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Bacterial resistance to oxytetracycline in Chilean salmon farming

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Abstract

The use of oxytetracycline for preventing and controlling bacterial pathogens in salmon farming is frequent in Chile, yet no studies have been performed to evaluate the ecological impact of its intensive and prolonged use. In this work, the frequency of oxytetracycline-resistant bacteria from water, pelletized feed and fingerlings from four Chilean freshwater Atlantic salmon farms, as well as the level of resistance of selected strains was investigated. Viable bacterial counts were performed by a spread plate method and antibiotic-resistant bacteria were counted in culture media supplemented with the selected antibiotic. Resistance levels of selected strains isolated in media containing antibiotic were determined using an agar plate dilution method. High proportions of low- and high-level oxytetracycline-resistant bacteria (selected in agar plates containing 30 and 100 μg/ml, respectively), mainly from pelletized feed and effluent samples of the fish farms were found. The highest proportions of resistant bacteria were found in the effluent samples, and were significantly higher ($P < 0.05$, Tukey's test) than those from the other samples studied. On the contrary, influent samples exhibited the lowest proportions of resistant bacteria. One hundred and three resistant Gram-negative isolates, which represented the oxytetracycline-resistant bacterial population, were randomly selected on TSA containing 30 or 100 μg/ml of oxytetracycline, from salmon farms and pellet samples, and streaked for purification on TSA plates without oxytetracycline. A large number of non-fermenting bacteria (77.7%) were identified. Among these, *Pseudomonas fluorescens* (28.2%), *Stenotrophomonas maltophilia* (5.8%), *Sphingomonas paucimobilis* (5.8%), *Acinetobacter lwoffii* (4.8%), and *Pseudomonas putida* (4.8%) were the most frequent. Also, an important number of strains of *Aeromonas hydrophila* (9.7%), *Burkholderia cepacia* (3.9%), *Brevundimonas vesicularis* (3.9%), *Acinetobacter johnsonii* (2.9%), *Pantoea* sp. (2.9%) and *Moraxella* sp. (2.9%)

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were found. *P. fluorescens* and *A. hydrophila* predominated in salmon fingerlings, whereas *A. lwoffii* and *S. maltophilia* were predominant in pellet samples. Selected strains exhibited high levels of oxytetracycline resistance, with minimum inhibitory concentrations (MICs) ranging from 64 to 2048 μg/ml, whereas MIC_{90} of oxytetracycline varied between 1024 and 2048 μg/ml. This study shows the presence of an important population of oxytetracycline-resistant bacteria in the microflora of Chilean salmon farms. Therefore, the environment of these farms might play important roles as reservoirs of bacteria carrying genetic determinants for high-level tetracycline resistance, prompting an important risk to public health for workers involved in fish culturing and processing.

Keywords: Oxytetracycline; Bacteria; Antimicrobial resistance; Atlantic salmon; Fish farms

1. Introduction

Worldwide production of salmon and trout can be estimated in 1.8 million tons for the year 2000, and approximately 60% of which is farmed. Chile is the second largest producer of farmed salmon in the world, second only to Norway, with an estimated production of approximately 300,000 tons during the year 2000 (Wurmann, 2000). In Chile, salmonid farms are concentrated in the southern coast of the country where the lakes for smolt production are also located (Petersen, 2000).

Intensive fish farming in Chile has resulted in growing problems of bacterial diseases, which, in turn, have lead to a widespread antibiotic use for their treatment and prevention (Alvarado et al., 1990). Various authors have emphasized the putative negative impact derived from the use of antimicrobial agents in fish farms (Aoki, 1992; Lewin, 1992; Lunestad, 1992; Midtvedt and Lingaas, 1992; Smith et al., 1994; Weston, 1995; Alderman and Hastings, 1998).

In Chile, a high frequency of antibiotic-resistant bacteria has been reported in polluted and unpolluted freshwater (Miranda and Castillo, 1998), and in commercial fish residing waters near the effluents through which urban sewage is disposed (Miranda and Zemelman, 2001).

Oxytetracycline is the most frequently used antibacterial in the Chilean salmonid industry because of its broad spectrum of activity and low cost. This antibiotic binds to the 30S subunit of the microbial 70S ribosomes inhibiting protein synthesis by blocking the attachment of aminoacyl-tRNA units (Levy, 1984; Chopra, 1985; Roberts, 1996). However, the intensive use of this antibacterial has resulted in a growing selection of microbial resistance, involving either active efflux of the drug; ribosomal protection or enzymatic drug modification (Salyers et al., 1990; Speer et al., 1992; Levy, 1992a; Roberts, 1994; Taylor and Chau, 1996; Chopra and Roberts, 2001).

