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Increase in the prevalence of oxolinic acid resistant *Acinetobacter* spp. observed in a stream receiving the effluent from a freshwater trout farm following the treatment with oxolinic acid-medicated feed

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Abstract

Acinetobacter spp. were used as bacterial indicators to monitor antimicrobial resistance in a freshwater trout farm before and after treatment with oxolinic acid (OA)-medicated feed. The level of susceptibility to four antimicrobial agents was determined in 331 *Acinetobacter* isolates obtained by a selective procedure from a pond ($n = 100$), the inlet channel ($n = 105$), and the stream receiving the farm effluent ($n = 126$). Before treatment, OA resistance was detected only among isolates from the pond (40%), in which the last medication with OA was dated back to 6 months before. Following treatment, a high prevalence of OA resistance was observed among isolates from both the pond (33–53%) and the effluent recipient (21–55%). In contrast, no OA resistant strains were isolated from the inlet channel. OA resistant strains were significantly more resistant to oxytetracycline (OT) compared with OA sensitive strains ($P < 0.0001$, odds ratio = 43.6). Furthermore, OA resistant isolates showed an increase of at least 10-fold times in the MIC values for ciprofloxacin (CIP) resistance compared with sensitive isolates. Phenotypic characterisation and PCR-based fingerprinting analysis showed lower diversity among *Acinetobacter* isolates from the pond and the stream receiving the farm effluent in comparison with isolates from the inlet channel. Since the two biotypes predominant in the pond and the effluent recipient included the vast majority of OA resistant isolates, the low diversity of *Acinetobacter* spp. observed at these sites could have been enhanced by exposure to the drug. The results of this study indicate that the use of OA in inland farms can affect the levels of antimicrobial resistance and

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diversity in the microflora of natural aquatic habitats situated downstream. Since the ecological consequences deriving from changes of the indigenous aquatic microflora are unknown, the careful consideration of the use of OA in aquaculture was suggested. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Acinetobacter*; Aquatic bacteria; Aquaculture; Antimicrobial resistance; Oxolinic acid

1. Introduction

Oxolinic acid (OA) is an older member of the quinolones, a class of synthetic antimicrobial agents which include important agents for control of bacterial disease in human medicine. Although new generations of quinolones out-perform OA with respect to both bactericidal activity and bioavailability, its relatively modest cost, low fish toxicity and satisfactory performance render it a widely used drug in aquaculture (Wells, 1995).

Both laboratory and field experiments have shown that the concentration of OA in marine sediment is not significantly reduced 180 days after medication (Samuelsen et al., 1994; Hektoen et al., 1995). The exposure to OA has been shown to affect the bacterial community of marine sediment with respect to bacterial numbers, degradation of organic material and development of resistance (Nygaard et al., 1992; Hansen et al., 1993).

Previous studies have shown that higher numbers of antibiotic resistant bacteria are likely to be present in aquaculture habitats treated with antimicrobial agents compared with the same habitats prior to treatment (DePaola et al., 1995), similar aquaculture habitats treated with a smaller amount of antibacterials (Hervig et al., 1997), or aquaculture habitats with no recent history of antimicrobial use (McPhearson et al., 1991). The method traditionally used for the measurement of antimicrobial resistance at the population level consists in bacteriological counts on agar plates containing a particular concentration of the antimicrobial agent. The main drawback of this method is the use of a single arbitrary breakpoint for the determination of antimicrobial resistance in different bacterial taxa. In fact, the use of a single breakpoint, corresponding to the amount of antimicrobial agent added to the medium, does not take into account the variability in the levels of antimicrobial susceptibility existing among different bacterial taxa. Furthermore, the selective count of resistant bacteria on agar plates containing antimicrobial agents is affected by the bacterial composition of the sample and seems to overestimate the number resistant bacteria (Jones et al., 1986).

Bacteria belonging to the genus *Acinetobacter* are non-motile, non-fermentative, Gram-negative coccobacilli that can be easily isolated from soil, water, sewage, human skin and a large variety of foodstuffs (Towner, 1996). *Acinetobacter* spp. have been found at densities of 10^4 organisms per 100 ml in freshwater ecosystems (LaCroix and Cabelli, 1982) and described as one of the major taxa among aerobic heterotrophic bacteria isolated from freshwater fish farms (Allen et al., 1983). In the present study, *Acinetobacter* spp. were used as bacterial indicators for monitoring antimicrobial resistance in a freshwater trout farm undergoing treatment with OA. Spatial and

temporal differences in the levels of antimicrobial resistance of aquatic *Acinetobacter* spp. were considered indicative of the selective pressure exerted on the entire aquatic microflora. The choice of *Acinetobacter* spp. as bacterial indicators was prompted by their ubiquitous distribution and particular ability to develop antibiotic resistance in environments subjected to antibiotic selective pressure (Towner, 1997; Guardabassi et al., 1998). *Acinetobacter* spp. were isolated using a genus-specific selective procedure and the levels of susceptibility to OA and three other antimicrobial agents were compared between different sampling sites and times. In addition, phenotypic characterisation and PCR fingerprinting were used to detect temporal and spatial variations in the distribution of *Acinetobacter* spp.

