never have had any value in combating *A. salmonicida* infections. As this conclusion is contrary to experience, it is argued that examination of the published data reinforces the conclusion that the 4:1 ratio has little value in the oral therapy of fish disease.

1. Introduction

In a therapeutic context, a bacterium must be classified as resistant if the concentration of the agent required to inhibit it is greater than the concentration that can be achieved in the treated host as a result of a standard therapy. Methods that allow the quantitation of the concentration of an antibacterial agent (MIC) required to inhibit bacteria associated with fin-fish disease have been developed (Alderman and Smith, 2001). Equally, a number of methods that allow the quantitation of the concentration of antimicrobial agents in fish have been developed and validated. This might suggest that, in any specific situation, the resistance or sensitivity of a bacterium could be assessed simply by a direct comparison of its MIC to the concentrations of the agent that can be expected in the host. However, a number of considerations indicate that a simple arithmetic equality would be an excessively simplistic representation of the relationship required between these parameters (McCabe and Treadwell, 1986). One set of complications arises because the interaction between bacterium and an antimicrobial agent is context dependent (Smith et al., 1994). Neither the susceptibility of a bacterium nor the biological activity of antimicrobial agent will be the same in a microbial laboratory as they will be in a fish. Thus, the laboratory determined MIC could provide only an indirect measure of the susceptibility that can be expected in the host. Other complications derive from the various meanings given to the word inhibition. With respect to laboratory MIC determinations, the term inhibition refers to total loss of the ability to multiply. In therapy, however, the function that has to be inhibited is the bacterium's ability to contribute to the disease process. There are no grounds for believing in order to influence a bacterium's contribution to a disease process it would always be necessary to totally inhibit its ability to undergo cell division (O'Reilly and Smith, 1999). Thus, a laboratory-determined MIC can represent only an indirect measure of the clinically relevant properties of a bacterium (McCabe and Treadwell, 1986).

In large animal medicine the complexity of the relationships between susceptibility data generated in the laboratory and the pharmacokinetic data derived from the host has often been resolved by the application of a general rule of thumb. This approximation suggests that it is reasonable to predict that the outcome of a therapy will be beneficial for the host if the maximum plasma concentration achieved by that therapy (plasma C_{max}) is four times greater than the MIC determined in the laboratory (BSAC, 1991). This 4:1 ratio, therefore,

provides a useful, simple but essentially pragmatic definition of resistance. A bacterium must be classified as resistant if its MIC, as determined by a standard laboratory method, is greater than a breakpoint defined as one quarter of the plasma C_{max} that can be predicted for any particular therapeutic administration (Stamm, 1989). Smith (2001, 2003) has, however, argued that, although the 4:1 ratio has proved to be of value in large animal medicine, there are, as yet, no data that demonstrate that its application in fin-fish therapies is valid.

There are grounds for believing that there are fundamental differences between the aims of antimicrobial therapies given to large animals and the aims of those given to populations of fish in commercial farms. In the case of large animals, the aim of the majority of therapeutic administrations is to combat an existing infection in a particular host. In contrast, the data of Coyne et al. (in press(a)) have demonstrated that, in orally administered therapeutic treatments of fish, the fraction of the population that is actually experiencing infection rarely receive any significant concentrations of the agent. Rather they suggest that the success of therapy of fish populations should be modelled as a function of their ability to prevent the initiation of infection in healthy fish. These differences in the aims of antimicrobial therapies of large animals and of fish populations represent theoretical grounds for questioning the appropriateness of applying the 4:1 ratio to fish therapies without some experimental validation of its relevance in this context.

The work presented here reports an investigation of a therapeutic treatment of an outbreak of furunculosis in Atlantic pre-smolts in a commercial hatchery with a course of orally administered oxytetracycline (OTC). The parameters of the treatment investigated included the susceptibility of the bacterium, the concentrations of OTC in the host and the impact of the therapy on the patterns of mortality. These data were then applied to an investigation of the value of the 4:1 ratio as a method of predicting the clinical outcome of antimicrobial therapies in fin-fish farming or as a method of generating a clinically relevant definition of bacterial resistance. Experiments were also performed with pre-smolts in the laboratory to determine the extent that data generated following the administration of OTC to laboratory held fish could accurately predict those concentrations occurring in the field.

