

Changes in oxytetracycline resistance of intestinal microflora following oral administration of this agent to Atlantic Salmon (*Salmo salar* L.) smolts in a marine environment

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Accepted 16 June 1997

Abstract

Atlantic salmon were held in experimental tanks and oxytetracycline–HCl was administered at 112.5 mg/kg body weight per day for 12 days via medicated feed. The frequency of resistant strains, defined as those capable of colony formation on 2216V medium containing 25 µg/ml oxytetracycline, was monitored in samples taken from the intestinal contents of the fish ($n = 5$). No evidence for a selection of resistant strains in these intestinal microflora was detected either during the period of administration or in the subsequent 16 days during which the fish were fed unmedicated feed. The range of the mean frequencies of resistance of the intestinal flora in the period after medication (0.1%–9.9%) were always lower than the frequency of resistance in the microflora of the feed (16.0%) which was fed to them. The analysis of the water samples ($n = 5$) obtained on each sampling day showed that frequency of resistance increased significantly during the experimental period (28 days). It is argued that the high frequencies of resistance that were detected in some of the water samples taken towards the end of the experimental period may have been the result of increasing accumulation of uneaten feed in the tanks, rather than a consequence of the presence of oxytetracycline. © 1997 Elsevier Science B. V.

Keywords: Oxytetracycline; Resistance; Atlantic salmon; Marine environment; Feeding; Intestinal microflora

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1. Introduction

The emergence of strains of bacteria resistant to antimicrobial agents is one of the main factors that limits their value in the control of bacterial diseases of fish (Smith et al., 1994). It is possible that selection for these resistant strains occurs in the environment of fish farms (Austin, 1985; Husevåg et al., 1991; Samuelsen et al., 1992; Kerry et al., 1994) or in non-target organisms (Ervik et al., 1994a,b; Kerry et al., 1995). The fact that the primary aim of antimicrobial therapy is to deliver the agents to the fish undergoing treatment would suggest, however, that selection within the target organism would also play an important role in the emergence of resistant strains.

A number of authors have investigated the emergence of resistance strains within the intestinal flora of fish which have received oral antimicrobial therapy. Takemaru and Kusuda (1988) have reported on studies of the use of josamycin in yellowtail and Sugita et al. (1988, 1989) have studied the consequence of oxytetracycline and oxolinic acid therapy in goldfish. An increase in the frequency of oxytetracycline resistance following the use of this agent was detected (Sugita et al., 1988) but little change in resistance patterns was detected following the use of oxolinic acid (Sugita et al., 1989). Austin and Al-Zahrani (1988) studied the frequencies of resistance in the intestinal microflora of rainbow trout held in fresh water following the administration of erythromycin, oxolinic acid, oxytetracycline and sulphafurazole. During the administration of oxytetracycline, the frequency of resistance increased from an initial value of 15% to 85% after 10 days of therapy. The selection for increased resistance frequencies detected following the oral administration of other agents was, however, significantly less. DePaola (1995) studied the consequences of oral oxytetracycline therapy of aquarium held channel catfish. Prior to the administration of oxytetracycline, the frequency of resistance in the fraction of the intestinal flora that could be cultured on MacConkey agar varied from 20–40% but at the end of a ten day therapy it had risen to 100%. DePaola et al. (1995) studied the consequences of oxytetracycline therapy in catfish in a farm environment during two different seasons. Using a direct method of assessing resistance they detected a rise from 0.3% to 48% in the autumn and from 3.9% to 82% in the spring. When they used a more sophisticated method that allowed them to detect inducible resistance, the respective increases were from 34% to 84% in the autumn, and from 23% to 89% in the spring.

All the above studies of the consequences of the use of oxytetracycline, for the emergence of resistant intestinal microflora, have followed its use in freshwater fish. In the studies reported here, the frequency of oxytetracycline resistance in the intestinal microflora of salmon smolts being held in seawater flow-through tanks was monitored following the oral administration of oxytetracycline–HCl.

2. Material and methods

2.1. Experimental fish

Healthy Atlantic salmon (*Salmo salar* L.) smolts ($n = 150$) were held in a seawater flow-through tank ($3.85 \times 0.8 \times 0.75$ m). The level of water in the tank was maintained at a height of 0.5 m and the flow was sufficient to provide a total water exchange every 4.5 h. During the experimental period, the seawater flowing through the tanks had a

salinity of 30.5%–32.5‰, water conductivity of 36–41 mS/cm, a dissolved oxygen level of 9.4–12.2 mg/l, pH 8.2–8.5 and a temperature of 11.0–15.5°C.