2. Materials and methods

2.1. Sampling sites, times and methods

A freshwater rainbow trout (*Oncorhynchus mykiss*) farm situated in Denmark was selected for sampling. The trout farm was structured as a typical Danish earth pond system, composed of an inlet channel, 44 earth ponds, an outlet channel, a sedimentation pond and a sheltered section intended for eggs and fingerling production. The water supply was an unpolluted stream which did not receive water from other fish farms or potential sources of antibiotic resistance. The farm effluent was discharged into the stream serving as the water supply, allowing comparison of the distribution and antibiotic resistance levels of *Acinetobacter* spp. isolated from sites situated upstream and downstream from the trout farm. The effluent treatment consisted of a system of micro sieves separating sludge from water and a sedimentation pond in which water was collected prior to release into the recipient stream.

Due to the occurrence of enteric red mouth disease outbreaks caused by *Yersinia ruckeri*, ponds had been medicated previously with either potentiated sulfonamides or OA in the form of medicated feed. Limited to the fingerling section, amoxicillin (AM) and oxytetracycline (OT) had been also employed sporadically for treatment. Sampling was performed in March 1998, corresponding with the occurrence of a new outbreak of enteric red mouth disease. At the time of sampling, the last medication with OA was dated back to August 1997. The medicated feed used at the farm (© Branzil) contained 0.125% of OA. The feed was administered at the dosage recommended by the manufacturer (1% of fish biomass for 7 days). About 60% of the feed was administered by hand, and the remaining part by automatic feeders. The total amount of medicated feed used during the period of study was approximately 1.9 kg, corresponding to 2375 mg of active product.

Samples were collected at four times: immediately before the beginning of the treatment period (day 0), immediately after the suspension of the treatment period (day 7), 14 days after suspension of the treatment (day 21) and 21 days after suspension of the treatment (day 28). The designations shown in brackets are used throughout the text to indicate the different sampling days. Samples were collected from a fishpond undergoing treatment, the inlet channel and the recipient stream in proximity of the farm

outlet. Limited to day 28, samples were additionally collected from the recipient stream approximately 300 m downstream from the outlet.

Samples of water mixed with sediment particles were aspirated from the bottom of each sampling site using sterile 150 ml syringes and catheters. Samples were particularly rich of sediment particles from the surface layer. Based on visual examination, the amount of suspended solids did not vary between different sampling times and sites. Residues of feed pellets were visible in the samples collected from the pond.

2.2. Bacteriological methods

For each sample, 20 to 30 subsamples of 1 ml were inoculated in situ into sterile tubes containing 9 ml of Baumann medium, a selective enrichment for *Acinetobacter* (Baumann, 1968), and transported to the laboratory for analysis. Isolation and identification of *Acinetobacter* spp. were performed as previously described (Guardabassi et al., 1998). Briefly, following selective enrichment and subculture of the enrichment cultures on Luria Bertani agar (Maniatis et al., 1989), two to four representative colonies were selected from each plate. *Acinetobacter* spp. were identified by colony hybridisation using a genus-specific 16S rRNA-targeted oligoprobe (Wagner et al., 1994) labelled with phosphatase alkaline (DNA Technology, Aarhus, Denmark). The combination of selective enrichment with colony hybridisation was previously demonstrated to allow a rapid and reliable identification of *Acinetobacter* spp. in aquatic samples (Guardabassi et al., 1999).

The level of susceptibility to four antimicrobial agents was measured by the disc diffusion method following the recommendations of the Swedish Reference Group for Antibiotics (Olsson-Liljequist et al., 1997). OA, AM, OT and sulfamethoxazole (SU) were included in the susceptibility testing as these drugs had been used at the fish farm. The following disc concentrations were used: AM, 10 µg; OA, 2 µg; OT, 30 µg; SU, 25 µg. Inhibition zone diameters were measured after 18–24 h of incubation at 30°C. Strains were defined resistant when inhibition zone diameters were ≤ 13 mm for AM, ≤ 16 mm for OA, ≤ 19 mm for OT and ≤ 12 mm for SU. After examination of the histograms representing the distribution of zone diameters (Fig. 1), the above breakpoint values were selected as the zone sizes separating between populations of resistant and sensitive strains. These values were in accordance with the interpretative breakpoints indicated by standard methods for disc susceptibility testing (Casals and Pringler, 1991; Olsson-Liljequist et al., 1997).

Due to the high levels of OA resistance observed in the first three sampling times, ciprofloxacin (CIP, 5 µg) was included in the disc sensitivity tests of isolates from day 28 in order to detect differences in the levels of resistance to fluoroquinolones between OA resistant and sensitive *Acinetobacter* isolates. Additionally, MIC values were determined for OA and CIP according to the National Committee for Clinical Laboratory Standards (1990). The range of concentrations used for MIC determination was from 0.0625 to 8 µg/ml for both drugs.