2. Materials and methods

2.1. Commercial farm study

The study was performed on fish in a single tank (tank 17) at a fresh water hatchery which contained approximately 25,000 Atlantic salmon (*Salmo salar*) pre-smolts with an average weight of 10 g. Samples were collected on the day after a 10-day course of orally administered oxytetracycline that had been prescribed in response to rising mortalities associated with the isolation of *Aeromonas salmonicida*. The days were arbitrarily numbered from the first day of OTC administration (Day 1) and samples were, therefore, collected on Day 11. The fish in this tank had previously experienced an epizootic of furunculosis that peaked on Day −37 and antimicrobial therapy was not administered. Following this epizootic, the average daily mortalities had fallen to single figures in the

week Day −13 to Day −7. In response to an increase in daily mortalities, first observed on Day −2, a course of oral OTC therapy at 75 mg/kg body weight/day was prescribed and medicated feed pellets were fed at 1% body weight on Day 1 through to Day 10. The average water temperature during the period of the therapy was 13.3 °C. Both before and during therapy, the daily mortalities were monitored, counted and recorded by farm personnel.

2.2. Sample collection

Farm personnel collected sample fish from the tank on Day 11 and classified them into categories of healthy and moribund, on the basis of their behaviour and in addition, collected dead fish. Fish swimming normally were removed by random netting and were classified as healthy. The key characteristic used to identify moribund fish were slow swimming, swimming close to the surface, swimming away from the general school of fish and/or darkened skin colour. In total, 26 (healthy fish (H1–H26), 2 moribund (M1–M2) and 10 dead (D1–D10) fish were collected.

2.3. Laboratory study

A total of 30 disease-free Atlantic salmon (*Salmo salar*), weight 92 ± 17 g, were held in dechlorinated freshwater at 12.8 °C at a flow rate of 10 l/min in a 200-l tank, Institute of Marine Research, Bergen, Norway. The OTC dosage regimen was the same as that applied to the fish in the commercial farm. Uneaten medicated feed pellets were siphoned off at the end of each feeding period and the pellets were counted to estimate the percentage of uneaten food.

2.4. Sample processing

Fish were sacrificed by a blow to the head and their blood was sampled from the caudal vein using a 1 ml heparinized syringe. The blood samples were maintained on ice, covered in tin foil and were centrifuged on site using a Labofuge 200 (Heraeus Sepatech, Dip Ino Holum, Grefsenvn 64, Oslo) at 10,000 rpm for 5 min. The external signs of the fish and internal observation of their stomach contents and signs of disease were recorded. Bacterial examination was performed at the farm site.

The fish samples were transported on ice and frozen ice blocks to the laboratory and stored at −20 °C until analysis. The bacteriological plates were transferred to an incubator at 22 °C.

2.5. Microbiology

Bacteriological examination of fish was performed by sampling from the kidney according to the method of Coyne et al. (2004) with the modification of using a 1 μl standard loop. These samples were inoculated onto Tryptone Soya agar (TSA) (Oxoid, Basingstoke, Hampshire, England) and the plates were read after 2 and 5 days incubation at 22 °C. Plates were examined for colonies with morphology, consistency and pigmen-

tation typical of *A. salmonicida* (Bernoth, 1997). Randomly selected colonies were purified and tested for their colonial morphology on Coomassie Brilliant Blue Agar (CBBA), according to the method of Cipriano and Bertolini (1988) and for their reaction with *A. salmonicida* antisera (Bionor, Mono-As, BioNor Aqua, Skien, Norway). Minimum inhibitory concentration (MIC) of OTC against strains isolated in this work was established using the protocols of Alderman and Smith (2001) using Muller Hinton agar (Difco, Sparks, USA).

A presumptive *A. salmonicida* isolated from a fish sampled before the start of antimicrobial therapy was made available by the veterinarian in charge.

2.6. HPLC system and analysis

Fish plasma were analysed according to the method of Iversen et al. (1989). The HPLC system used consisted of a Spectra Physics SP 8800 ternary HPLC-pump (Spectra-Physics, San Jose, CA, USA) connected to a Gilson 234 Autoinjector (Gilson, Middleton, WI, USA) with a 20 μl loop and a Spectra-Physics SP 8480 UV-detector operating at a wavelength of 353 nm. The integrator was model SP-4270, from Spectra-Physics. The analytical column used was 250×4.6 mm Supelcosil LC-PCNC 5 μm (Supelco, Supelco Park, Bellefonte, PA 16823, USA).

