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Phenotypic and Genetic Characterization of *Lactococcus garvieae* Isolated in Spain from Lactococcosis Outbreaks and Comparison with Isolates of Other Countries and Sources

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The phenotypic and genetic analysis results for 84 isolates of *Lactococcus garvieae* (including 62 strains from trout with lactococcosis from four different countries, 7 strains from cows and water buffalos with subclinical mastitis, 3 from water, and 10 from human clinical samples) are presented. There was great phenotypic heterogeneity (13 different biotypes) based on the acidification of saccharose, tagatose, mannitol, and cyclodextrin and the presence of the enzymes pyroglutamic acid arylamidase and *N*-acetyl-β-glucosaminidase. *L. garvieae* also exhibited high genetic diversity by pulsed-field gel electrophoresis (PFGE), with 19 different pulsotypes among the isolates of *L. garvieae* studied. Only epidemiologically related strains, like the Spanish and Italian fish isolates and the cow and water buffalo isolates, displayed a close genetic relationship by PFGE, while the strains isolated from sporadic clinical cases, like the human isolates, were genetically unrelated. Overall, a general correlation between phenotypic and genetic data was observed. Epidemiological analysis of biotype and PFGE results indicated that the trout lactococcosis outbreaks in Spain and Portugal and those in France and Italy were produced by genetically unrelated clones. In Spain, two different clones were detected; the outbreaks diagnosed from 1995 onward were produced by a clone (biotype 2, pulsotype A1) which, although genetically related, was different from the one that was responsible for the outbreaks studied between 1991 and 1994 (biotype 1, pulsotype B). The Portuguese isolate had a biochemical profile identical to that of the Spanish strain isolated from 1995 onward and is also genetically closely related to this strain (pulsotype A2). There was a close relationship between the two pulsotypes (E and F) found in the Italian isolates. The French isolate (biotype 3, pulsotype D) was not genetically related to any other *L. garvieae* fish isolate. These results suggest the existence of diverse infection sources for the different lactococcosis outbreaks.

Lactococcus garvieae, *L. lactis* subsp. *lactis*, and *L. piscium* are the species of the genus *Lactococcus* with clinical significance in humans and animals (1, 15). *L. garvieae* is responsible for mastitis in cows and buffalos (9, 32), and it has been isolated from clinical specimens of human blood, urine, and skin (14–16). For this reason, *L. garvieae* is considered to be an emerging pathogen of increased clinical significance in both veterinary and human medicine.

L. garvieae is also a well-recognized bacterial fish pathogen. The first description in Europe of *L. garvieae* as a fish pathogen was in 1993 (27). Bacteriologic and molecular studies confirmed *L. garvieae* as the etiological agent of a hemorrhagic septicemia in farmed trout that was characterized by bilateral exophthalmos; darkening of the skin; congestion of the intestine, liver, kidney, spleen, and brain; and a characteristic hemorrhagic enteritis (10). Previously, in 1991, a new enterococcal species, *Enterococcus seriolicida*, was described as a new pathogen responsible for an infection of eels and yellowtail with symptoms identical to those produced by *L. garvieae* in trout (19). Further biochemical, protein profile, 16S rRNA sequencing, and DNA hybridization studies confirmed that *L. garvieae* and *E. seriolicida* are the same species (10, 12, 32). The septicemic infection produced by *L. garvieae* was termed lactococcosis (27) to differentiate it from infections produced by other taxa of gram-positive, catalase-negative cocci, such as *Streptococcus iniae*, *S. parauberis*, or *Vagococcus salmoninarum*, that are usually referred to by the generic term streptococcosis (3, 11, 23, 30, 35). During the last decade, infections by *L. garvieae*, or the synonymous bacterium *E. seriolicida*, have been diagnosed in many countries (12, 13, 19, 20). Lactococcosis is actually a worldwide bacterial disease affecting different fish species such as eels, yellowtail, or prawns, although the highest sanitary and economic impact is that on the trout farming industry (8, 12, 20, 26). Despite the increased clinical significance of *L. garvieae*, studies on the characterization and epidemiological relationship of isolates of this microorganism from different species and/or clinical samples are very limited (13, 32).

