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Genetic diversity and antimicrobial resistance of *Flavobacterium psychrophilum* isolated from cultured rainbow trout, *Onchorynchus mykiss* (Walbaum), in Spain

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Abstract

Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease (CWD) and rainbow trout fry syndrome (RTFS) in salmonids. These diseases are a major problem in the aquaculture industry in Spain, and a better understanding of the epidemiology of *F. psychrophilum* isolates is necessary to improve management strategies. In this study, to investigate genetic variability of this bacterium, pulsed-field gel electrophoresis after DNA digestion with endonuclease *Stu*I, plasmid profiling analysis and antimicrobial susceptibility testing were undertaken with 25 isolates of *F. psychrophilum* from Spain. These isolates were classified into 17 patterns by PFGE analysis, which were grouped into four clusters and seven independent branches. Twenty isolates (80%) possessed plasmids of 3.5 kb (n = 13) or 5.5 kb (n = 7). No plasmids were associated with antibiotic resistance to oxytetracycline (OTC) or florfenicol (FLO). Twenty isolates (80%) had minimum inhibitory concentrations (MICs) to OTC of between 2.4 and 9.7 μg mL^{-1}, and all isolates were susceptible to FLO. A relationship between the origin of the isolates and PFGE genotypes was found. Plasmid profile typing correlated with PFGE profile typing, whereas no correlation was found between antimicrobial susceptibility testing and PFGE profiles. These results suggest that the population of *F. psychrophilum* with pathogenic potential in northern Spain is quite heterogeneous.

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Introduction

Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease (CWD) and rainbow trout fry syndrome (RTFS) in salmonids. Juvenile fish are primarily affected, with high mortalities in fry. It has been recognized as a worldwide occurring pathogen in freshwater aquaculture, causing substantial economic losses (Dalsgaard 1993). Although outbreaks are more prevalent in winter and spring when water temperature is below 10 °C (Nematollahi, Decostere, Pasmans & Haesebrouck 2003), we have detected some cases during summer with water temperatures above 18 °C. In Spain, rainbow trout, *Onchorynchus mykiss* (Walbaum), farming is an important industry with 25 000 tons produced per year, and infection by *F. psychrophilum* has been observed in freshwater farms since the mid-eighties. Despite the importance of CWD/RTFS in local rainbow trout fisheries, there is a lack of epidemiological studies on *F. psychrophilum* in Spain.

Several typing methods, such as serotyping (Mata, Skarmeta & Santos 2002), random amplified polymorphic DNA (RAPD) (Chakroun, Urdaci, Faure, Grimont & Bernardet 1997), restriction fragment length polymorphism of PCR products (PCR-RFLP) (Izumi, Aranishi & Wakabayashi 2003), total plasmid profile analysis (Izumi & Aranishi 2004), ribotyping (Chakroun, Grimont, Urdaci & Bernardet 1998), polymorphism in 16s rRNA genes

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(Soule, LaFrentz, Cain, Lapatra & Call 2005a), reciprocal suppression subtractive hybridization by microarray analysis (Soule, Cain, LaFrentz & Call 2005b) and pulsed-field gel electrophoresis (PFGE) (Arai, Morita, Izumi, Katagiri & Kimura 2007; Chen, Davis, LaPatra, Cain, Snekvik & Call 2008) have been used for molecular characterization of *F. psychrophilum*. These methods vary in their reproducibility and discriminatory ability with PFGE reported to be superior to the others (Barbier, Saulnier, Chachaty, Dumontier & Andremont 1996; Chiew & Hall 1998). Therefore, PFGE is currently considered to be the 'gold standard' for bacterial pathogen subtyping and has been used extensively for molecular epidemiological characterization of numerous pathogens (Kawanishi, Kojima, Ishihara, Esaki, Kijima, Takahashi, Suzuki & Tamura 2005; Barrett, Gerner-Smidt & Swaminathan 2006; Lopez-Cánovas, Bravo, Herrera, Riveron, Javer, Sanchez, Fando, Noa & Fernandez 2006).