Isolates from day 0 and day 28 were characterised phenotypically by the following tests: glucose O/F, growth in brain heart infusion broth (BHI) at 37°C, sheep blood

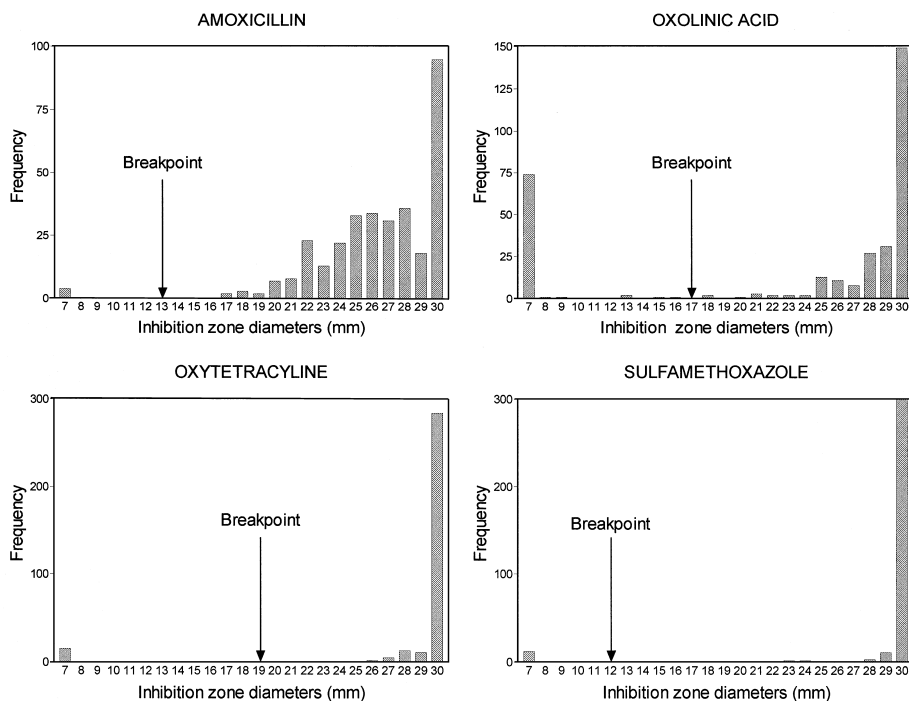


Fig. 1. Frequencies of inhibition zone diameters observed for AM, OA, OT and SU among 331 *Acinetobacter* isolates. The arrows indicate the zone diameter breakpoints selected for definition of resistance to each antimicrobial agent.

haemolysis, utilisation of azelate, citrate, L-histidine and DL-lactate (Guardabassi et al., 1999).

2.3. Statistical analysis

Logistic regression analysis by Statistix Analytical Software (Tallahassee, USA) was used to evaluate the effect of multiple independent variables (sampling site, sampling time and resistance to antimicrobial agents other than OA) on the outcome variable (OA resistance). Logistic regression analysis was chosen because it allows to indicate whether an independent variable can significantly contribute to the prediction of the outcome variable after all other variable in the model have been taken into account. In fact, some variables, which might be significant on their own, may not be significant if other variables are included in the model. In order to detect an eventual effect of the treatment with OA on the prevalence of OA resistance in the stream receiving the farm effluent, only data from the inlet and the outlet were included in the model, as both sites were located in the same stream. Odds ratios were calculated in order to measure the strength of statistically significant associations. For each sampling site, chi-square analysis was used to detect statistically significant differences in the frequency of OA resistance between different sampling times.

2.4. PCR-based fingerprinting and cluster analysis

Limited to the last sampling time (day 28), *Acinetobacter* isolates were analysed by PCR-based fingerprinting using the universal primer M13 (Grundman et al., 1997). The reproducibility of this method for typing of *Acinetobacter* spp. was previously demonstrated in an inter-laboratory study as reported by Grundman et al. (1997). After suspension of colonies in 100 µl of sterile distilled water, crude DNA extracts were obtained by heating for 15 min at 95°C and centrifugation at 12,000 × g for 20 s to remove cell debris. In order to ensure the maximum possible standardisation, all reaction mixtures were based on Ready-To-Go RAPD Analysis Beads (Amersham Pharmacia Biotech, Denmark). The following PCR conditions were used: 95°C for 5 min, followed by 35 cycles at 95°C for 20 s, 50°C for 1 min, and 72°C for 20 s, followed by a final extension at 72°C for 5 min. PCR products were analysed by electrophoresis in 2% agarose gels (w/v) prepared in Tris–borate electrophoresis buffer (TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA), including a size marker (100 Base-Pair Ladder, Boehringer Mannheim, Germany) added at regular intervals.

Cluster analysis of genotypic data was performed by the unweighted pair group method using average linkages (UPGMA)(Gel Compar program, Applied Maths, Kortrijk, Belgium). Gel pictures were scanned and normalised according to the reference size marker. Levels of similarity between fingerprints were calculated based on the Dice coefficient.

3. Results

Among a total of 569 isolates tested by colony hybridisation, 331 isolates reacted positively with the genus-specific oligonucleotide probe, including 105 isolates from the inlet channel, 100 isolates from the pond and 126 isolates from the recipient stream. The numbers of *Acinetobacter* isolates in relation to sampling times and sites are shown in Table 1.

Table 1

Numbers of *Acinetobacter* isolates in relation to sampling times and sites

Day 0, beginning of the treatment period; day 7, end of the treatment period; day 21, 2 weeks after suspension of the treatment; day 28, 3 weeks after suspension of the treatment.