This report describes the phenotypic and genetic characterization of *L. garvieae* isolates from trout with lactococcosis in Spain between 1992 and 1998 and their comparison to the strains of *L. garvieae* isolated from cases of lactococcosis in other European countries, as well as with *L. garvieae* isolates from human clinical samples and from cows and water buffalos with subclinical mastitis.

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MATERIALS AND METHODS

***L. garvieae* isolates.** Eighty-four isolates of *L. garvieae* were studied (Table 1). Sixty-two isolates were recovered from diseased trout with lactococcosis. The 54

TABLE 1. Data on the *L. garvieae* strains analyzed in this study

Isolate(s)	Source	Country[a]	Yr of isolation	Phenotypic characteristics[b]						Bio-type	Pulso-type
				Sac	Tag	Man	Cedex	Pyra	β-Nag		
CP1	Trout	Spain/SF1	1991	+	+	+	−	+	−	1	B
CP2	Trout	Spain/SF1	1992	+	+	+	−	+	−	1	B
94/32	Trout	Spain/SF1	1994	+	+	+	−	+	−	1	B
1581, 95/861	Trout	Spain/SF2	1995	+	+	+	+	+	−	2	A1
1336	Trout	Spain/SF3	1996	+	+	+	+	+	−	2	A1
1182	Trout	Spain/SF4	1996	+	+	+	+	+	−	2	A1
1184, 1185	Trout	Spain/SF5	1996	+	+	+	+	+	−	2	A1
1987, 1999, 2000, 2013, 2014, 2001, 2002, 2007, 2005, 2006, 4316	Trout	Spain/SF1	1998	+	+	+	+	+	−	2	A1
1982	Trout	Spain/SF6	1998	+	+	+	+	+	−	2	A1
1989, 1990, 1919, 1758, 1964, 1759, 1940, 1944, 1957, 1967	Trout	Spain/SF7	1998	+	+	+	+	+	−	2	A1
1935, 1936, 1909, 1893, 1934, 1955, 1954, 1924, 1925	Trout	Spain/SF8	1998	+	+	+	+	+	−	2	A1
1958, 1981, 1956, 1975, 2016, 2017	Trout	Spain/SF9	1998	+	+	+	+	+	−	2	A1
1960, 1961, 1988	Trout	Spain/SF10	1998	+	+	+	+	+	−	2	A1
1966	Trout	Spain/SF11	1998	+	+	+	+	+	−	2	A1
1959, 1986, 1985, 4294	Trout	Spain/SF12	1998	+	+	+	+	+	−	2	A1
864	Trout	Portugal	1995	+	+	+	+	+	−	2	A2
2398	Trout	France	1998	−	+	+	−	+	−	3	D
1691	Trout	Italy/IF1	1997	−	−	+	−	−	−	4	F2
1683	Trout	Italy/IF2	1997	−	−	+	−	−	−	4	F1
1684	Trout	Italy/IF3	1997	−	−	+	−	−	+	5	E
1685, 1689	Trout	Italy/IF4	1997	−	−	+	+	−	+	6	F1
1687	Trout	Italy/IF5	1997	−	−	+	+	−	+	6	E
2008, 4284	Water	Spain/SF1	1998	+	+	+	+	+	−	2	A1
4289	Water	Spain/SF1	1998	−	+	+	−	+	−	7	K
34, 41, 42	Cow	Spain	1999	−	+	+	−	+	−	7	J
1205, 1193, 1183	Buffalo	Brazil	1998	−	−	−	−	+	−	8	P1
1195	Buffalo	Brazil	1998	−	−	−	−	+	+	9	P2
IM-86	Human	Brazil	1998	−	−	+	+	−	−	10	Q
306-79	Human	United States	1997	−	+	+	+	+	−	11	S
673-80, 588-80	Human	United States	1980	−	−	+	+	−	−	10	R
2182-81	Human	United States	1981	+	+	+	−	+	−	1	H
1108-86	Human	United States	1986	+	+	+	−	+	−	1	N
2486-87	Human	United States	1987	+	+	+	+	+	−	2	O
364-88	Human	United States	1988	+	+	+	−	−	−	12	G
240-88	Human	United States	1988	+	+	+	−	−	−	12	L
66-90	Human	United States	1990	−	+	+	−	−	−	13	M
L. garvieae ATCC 43931^T	Cow	United Kingdom	1984	−	−	+	−	−	−	4	I
E. seriolicida ATCC 49156^T	Yellowtail	Japan	1991	−	−	+	+	−	−	10	C