The use of oxytetracycline in fish farming has been demonstrated to coincide with an increased frequency of oxytetracycline-resistant microorganisms (DePaola et al., 1988, 1995; Torsvik et al., 1988; Samuelsen et al., 1992; Hansen et al., 1993; DePaola, 1995; Kerry et al., 1996a), and of oxytetracycline-resistant fish pathogens (Björklund et al., 1991). Clearly, the data suggest that numbers of oxytetracycline-resistant bacteria are

usually higher in fish farms undergoing antimicrobial therapy because susceptible microorganisms are inhibited, thus allowing colonization by a resistant microflora (Samuelsen et al., 1992).

Because oxytetracycline is very poorly absorbed through the intestinal tract of fish (Cravedi et al., 1987; Jacobsen, 1989; Björklund and Bylund, 1990), it has to be administered at a high dosage rate of 100–150 mg per kg fish per day for 10–15 days, causing consequently a slow excretion of large amounts of this antibiotic (Rogstad et al., 1991), thus increasing the selective pressure which might lead to selection of oxytetracycline-resistant bacteria in faecal and gastrointestinal microflora of fish following oral oxytetracycline therapy (Austin and Al-Zahrani, 1988; Sugita et al., 1988a,b). Also, DePaola (1995) and DePaola et al. (1995) have presented evidence that suggests a link between administration of oxytetracycline-medicated and oxytetracycline-contaminated feeds and the frequency of oxytetracycline resistance in intestinal and aquatic microflora of catfish. It is likely that the gastrointestinal tract of fish also serves as a niche in which selection of resistant microorganisms might take place due to the high concentration of antibiotics in this environment during periods of medication. On the contrary, Kerry et al. (1997) found no evidence for a selection of resistant strains in intestinal microflora of Atlantic salmon smolts treated with this agent.

The frequency of resistance to oxytetracycline in marine environments has been extensively studied (Torsvik et al., 1988; Samuelsen et al., 1992; Nygaard et al., 1992; Hansen et al., 1993; Sandaa et al., 1992; Ervik et al., 1994; Kerry et al., 1994), but only a few of studies dealing with this problem have been performed in freshwater systems (Austin, 1985; McPhearson et al., 1991; Spanggaard et al., 1993).

The use of oxytetracycline in aquaculture might produce some negative impact on the treatment of human infections as a consequence of either direct transmission of resistant pathogens to humans, or indirectly through the transfer of resistance genes from environmental bacteria to human pathogens (Smith et al., 1994). Rhodes et al. (2000), using different *Aeromonas* species, have provided evidence of the dissemination of determinants of tetracycline resistance between the human and aquaculture environments, and Schmidt et al. (2001) suggested the occurrence of horizontal transfer of antibiotic resistance genes among the aeromonads found in and around the sampled fish farms. On the other hand, the use of oxytetracycline-supplemented feed in chicken farms has been shown to contribute to the selection of resistant intestinal flora of farm personnel (Levy et al., 1976).

Frequent prophylactic use of antibiotics in Chilean aquaculture increases the risk of transfer of antibiotic resistance to human pathogens associated with fish consumption. At present, no studies on antibiotic-resistant bacteria in Chilean salmon farming have been performed, despite the importance of this industry in this country (Petersen, 2000; Wurmann, 2000). Therefore, it was considered important to evaluate the possibility that salmonid culture environments might behave as reservoirs of tetracycline-resistant bacteria, thus increasing the risk of transfer of the resistance encoding genes into fish and human pathogens.

The aims of this study were to determine the frequency of oxytetracycline-resistant bacteria in Chilean freshwater salmon farming, to identify the representative resistant Gram-negative bacilli and to determine their levels of resistance to this antibiotic.

2. Materials and methods

2.1. Sampling sites

Four freshwater Chilean Atlantic salmon (*Salmo salar* L.) farms with no history of recent oxytetracycline exposure, located at the South of the country were considered in this study. Sampled farms were not exposed to antibacterial therapy for more than 6 months before the sampling period. The geographic location of the studied farm sites is shown in Fig. 1.