The use of antimicrobial agents in both human and veterinary medicine exerts a strong selective pressure, inducing resistance to antimicrobial agents. For this reason, farmed fish and terrestrial animals should be monitored and kept under surveillance for the presence of resistant organisms (Franklin, Acar, Anthony, Gupta, Nicholls, Tamura, Thompson, Threlfall *et al.* 2001). In aquaculture systems, oxytetracycline (OTC) incorporated into fish feed has been used to control bacterial diseases. The wide use of OTC has, however, reduced its efficacy and has increased the occurrence of tetracycline-resistant bacterial fish pathogens such as *F. psychrophilum* (Bruun, Schmidt, Madsen & Dalsgaard 2000), *Vibrio* sp. and *Lactococcus garvieae* (Kim, Nonaka & Suzuki 2004). As an alternative, florfenicol (FLO) has become the drug of choice for treating CWD and RTFS outbreaks. There are studies on antimicrobial resistance patterns of *F. psychrophilum* isolated in northern Europe (Rangdale, Richards & Alderman 1997; Bruun, Schmidt, Madsen & Dalsgaard 2000), but there is a lack of knowledge about the situation in Spain.

The aim of this study was to characterize *F. psychrophilum* isolates obtained from 23 CWD/RTFS outbreaks in Spain using antimicrobial susceptibility testing and molecular typing methods, i.e. PFGE and total plasmid profile, to investigate the bacterial susceptibility to some antimicrobial agents and to assess the epidemiological relationships among the isolates.

Materials and methods

Bacteria and culture conditions

Twenty-five *F. psychrophilum* strains were isolated from the liver and/or kidney of diseased salmonids, from 12 fish farms situated throughout northern Spain during 2005–2008 (Table 1). The reference strain, NCIMB 1947^T (kidney of salmon, United States 1955), was included for comparative purposes. All the strains were confirmed as *F. psychrophilum* using the PCR method described by Del Cerro, Márquez & Guijarro (2002) (data not shown). For all experiments, the strains were routinely grown on tryptone yeast extract salts medium (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulphate, pH 7.2, Holt, Rohovec & Fryer 1993) and nutrient broth (NB; Pronadisa) and incubated at 15–17 °C for 48 h. Stock cultures were maintained frozen at −80 °C in NB with 15% glycerol.

PFGE fingerprinting

Each strain of *F. psychrophilum* was grown in NB at 17 °C for 48 h. Bacterial cells from 1-ml aliquots were harvested, washed twice with cell suspension buffer (CSB: 0.1 M Tris, 0.13 M EDTA, pH 8) and resuspended in the same buffer until an optical density (OD) of 2.5 was obtained. The OD was measured at 525 nm. Bacterial suspensions were incubated with 0.8 mg mL^{-1} of proteinase K (Roche Diagnostics) at 37 °C for 15 min, mixed with an equal volume of 1% chromosomal grade agarose (Seakem GTG agarose, Cambrex Corporation) and immediately loaded into a disposable plug mould (Bio-Rad Laboratories) and allowed to solidify at room temperature. Bacterial cells in each plug were then lysed in lysis buffer (50 mM Tris–HCl [pH 8], 0.06 M EDTA, 1% sarcosyl [GE Healthcare Bio-Sciences Corp.] and proteinase K [0.5 mg mL^{-1}] for 2–3 h at 54 °C with shaking. Prior to digestion with restriction enzyme, the plugs were washed twice for 30 min with distilled water at 50 °C and four times for 15 min with Tris–EDTA buffer (TE: 10 mM Tris–HCl [pH 8], 0.1 mM EDTA). The plugs were stored in TE at 4 °C until use. The DNA in the plugs was restriction digested overnight at 37 °C with 30 U of *Stu*I according to the manufacturer's instructions (Takara Bio Inc.). The resultant DNA fragments were electrophoresed on a 1.2% Seakem Gold agarose gel (Cambrex Corporation) in 0.5× Tris–Borate–EDTA buffer with 8 g L^{-1} of thiourea