[a] SF and IF are different fish farms in Spain and Italy, respectively. Fish farms SF1, SF3, SF4, and SF6 are located in the central region of Spain; SF2, SF5, and SF12 are located in the north of Spain; SF7 is in the northwest of Spain; SF8, SF9, and SF10 are located in the west of Spain; SF11 is located in the south of Spain. The IF fish farms are located in the north of Italy.

[b] Sac, Tag, Man, and Cedex, acidification of saccharose, tagatose, mannitol, and cyclodextrin, respectively. Pyra and β-Nag, presence of the respective enzymes.

Spanish isolates were collected between 1992 and 1998 from fish farms in different geographic areas. The Portuguese, the French, and the six Italian isolates were also recovered from trout with lactococcosis. Three isolates of *L. garvieae* were recovered from the water pond of a fish farm with chronic lactococcosis. Three *L. garvieae* isolates from cows, 4 from water buffalo with subclinical mastitis, and 10 from humans and two type strains (*L. garvieae* ATCC 43921^T and *E. seriolicida* ATCC 491561^T) were also included in the study. *E. seriolicida* ATCC 491561^T was purchased from the American Type Culture Collection. All isolates, stored frozen (−80°C), were grown on Columbia blood agar (bioMérieux España, S.A.) at 30°C for 24 h.

Biochemical and enzymatic characterization and PCR assay. Biochemical and enzymatic tests were performed with the Rapid ID 32 Strep and API 50CH systems (bioMérieux España, S.A.) by following the manufacturer's instructions, except for the temperature of incubation, which was always 30°C. The identification of the *L. garvieae* isolates was confirmed by PCR assay as described by Zlotkin et al. (36).

Analysis of chromosomal DNA restriction patterns by PFGE. *Lactococcus* cells were grown aerobically in brain heart infusion broth (Difco) at 37°C to an A_{610} of approximately 0.6. The cells were harvested by centrifugation (3,500 × *g*, 10 min, 4°C) and washed twice with buffer (10 mM Tris-HCl, 10 mM EDTA, 10 mM EGTA, 1 M NaCl [pH 8]). Agarose plugs were made from a 1:1 mixture of 2% low-melting-point agarose and the cell suspension. The plugs were lysed in buffer (6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, 0.5% [wt/vol] Brij 58, 0.2% [wt/vol] sodium deoxycholate, 0.5% [vol/vol] lauroyl sarcosine, lysozyme at 5 mg/ml) for 24 h at 37°C. The cells were treated for 50 h at 56°C with the same volume of a solution (0.25 M EDTA, 20 mM NaCl, 1% lauroyl sarcosine [pH 9]) containing proteinase K at 2.5 mg/ml and washed three times with Tris-EDTA buffer for 1 h at 4°C. *Apa*I (Promega Co.) was used for restriction endonuclease digestion in accordance with the manufacturer's instructions. The fragments were resolved by pulsed-field gel electrophoresis (PFGE) with electrophoresis-grade agarose (1%; Boehringer Mannheim) by using a CHEF-DR III System (Bio-Rad). The following parameters were used: running time, 21 h; temperature, 14°C; voltage gradient, 200 V; initial pulse time, 0.1 s; final pulse time, 25 s; included angle, 120°. The gels were stained with ethidium bromide (0.5 μg/ml) for 15 min, destained in distilled water, and photographed under UV light. A lambda ladder PFGE marker (Boehringer Mannheim) was used for molecular size determination.