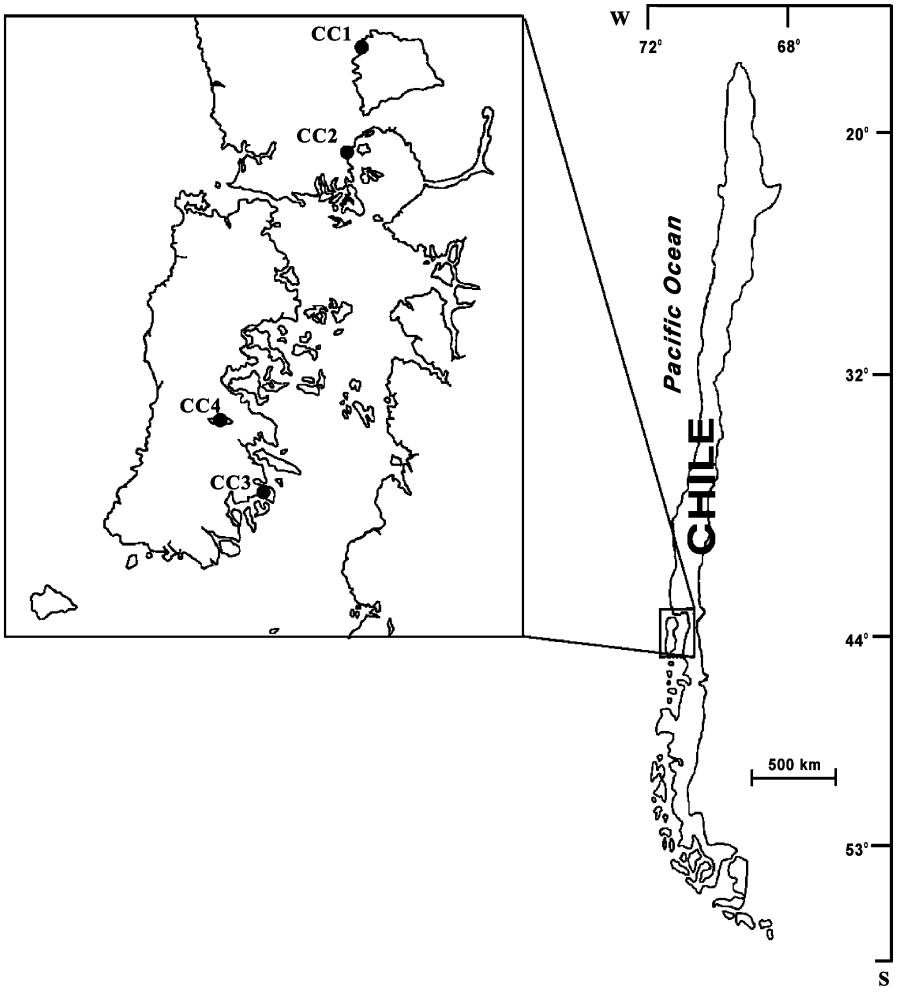

Fig. 1. Geographic location of studied Chilean salmon farms (CC1–CC4).

2.2. Sampling

Water samples from fish farm influents, salmon fingerling culture tanks, and farm effluents were considered for land-based culture centers (CC1–CC3), while surface water around salmon cages was considered for culture located at Natri Lake (CC4). Also, salmon fingerling and unmedicated fish food pellet samples from all sampled centers were analyzed. The fish feed was a commercial pellet (ALITEC^{TM}), currently used in each farm.

Water samples were collected in sterile water sampling bottles (APHA, 1992), whereas food pellet and fingerling samples were packed in sterile plastic bags. Samples were placed on ice, immediately transported to the laboratory and processed within 24 h after collection.

2.3. Processing of samples

Salmonid fingerlings were externally washed with sterilized distilled water to reduce potential contamination with skin bacteria. Salmon fry and pelletized feed were placed on sterile petri dishes, weighted and grinded by hand using a sterile glass digester. Homogenates of these samples were made in microfiltered (0.22 μm) distilled water.

2.4. Oxytetracycline

Oxytetracycline was obtained from Sigma (St. Louis, MO) and stored in the dark at 4 °C. Filter-sterilized oxytetracycline stock solutions were prepared by the addition of 0.1 M HCl (Merck) dropwise to a drug suspension in distilled water until the compound was dissolved. Stock solutions were adjusted to pH 7.0 with 0.1 N NaOH (Sigma) and filter-sterilized (0.22 μm) before use.

2.5. Total and resistant bacterial viable count

Total viable counts of heterotrophic bacteria were performed by a spread plate method using Tryptic soy agar (TSA, Difco labs). The number of low- and high-level resistant bacteria was determined on TSA medium supplemented with oxytetracycline at concentrations of 30 and 100 μg/ml, respectively (Hansen et al., 1993). Aliquots (100 μl) of appropriate dilutions of either homogenates of the pelletized feed and fingerling or water samples were spread onto plates in triplicate, and incubated for 7 days at 20 °C.