2.7. Statistical analysis

Statistica (Version 6) was employed to establish means, medians and the standard deviation (S.D.) of the distributions of OTC concentration in plasma and to run the Kolmogorov–Smirnov test. The coefficient of variation was calculated by expressing the standard deviation (S.D.) as a percentage of the relevant mean. The 95% confidence interval was calculated as the mean $\pm 1.96 \times$ S.D. To calculate the 80th percentile, the data were rank-ordered. The 80th percentile, the concentration achieved in at least 80% of the fish, was then represented by the concentration in the $(80 \times (n-1))/100$th item in the order.

With respect to any data set, outlier were identified as data points that lay outside the range specified by the mean $\pm$ three times the standard deviation of the other members of that set. Outliers were not considered in calculating means and 80th percentile values.

3. Results

3.1. Pattern of mortality

The mortality for the fish in tank 17 is presented in Fig. 1. This figure shows the daily percentage mortality recorded both before and during the epizootic. The daily mortalities were expressed as a percentage of the stock estimated to be present on the previous day. The mortality rose from Day −2 and reached a peak on Day 4. From Day 5 through to Day 11 the mortalities showed a steady decline. Unfortunately, the hatchery studied has now ceased trading and it has proved impossible to obtain the mortality data for the period after Day 11.
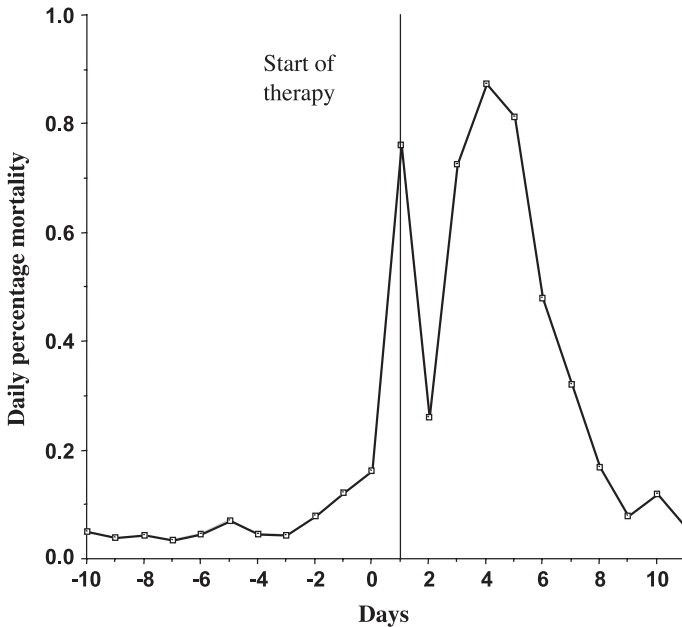
Fig. 1. Changes in daily percentage mortality in Tank 17 over time.

3.2. Bacteriology

Presumptive *A. salmonicida* colonies were not detected in bacteriological examination of kidney sampled from healthy fish. Two moribund fish, M1 and M2, yielded growth of *A. salmonicida*. The majority of the dead fish were too small for bacterial examination of their kidneys but presumptive *A. salmonicida* colonies were isolated from one of them (D3). From the fish farm investigation sampling, in total, eight presumptive *A. salmonicida*, isolated from three different fish, were examined and all gave colonies typical of A-layer positive strains on CBBA and positive agglutination with the *A. salmonicida* antisera.

Disc diffusion and MIC broth dilution methods were used to determine the susceptibility to OTC of the eight *A. salmonicida* strains isolated in this work and to one strain isolated by the veterinarian in charge at an earlier stage in the epizootic. The disc diffusion zone values for the isolates ranged between 36 and 55 mm and all nine isolates had MIC values of 0.5 mg/l OTC.