Sampling time	No. of <i>Acinetobacter</i> isolates				
	Total	Inlet	Pond	Outlet 1 ^a	Outlet 2 ^b
Day 0	76	24	20	32	— ^c
Day 7	77	31	21	25	— ^c
Day 21	93	33	32	28	— ^c
Day 28	85	17	27	20	21

^aSite situated in proximity of the outlet of the farm effluent.

^bSite situated about 300 m downstream from the outlet of the farm effluent.

^cSamples not collected at this site.

Table 2

Antimicrobial resistance (%) among *Acinetobacter* spp. isolated throughout the study from the inlet channel (inlet), a pond treated with OA (pond), and the stream receiving the farm effluent (outlet)

Antimicrobial agent	Total (n = 331)	Inlet (n = 105)	Pond (n = 100)	Outlet 1 ^a (n = 105)	Outlet 2 ^b (n = 21)
Amoxicillin	1.2	1.9	2	0	0
Oxolinic acid	24.2	0	46	21.9	52.4
Oxytetracycline	4.5	0.9	4	10.5	0
Sulfamethoxazole	3.6	2.9	0	7.6	4.8

^aSite situated in proximity of the outlet of the farm effluent.

^bSite situated about 300 m downstream from the outlet of the farm effluent. Samples were collected at this site limited to the last sampling time.

The highest overall prevalence of antibiotic resistance was shown for OA (23.8%). Relatively low levels of resistance (< 5%) were observed for the other antibacterial agents (Table 2). OA resistant strains were not isolated from the inlet channel, whereas 46% and 21.9% among isolates from the pond and the stream receiving the farm effluent were OA resistant, respectively.

At day 0, OA resistance was detected only among pond isolates (40%) but not among outlet isolates (Table 3). However, relatively high prevalences of OA resistance were observed among outlet isolates at day 7 (24%), day 21 (21%) and day 28 (55%). At day 28, a similar prevalence of OA resistance was found also among isolates from the site situated 300 m downstream from the outlet (11/21 isolates = 52%). The prevalences of OA resistance observed at different days among isolates from the pond and the outlet are shown in Fig. 3.

Logistic regression analysis showed that the outcome variable OA resistance was statistically associated with OT resistance ($P < 0.0001$) and sampling time ($P < 0.0042$). The OR measuring the association between OA and OT resistances was on average 43.6 (range 8–237.9, 95% confidence interval), indicating that the odds of being OT resistant were 43.6 times higher in OA resistant strains compared with OA sensitive strain. The

Table 3

Prevalence of OA resistance observed among *Acinetobacter* spp. isolated from the pond and the outlet at different sampling times

Day 0, immediately before treatment; day 7, immediately after the suspension of the treatment; day 21, 14 days after the suspension of the treatment; day 28, 21 days after the suspension of the treatment.

Day	Pond	Outlet
0	8/20 (40%)	0/32 (0%)
7	7/21 (33%)	6/25 (24%) ^a
21	17/32 (53%)	6/28 (21%)
28	14/27 (52%)	11/20 (55%) ^b

^aStatistical significant increase in the prevalence of OA resistance compared with day 0 (chi-square, $P = 0.0034$).

^bStatistical significant increase in the prevalence of OA resistance compared with day 21 (chi-square, $P = 0.0165$).

OR measuring the association between OA and sampling site was 2.3 (range 1.3–4.1, 95% confidence interval), indicating a positive association between the two variables. Among isolates from the outlet, there was a statistically significant increase in the prevalence of OA resistance between days 0 and 7 (chi-square, $P < 0.005$) and between days 21 and 28 (chi-square, $P < 0.05$). Among isolates from the pond, no statistically significant increase in the prevalence of OA resistance was observed between different sampling times.

OA resistant strains showed smaller inhibition zone diameters for CIP (24–28 mm) compared to OA sensitive strains (> 30 mm). The MIC values for OA were $8 \mu\text{g/ml}$ among resistant strains and varied from 0.125 to $0.250 \mu\text{g/ml}$ among sensitive strains. MIC values for CIP were $\geq 0.0625 \mu\text{g/ml}$ among OA sensitive strains and $0.5 \mu\text{g/ml}$ among OA resistant strains.

Based on phenotypic characterisation, the majority of OA resistant isolates collected at days 0 and 28 belonged to two biotypes, A (43.2%) and B (54.5%). At day 0, a certain phenotypic diversity was observed among isolates from all sites (Table 4). In contrast, at day 28 biotypes A and B were highly predominant among isolates from the pond (27/27 isolates) and the stream receiving the farm effluent (39/41 isolates). All OA resistant isolates except one belonged either to biotype A (19/44 isolates) or biotype B (24/44 isolates). Both biotypes were characterised by the inability to grow in BHI at 37°C , haemolyse blood and acidify glucose. Among the four tested carbon sources, biotype A strains were able to utilise only lactate, whereas biotype B strains were able to utilise lactate and histidine. According to the identification table proposed by Dijkshoorn (1996), biotype A corresponded to the group including genomic species 7 (*Ac. johnsonii*) and 15 (unnamed), whereas biotype B remained ungrouped.