Table 1 Data on *Flavobacterium psychrophilum* strains analysed in this study

Cluster	PFGE patterns	Strains	Origin (fish farm)	Plasmid (kb)	MIC_{OTC} (μg mL^{-1})	MIC_{FLO} (μg mL^{-1})
	S1	253/08	GAL (1)	3.5	2.44	< 0.61
	S2	42/07	AS (2)	–	4.88	< 0.1
	S3	263/06	AS (3)	–	2.44	0.4
I	S4	264/08	AS (4)	–	2.44	< 0.61
		154/08	AS (3)	–	2.44	< 0.61
I	S5	2/05	AR (5)	–	4.88	1.22
II	S6	258/08	GAL (6)	3.5	9.74	1.22
II	S7	157/06	AS (7)	3.5	< 0.05	< 0.1
		85/07	AS (3)	3.5	< 0.61	< 0.1
		65/08	AS (3)	3.5	< 0.61	1.22
		81/08	AS (3)	3.5	< 0.61	< 0.61
	S8	141/08	AS (2)	5.5	9.74	< 0.61
	S9	35/07	C (8)	3.5	> 9.74	< 0.1
	S10	51/07	AS (9)	3.5	4.88	< 0.1
		25/08	AS (9)	3.5	4.88	< 0.61
III	S11	153/07	GAL (10)	3.5	4.88	0.4
III	S12	205/08-1	GAL (6)	3.5	9.74	2.44
		205/08-2	GAL (6)	3.5	9.74	1.22
		266/08	GAL (6)	3.5	9.74	2.44
III	S13	117/07	AS (3)	5.5	9.74	0.4
		33/08	GAL (11)	5.5	9.74	< 0.61
IV	S14	221/08	AS (7)	5.5	1.22	< 0.61
IV	S15	101/07	GAL (12)	5.5	2.44	< 0.1
		234/08	AS (7)	5.5	4.88	< 0.61
IV	S16	205/08-5	GAL (6)	5.5	4.88	2.44
		NCIMB 1947^T	US	2.7	< 0.61	0.4

GAL, Galicia (provided by ATRUGAL); AR, Aragón (provided by Universidad de Zaragoza); AS, Asturias; C, Cantabria (provided by Biomar); US, United States; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; FLO, florfenicol; MIC, minimum inhibitory concentrations; OTC, oxytetracycline.

(Merck KGaA,) using a CHEF-DR III system (Bio-Rad Laboratories). The running conditions were 6 V cm^{-1} at 14 °C for 22 h. The pulse times ranged from 0.2 to 5 s over 10 h and from 5 to 15 s over 12 h. The gels were stained for 15 min with ethidium bromide and photographed under UV light (Gel-Doc, Bio-Rad). Two strains were considered different when they differed in at least one band. Lambda ladder PFG marker was included as control.

Computer data analysis

All the gels were scanned, and the images were captured using the Gel Doc-2000 gel documentation system (Bio-Rad Laboratories). For analysis and comparison of the PFGE patterns, data analysis was performed using the Diversity Database software (Bio-Rad Laboratories). Similarity between profiles was evaluated using Jaccard's coefficient (S), and clustering was performed by the unweighted pair group method of analysis with arithmetic averages (UPGMA), using the software program MVSP (Multivariate Statistics Package ver. 3.1, Kovach Computing Services). Profiles with similarity coefficients > 0.8 were considered as members of the same cluster.

Antibiotic resistance

The antimicrobial agents selected to be tested in this study were oxytetracycline (OTC) and florfenicol (FLO), because they are currently used in freshwater aquaculture in Spain. OTC hydrochloride and FLO were purchased from Sigma. MIC values were determined using a broth macrodilution method as described by Alderman & Smith (2001).

Plasmid profile

Plasmid DNA was isolated using the QIAprep Spin kit (QIAGEN GmbH) and digested with *EcoR*I and *Hind*III (Takara Bio Inc.). Plasmids were separated in 0.8% agarose gels in 1× TAE buffer, and plasmid profiles were identified following visual examination for differences in size and band intensity.

Results

PFGE analysis

A total of 17 restriction enzymes (*Apa*I, *BamH*I, *Bgl*I, *Hind*III, *Kpn*I, *Mlu*I, *Nco*I, *Nhe*I, *Not*I, *Pvu*I, *Sal*I, *Sma*I, *Spe*I, *Sph*I, *Stu*I, *Xba*I and *Xho*I) were