PFGE pattern analysis. The similarities between restriction endonuclease digestion profiles (REDPs) were expressed as Jaccard similarity indexes determined by the numerical taxonomy program TAXAN (Information Resources Group, Maryland Biotechnology Institute, University of Maryland, College Park). A similarity matrix was computed and transformed into an agglomerative cluster using the unweighted pair group method with arithmetic averages (UPGMA) (31).

RESULTS

Phenotypic studies and PCR identification. All of the *L. garvieae* isolates studied gave the expected 1,100-bp PCR amplification product, which is specific for this microorganism (36), confirming the preliminary biochemical identification. There was great biodiversity among the *L. garvieae* isolates, with 13 different biotypes, based on the acidification of saccharose, tagatose, mannitol, and cyclodextrin and the presence of the enzymes pyroglutamic acid arylamidase (Pyra) and *N*-acetyl-β-glucosaminidase (β-Nag) (Table 1). All of the strains of *L. garvieae*, except the water buffalo isolates, produced acid

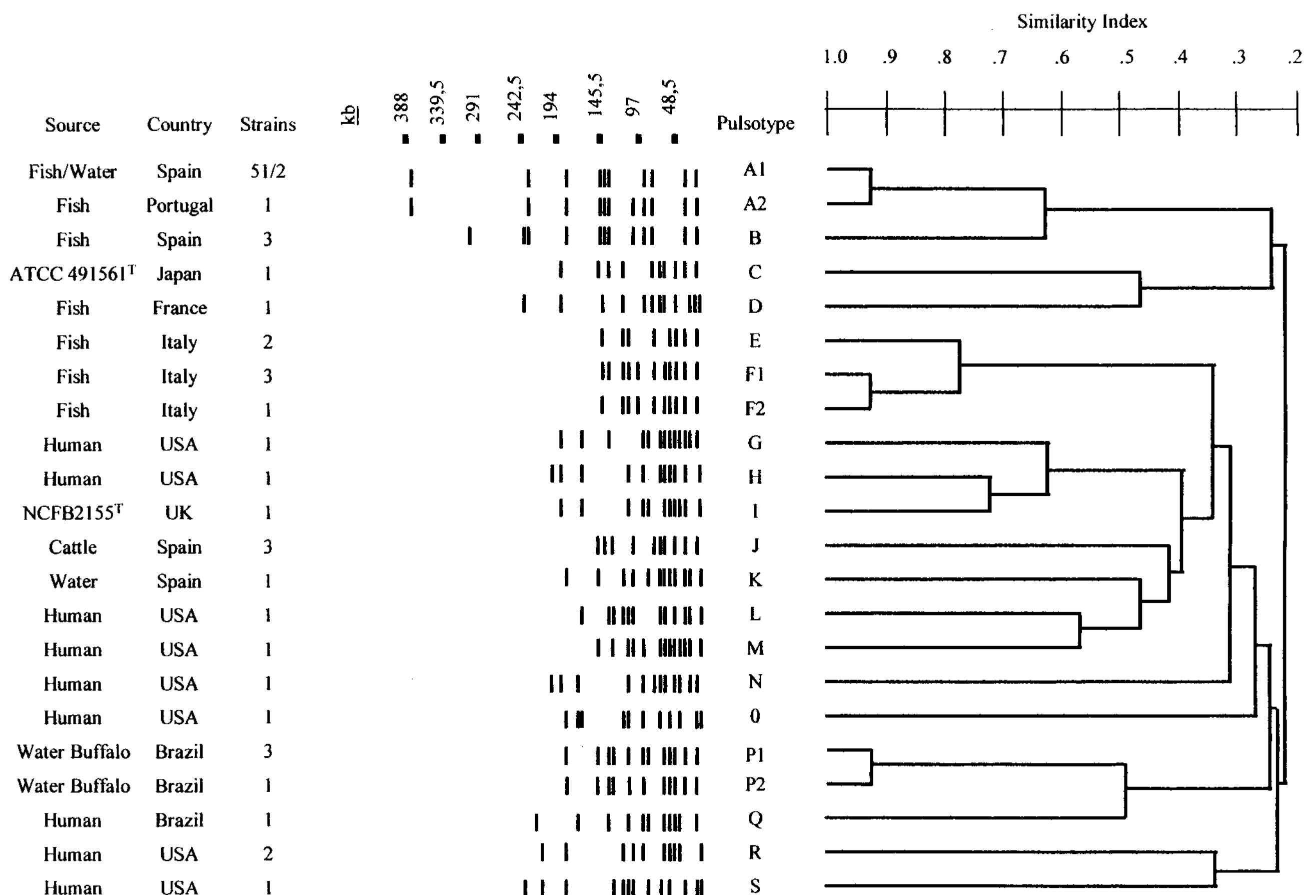

FIG. 1. Dendrogram of *L. garvieae* isolates based on UPGMA cluster analysis of the 20 different *Apa*I pulsotypes obtained in this study.

from mannitol. The enzyme Pyra was present in all of the trout isolates except the Italian strains. The cow and water buffalo isolates, as well as 6 out of the 10 human isolates analyzed, were Pyra negative. All of the Spanish fish isolates and the Portuguese isolate produced acid from sucrose and tagatose. The Spanish isolates recovered in 1991, 1992, and 1994 did not produce acid from cyclodextrin, and the enzyme β-Nag was not present (biotype 1). On the other hand, the Portuguese isolate and Spanish strains isolated after 1995 produced acid from cyclodextrin (biotype 2). The French isolate produced acid only from tagatose, and it did not produce acid from cyclodextrin, nor did it