3.3. External and internal macroscopic examination of farmed fish

Food was present in the intestines of all fish classified as healthy while neither of the moribund nor 7 of the 10 dead fish showed recent signs of feeding. Externally, one of the moribund fish (M1) exhibited signs of pectoral erosion while internal examination of these two salmon revealed no macroscopic signs of disease. Dead fish showed varying external

signs, with two displaying blistering without haemorrhaging, two with dorsal fin erosion, three with bloody fins, one with a swollen abdomen, one with slight haemorrhaging under the jaw, one with a classic case of furuncles and the remaining three fish exhibited no external signs of disease. Internal examination of these dead smolts revealed no macroscopic signs of disease.

3.4. Oxytetracycline analysis

Standard curves using plasma spiked with OTC were constructed by linear regression analysis and were used to calculate the concentration of OTC in the samples and the standard curves gave a correlation with linearity of $r^2 > 0.99$. The limit of quantitation (LOQ) was 0.03 mg/l. The concentration of OTC in plasma and tissues is shown in Table 1.

3.5. Plasma OTC concentrations in farmed fish

Sufficient plasma to allow analysis of OTC concentrations was obtained from 26 healthy and one moribund fish. Quantifiable concentrations were detected in the plasma of all 26 fish analysed and the Kolmogorov–Smirnov test (K–S $d = 0.075$, $p > 0.2$) demonstrated that the distribution of the concentrations was not significantly different from normal. With respect to the healthy fish, all of which had been recently feeding, the mean concentration was 0.25 mg/l and the standard deviation was 0.06 mg/l, giving a percentage coefficient of variation of 22% (Table 1). The 80th percentile of the concentrations in the healthy fish was calculated to be 0.21 mg/l. The one moribund fish that was analysed, showed no signs of recent feeding and contained 0.21 mg/l OTC in its plasma.

3.6. Plasma OTC concentrations in laboratory fish

In the laboratory experiments, it was estimated that 70% of the feed presented was consumed and all fish examined showed some food in their intestines. Plasma samples

Table 1

Measures of plasma oxytetracycline concentrations (mg/l) in farmed and laboratory held fish and the breakpoint MIC (mg/l) values estimated from them

Parameter	Farm ($n = 26$)	Laboratory ($n = 26$)
Mean	0.25	0.21
Standard deviation	0.06	0.06
Coefficient of variation	22	30
Median	0.25	0.22
Range	0.14–0.39	0.11–0.37
80th percentile	0.21	0.15
Breakpoint MIC values[a]		
Based on mean	0.0625	0.03125
Based on 80th percentile	0.03125	0.03125

[a] Breakpoint MIC values estimated as the doubling dilution immediately below the concentration estimated by dividing the parameter by four.

were analysed from all 30 fish and quantifiable concentrations were detected in 26 of them. The remaining four fish (OTC < 0.03 mg/l) were treated as outliers. The mean plasma concentration in the laboratory fish was 0.21 mg/l ($n = 26$) and the standard deviation was 0.06 mg/l, giving a percentage coefficient of variation of 30% (Table 1).

4. Discussion

4.1. Estimating plasma concentration in farmed fish from laboratory data

Any attempt to apply pharmacokinetic data to the prediction of the outcome of a therapy requires a quantitative estimate of the susceptibility of the bacterium and an estimate of plasma concentrations (C_{max}) of the agent that will be achieved in the host. The data on bacterial susceptibility can be obtained before any therapy is initiated, but the values of the plasma C_{max} that will be achieved cannot be measured in advance. Thus, the ability of the 4:1 ratio or any other relationship between C_{max} and susceptibility to predict the outcome of a particular therapy will be a function of our ability to generate reasonably accurate predictions of the plasma concentrations that will result from that therapy.

In this work, a laboratory trial was designed to mimic the on-farm treatment with respect to dose regimen and water temperature. Although the fish in the laboratory trial were heavier than those in the commercial farm, there was a considerable degree of agreement between the distributions of plasma OTC concentrations in the two situations (Table 1). This correspondence between laboratory and field data confirms the conclusions of Coyne et al. (in press(b)) that appropriately designed laboratory trials can provide useful predictions as to the plasma concentration that will result from a particular antimicrobial therapy administered under commercial farming conditions. Coyne et al. (in press(b)) found that laboratory studies could provide a reasonably accurate prediction of the mean C_{24} but tended to underestimate the coefficient of variation. In this work, the laboratory studies provided a reasonably accurate measure of both these parameters.