tested, but the best discrimination between strains with the most easily interpreted patterns was provided by *Stu*I. A total of 17 different PFGE patterns were identified with this enzyme, with bands ranging from < 24 to 200 kb (Fig. 1). Only DNA fragments greater than 50 kb in length were scored in this analysis; the density of bands below this size meant that they could not be sufficiently distinguished. Variations in band intensity were not counted as a difference. Although none of the profiles included a high number of isolates, the most frequent were S7 and S12 (four and three isolates, respectively). The rest were represented by one or two isolates. The similarity dendrogram constructed based on the PFGE patterns identified four clusters, each of them having more than 80% pattern similarity (S = 0.8), and seven independent branches corresponding to patterns S1, S2, S3, S8, S9, S10 and the type strain (Fig. 2). Clusters I and II included two patterns each (S4 and S5, 12% of the isolates, $S \geq 0.94$; S6 and S7, 20% of the isolates, $S \geq 0.82$), whereas clusters III and IV included three patterns each (S11, S12 and S13, 24% of the isolates, $S \geq 0.82$; S14, S15 and S16, 16% of the isolates, $S \geq 0.86$). The high genomic diversity observed between the isolates is shown by the low value of S between all the patterns (S = 0.12; corresponding to the last knot of the dendrogram).

Cluster I comprised two isolates from Asturias and one from Aragón, all the isolates from cluster II, except one, were from Asturias, all the isolates from cluster III, except one, were from Galicia, and isolates from cluster IV were from Asturias and Galicia. The independent branches included isolates from Asturias, Galicia and Cantabria.

MIC

The MIC values for the bacterial isolates are shown in Table 1. More than 80% of the isolates had MIC of between 2.4 and 9.7 μg mL^{-1} for OTC. In the case of FLO, no resistant isolates were detected, with MICs between ≤0.1 and 2.44 μg mL^{-1}.

Plasmids

Three different plasmid profiles were detected among the 25 Spanish isolates, no plasmid, and 3.5 or 5.5 kb plasmids (Table 1). Twenty isolates (80%) had a plasmid, of which 65% had a 3.5 kb plasmid, and the remainder a plasmid of 5.5 kb. The reference strain harboured a 2.7 kb plasmid.

Discussion

Cold-water disease and rainbow trout fry syndrome caused by *F. psychrophilum* are infectious diseases with significant economic and health repercussions for rainbow trout farms worldwide. So far, there are only two studies concerned with the molecular characterization by PFGE of *F. psychrophilum* (Arai *et al.* 2007; Chen *et al.* 2008). This study describes the genotypic analysis of 25 isolates of *F. psychrophilum* from naturally occurring outbreaks in different fisheries in northern Spain.

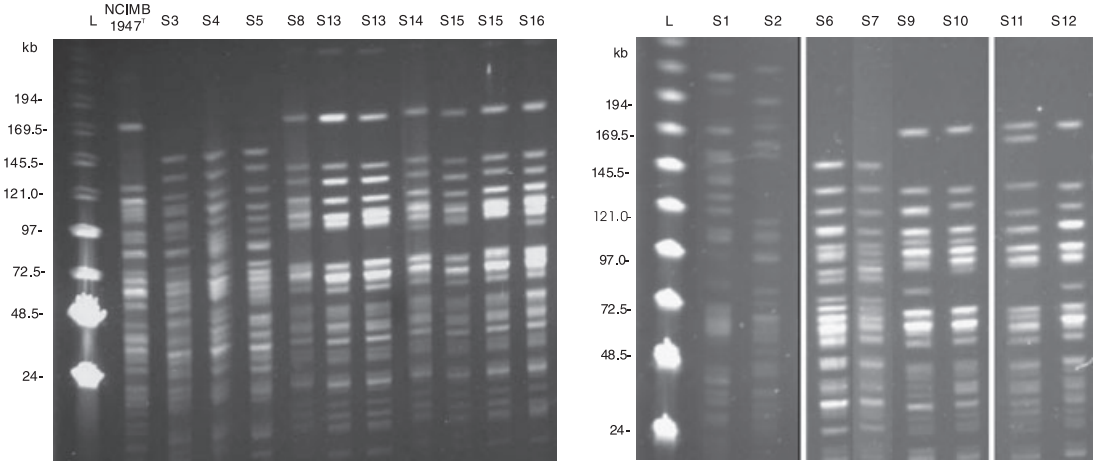

Figure 1 PFGE patterns of *Stu*I-digested total cellular DNA from *Flavobacterium psychrophilum* isolates from Spain and the type strain NCIMB 1947^T. L is the molecular weight marker (Mid Range PFG Marker, BioLabs).