Based on PCR fingerprint analysis, high genetic diversity was observed among isolates from the inlet channel, as most strains showed a unique pattern with less than 70% similarity to the pattern of any other strain. On the contrary, less diversity was observed among isolates from other sites, with two main clusters including 25/27 isolates from the pond, 14/20 isolates from the outlet and 9/21 isolates from the site

Table 4

Distribution and antibiotic resistance of different biotypes observed among *Acinetobacter* isolates before and after treatment with oxolinic acid

Day 0, immediately before treatment; day 28, 21 days after suspension of the treatment period.

Biotype	Phenotypic pattern ^a	Day 0 ($n = 76$)			Day 28 ($n = 85$)			OA resistant isolates
		Inlet	Pond	Outlet	Inlet	Pond	Outlet ^b	
A	Lac	6	5	8	4	15	23	19
B	Lac, His	2	3	2	1	12	16	24
C	Lac, T	4	3	9	2	0	1	–
D	Cit, Aze, Lac, T	4	4	1	0	0	0	1
E	Lac, Cit, T	1	2	3	0	0	0	–
Other		7	3	9	10	0	1	–

^aUtilisation of carbon sources: Lac, lactate; His, histidine; Cit, citrate; Aze, azelate; other tests: T, growth at 37° in brain heart infusion broth; glucose acidification; sheep blood haemolysis.

^bIncluding isolates from the site situated 300 m downstream from the outlet.

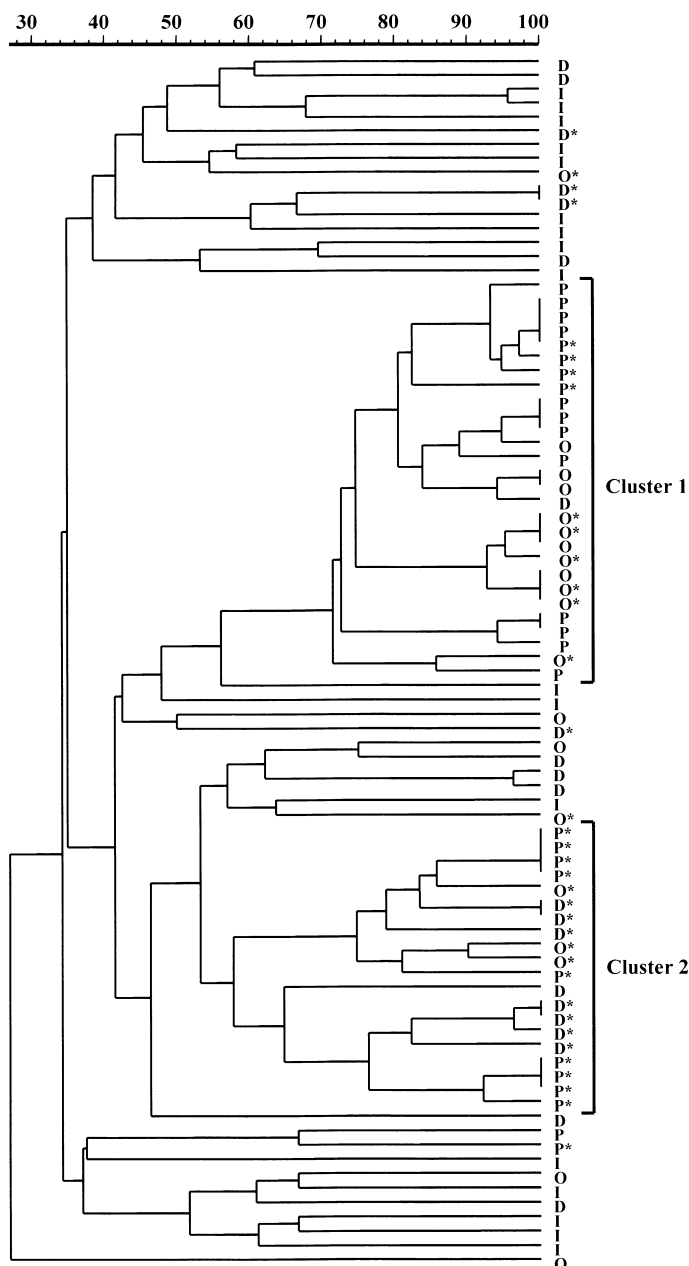


Fig. 2. Dendrogram obtained from PCR fingerprints of *Acinetobacter* isolates obtained 21 days after suspension of OA. Cluster analysis was performed by UPGMA using the Dice similarity coefficient. It should be noted that most OA resistant isolates (30/36) belonged to clusters 1 and 2. Symbols: I, isolates from the inlet channel; P, isolates from the pond; O, isolates from the site situated in proximity of the farm outlet; D, isolates from the site situated 300 m downstream from the farm outlet; *, OA resistant isolates.