2.6. Bacterial strains

One hundred and three Gram-negative bacilli were picked at random from plates of TSA supplemented with oxytetracycline to represent oxytetracycline-resistant bacterial community from each salmon farm. From these, 32 strains were recovered from farm CC1, 24 strains from farm CC2, 26 strains from farm CC3, and 21 strains from farm CC4 (Table 1).

Isolates were purified onto Tryptic soy agar (Difco) and maintained at −70 °C in Brain Heart Infusion broth (Difco), containing 10% glycerol. When needed, frozen organisms

Table 1
Source of resistant strains recovered from Chilean salmon farms

Source	Number of strains			
	Salmon farm			
	CC1	CC2	CC3	CC4
Influent	4	5	4	–
Tank water[a]	6	4	5	8
Effluent	5	5	6	–
Pellet	1	5	5	3
Fingerling	16	5	6	10
Total	32	24	26	21

[a] CC4 corresponds to surface lake water from salmon cages.

were recovered by streaking onto TSA plates (Difco) containing oxytetracycline (30 μg/ml) which were incubated at 20 °C for 24–48 h.

2.7. Bacterial identification

Phenotypical characteristics, Gram stain, oxidase production, oxidative/fermentative utilization of glucose (OF medium), motility and growth on MacConkey agar (Difco), were determined for every selected isolate according to Cowan (1974). Carbohydrate fermenters and non-fermenters resistant strains were further characterized by using the API 20E and API 20NE kits (bioMérieux, Marcy-l'Etoile, France), respectively. Tests were performed according to the instructions of the manufacturer, and incubated at 22 °C. Results were recorded after 24 and 48 h, but only results after 48 h of incubation were considered. Bacterial identification was performed by using the APILAB Plus identification software (bioMérieux). A number of isolates (20%) were re-examined to check reproducibility of the assay.

2.8. Minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations (MICs) of oxytetracycline (Sigma) against all strains were determined by an agar plate dilution method, as recommended by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards (NCCLS), 1997). A serial twofold dilution pattern of oxytetracycline were added into Mueller–Hinton agar (Difco) as to obtain final concentrations ranging from 0.5 to 2048 μg/ml. Triplicate plates were inoculated by using a Steers replicator apparatus (Steers et al., 1959), delivering approximately 10^4 colony-forming units per spot, and incubated for 72 h at 22 °C. Bacterial suspensions were prepared in sterile 0.85% saline, adjusting their turbidity to match a 0.5 McFarland standard (bioMérieux), corresponding approximately to 10^8 colony-forming units/ml. Adequate serial dilutions were prepared prior to their use. MIC was defined as the lowest concentration of oxytetracycline producing absence of growth at least in two of the three plates after 72 h.

Reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, recommended by NCCLS (1998), were used as quality control organisms for verification of MIC ranges on Mueller–Hinton agar plates. Strains were incubated for 24 h at 35 °C.

2.9. Statistical analysis

A Tukey-type multiple comparison testing among proportions (Zar, 1984), was used to determine significant differences among the proportions of antibacterial-resistant bacteria from different fish farm sources. Probabilities <0.05 were considered significant.

3. Results

3.1. Total and resistant bacterial viable count

Overall variations in numbers of oxytetracycline-resistant bacteria of water samples through the fish farm system were quite similar in the four salmon farms, showing increasing values in the influent, culture tank and effluent samples (Table 2).

Farm CC1 showed the highest proportions of bacteria resistant to oxytetracycline at both concentrations (30 and 100 μg/ml) in all studied samples and only samples of pelletized feed from farm CC4 exhibited higher proportions of resistant bacteria (Table 2). The proportions of low- and high-level oxytetracycline-resistance in samples from farms CC2 and CC3 were rather low and only in effluent samples high proportions of resistance to this antibiotic were found. Samples from farm CC3 evidenced very low proportions of high-level resistant bacteria (Table 2).

The proportions of bacteria resistant to low concentrations of oxytetracycline in salmon fingerling and pellet samples from farm CC4 were high, which were significantly higher ($P<0.05$, Tukey's test) than those obtained from lake water samples.

In general, effluent samples from all fish farms showed the highest proportions of oxytetracycline-resistant bacteria. On the contrary, the lowest proportions of resistant bacteria were observed in the influent samples (Table 2).