have the enzyme β-Nag (biotype 3). The Italian strains did not produce acid from either sucrose or tagatose. Two strains neither produced acid from cyclodextrin nor had the enzyme β-Nag (biotype 4), one strain did not produce acid from cyclodextrin but did have the enzyme β-Nag (biotype 5), and the other three strains produced acid from cyclodextrin and had the enzyme β-Nag (biotype 6).

The strains isolated from cows produced acid from tagatose but not from sucrose and cyclodextrin, and the enzyme β-Nag was not present (biotype 7). The water buffalo strains did not acidify sucrose, tagatose, or cyclodextrin. Three strains did not have the enzyme β-Nag (biotype 8), while the remaining strain produced this enzyme (biotype 9). Human isolates exhibited the greatest diversity, with eight different biochemical-enzymatic profiles (biotypes 1, 2, and 10 to 13). Three of the *L. garvieae* strains from water exhibited biotype 2, and the other strain had biotype 7.

PFGE analysis. The *Apa*I enzyme generated 19 different DNA fragment profiles, designated pulsotypes A to S, with 7 to 13 bands over a size ranging of about 25 to 343 kb. Figure 1 shows the dendrogram obtained with the 19 different patterns after UPGMA clustering. The Spanish clinical isolates exhibited two different *Apa*I patterns: pulsotype B for the strains isolated in 1991, 1992, and 1994 and pulsotype A (subtype A1) for all of the *L. garvieae* strains isolated from 1995 onward, irrespective of geographic origin. The Portuguese strain was closely related to the Spanish isolates of pulsotype A, differing from the pattern displayed by these strains by only one restriction fragment and referred to as subtype A2. The Italian strains displayed two different patterns, designated pulsotypes E and F, that clustered in a genetically related cluster (0.8 index similarity). Pulsotype F had two subtypes (F1 and F2). The French isolate belonged to a different group (pulsotype D).