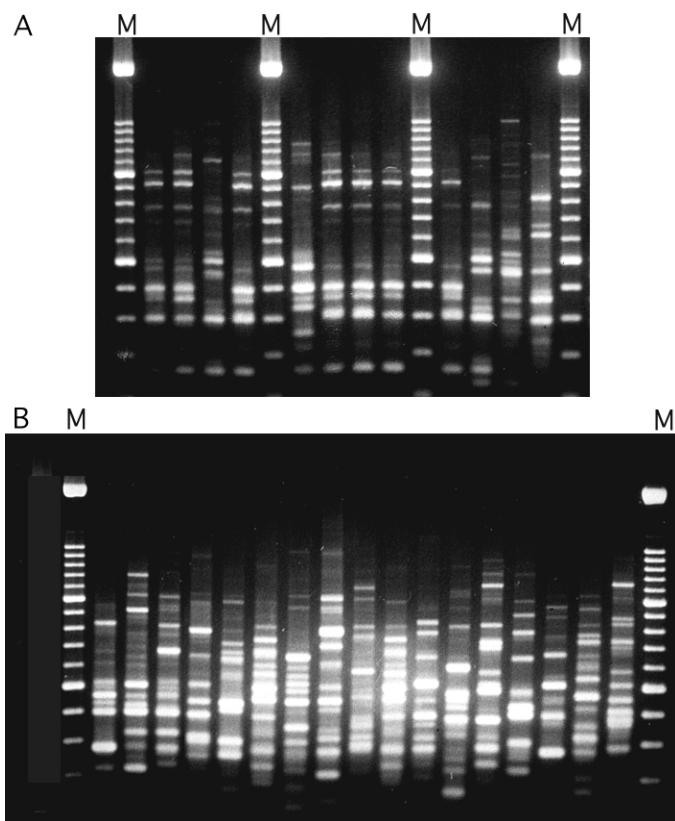


Fig. 3. Examples of PCR fingerprints of *Acinetobacter* isolates from the stream receiving the farm effluent (A) and from the inlet channel (B). Lanes M, 100 bp size marker.

situated 300 m downstream (Fig. 2). Among the 36 OA resistant isolates from day 28, 10 isolates were included in cluster 1 and 19 isolates in cluster 2, whereas seven isolates were not comprised in the two main clusters (Fig. 2). In general, the two clusters contained strains of biotype A and B, respectively (data not shown). An example of PCR fingerprints of isolates from the inlet channel and from the effluent recipient is illustrated in Fig. 3.

4. Discussion

Our investigation indicates that the use of OA-medicated feed in the trout farm affected the level of antimicrobial resistance of *Acinetobacter* spp. in the stream receiving farm effluent. Before treatment, OA resistant isolates could not be detected in

the outlet, whereas following treatment relatively high prevalences of OA resistance (22–55%) were observed among isolates from this site. The lack of OA resistance among strains isolated from the stream at sites situated upstream from the farm indicates that the background level of OA acid resistance among aquatic *Acinetobacter* was below the detection limits in a freshwater habitat not exposed to this drug. The high prevalence of OA resistance observed at the site situated 300 m downstream from the outlet three weeks after the suspension of the treatment (day 28) suggests that the use of OA in inland-based farms could affect the levels of antimicrobial resistance also in the bacterial populations of distant aquatic habitats. However, the prevalence of OA resistance was likely to decrease with time following the suspension of treatment, since OA resistant strains were not isolated from the outlet at day 0, 6 months after the previous treatment.

A previous study by Spanggaard et al. (1993) reported that OA resistance was more prevalent among bacteria isolated from freshwater trout farms (27%) compared with bacteria isolated from an unpolluted stream (16%). However, the differences found in the frequencies of OA resistance between the fish farms and the unpolluted stream were not statistically significant (Spanggaard et al., 1993). Furthermore, although all the three fish farms included in the study regularly used OA, the study was not performed in correspondence with a period of treatment and did not investigate the eventual effects on the bacterial flora of the effluent recipients.

The high prevalence of OA resistance observed after treatment in the stream receiving the farm effluent could have been determined by the presence of either OA residues or OA resistant strains in the farm effluent. A previous study by Smith et al., (1994a,b) suggested the importance of effluent treatment systems to reduce the environmental impact of antimicrobial usage in land-based fish farms. The ability of different effluent treatment systems to detain antimicrobial agents and bacteria is an interesting subject for future research.

At day 0, 6 months after the previous treatment with OA, 40% of the isolates from the pond were resistant to OA, indicating that in a pond treated with OA high numbers of resistant bacteria can persist for at least 6 months after treatment. The variations in the prevalence of OA resistance observed in the pond at different sampling times were not statistically significant, indicating that the prevalence of OA resistant *Acinetobacter* spp. in the pond was not significantly affected by the treatment. This could be due to the fact that the pond had been previously treated with OA, and consequently the indigenous microflora was less susceptible to the selective effect of the drug. Furthermore, the presence of feed residues in samples collected from the pond could influence the recovery of resistant isolates at different days.

The MIC value observed for OA resistant *Acinetobacter* isolates (8 µg/ml) is above the breakpoint concentration of 0.5 µg/ml indicated for determination of OA resistance in aquatic bacteria (Smith, 1998). OA resistant strains in comparison with OA sensitive strains demonstrated to be less susceptible to CIP, an important member of the quinolones used in human medicine. Low-level resistance to CIP MIC has previously been shown to be associated with mutations in the *gyrA* gene (Everett et al., 1996). While a single mutational event may lead to complete resistance to older quinolones, full resistance to newer quinolones is mediated by multiple mutations of genes involved in

the synthesis of DNA-gyrase and changes of the cell envelope (Baquero, 1990). Thus, the use of OA in aquaculture could determine conditions of selective pressure that allow low-level CIP resistant mutants to arise and establish themselves in aquatic bacterial populations.