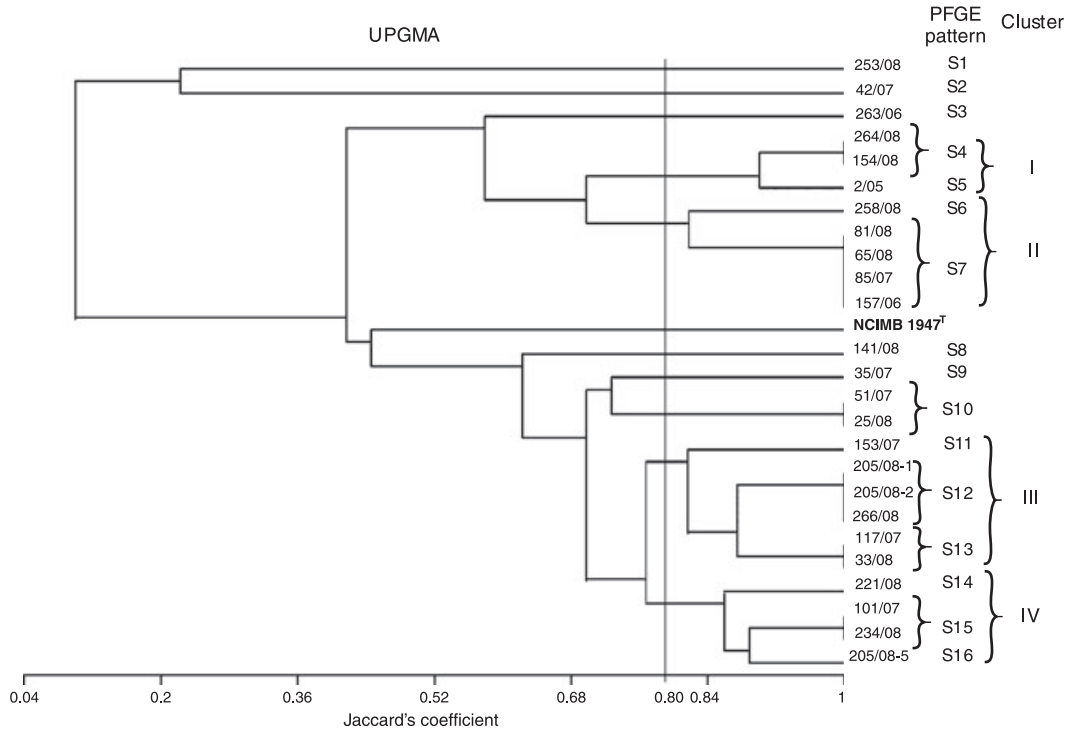

Figure 2 Dendrogram of Spanish *Flavobacterium psychrophilum* isolates and the type strain NCIMB 1974^T, based on UPGMA cluster analysis of the 17 PFGE band patterns obtained using *Stu*I. PFGE band similarity exceeding 80% was used as the criterion for cluster formation, and the isolates were classified into four clusters and six independent branches.

Pulsed-field gel electrophoresis is a valuable typing method that has been successfully used for the epidemiological investigation of many species of pathogenic bacteria (Tenover, Arbeit, Goering, Mickelsen, Murria, Persing & Swaminathan 1995). The results obtained in this work using PFGE analysis highlight the genetic heterogeneity among *F. psychrophilum* isolates recovered from locations in northern Spain. A total of 17 PFGE band patterns were confirmed in these isolates which were grouped into four clusters and seven independent branches in a dendrogram based on these PFGE patterns. There was a relationship between the origin of the isolates and the PFGE patterns obtained. The patterns S2, S3, S4, S7, S8, S10 (two isolates from the same farm) and S14 were only detected in isolates from Asturias; patterns S1, S6, S11, S12 (three isolates from the same farm) and S16 were found in Galicia; and pattern S9 in Cantabria.