3.2. Bacterial identification

Phenotypical characterization of resistant isolates recovered from various sources of the four culture centers, showed predominance of non-fermenting bacteria, which represented 77.7% of the total isolates. The identification of isolates, as displayed in Table 3, showed a high frequency of *Pseudomonas fluorescens* (28.2%), followed by *Aeromonas hydrophila* (9.7%), *Stenotrophomonas maltophilia* (5.8%) and *Sphingomonas paucimobilis* (5.8%) species. Also, an important frequency of the bacterial species *Pseudomonas putida*, *Ralstonia pickettii*, *Burkholderia cepacia*, *Brevundimonas vesicularis* and *Moraxella* sp. was found (Table 3).

The most prevalent species, *P. fluorescens* was recovered from all culture centers, whereas *A. hydrophila* was isolated from all fish farms with the exception of farm CC3. A

Table 2
Total and oxytetracycline-resistant viable bacterial concentrations from various Chilean salmon farms

Source	Viable count (CFU/ml or CFU/g)											
	Farm CC1			Farm CC2			Farm CC3			Farm CC4		
	Total	Resistant to OT (%)		Total	Resistant to OT (%)		Total	Resistant to OT (%)		Total	Resistant to OT (%)	
		30 μg/ml	100 μg/ml		30 μg/ml	100 μg/ml		30 μg/ml	100 μg/ml		30 μg/ml	100 μg/ml
Influent	3.99×10^3	16.72	7.24	1.58×10^3	5.87	0.82	4.44×10^3	0.22	0.00	ND	ND	ND
Tank water[a]	4.20×10^3	18.52	7.90	5.40×10^3	3.15	1.17	1.12×10^5	6.93	0.95	3.61×10^3	7.00	3.79
Effluent	9.47×10^3	69.22	39.75	8.58×10^3	12.78	3.81	2.15×10^4	8.33	0.58	ND	ND	ND
Pelletized food	1.12×10^4	24.20	7.97	2.61×10^5	5.49	3.83	4.56×10^6	0.39	0.22	2.93×10^4	59.85	34.28
Fingerling	1.48×10^4	32.25	19.24	5.74×10^4	1.07	0.21	6.74×10^4	3.45	0.30	5.75×10^4	16.01	3.71

[a] CC4 corresponds to surface lake water from salmon cages; CFU: colony-forming units; ND: not determined.

Table 3
Frequency of bacterial isolates from salmon farm source

Species	Water				Pellet	Fingerling	Total
	Influent	Tank	Effluent	Lake			
Acinetobacter johnsonii		2				1	3
Acinetobacter lwoffii			1		4		5
Acinetobacter radioresistens				1			1
Acinetobacter sp.				1		1	2
Aeromonas hydrophila		1	2	1		6	10
Brevundimonas vesicularis	1			1	1	1	4
Burkholderia cepacia		1	2			1	4
Citrobacter koseri			1				1
Enterobacter sakazaki						1	1
Escherichia coli	2						2
Klebsiella pneumoniae						1	1
Moraxella sp.			3				3
Morganella morganii					1		1
Pantoea sp.					1	2	3
Providencia rettgeri			2				2
Pseudomonas aeruginosa						1	1
Pseudomonas fluorescens	4	5	2	3	2	13	29
Pseudomonas pseudoalcaligenes						1	1
Pseudomonas putida	2		1			2	5
Pseudomonas sp.		1				4	5
Ralstonia pickettii		1				1	2
Serratia liquefaciens			2				2
Sphingobacterium spiritivorum		1					1
Sphingomonas paucimobilis	2	2		1	1		6
Stenotrophomonas maltophilia	2	1			3		6
Not identified					1	1	2
Total no.	13	15	16	8	14	37	103

high proportion of these species was recovered from fingerling samples. Also, 13 strains of enterobacteria (12.6%) were identified, mainly belonging to the species *E. coli*, *Pantoea* sp., *Proteus rettgeri*, *Serratia liquefaciens* and *Morganella morganii* (Table 3). On the other hand, different *Acinetobacter* species, especially *Acinetobacter lwoffii*, were isolated from various culture centers, mainly from pelletized feed samples used in the farm CC3. Only two strains, recovered from the farm CC4, were not identified by using the APILAB Plus identification program (bioMérieux).