4.2. Estimating a population C_{max}

In its original formulation for large animal therapies, the 4:1 ratio employs the parameter C_{max} to refer to the peak plasma concentration achieved in an individual. In fish farming, however, antimicrobial therapy is administered to a population of fish and the statistic chosen to characterise the parameter C_{max} must reflect the distribution of antimicrobial agent concentrations in the treated population. One possible way of reflecting the distribution within the population would be to use the estimated mean of C_{max} values determined in a sufficiently large (Council Regulation, 1996; NicGabhainn et al., 1996) sample of a population. However, provided that the distribution of OTC concentrations in the population is normal, then half the population will contain less than the mean concentration. Coyne et al. (in press(a)) have suggested that for estimating breakpoint MIC values, the concentration estimated to be present in at least 80% (C^{80th}_{T24h}) of the treated population might provide a better measure of the concentrations achieved.

4.3. Estimating a breakpoint MIC value

In its original formulation the 4:1 ratio should be applied to C_{max} values. In this work, data on the plasma concentration achieved 24 h after the end of therapy (C_{24}) was collected. The work of Elema et al. (1996), Uno et al. (1992), Jacobsen (1989) and Bjørklund and Bylund (1990) suggest that a reasonable estimate for the T_{max} for orally administered OTC would be at 24 h and therefore that it is reasonable to employ C_{24} as a proxy measure of C_{max}.

An estimate of an appropriate breakpoint MIC value could be made by applying the 4:1 ratio to either the mean or the 80th percentile of the OTC C_{24} values and it could be made using the data generated in the field or laboratory studies. In this work, the similarity and homogeneity of the OTC plasma concentration data sets mean that all the four possible breakpoints would be remarkably close to each other. As the Alderman and Smith (2001) protocols require that MIC values are determined using doubling dilution series that include 1 mg/l, then these breakpoint MIC values would be either 0.0625 or 0.03125 mg/ l (Table 1). In other words, the application of the 4:1 ratio to the data generated in this work would predict that the therapy would have been beneficial only if the MIC of the infecting bacterium had been lower than 0.0625 or 0.03125 mg/l.

The MICs of the nine isolates of *A. salmonicida* made from this disease event were all 0.5 mg/l or 8- to 16-fold greater than the breakpoint suggested by the application of the 4:1 rule. Thus, the application of this ratio would predict that the administration of OTC studied here would have had no beneficial effect and that the isolates of *A. salmonicida* should be classified as resistant.

4.4. Estimating the outcome of the therapy

The application of the 4:1 ratio to therapy in fish farming can only be considered valid if the predictions it makes are consistent with the observed facts. With respect to the disease event studied here, a comparison of the MIC values with either of the breakpoints generated by the ratio would predict that the therapy should have no impact on the mortalities. Thus, any demonstration that the therapy was beneficial, that it did, in reality, reduce the mortalities, would represent evidence that the application of the 4:1 rule was invalid.

The unambiguous demonstration of the efficacy of any therapeutic treatment performed in a commercial farm is rarely straightforward. In the commercial context, it is extremely rare that an untreated control group of fish is maintained on the farm. In the absence of such a control group, estimates of the efficacy of the therapy cannot be made by comparing the mortalities in populations that were not treated with OTC. The only way in which an estimate of the probable consequence of the therapy can be made is to predict the pattern of changes in mortality that might be expected from a successful therapy and then to compare these predictions with the changes observed in the farm.

Coyne et al. (in press(a)) have suggested that the primary mode of action of antimicrobial therapy of fish populations is to inhibit de-novo infections of healthy members of the treated population. They propose that moribund fish contained insufficient levels of OXA to provide protection against either the initiation or progression of an

infection. The infections in these fish would proceed without hindrance and the fish would, therefore, die during the period of therapy. The number of these deaths during therapy would be a function of the infection rate immediately before therapy was administered but would be independent of the efficacy of that therapy. Thus, even in a successful therapy, mortalities would continue to rise for the first few days of administration of the agent. If orally administered, therapy acts by preventing de-novo infections, then any impact on the patterns of mortality would be manifest only some days after the start of therapy. The length of this delay would be related to the mean time between the establishment of infection in a fish and the death of that fish as a consequence of that infection.