We have detected 7 PFGE patterns circulating in Galicia, representing the independent branch S1 and clusters CII, CIII and CIV. Three of these PFGE patterns appeared in isolates from the same fish farm (258/08, 205/08-1, 205/08-2, 266/08 and 205/08-5), which caused recurrent outbreaks of CWD/RTFS during the last 3 months of 2008. It is noteworthy that these isolates had different plasmid profiles, were resistant to OTC and the MICs of FLO, although still sensitive, were slightly higher than average (between 1.22 and 2.44). These three genomic groups, as well as five other different groups (S2, S3, CI, S8 and S10) were detected in Asturias. In three fish farms from this region, a mixture of genetically different isolates was detected in each one during the period of the study. Some of them were genetically related with more than 50% similarity, suggesting a common ancestor. The most distantly related isolates were 253/08 (from Galicia) and 42/07 (from Asturias), showing $<30\%$ PFGE-based similarity to all other isolates. Madsen, Møller & Dalsgaard (2005) suggested that strains with different levels of virulence could be isolated from a stock of rainbow trout, and it would be of interest to test the virulence of the strains from the same farm.

Differences in PFGE patterns between OTC resistant and susceptible isolates could not be

determined, indicating that no susceptibility to OTC correlation exists between the four clusters. However, it is noteworthy that all the isolates with lower MICs to OTC (< 0.05–1.2 μg ml^{-1}) appeared in two farms from Asturias, and most of them presented the same PFGE pattern (S7). Higher MICs (2.4–9.7) appeared mainly in isolates from Galicia, most in the same fish farm and belonging to cluster III.

Oxytetracycline has been widely used to treat rainbow trout for CWD/RTFS, and lately, because of the increasing number of resistant strains isolated, the FLO has been introduced. In this study, more than 80% of the isolates were resistant to OTC, and all were susceptible to FLO, which remains the best choice for antimicrobial treatment of CWD/RTFS. Nevertheless, some isolates showed MICs of between 1.2 and 2.4 for FLO, which are slightly higher than average and suggest resistance problems in the future. Bruun *et al.* (2000) detected MIC values between 1.0 and 2.0 in some of the 387 Danish isolates analysed, and they considered that an improvement in the husbandry methods in all stages of fish production is required to limit the use of antimicrobial agents in aquaculture, as well as an understanding of the mechanisms involved in resistance development. These mechanisms are still unknown, but the lack of resistance plasmids in *F. psychrophilum* indicates that resistance determinants may be carried in mobile genetic elements (such as transposons) located in the bacterial chromosome.

Plasmid profile analysis of Spanish strains confirmed earlier results according to which *F. psychrophilum* isolated from rainbow trout usually harboured low molecular weight plasmids of 3.5 kb (Chakroun *et al.* 1998; Madsen & Dalsgaard 2000; Izumi & Aranishi 2004). No correlation was found between plasmid profile and PFGE typing, because isolates from the same cluster had different plasmid profiles (for example isolates from CIII). In this study, another plasmid of 5.5 kb was found in isolates from genotypes S8, S13 and CIV. However, a relationship between plasmid and antibiotic resistance could not be established, because some isolates resistant to OTC did not have a plasmid, whereas isolates susceptible to OTC harboured a 3.5 kb plasmid.

As reported by other authors (Madsen *et al.* 2005), we also found genetically different isolates coexisting in the same fish farm, causing disease outbreaks at the same time in different fish ponds (isolates 205/08-1, 205/08-2 and 205/08-5). Madetoja, Dalsgaard & Wiklund (2002) found that a particular clone of *F. psychrophilum* can dominate in one fish farm, such that the same strain can persist over a period of time causing epizootics in consecutive years. This is also suggested by our study, in which, for example, isolates 51/07 and 25/08 appeared in the same fish farm from Asturias. This suggests that bacterial cells released by dead and moribund fish remain in the water column for a long time increasing the risk of infections. For this reason, good management of farmed rainbow trout is recommended, as well as avoiding unnecessary handling to reduce damage to the skin (Madetoja, Nyman & Wiklund 2000).

In conclusion, our data proved PFGE to be a useful, reliable and reproducible technique to distinguish intraspecific genetic variation within isolated *F. psychrophilum* from rainbow trout in Spain. Although the number of isolates tested is not very high, DNA fingerprints obtained by PFGE demonstrated large heterogeneity among them. The antimicrobial susceptibility results showed that resistance to OTC among isolates is high, while all isolates remain susceptible to FLO. Nevertheless, surveillance in the use of FLO is recommended to prevent resistance problems in the future.

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