OA resistance among *Acinetobacter* spp. isolated from the stream receiving the farm effluent was strongly associated with OT resistance. Previous studies have reported the occurrence of cross-resistance between OA and OT in bacteria from marine sediment (Nygaard et al., 1992) and *Aeromonas salmonicida* strains isolated from outbreaks of furunculosis (Barne et al., 1990). Although the two drugs are not related with respect to chemical structure and mechanism of action, cell envelope modifications of quinolones resistant mutants could determine permeation difficulties for other drugs such as tetracyclines, chloramphenicol and some β -lactams (Baquero, 1990). Alternatively, the use of OA and OT in the same environment could lead to the selection of genotypes resistant to both drugs.

Based on the analysis of a subset of isolates, a reduced phenotypic and genotypic diversity of *Acinetobacter* spp. was observed both in the pond and in the effluent recipient compared to the inlet channel. Since the two biotypes predominant in the pond and the stream included most OA resistant isolates, the observed reduction in the diversity of *Acinetobacter* spp. could have been enhanced by the presence of OA at these sites. It should be noted that quinolones are not only cytotoxic but also mutagenic (Couturier et al., 1998). Consequently, the mutagenic effects could accelerate the emergence of resistant genotypes in bacterial communities exposed to these drugs.

The use of a bacterial indicator offers various advantages in comparison with the traditional count of resistant bacteria on agar-plates containing antimicrobial agents. The isolation of bacteria makes possible the use of standardised methods for antimicrobial susceptibility testing, which allow the interpretation of a wider range of response compared with the traditional method. Appropriate breakpoints for the definition of antimicrobial resistance can be determined within a group of aquatic organisms based on the study of the distribution of the inhibition zone diameters (Fig. 1), avoiding the use of breakpoints determined arbitrarily. Furthermore, methods for bacterial typing can be usefully employed in association with antimicrobial susceptibility testing to detect the effects of antimicrobial agents on the distribution of different genotypes within the target bacterial population. The only limitation is that results based on the study of a single group of organisms may not be representative for other bacterial taxa. However, also when traditional methods for determination of antimicrobial resistance are employed, results are representative only for culturable bacteria, which represent a small fraction of the viable bacterial population in aquatic environments.

The use of antimicrobial agents in aquaculture could have a negative impact on the treatment of human infections either by the direct transmission of resistant pathogenic bacteria to humans, or indirectly by the transfer of resistance genes from environmental bacteria to human pathogens (Smith et al., 1994a,b). Although *Acinetobacter* spp. are considered as opportunistic human pathogens (Towner, 1997), the aquatic strains isolated in this study showed different phenotypic patterns in comparison to the species of medical interest, *A. baumannii*. Since OA resistance is generally assumed to be mediated by chromosomal mutations and not capable of genetic transfer, bacteria that

are not able to survive under environmental conditions, for example most human pathogenic bacteria, should not be affected by the use of OA in aquaculture. Based on these considerations, our investigation does not indicate any direct risk for human health.

In conclusion, the results of this study indicate that the use of OA in inland farms can affect the levels of antimicrobial resistance and diversity in the microflora of natural aquatic habitats situated downstream. Since the long-term consequences associated with changes in the natural microbial community are unknown, the authors suggest that the use of OA in aquaculture should be considered carefully. OA is likely to have a higher ecological impact compared with other drugs, as quinolones can persist in the aquatic environment for a long time and induce genotoxic effects on bacterial populations. The effects of different antimicrobial agents on natural microbial communities should be investigated in the future. Molecular techniques that have been developed to study bacterial diversity in microbial ecology, like Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993) or terminal-Restriction Fragment Length Polymorphism analysis (t-RFLP) (Wen-Tso et al., 1997), could be usefully employed to achieve this type of information.

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References

- Allen, D.A., Austin, B., Colwell, R.R., 1983. Numerical taxonomy of bacterial isolates associated with a freshwater fishery. *J. Gen. Microbiol.* 129, 2043–2062.
- Baquero, F., 1990. Resistance to quinolones in Gram-negative microorganisms: mechanisms and prevention. *Eur. Urol.* 17 (Suppl. 1), 3–12.
- Barne, A.C., Lewin, C.S., Hastings, T.S., Amyes, S.G.B., 1990. Cross resistance between oxytetracycline and oxolinic acid in *Aeromonas salmonicida* associated with alterations in outer membrane proteins. *FEMS Microbiol. Lett.* 72, 337–340.
- Baumann, P., 1968. Isolation of *Acinetobacter* from soil and water. *J. Bacteriol.* 96, 39–42.
- Casals, J.B., Pringler, N., 1991. Antibacterial/Antifungal Sensitivity Testing Using Neo-Sensitabs. Rosco Diagnostica, Taastrup, Denmark.
- Couturier, M., Bahassi, E.M., Van Melderren, L., 1998. Bacterial death by DNA gyrase poisoning. *Trends Microbiol.* 6, 269–275.
- DePaola, A., Peeler, J.T., Rodrick, G.E., 1995. Effect of oxytetracycline-mediated feed on antibiotic resistance of gram-negative bacteria in catfish ponds. *Appl. Environ. Microbiol.* 61, 2335–2340.
- Dijkshoorn, L., 1996. *Acinetobacter*-microbiology. In: Bergogne-Bérézin, E., Joly-Guillou, M.L., Towner, K.J. (Eds.), *Acinetobacter: Microbiology, Epidemiology, Infections, Management* CRC Press, Boca Raton, 37 pp.
- Everett, M.J., Fang Jin, Y., Ricci, V., Piddock, L., 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrob. Agents Chemother.* 40, 2380–2386.

