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Effects of three dosages of oral oxolinic acid treatment on the selection of antibiotic-resistant *Aeromonas*: Experimental approach in farmed trout

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Abstract

To determine the effect of oxolinic acid (OA) dosage treatment on the selection of antibiotic-resistant bacteria in farmed trout, three different dosages of OA were administered over seven consecutive days, *via* medicated feed, to healthy rainbow trout reared in experimental tanks supplied with river water. OA dosage licensed in France (12 mg/kg/day), over-dosage (24 mg/kg/day) and under-dosage (6 mg/kg/day) were tested. The importance of trout gut and rearing waters as potential sites of selection for antibiotic-resistant bacteria in fish farming were investigated (i) by quantification of heterotrophic microbiota on GSP selective medium and of motile *Aeromonas* on TSA medium, and (ii) by characterisation of *Aeromonas* OA susceptibility levels before, during and until two weeks after treatment.

In the intestines of treated and control fish, bacterial counts were severely reduced during the entire experiment, and *Aeromonas* populations remained susceptible to OA. In contrast, bacterial counts in output water of both medicated and control tanks were significantly higher than in input water. OA minimum inhibitory concentration distributions of *Aeromonas* estimated with a replica plating method (GSP plates supplemented with OA concentrations from 0.03 mg/l to 2 mg/l) indicated that the treatment, whatever the dosage, had induced the emergence of OA-resistant *Aeromonas* in output water. OA MIC of resistant *Aeromonas* isolated on GSP agar (estimated MIC $\geq$ 4 mg/l) determined using an agar dilution method on Mueller–Hinton agar confirmed the presence in output water of highly OA-resistant *Aeromonas* (MIC $\geq$ 128 mg/l). Between one and two weeks after the end of treatment, those highly OA-resistant *Aeromonas* were all the more prevalent among total *Aeromonas* populations that treatment dosage was higher. Results suggested that selection and emergence of OA-resistant *Aeromonas* had probably occurred in faecal matters after excretion, rather than in the fish intestines.

Keywords: Antimicrobial resistance; Oxolinic acid; Rainbow trout, Dosage effect; *Aeromonas*

1. Introduction

To control bacterial infectious diseases and limit fish mortality, antimicrobial agents are widely used in fish farms. High-levels of antimicrobial resistance have been observed among fish pathogenic bacteria and more

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generally in aquatic bacterial flora isolated in and around fish farms, which are currently assumed to be linked to this antibiotic use (Akinbowale et al., 2006; Migliore et al., 2002; Miranda and Zemelman, 2002; Schmidt et al., 2000). Although direct causality or even simple correlation between antibiotic use and antibiotic resistance were often not well-established in previous studies (Alcaide et al., 2005; Schmidt et al., 2000), some authors consider that the overprescription and the misuse of antimicrobials are the major cause of bacterial resistance in the aquaculture context (Cabello, 2006).

In the European Union, the use of antimicrobial drugs is restricted to therapeutic use, and only few antimicrobial agents are licensed for fish application. For economic and practical reasons, drugs are mainly administered as medicated feed in fish production, so that the intake of antimicrobial drug directly depends on appetite, which is often lower for diseased fish than for healthy fish, and on fish position in the population hierarchy (McCarthy et al., 1992; Samuelsen, 2006). Therefore, some diseased fish could ingest medicated feed at levels unsufficient for obtaining a bacteriological cure. On the other hand, even in healthy fish, treatments may promote conditions to select antimicrobial-resistant bacteria among the intestinal microbiota (Giraud et al., 2006). At the same time, some part of the distributed drug is directly released into the aquatic environment, and since non-metabolised molecules and some active metabolites are excreted by the fish into the water, this may also contribute to the emergence and/or persistence of antimicrobial-resistant bacteria in microbiota of aquatic environments exposed to aquaculture activities (Gordon et al., 2006). It is likely that the selective effect exerted on the microbiota of fish intestines and of fish farms environment depends on the antimicrobial dosages which are used for fish treatment. Despite the importance of this issue, the effect of antimicrobial treatment dosage on bacterial resistance emergence has not been extensively explored *in vivo*. That is particularly true for the use of the quinolone oxolinic acid (OA), which is licensed in France for the treatment in farmed trout of furunculosis (*Aeromonas salmonicida*), haemorrhagic septicaemia due to motile *Aeromonas* spp. and yersiniosis (*Yersinia ruckeri*) episodes.

The objective of the present study was to determine the effect of over-, under- and French-licensed dosages of oxolinic acid treatment on the selection of antibiotic-resistant *Aeromonas* spp. in healthy rainbow trout gut and rearing waters, under experimental conditions. The importance of these potential sites of selection for antibiotic-resistant bacteria in fish farming was investigated by quantification of heterotrophic microbiota and of motile *Aeromonas*, a common bacteria found in the freshwater environment and in the trout intestinal tract, and by characterisation of the susceptibility to OA of *Aeromonas* populations before, during and after administration of medicated or non-medicated feed.

2. Materials and methods

2.1. Experimental treatments

Experimental treatments were conducted on September 2004 in the experimental facilities of the PEIMA (Pisciculture Experimentale INRA des Monts d'Arrée, Northern Brittany, France). Batches of 35 healthy rainbow trout were constituted and reared in indoor 200-L tanks, supplied with water from the Elorn River at a flow rate of 5.4 l/min. Water temperature was 17.3 ± 0.4 °C. At the beginning of the experiment, mean fish weight was 156 ± 2 g. Trout were fed a commercial diet (Ecolife BioMar, Nersac, France) at a rate of one percent of body weight per day, except during the antibacterial treatment, during which they were fed at a daily rate of 0.5 percent of body weight. Medicated feed was prepared by oxolinic acid oil-coating (OXOMID® acide oxolinique 240 Salmonidés, Virbac, France) on commercial feed pellets (BioMar, Nersac, France) extemporaneously. During the whole experiment, trout were fed once a day at mid-day. It was verified that all the feed, medicated or unmedicated, was consumed. A chase was performed each day just after the feeding in all tanks to eliminate faecal material accumulated during the previous 24 hours.

Three OA dosages were tested (12 mg per kg body weight per day according to the French marketing authorisation, 6 mg per kg body weight per day, and 24 mg per kg body weight per day) over seven consecutive days. Each OA dosage was tested in two tanks. Oxolinic acid was quantified by HPLC at 1.1 mg/g, 2.1 mg/g and 5.2 mg/g in the different medicated feed prepared for experimental treatments. Therefore, at a daily feed rate of 0.5%, OA treatments corresponded to expected dosages. Trout from two control tanks received unmedicated feed. OA was never detected in commercial diet distributed to the control trout and after the treatment period.

2.2. Sampling procedures

Sampling time points were defined as T0, T2, T5 (the second and the 5th day of OA treatment), T8, T11, T16, T23 (the first, the 4th, the 9th and the 16th day after the end of treatment). All samples were taken in the three hours before the mid-day feeding. Sampling consisted