The pattern of mortality shown in Fig. 1 demonstrated that, for the first 4 days after therapy was initiated, the mortalities rose, but after 4–5 days, fell dramatically. These changes in mortality would be reasonably consistent with those that would be expected for a successful therapy provided it was accepted that the time from infection to death was 4–5 days. The suggestion that, for Atlantic salmon pre-smolts at 13 °C, death might occur an average of 4–5 days after an infection had become established is not unreasonable (Bricknell, 1995).

It is not possible to demonstrate unambiguously that the therapy studied in this work was successful. All that can be claimed is that mortality data are not inconsistent with that expected following a successful therapy. It remains a theoretical possibility that the decline in mortality that occurred 4–5 days after the start of therapy is unrelated to the therapy and would have occurred even if no OTC had been administered.

If the therapy is considered to have been successful then this is in direct conflict with the prediction made by the application of the 4:1 ratio to the pharmacokinetic data. Thus, if the therapy is considered to have had a beneficial effect on the treated fish population, we must conclude that, in this context, the application of the 4:1 ratio was not valid.

4.5. Published MIC and C_{max} values

Uhland et al. (submitted for publication) have recently used the Alderman and Smith (2001) protocols to determine the MIC values of OTC with respect to 50 strains of *A. salmonicida*. The lowest MIC they detected was 0.125 mg/l. The breakpoints generated in this work by the application of the 4:1 ratio indicate that any strain of *A. salmonicida* for which the MIC of OTC was >0.0625 mg/l should be considered clinically resistant. Thus, all the *A. salmonicida* strains studied by Uhland et al. (submitted for publication) would have to be classified as resistant. The attribution of resistant is, of course, only valid with respect to a specified therapeutic context. In this case, it refers to the probable outcome of a therapy aimed at the control of furunculosis in Atlantic salmon pre-smolts at 13 °C by the administration of 75 mg/kg OTC for 10 days. Thus, the application of the 4:1 ratio suggests that the administration of OTC to treat furunculosis under these conditions could never be successful. This conclusion would appear to be totally contrary to all experience and, therefore, the arguments by which it was arrived at must at some point be flawed. The conclusion was the product of the application of a specific rule (the 4:1 ratio) for generating breakpoint MIC values to a specific set of pharmacokinetic data. Logically, if the conclusion is absurd, the flaw in the argument must lie either in the assumption that

the particular OTC concentrations detected in this work can be taken as typical of treatments in general or in the assumption that the 4:1 rule is a legitimate method of estimating breakpoint MIC values.

Reviews of the relevant literature (Stoffregen et al., 1996; Pursell, 1998) have, unfortunately, revealed very few reports of plasma concentrations achieved following multiple oral administrations of OTC-medicated feed to salmonid fish in fresh water. Jacobsen (1989) reported a mean ($n = 3$) blood concentration of 0.69 mg/l in rainbow trout fed OTC for 8 days at 50 mg/kg. Herman et al. (1969) fed OTC at 75 mg/kg for 14 days to various populations of trout and detected mean ($n = 5$) concentrations of 0.75, 0.9, 0.28 and 0.68 mg/l in their plasma. The mean plasma concentrations (C_{24}) detected in this work in farmed fish (0.25 mg/l, $n = 26$) and in laboratory held fish (0.21 mg/l, $n = 26$) are at the lower end of the range of previous published values. It could be argued that the concentrations measured in this work are not typical of those that might be expected in OTC therapies. However, it should be noted that the 4:1 ratio would only have predicted therapeutic success against a bacterium with an MIC of 0.5 mg/l if the predicted plasma concentrations had been >2.0 mg/l. None of the published studies provide any ground for suggesting that plasma concentrations of this level could be achieved by oral administration of OTC-medicated feed to freshwater salmonids. These considerations of published plasma concentrations again support the conclusion that, in the context of oral administration of OTC in fish farming, the application of the 4:1 ratio is not valid.