2.2. Experimental oxytetracycline therapy

The initial experimental design assumed that fish would feed at 3.2% body weight per day and it was intended that the daily dose of oxytetracycline–HCl would be 125 mg/kg. To this end, feed was surface coated with an oxytetracycline (Vetrapharm, UK) suspension in cod liver oil. Medicated feed was prepared on a daily basis and was administered twice daily to appetite. During the experimental period the average daily intake was 2.8% of body weight. Thus, oxytetracycline was administered at an average of 112.5 mg/kg per day over the 12-day period.

2.3. Sample collection

Samples were collected the day before medicated feed was administered (Day-12), during the administration of medicated feed on Day-7, and after medication ended on Days 1, 6, 11 and 16. On each sampling day, five fish and five water samples were taken randomly from the experimental tank. The five fish were placed in a 0.005% (w/v) benzocaine solution. Intestinal contents were collected from each fish by running two fingers along both ventral sides of the salmon, from just below the gills to the anal fin. The extruded material was collected in pre-weighed sterile containers and stored on ice for bacteriological analysis which, in each case, was carried out within 4 h. Fish whose intestinal contents had been extracted were not returned to the experimental tank.

2.4. Microbial analysis

Bacteriological analysis of the water, intestinal contents and the fish feed was performed using 2216V medium (Väättänen, 1977) with and without the addition of 25 µg/ml oxytetracycline. The protocols used have been described by Kerry et al. (1994, 1995).

2.5. Definition of resistance

In these experiments, the term resistant is defined as the ability to grow on 2216V medium containing 25 µg/ml oxytetracycline. The frequency of resistance in a sample is defined as one hundred times the number of colony forming units detected on 2216V medium containing 25 µg/ml oxytetracycline divided by the number on 2216V medium with no addition of oxytetracycline.

2.6. Criteria for evidence of selection

The criteria that the data would have to meet if the claim that they demonstrated evidence for selection of an increase in the frequency of resistance were set prior to the performance of these experiments. These were that there should be a statistically significant increase in the resistance frequency data collected on any one day when compared to the data collected prior to the treatment on Day-12.

3. Results

3.1. Analysis of feed

The mean number of colony forming units (cfu) per gram in 12 samples of the commercial feed pellets used in these experiments was $1.3 \pm 1.0 \times 10^4$ cfu/g and the mean frequency of resistance was $16.0 \pm 17.8\%$. After surface coating the pellets with oxytetracycline, no bacteria could be cultured from samples of the feed.

3.2. Analysis of frequency of resistance data

The data sets collected concerning the frequencies of resistance, in both the intestinal contents and the water, were tested for normality employing tests of skew and kurtosis (Tabachnik and Fidell, 1989). The findings were acceptable (< 1.96) at each level of the independent variable and, therefore, the data appeared to be amenable for parametric analyses using students *t*-tests. However, *F*-tests comparing the variances between groups indicated that the assumption of homoscedasticity was not met. Thus, the next most powerful inferential statistic for these data, the Mann–Whitney U (Siegal, 1956) was employed.

3.3. Analysis of intestinal contents

Visual examination of the intestinal contents of all fish sampled in this work demonstrated that they were actively feeding. The frequencies of resistance to oxytetracycline in the fraction of the intestinal microflora of the smolts that were cultured on each sampling day are shown in Fig. 1. Before medicated feed was given (Day-12) the mean (\pm standard deviation) frequency of resistance was $4.7 \pm 4.8\%$ and after being fed oxytetracycline-medicated feed for 5 days (Day-7), the mean frequency of resistance

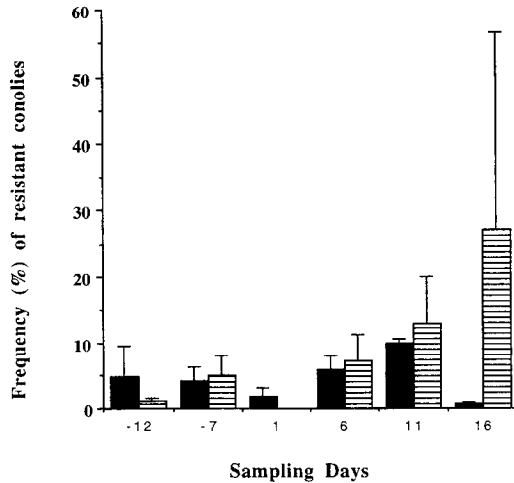


Fig. 1. Frequencies of resistance in microflora cultured on various days during and after a 12-day oral administration of oxytetracycline. Solid columns indicate mean frequencies in intestinal microflora ($n = 5$) and striped columns indicate mean frequencies in water samples ($n = 5$). Standard deviations are indicated by the error bars extending from each column. Data for the water samples collected on Day 1 are not included.

decreased very slightly to $4.1 \pm 2.1\%$. On the day after the end of the 12-day medication period (Day 1) the frequency of resistance was found to be $1.7 \pm 1.3\%$ and in the fish sampled 6, 11 and 16 days after the end of therapy the mean frequencies were $5.9 \pm 2.1\%$, $9.9 \pm 0.7\%$ and $0.1 \pm 0.1\%$, respectively. Using the non-parametric method of Mann–Whitney, none of the data sets were sufficiently different from those collected on Day-12 to justify the hypothesis that selection for increased resistance had occurred.