- Grundman, H.J., Towner, K.J., Dijkshoorn, L., Gerner Smidt, P., Maher, M., Seifert, H., Vaneechoutte, M., 1997. Multicenter study using standardized protocols and reagents for evaluation of reproducibility of PCR-based fingerprinting of *Acinetobacter* spp. J. Clin. Microbiol. 35, 3071–3077.
- Guardabassi, L., Dalsgaard, A., Olsen, J.E., 1999. Phenotypic characterisation and antibiotic resistance of *Acinetobacter* spp. isolated from aquatic sources. J. Appl. Microbiol. 87, 659–667.
- Guardabassi, L., Petersen, A., Olsen, J.E., Dalsgaard, A., 1998. Antibiotic resistance in *Acinetobacter* spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. Appl. Environ. Microbiol. 64, 3499–3502.
- Hansen, P.K., Lunestad, B.T., Samuelsen, O.B., 1993. Effects of oxytetracycline, oxolinic acid, and flumequine on bacteria in artificial marine fish farm sediment. Can. J. Microbiol. 39, 1307–1312.
- Hektoen, H., Berge, J.A., Hormazabal, V., Yndestad, M., 1995. Persistence of antibacterial agents in marine sediment. Aquaculture 133, 175–184.
- Hervig, R.P., Gray, J.P., Weston, D.P., 1997. Antibacterial resistant bacteria in surficial sediments near salmon net-cage farms in Puget Sound, Washington. Aquaculture 149, 263–283.
- Jones, J.G., Gardener, S., Simon, B.S., Pickup, R.W., 1986. Factors affecting the measurement of antibiotic resistance in bacteria isolated from lake water. J. Appl. Bacteriol. 60, 455–462.
- LaCroix, S.J., Cabelli, V.J., 1982. Membrane filter method for enumeration of *Acinetobacter calcoaceticus* from environmental waters. Appl. Environ. Microbiol. 43, 90–96.
- Maniatis, T., Fritsch, E.F., Sambrook, J., 1989. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- McPhearson, R.M., DePaola, A., Zywno, S.R., Motes, M.L. Jr., Guarino, A.M., 1991. Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds. Aquaculture 99, 203–211.
- Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.
- National Committee for Clinical Standards, 1990. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standards M7-A2. NCCLS, Villanova, PA.
- Nygaard, K., Lunestad, B.T., Hektoen, H., Berge, J.A., Hormazabal, V., 1992. Resistance to oxytetracycline, oxolinic acid and furazolidone in bacteria from marine sediments. Aquaculture 104, 31–36.
- Olsson-Liljequist, B., Larsson, P., Walder, M., Miörner, H., 1997. Methodology for susceptibility testing. Scand. J. Infect. Dis., 13–23.
- Samuelsen, O.B., Lunestad, B.T., Ervik, A., Fjelde, S., 1994. Stability of antibacterial agents in an artificial marine aquaculture sediment studied under laboratory conditions. Aquaculture 126, 283–290.
- Smith, P., 1998. Towards the establishment of a breakpoint concentration for the determination of resistance to oxolinic acid in marine microflora. Aquaculture 166, 229–239.
- Smith, P., Donlon, J., Coyne, R., Cazabon, D.J., 1994a. Fate of oxytetracycline in a freshwater fish farm: influence of effluent treatment systems. Aquaculture 120, 319–325.
- Smith, P., Hiney, M.P., Samuelsen, O.B., 1994b. Bacterial resistance to antimicrobial agents used in fish farming: a critical evaluation of method and meaning. Annu. Rev. Fish Dis. 4, 273–313.
- Spanggaard, B., Jørgensen, F., Gram, L., Huss, H.H., 1993. Antibiotic resistance in bacteria isolated from three freshwater fish farms and an unpolluted stream in Denmark. Aquaculture 115, 195–207.
- Towner, K.J., 1996. Biology of *Acinetobacter* spp. In: Bergogne-Bérézin, E., Joly-Guilou, M.L., Towner, K.J. (Eds.), *Acinetobacter: Microbiology, Epidemiology, Infections, Management* CRC Press, p. 71, Boca Raton, FL.
- Towner, K.J., 1997. Clinical importance and antibiotic resistance of *Acinetobacter* spp. J. Med. Microbiol. 46, 721–746.
- Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., Schleifer, K.H., 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. 60, 792–800.
- Wells, R., 1995. Oxolinic acid. FAO Food Nutr. Pap. 41 (7), 69–88.
- Wen-Tso, L., Marsh, T.L., Cheng, H., Forney, L.J., 1997. Characterization of microbial diversity by determining terminal restriction length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63, 4516–4522.