Tsoumas et al. (1989) approached the setting of a breakpoint MIC for OTC against *A. salmonicida* by examining the frequency of distributions of MIC values determined using Difco Antibiotic Medium 3. Their work suggested that any strain with an MIC ≤ 1 mg/l should be considered as sensitive. This observation could only be compatible with the 4:1 ratio if plasma concentrations ≥ 4 mg/l could be regularly achieved. Even in the nine reports where oral intubation has been the method of administration (Strasdine and McBride, 1979; Nouws et al., 1992; Elema et al., 1996; Rogstad et al., 1991; Uno et al., 1992; Bjørklund and Bylund, 1990, 1991), concentrations >3.5 mg/l have only been reported on one occasion (Bjørklund and Bylund, 1990). Thus, the breakpoints of Tsoumas et al. (1989) are incompatible with the application of the 4:1 ratio to the published data on the plasma concentrations of OTC that can be achieved by any form of oral administration.

5. Conclusions

There must be a relationship between the concentration of any therapeutic agent that is achieved during therapy, the susceptibility of any invading bacterium and the outcome of the therapy. It is possible to obtain data on the concentrations of antimicrobial agents that can be achieved in fish following multiple oral administrations of medicated feed to fish in commercial farms. It is also possible to obtain data on the susceptibility of bacteria infecting fish. At present, however, there is no validated formula for using these two sets of data to make predictions as to the outcome of any therapy. In large animal medicine, it has been found that if the plasma concentration is at least four times the MIC of the infecting bacteria, it is reasonable to predict that the therapy will be beneficial to the host.

The work presented here demonstrated that there are strong reasons to suggest that the application of this 4:1 ratio in the context of the oral administration of OTC to fish is not valid.

Acknowledgements

References

Alderman, D., Smith, P., 2001. Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish disease. Aquaculture 196, 211–243.

Bernoth, E.-M., 1997. Diagnosis of furunculosis: the tools. In: Ellis, A.E., Bernoth, E.-M., Midtling, P., Olivier, G., Smith, P. (Eds.), Furunculosis: Multidisciplinary Fish Disease Research. Academic Press, London, pp. 98–158.

Bjørklund, H., Bylund, G., 1990. Temperature-related absorption and excretion of oxytetracycline in rainbow trout (Salmo gairdneri R.). Aquaculture 84, 363–372.

Bjørklund, H., Bylund, G., 1991. Comparative pharmacokinetics and bioavailability of oxolinic acid and oxytetracycline in rainbow trout (Oncorhynchus mykiss). Xenobiotica 21, 1511–1520.

Bricknell, I.R., 1995. A reliable method for the induction of experimental furunculosis. J. Fish Dis. 18, 127–133.

BSAC, 1991. A guide to sensitivity testing. Report of the working party on antibiotic sensitivity testing of the British Society for Antimicrobial Chemotherapy. J. Antimicro. Chemother. 23 (Suppl. D), 1–47.

Cipriano, R.C., Bertolini, J., 1988. Selection for virulence in the fish pathogen Aeromonas salmonicida using Coomassie brilliant blue agar. J. Wildl. Dis. 24, 672–678.

Council Regulation 281/96, 1996. Amendment to Council Regulation (EEC) 2377/90 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Communities, L 37, 9–11.

Coyne, R., Hjeltnes, B., Bergh, Ø., Andersen, K., Rudra, H., Smith, P., 2004. Quantitative properties of data generated by the examination of Aeromonas salmonicida infected fish by the standard bacteriological loop. Aquaculture, 236, 27–35.

Coyne, R., Bergh, Ø., Samuelsen, O., Andersen, K., Lunestad, B.T., Nilsen, H., Dalsgaard, I., Smith, P., in press(a). Attempt to validate breakpoint MIC estimated from pharmacokinetic data obtained during oxolinic acid therapy of winter ulcer disease in Atlantic salmon (Salmon salar).

Coyne, R., Samuelsen, O., Kongshaug, H., Andersen, K., Dalsgaard, I., Smith, P., Bergh, Ø., in press(b). A comparison of oxolinic acid concentrations in farmed and laboratory held rainbow trout (Oncorhynchus mykiss) following oral therapy. Aquaculture.

Elema, M.O., Hoff, K.A., Kristensen, H.G., 1996. Bioavailability of oxytetracycline from medicated feed administered to Atlantic salmon (Salmo salar L.) in seawater. Aquaculture 143, 7–14.

Herman, R.L., Collis, D., Bullock, G.L., 1969. Oxytetracycline residues in different tissues in trout. U.S. Wildlife Service Technical Paper 37, 1–6.

