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Antimicrobial resistance of *Aeromonas* spp. isolated from the growth pond to the commercial product in a rainbow trout farm following a flumequine treatment

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ABSTRACT

This study was intended to address, in the field conditions of a rainbow trout farm, the effect of a flumequine treatment on the antimicrobial resistance of *Aeromonas* spp. isolated, during the production process, from different compartments (gut content and skin of treated fish, pond water and wall biofilm), and after the slaughtering process (36 days after the antimicrobial treatment end), from trout fillets destined to human consumption. *Aeromonas* were isolated on glutamate starch phenol-red selective agar supplemented or not with 8 mg/L of flumequine. Antimicrobial resistance patterns were defined by determination of minimal inhibitory concentrations of ten antimicrobial drugs, and bacteria with the same pattern were compared by pulsed-field gel electrophoresis (PFGE). Results indicated that this treatment effectively caused a transient increase of proportion of flumequine resistant *Aeromonas* in all compartments (production pond and fish). Their highest proportion was observed 24 h after the end of treatment, particularly in gut content and skin of trout and in pond biofilm. Resistant strains were still present 15 days after the end of treatment, particularly in the pond biofilm and fish skin. The antimicrobial resistance patterns of these strains showed a high diversity. Most strains were multidrug resistant, and some were resistant to quinolones and fluoroquinolones, streptomycin, oxytetracycline, chloramphenicol, florfenicol, sulfamethoxazole and trimethoprim. Multidrug resistant *Aeromonas* spp. clones characterized by their PFGE pattern were isolated in various compartments and/or at different sampling times, showing their capacity to persist and circulate in the trout farm environment and eventually end up, for a particular clone, on trout fillets ready for commercialisation.

1. Introduction

As a consequence of the wide use of antimicrobial agents to control human and animal infections, antimicrobial resistance is increasingly reported not only in pathogenic bacteria, but also in commensal and environmental bacteria. Resistant bacteria and resistance determinants efficiently circulate between ecological niches and bacterial genus, respectively. Therefore, the increasing rates of antimicrobial resistance observed among all types of bacterial communities are perceived as a potential danger for human health. This has led to the questioning of human activities involving the use of antimicrobials for their participation to the global increase of antimicrobial resistance. As well as other types of livestock farming, fish farming is pointed at for its possible participation to the selection and the spread of environmental and pathogenic antimicrobial resistant bacteria (Cabello, 2006; Sapkota et al., 2008; Heuer et al., 2009; Martinez, 2009). However, ecological, epidemiological or molecular data have not raised definitive evidences for this participation, which remains an open field for controversy (Smith, 2008; Cabello, 2009).

Because of their ubiquitous distribution in freshwater environment, their natural susceptibility to most antimicrobial agents and their ability to develop single or multiple antimicrobial resistance, aeromonads can be used as indicators in resistance surveys, for instance in ecological impact assessments of urban, livestock or fish farming effluents. In addition, many members of this group are recognized as primary or secondary pathogens to a wide range of vertebrates, such as fish, frogs, birds, domestic animals and humans. Species such as *A. salmonicida* (which is the aetiological agent of furonculosis) and sometimes *A. hydrophila* are responsible of disease outbreaks in fish. In humans, some species (in particular *A. hydrophila*, *A. caviae* and *A. veronii* biotype *sobria*) are associated with food borne gastroenteritis and with wound infections acquired via contaminated water and are recognized as opportunistic pathogens (Deodhar et al., 1991; Figueras et al., 2000; Krovacek et al., 1994). Moreover, Bruun et al. (2003) have suggested the

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possible role of environmental aeromonads as resistance genes suppliers for other bacterial pathogens.

In previous studies conducted in experimental conditions, we showed that treatments with oxolinic acid applied to trout resulted in high proportions of OA-resistant aeromonads in deposited faecal matters and in water flowing from the rearing tanks (Naviner et al., 2007; Le Bris et al., 2007). In order to precise the effects of a quinolone treatment in field conditions, we conducted the present study in a commercial rainbow trout farm. As this farm was affected by a bacterial outbreak, we took advantage of a treatment with flumequine (another quinolone) to investigate the evolution of antimicrobial-resistance of *Aeromonas* spp. isolated from the reared trout and from their direct pond environment. We also investigated the clonal diversity of quinolone-resistant *Aeromonas* spp. strains isolated throughout the production process, from the growth pond were the treatment was applied to the slaughter unit and the final product ready to be commercialised for human consumption.

2. Materials and methods

2.1. Study site description and flumequine treatment conditions

The study was conducted in a commercial rainbow trout farm located along a coastal river in Brittany, a region of France known for the intensity of its livestock farming activities and where freshwater fish farming is also developed. This fish farm is composed of a hatching unit, a fry house and two successions of ponds, the up-stream ones for juvenile trout and the down-stream ones for the growth of fish until they reach the commercial weight. A fish slaughter unit is also present on the site. The fish farm is fed by the river water, except the hatching unit which is supplied with spring water. Three other fish farms are located up-stream along the same river. Stock poultry farms are also present near the river, up-stream the studied structure.

The rainbow trout (*Oncorhynchus mykiss*) we studied were reared in one of the down-stream ponds (800 m^3), and were intended to be soon slaughtered and commercialised as fillets. These fish had been previously treated twice with flumequine, four and two months before our study. Antimicrobial treatments had also been performed four and three month before in one of the up-stream ponds.