3.4. Analysis of holding water samples

Seawater samples ($n = 5$) were collected on the same days as were the fish samples and again the frequencies of resistance to oxytetracycline in the fraction of the microflora that were cultured on these days are shown in Fig. 1. Before the commencement of therapy, the mean frequency of oxytetracycline resistance in the water samples was $1.0 \pm 0.5\%$. By Day-7, the frequency of resistance significantly increased to $4.9 \pm 3.1\%$. In three of the five water samples taken at the end of therapy (Day 1), no resistant colony forming units were detected. In these samples, however, the total numbers of bacteria counted were low (mean 1.2×10^2 cfu/ml) compared to those determined on all other sampling days (mean 2.0×10^3 cfu/ml). As a consequence, the limit of detection of the frequency of resistance in these samples was unacceptably high and the data collected on this day were not analysed further. The frequency of resistance determined 6, 11 and 16 days after the end of therapy were $7.2 \pm 3.9\%$, $12.8 \pm 7.3\%$ and $27.0 \pm 29.5\%$, respectively.

The differences between the data obtained prior to the start of therapy and those obtained on Days-7, 6, 11 and 16 were analysed using the Mann–Whitney test. On all sampling days following the commencement of treatment, the data collected were sufficiently different to those data collected prior to treatment to allow the acceptance of the hypothesis that selection for increased resistance had occurred. This analysis also demonstrated that the resistance frequencies determined at the end of the experiment (Day 16) were significantly higher than those determined 6 days after the end of the therapy.

4. Discussion

The data presented in this paper were collected in order to detect whether the administration of oxytetracycline to the fish resulted in a selection for an increase in the frequency of resistance. On no occasions, after the commencement of the oral therapy, did the data generated from the culturable fraction of the intestinal microflora meet these conditions. Thus the data presented here provide no evidence that selection for increased resistance occurred in the fishes intestine either during the 12 days when oxytetracycline was administered at 112 mg/kg/day or during the subsequent 16 days.

In interpreting the significance of this observation two related factors must be born in mind. Firstly the number of fish sampled at any one time was small (CVMP, 1993; NicGabhainn et al., 1996). The influence of this small sample size was greater because of the extent of fish to fish variation in the data collected on a number of sample days. In this regard the variation in the data collected on the day prior to the commencement of the administration of oxytetracycline was of particular significance.

The failure to detect evidence for selection in the experiment reported here can be contrasted with the data presented in other studies of oxytetracycline administration to fish. Large and significant changes in the frequency of resistance were detected by Austin and Al-Zahrani (1988) following the administration of oxytetracycline to rainbow trout at 75 mg/kg and by DePaola (1995) and DePaola et al. (1995) following its administration to catfish at 50 mg/kg. Equally the data presented here would appear to be at odds with the data used to calculate the no-effect concentration of oxytetracycline in mammals (WHO, 1990). In these studies administration of 2 mg/kg oxytetracycline to rats was shown to have no effect on the frequency of resistance in their intestinal flora but changes in this parameter were detected after the administration of 20 mg/kg. In humans the no-effect concentration was established, using the same resistance criteria, to be 3 mg/kg.

The apparent contrast between these published data and those presented here may be a function of the experimental conditions employed in the various studies. The most obvious difference in these experimental conditions would be the presence of seawater in the experiments reported here. Lunestad and Goksøyr (1990), Barnes et al. (1995), Pursell et al. (1996) and Smith et al. (1996) have demonstrated that, in the presence of the divalent ions that occur in seawater, the biological activity of oxytetracycline is dramatically reduced. Smith et al. (1996) have estimated that in the presence of these ions, at the concentrations they achieve in seawater, the bioavailability of oxytetracycline would be approximately 2–7%. Pursell and Smith (1994) have further argued that in salmon living in the marine environment the concentrations of divalent cations present in the intestines would be equal to, or would exceed, those present in the seawater itself. Thus, it is reasonable to postulate that 125 mg/kg oxytetracycline in the matrix of the salmon intestine would be equivalent to 2.5 ($125 \times 2/100$) mg/kg in a terrestrial or fresh water matrix. These considerations would, therefore, suggest that there is not necessarily any major or inexplicable contradiction between the conclusion of the various studies that have been performed on the consequences of oxytetracycline administration to fish and mammals.