3.3. Minimum inhibitory concentrations

The range of minimum inhibitory concentrations (MICs) of oxytetracycline against the oxytetracycline-resistant strains is shown in Fig. 2. High levels of oxytetracycline resistance were observed for the selected bacterial strains, with MIC values ranging from 64 to 2048 μg/ml. MIC_{90} values of 1024 μg/ml for the CC2 and CC3 farms, and 2048 μg/ml for the farms CC1 and CC4 were observed. The lowest MICs were observed against resistant strains recovered from the farm CC4 located at lake, where more than

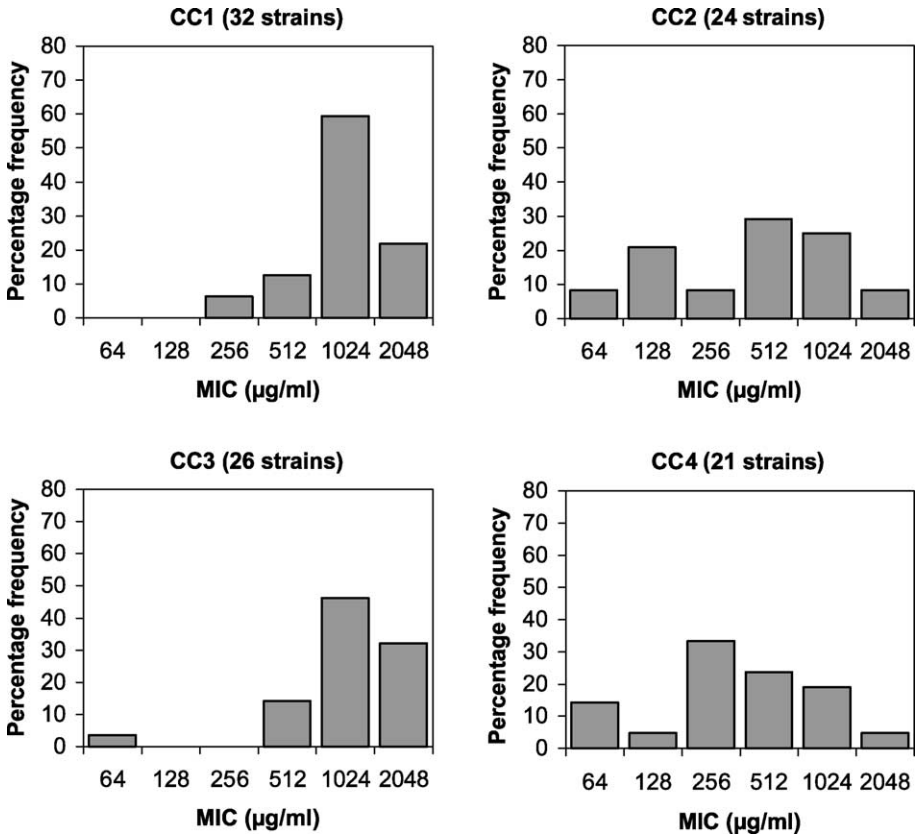

Fig. 2. Distribution of minimum inhibitory concentrations (MICs) of oxytetracycline values of resistant bacteria isolated from the Chilean salmon farms (CC1–CC4).

50% of these strains exhibited ≤ 256 μg/ml values. On the contrary, the highest MICs were found against strains from farm CC3, showing 2048 μg/ml values in approximately 30% of strains (Fig. 2). No correlation between MIC values and bacterial species was observed.

The strains of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, used as reference controls showed MIC values of 2 and 8 μg/ml, respectively, which were acceptable quality control ranges for tetracyclines (NCCLS, 1998).

4. Discussion

Bacterial resistance to tetracyclines might be considered as an important property related to Chilean salmon industry since oxytetracycline is used to treat a variety of salmon diseases, including enteric redmouth disease caused by *Yersinia ruckeri* and flavobacteriosis disease caused by *Flavobacterium psychrophilum* and *F. columnare*. In addition, this

antibiotic is also currently administered as a preventive treatment before the smoltification process.

Since oxytetracycline was not currently used during sampling period in this study, the high frequency of resistant bacteria found in the samples as well as the high resistance levels of the microorganisms might be a consequence of previous use of this compound in the fish farms, having persisted a selective effect during the period in which no antibiotic has been used, or because a high prevalence of resistant bacteria with tetracycline resistance determinants in these environments.

The important incidence of oxytetracycline-resistant strains in influents of farms CC1 and CC2 could be explained by the acquisition of genes for resistance that very likely protect them from the killing effects of antibiotics that are produced in nature by other bacteria and other naturally occurring substances (Kadavy et al., 2000). Once required, these resistance genes could be maintained within a population, protecting the bacteria from antibiotics produced by other members of the microflora or residues in agricultural or domestic effluents.