The strains isolated from water buffalos with subclinical mastitis displayed two very similar REDPs with a similarity index of 0.95. The two REDPs differed by only one band and were considered to be two subtypes (P1 and P2) of the same group (pulsotype P). The three strains isolated from cows had the same pulsotype (J). Two of the strains isolated from water exhibited the same pulsotype as the Spanish clinical isolates (A1); the other one was not genetically related (pulsotype K). The human isolates exhibited great diversity, with nine different REDPs (pulsotypes G, H, L, M, N, O, Q, R, and S) with a

low level of similarity, which agrees with the phenotypic diversity of these isolates.

DISCUSSION

Lactococcosis is one of the infectious diseases with significant economic and sanitary repercussions for trout farms in Mediterranean countries during the summer months (12, 13, 19, 20, 26, 30). Only two studies concerned with the epidemiological characterization of *L. garvieae* have been published until now. In the first one, isolates responsible for subclinical mastitis in water buffalos, together with human clinical isolates, were characterized by PFGE (6) and in the second one, a total of 15 strains isolated from diseased fish from different countries were analyzed by ribotyping (13). This epidemiological study was based on PFGE results because of the high discriminative power of PFGE (2, 18, 21, 22), and the phenotypic characterization was done to correlate the phenotypic and genetic data. With a total of 84 isolates of *L. garvieae* analyzed, this is the largest phenotypic and genetic comparative study trying to correlate these data and it is also the first one including isolates representative of the main diseases caused by *L. garvieae* and the species in which they have been diagnosed.

Most of the papers published to date have used the API 50CH and API 20 Strep systems for the phenotypic characterization of *L. garvieae* (7, 8, 13, 26, 34). Our results for those tests included in both of these commercial systems are identical to those reported by the authors of those papers, as well as to those of studies in which conventional biochemical characterization was used (5). The presence of the enzymes Pyra (83% of positive strains), β-Nag (6% of positive strains), alanine-phenylalanine-proline arylamidase (100% of positive strains), glycyl-tryptophan arylamidase (0% of positive strains), and urease (0% of positive strains) and the acidification of cyclodextrin (75% of positive strains), pullulan (0% of positive strains), methyl-β-D-glucopyranoside (100% of positive strains), and β-mannosidase (0% of positive strains) are included only in the Rapid ID 32 Strep system. Although it is not possible to compare the results of these tests with those obtained by other authors, our results agree with those of the bioMérieux database for *L. garvieae*. With the Rapid ID 32 Strep identification system, all of the Spanish, French, and Portuguese isolates, as well as the type strains of *L. garvieae* and *E. seriolicida*, gave negative results for the acidification of ribose. However, all of the strains were systematically positive for this test with the API 50CH system, which agrees with previous descriptions of this species (8–10, 12, 32). These results should be taken into account when this system is used for the routine identification of clinical isolates of *L. garvieae*.

Our results indicated great phenotypic heterogeneity and genetic diversity among the *L. garvieae* isolates studied, with a generally good correlation between the phenotypic and genetic properties of *L. garvieae*. This correlation was especially evident for the trout, cow, and water buffalo isolates. However, two human strains (2182-81 and 1108-86) had the same biotype as the 1991 to 1994 Spanish fish isolates but very different pulsotypes. Similarly, human isolate 2486-87 and the Spanish fish isolates recovered in 1995 also had the same biotype but different pulsotypes (Table 1; Fig. 1). The existence of different isolates that have the same biotype but differ by genetic methods has also been observed in other microorganisms (25, 28) and can be explained by the much higher discriminatory power of PFGE than biotyping (2).