Smith et al. (1994) have stated that resistance is not an objective property of a bacterial strain but is a relative and context dependent property. Therefore, in any comparison of data on frequencies of resistance, attention must be paid to the empirical definitions of resistance used to generate the data being compared. In the work of DePaola (1995) and DePaola et al. (1995) resistance is defined as the ability to form colonies on MacConkey agar in the presence of 25 $\mu\text{g}/\text{ml}$ of the agent. In the work reported here resistance was also defined as the ability to form colonies in the presence of 25 $\mu\text{g}/\text{ml}$ oxytetracycline but in this case the medium employed was 2216V. Pursell et al. (1996) have presented data showing that, in this medium, the biological activity of oxytetracycline is reduced to 6.4–25% of its activity in Mueller–Hinton agar. Thus, the inclusion of 25 $\mu\text{g}/\text{ml}$ oxytetracycline in 2216V medium exerts a selection equivalent to approximately 6.2–1.6 $\mu\text{g}/\text{ml}$ oxytetracycline in Mueller–Hinton agar. Tsoumas et al. (1989) suggested that concentrations in the range 2–4 $\mu\text{g}/\text{ml}$ oxytetracycline in Antibiotic Medium 3 (Difco, Detroit, USA) would represent a suitable breakpoint for differentiation between sensitive and clinically resistant strains of *Aeromonas salmonicida*. If we postulate that the activity of oxytetracycline in Mueller–Hinton and

Antibiotic Medium 3 is similar, this would suggest that the selection conditions used in the present work would have selected the majority of strains which possessed clinically significant resistance. The same arguments would suggest that the selection conditions used by DePaola et al. (1995) would have resulted in an underestimate of the frequencies of such strains. Given the fact that the definition of resistance used in this work is more inclusive than that used by DePaola (1995) and DePaola et al. (1995) then the differences in the results presented, particularly the dramatically lower frequencies obtained in this work, cannot be a function of any differences in empirical definitions of resistance.

The presence of a significant (16%) frequency of resistance in the microflora of the feed administered to the fish is a complicating factor in analysing the results obtained in this work. DePaola (1995) faced a similar problem but his analysis suggested that the resistant flora present in the feed he used did not play a significant role in the resistant flora he isolated from the intestines of the catfish receiving that food. The data presented by Kerry et al. (1995) would also suggest that resistant microorganisms that are found in fish feed have a very limited ability to survive in the marine environment. Although the design of the experiments reported here does not allow any detailed elucidation of the role of these food derived bacteria, two observations can be made. The first is that on all sampling days the frequency of resistance in the intestinal microflora of the fish was lower than that in the flora of feed being administered to them. The second is that any debate concerning the possible contribution of the feed derived resistant microorganisms to an increase in the frequency of resistance in the intestinal flora is, in this case, unnecessary. No evidence of such an increase was obtained in this work.

With respect to the frequencies of resistance detected in the microflora that could be cultured from water samples, DePaola et al. (1995) reported a dramatic but transitory increase coinciding with the period of therapy. Interestingly, they also reported an increase in resistance frequency in the flora from untreated control ponds. The data collected in the work reported here provides evidence that a significant increase in resistance frequency was detected during the period of therapy. In contrast to the data of DePaola et al. (1995), our data would also support the hypothesis that the frequency of resistance continued to increase after the end of the therapy period. In interpreting data on changes in the frequency of resistant strains, care must be taken to avoid making simple causal statements linking these changes to a putative selective pressure exerted by the presence of oxytetracycline in the system. McPhearson et al. (1991), Kapetanaki et al. (1995) and Vaughan et al. (1996) have all demonstrated that increased frequencies of strains resistant to oxytetracycline can occur in the absence of the agent itself. Kapetanaki et al. (1995) and Vaughan et al. (1996) have presented evidence to support the hypothesis that elevations of the frequencies of resistance to oxytetracycline may be expected in any aquatic environment where there is accumulation of fish feed. Visual monitoring of the water quality in the fish tanks used in the study reported here suggested that there was an increase, over the duration of the experiment, in the amount of uneaten feed accumulating in the experimental tanks. In such an environment the data of Kapetanaki et al. (1995) and Vaughan et al. (1996) would predict that an increase in the frequency of resistance to oxytetracycline in the water microflora would occur in the absence of any oxytetracycline.

In conclusion, the data presented in this paper demonstrate that the emergence of strains resistant to oxytetracycline in the microflora of the intestines of Atlantic salmon held in seawater is not a necessary consequence of the oral administration of this agent. This work provides no empirical data on the factors that underlie this slightly surprising result. The published data would suggest that strains of pathogenic bacteria, that possess clinically significant resistance to oxytetracycline, do emerge as a consequence of therapy with this agent in the marine environment (Smith et al., 1994). The data presented in this paper fail to identify, in an unambiguous manner, where such a selection occurs.

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