The proportions of oxytetracycline resistance found in this study are remarkably higher than those observed in environmental bacteria associated with four Danish rainbow trout farms (Schmidt et al., 2000) and bacteria from sediment beneath a marine salmon farm following oxytetracycline therapy (Kerry et al., 1996a).

Frequencies of oxytetracycline-resistant bacteria in salmon fingerling and pellet samples are high when compared to those reported in previous studies (Kerry et al., 1995, 1997). With exception of farm CC3, pellet samples showed higher low- and high-level oxytetracycline resistance than those observed in the fingerling ones. A finding in agreement with the results reported by Kerry et al. (1997), who reported mean frequencies of resistance of 0.1% to 9.9% among bacteria from the intestinal microflora, in comparison with resistance frequencies of 16% among bacteria from the microflora of the feed.

The important frequency of bacteria resistant to oxytetracycline from unpolluted influent samples, mainly from farms CC1 and CC2 (16.7% and 5.9%, respectively), agrees with the results of Spanggaard et al. (1993) and Nygaard et al. (1992) who noted that more than 6% of bacterial isolates obtained from unpolluted aquatic environments were resistant to oxytetracycline. Also, Magee and Quinn (1991) found an important frequency of oxytetracycline-resistant *Pseudomonas* species in freshwater without anthropogenic influence, suggesting that an intrinsic and non-transferable resistance mechanism was involved.

The basis for the high intrinsic resistance of most non-fermentative Gram-negative bacilli (especially *P. aeruginosa*, *S. maltophilia*, *B. cepacia* and *Acinetobacter*) is the low outer-membrane permeability, coupled with secondary resistance mechanisms such as antibiotic efflux pumps, which take advantage of low outer-membrane permeability (Hancock, 1998). The efflux pumps of Gram-negative bacteria expel a remarkably broad range of antimicrobial compounds, including antibiotics, detergents, dyes, and organic solvents (Nikaido, 1996; Zgurskaya and Nikaido, 2000).

Factors responsible for the occurrence of antibiotic resistance in absence of antibiotic use are still unclear. Some reports indicate that relatively high levels of nutrients may give rise to increases in the frequency of resistant bacteria in aquatic environments in the absence of antibiotic use (Husevag et al., 1991; McPhearson et al., 1991; Vaughan et al., 1996). These bacteria appear to be tolerant to antibiotic as a consequence of mutations

producing alterations in outer membrane proteins (Barnes et al., 1990; Hansen et al., 1993; Smith et al., 1997; Chopra and Roberts, 2001).

These findings are in accordance with results reported by Kapetanaki et al. (1995), who recovered an important frequency of bacteria resistant to high levels (>512 μg/ml) of oxytetracycline from tanks containing marine sediments overlaid only with high levels of sterilized commercial fish food pellets and seawater. Also, Kerry et al. (1996b) observed no increase in oxytetracycline-resistant bacteria in the absence of fish feed, even in the presence of 25 μg/ml oxytetracycline using small-scale marine sediment microcosms. Kerry et al. (1995) reported that accumulation of uneaten feed rather than the presence of oxytetracycline might be responsible for high frequencies of resistance in fish farms.

Fish farm effluents can cause immediate increases in the abundance and specific growth rate of suspended bacteria in recipient rivers and lakes (Bedwell and Goulder, 1996, 1997). If so, the increase in oxytetracycline-resistant bacteria in the fish tanks is probably accounted for their enrichment with organic carbon, especially in the pellet and effluent samples.

The high frequency of oxytetracycline-resistant bacteria observed in feed samples agree with data obtained by Kerry et al. (1995) and DePaola (1995), who found high numbers of oxytetracycline-resistant Gram-negative bacteria in both medicated and unmedicated pelletized feeds, a fact that strongly support the hypothesis that the resistant microflora introduced in the feed might be one of the most important sources of the elevated frequency of resistance in these systems.

Bacterial species identified in this study are similar to those isolated by Spanggaard et al. (1993) from Danish freshwater rainbow trout farms exhibiting resistance to both oxytetracycline and oxolinic acid. These isolates were mainly identified as Vibrionaceae, Enterobacteriaceae, *Acinetobacter* spp., *Pseudomonas* spp. and *Moraxella* spp. DePaola et al. (1988) and DePaola and Roberts (1995) reported an important predominance of *A. hydrophila* among tetracycline-resistant catfish pond bacteria. Also, oxytetracycline-resistant bacteria identified in this study are in accordance with those recovered from Danish and Norwegian marine polluted sediments, where *Aeromonas* species had a dominant role, and *S. maltophilia* was the dominant species identified from unpolluted Danish sediments (Andersen and Sandaa, 1994).