The study was carried out during a red mouth disease outbreak (caused by the bacteria *Yersinia ruckeri*) occurring in a rainbow trout lot. The fish were treated on decision of the veterinarian with flumequine at 12 mg per kg of body weight per day during eight consecutive days. The trout were fed the flumequine-supplemented food at a rate of 0.5% of body weight per day. At the onset of treatment, mean fish weight was approximately 400 g and water temperature was about 13 °C. This treatment was successful in curing the affected trout lot. The first fish intended to be commercialised were slaughtered from 37 days after the end of the flumequine treatment (flumequine withdrawal period is 2 days in France). At the end of the study period, water temperature in the farm environment was about 5 °C.

2.2. Sampling procedures

Sampling time points were defined as T0, T9, T22 and T44 (before, then the 9th, the 22nd and the 44th days after the flumequine treatment start). The last sampling (T44) was performed on slaughtered trout.

During the production process (T0, T9 and T22), samples consisted of water, biofilm from pond walls and trout. Water samples from about 10 cm beneath the surface were collected in sterile bottles of 0.5 L at the entry of the fish farm, and in the production pond. Biofilm of the production pond walls was sampled from about 10 cm beneath the water surface with dry drag-swabs (Sodibox, Névez, France). Ten trout were captured and were first wiped off with a sterile drag-swab (5 trout for one drag-swab) to collect samples of the mucous layer of the skin. Trout were then killed in order to collect the gut contents. Gut contents were taken between the last pyloric caecum and the anus, and pooled to constitute the intestinal sample to analyse.

During the slaughter process (T44), five trout were immediately set apart after the capture to collect samples of the mucous layer of the skin and gut contents. Ten surface samples were taken with drag-swabs, before and after slaughtering operations, on the slaughter line material and various instruments (saws used for the remove of the fishes' heads or for threading, knifes). Eight surface samples of trout fillets (four collected during filleting operations and four collected from expedition boxes) were also taken by wiping with sterile drag-swabs. Samples were placed on ice and transported to the laboratory within 4 h for bacteriological processing.

2.3. Total heterotrophic aerobic microbiota and Aeromonas spp. counts

Total heterotrophic aerobic microbiota (cultivable bacteria) and *Aeromonas* spp. were isolated, respectively, on Tryptone Soy Agar (TSA, Biokar Diagnostics) and on Glutamate Starch Phenol-red selective agar (GSP, Merck). On GSP plates, yellow colonies were considered as presumptive *Aeromonas* spp. (Kilwein, 1969). Based on the flumequine MICs we had previously observed in *Aeromonas* clinical and environmental isolates, we supplemented selective agar plates with 8 mg/L of flumequine (Sigma-Aldrich) (Giraud et al., 2004; Naviner et al., 2007). Strains growing on those flumequine-supplemented plates were considered as resistant, which was confirmed by MICs determinations.

Non-filtered water samples were serially ten-fold diluted in sterile physiological saline solution (0.9% NaCl). Gut contents were brought together for each sampling day and diluted to 10^{-1} in saline sterile water with 0.1% peptone. Samples were serially ten-fold diluted in sterile saline solution up to 10^{-5}. Drag-swabs were placed individually in sterile bags with 150 mL of sterile alkaline peptonic water (10 g/L peptone, 10 g/L NaCl, pH 8.5). Homogenisation was performed for 3 min with a Stomacher. The suspension was then incubated for 18 h with agitation at 22 °C and ten-fold diluted in sterile physiological saline solution up to 10^{-7} before plating.

Water and gut content samples were spread on both TSA and GSP plates. For each sample, 100 μL of each dilution was spread in duplicate and incubated at 22 °C for 48 h. Bacterial counts were expressed as the number of colony forming units per mL of water (CFU/mL) or per gram of gut content (CFU/g). Proportions of resistant bacteria among presumptive *Aeromonas* spp. microbiota were estimated by the ratio of CFU counts on flumequine-supplemented GSP agar to the CFU counts on flumequine-free GSP.

Samples collected with drag-swabs and submitted to enrichment were spread only on GSP plates (supplemented with flumequine or not). We assumed that the growth of flumequine-resistant and -susceptible *Aeromonas* was similar during the enrichment step and therefore assumed that this enrichment conserved the proportion of resistant *Aeromonas* among total *Aeromonas*.

2.4. Identification and typing of flumequine resistant Aeromonas

For each sampling time and in each type of sample, about 10 presumptive *Aeromonas* spp. colonies isolated on flumequine-supplemented GSP agar were subcultured on TSA plates. Presumptive *Aeromonas* identification was confirmed by standard PCR. For the *Aeromonas* genus, we used the previously described primers AerF (5′-CTACTTTTGCCGGC-GAGCGG-3′) and AerR (5′-TGATTCCCGAAGGCACTCCC-3′) which produce a 953-bp amplicon (Lee et al., 2002). Isolates positive for this *Aeromonas* genus-specific PCR were further tested using *A. hydrophila/caviae*-specific primers AH1 (5′-GAAAGGTTGATGCCTAATACGTA-3′) and AH2 (5′-CGTGCTGGCAACAAAGGACAG-3′), which produce a 625-bp amplicon (Nielsen et al., 2001).

Several phenotypic tests were also used in order to differentiate members of the *A. hydrophila/A. caviae* complex (Abbott et al., 2003), in particular Voges–Prokauer test, production of gas in glucose, acid