Iversen, B., Aanesrud, A., Kolstad, A.K., 1989. Determination of oxytetracycline in plasma from rainbow trout using high performance liquid chromatography. J. Chromatogr. 493, 217–221.

Jacobsen, M.D., 1989. Withdrawal times of freshwater rainbow trout (*Salmo gairdneri* Richardson) after treatment with oxolinic acid, oxytetracycline and trimethoprim. J. Fish Dis. 12, 29–36.

McCabe, W.R., Treadwell, T.L., 1986. In-vitro susceptibility tests; correlations between sensitivity testing and clinical outcome in infected patients. In: Lorian, C. (Ed.), Antibiotics and Laboratory Medicine. Williams and Wilkins, Baltimore, pp. 925–937.

NicGabhainn, S., Pursell, L., McCormack, F., Barker, G., Alderman, D., Smith, P., 1996. How many fish are there in an adequate sample? In: Haagsma, N., Ruiter, A. (Eds.), Proceedings of the EuroResidue III Conference: Residues of Veterinary Drugs in Food, pp. 755–759. Veldhoven, The Netherlands.

Nouws, J.F.M., Grondel, J.L., Boon, J.H., van Ginneken, V.J.T., 1992. Pharmacokinetics of antimicrobials in some freshwater fish species. In: Alderman, D.J., Michel, C.M. (Eds.), Chemotherapy in Aquaculture: From Theory to Reality. Office International des Epizooties (OIE), Paris, pp. 437–447. March.

O'Reilly, A., Smith, P., 1999. Development of methods for predicting the minimum concentrations of oxytetracycline capable of exerting a selection for resistance to this agent. Aquaculture 180, 1–11.

Pursell, L., 1998. Comparison of the kinetics and efficacy of oxytetracycline salts in farmed fish and the validation of a bioassay technique for their detection in salmonids. PhD Thesis, National University of Ireland, Galway.

Rogstad, A., Hormazabal, V., Ellingsen, O.F., Rasmussen, K.E., 1991. Pharmacokinetic study of oxytetracycline in fish: 1. Absorption, distribution and accumulation in rainbow trout in freshwater. Aquaculture 96, 219–226.

Smith, P., 2001. Accuracy, precision and meaning of antimicrobial agent susceptibility testing of bacteria associated with fish diseases. Aquaculture 196, 253–266.

Smith, P., 2003. Towards rational decision making in antimicrobial therapy. Proceedings of the Seminar of the CIHEAM Network TECAM, Izmir 21–23 May 2003. Options Mediterraneennes. Serie Cahiers. Zaragoza: CIHEAM, FAO, MARA, TFF. Under preparation.

Smith, P., Hiney, M.P., Samuelsen, O.B., 1994. Bacterial resistance to antimicrobial agents used in fish farming: a critical evaluation of method and meaning. Annu. Rev. Fish Dis. 4, 273–313.

Stamm, J.M., 1989. In vitro resistance by fish pathogens to aquacultural antibacterials, including the quinolones difloxacin (A-56619) and sarafloxacin (A-56620). J. Aquat. Anim. Health 1, 135–141.

Stoffregen, D.A., Bowser, P.R., Babish, J.G., 1996. Antibacterial chemotherapeutants for fin-fish aquaculture: a synopsis of laboratory and field efficacy studies. J. Aquat. Anim. Health 3, 181–207.

Strasdine, G.A., McBride, J.R., 1979. Serum antibiotic levels in adult sockeye salmon as a function of route of administration. J. Fish Biol. 15, 135–140.

Tsoumas, A., Alderman, D.J., Rodgers, C.J., 1989. *Aeromonas salmonicida*: development of resistance to 4-quinolone antimicrobials. J. Fish Dis. 12, 493–507.

Uhland, C.F., Higgins, R., Hélie, P., submitted for publication. Comparison of antimicrobial disk diffusion and dilution susceptibility tests, for *Aeromonas salmonicida* and the tetracycline family (submitted to Aquaculture).

Uno, K., Aoki, T., Ueno, R., 1992. Pharmacokinetics study of oxytetracycline in cultured rainbow trout, amago salmon and yellowtail. Jap. Soc. Sci. Fish 58, 1151–1156.