The *Acinetobacter* species identified in this study agree with the report of Guardabassi et al. (1999), who found that *Acinetobacter* isolated from freshwater aquaculture habitats mainly belonged to the *A. johnsonii* and *A. lwoffi* species. *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). This genus has been used as bacterial indicator for monitoring antimicrobial resistance in a freshwater trout farm because of their ubiquitous distribution and particular ability to develop antibiotic resistance in environments subjected to antibiotic selective pressure (Towner, 1997; Guardabassi et al., 1998).

The high incidence of *A. lwoffi* and *A. hydrophila* in pellet and fingerling samples, respectively, must provide some concern about the possibility of its transmission to humans considering that these samples are highly manipulated by farm workers. Besides, these bacterial species grow very well in these sites because of the high availability of nutrients.

Levels of resistance of microorganisms under assay were much higher than those usually reported in fish pathogenic bacteria and *Pseudomonas* spp. isolated from fish (Ledo et al., 1987; Stamm, 1989; Rodgers, 2001). Also, remarkably lower MICs of oxytetracycline against 29 oxytetracycline-resistant bacterial strains isolated from three freshwater fish farms in Denmark were found (Spanggaard et al., 1993). This may be because an important proportion of the resistant isolates used in this study were initially selected using high oxytetracycline concentrations (100 μg/ml). On the other hand, the significance of using media supplemented with oxytetracycline must be considered, because resistance to this antibiotic has been probed to be inducible (Marshall et al., 1986), showing that strains previously exposed to this antibiotic can increase significantly their minimum inhibitory concentrations.

The level of antibiotic resistance expressed by the majority of studied bacteria, is far higher than what is actually needed to resist a therapeutic dose of this antibiotic, suggesting that the evolution of resistance determinants was probably not merely a response to accommodate the antibiotic levels used in Chilean salmon farming. Levy (1992b) suggested that these resistance determinants may actually have another function in nature that eludes scientific investigators.

The results obtained in this study suggest that high numbers of resistant bacteria exist in Chilean salmon farming environment, a fact which might increase the possibilities of transfer of resistance determinants into fish pathogenic bacteria (Sandaa et al., 1992; Sandaa and Enger, 1994). Additionally, returning of resistant enteric bacteria into fish consumers might occur (Midtvedt and Lingaas, 1992). For this reason, further epidemiological and molecular investigations are needed in order to evaluate the presence of genetically mobile antibiotic resistance genes in the human and animal food chain (Levy, 1989; Young, 1993; Sørum, 1998). Also, this results prompt the necessity towards establishing antibiotic policies (Gould, 1999), in order to reduce current levels of resistance in salmon industry.

The results reported in this work increase concern on the creation of reservoirs of transferable antibacterial resistance in Chilean salmon culture systems and the importance of evaluating the role of these environments in the dissemination and evolution of oxytetracycline resistance genes and its vectors. This is an antibacterial for which plasmid-encoded resistance has been found in some cases (Sandaa and Enger, 1994; Adams et al., 1998). Furthermore, Sandaa et al. (1992) reported a high frequency of resistance to oxytetracycline in fish farm environments, where *Vibrio* and *Pseudomonas* strains were able to transfer resistance into a recipient *E. coli* strain.

The presence of high numbers of oxytetracycline-resistant bacteria in salmon farming has ecological and public health implications and emphasizes the need for further studies in relation to the genes encoding resistance in different bacterial species as well as on the possibility of the returning of resistance genes to the human population through fish consuming.

5. Conclusions

This study demonstrates that Chilean freshwater salmon farms exhibit significant frequencies of bacteria with low- and high-level resistance to oxytetracycline, mainly in

pelletized feed and effluent samples. The high percent of oxytetracycline-resistant bacteria, which was observed in Chilean salmon farms with no recent history of antibiotic use, suggests that antibiotic resistance can be promoted and maintained due to factors other than the presence of drug in the corresponding environment, providing evidence that salmon culture centers may play an important role as reservoirs of antibiotic-resistant bacteria and thereby increase a potential public health hazard.

Further studies are necessary to determine the potential public health significance of important numbers of resistant bacteria in Chilean salmon farming to elucidate the possibility of transmitting multiresistant bacteria to salmon farm personnel or human consumers.

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