Only epidemiologically related strains, like the Spanish and Italian fish isolates and the cow and water buffalo isolates, displayed a close genetic relationship by PFGE (Fig. 1). The close relationship of the Italian isolates found by us with PFGE is similar to that of other Italian *L. garvieae* strains found by ribotyping (13). The single French isolate was not genetically related to any other *L. garvieae* fish isolate. In Spain, two different clones were implicated in the lactococcosis outbreaks between 1991 and 1998. The outbreaks diagnosed since 1995 were produced by a clone (biotype 2, pulsotype A1) which, according to Tenover et al. (33), although genetically related, was different from the one responsible for the outbreaks studied between 1991 and 1994 (biotype 1, pulsotype B). The single Portuguese isolate analyzed had a biochemical profile identical to that of the Spanish strain isolated from 1995 onward and is also closely genetically related to this strain, indicating a clonal relationship between them. Three biotypes had been established for the *L. garvieae* strains isolated from fish based on their ability to acidify sucrose and/or tagatose (13). The 62 Spanish trout isolates studied by us all produced acid from both sugars, while the 6 Italian isolates did not acidify either sugar. Identical results have been described for other Spanish and Italian *L. garvieae* fish isolates (13, 26). Thus, the phenotypic differences observed between the clinical fish isolates from different geographical areas, in correlation with the PFGE results, could be useful as phenotypic epidemiological markers for lactococcosis outbreaks. On the other hand, the strains isolated from sporadic clinical cases, like the human isolates, were genetically unrelated, with a similarity index usually lower than 0.5 (33), results that agree with previous reports for other widely distributed pathogens (17). The low genetic relatedness observed between different isolates may suggest that some of the most diverse strains are not *L. garvieae*. This low genetic relatedness might reflect bacterial populations genetically isolated but showing a high recombination rate. So, after long-term genetic isolation, produced by a difference in geographical or habitat distribution, the bacterial populations show a great divergence. Similar low genetic relatedness has been observed in *Neisseria* isolates by using PFGE analysis (4). Nevertheless, the identities of those most diverse isolates had been previously confirmed. Thus, the identities of the CP1 and CP2 Spanish trout isolates of pulsotype B were confirmed by sequencing of their 16S rRNA genes (10), and those of the buffalo isolates (pulsotypes P1 and P2) were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell protein and DNA-DNA hybridization analysis (32). In addition, all of the strains included in the study gave the expected 1,100-bp PCR amplification product with the specific *L. garvieae* PCR assay (36), all which precludes any doubt about the identity of all of the strains included in the study as *L. garvieae*.

Unlike other highly host-adapted bacterial fish pathogens, *L. garvieae* is pathogenic for several fish species, ruminants, and humans and could therefore be considered potentially zoonotic. However, the low genetic relatedness between the animal and human isolates included in this study (Fig. 1) does not allow confirmation of this hypothesis. Asymptomatic infected fish can act as carriers (24), contributing to the dissemination and transmission of *L. garvieae* through the trade of livestock. This fact could explain the rapid spread of the *L. garvieae* pulsotype A strain, first isolated in the north of Spain in 1995, throughout different Spanish regions in only 4 years. *L. garvieae* is eliminated through the feces of diseased or carrier fish (24). This is probably the origin of the 98/4284 and 2008 *L. garvieae* strains, which had the same biotype and pulsotype as the clinical isolates (2; A1) and were isolated from the water of a fish farm in which lactococcosis was endemic during the latter years. Therefore, the contaminated water seems to play an important role in the horizontal transmission

of lactococcosis, probably through the fecal-oral route (29). *L. garvieae* is also widespread in the environment (19), and this could be the origin of strain 98/4289. However, from the facts that this strain had the same biotype (biotype 7) as the cow isolates and was genetically more closely related to these strains and some of the human isolates than to the Spanish fish clinical strains (Table 1; Fig. 1) arise the possibilities that some environmental *L. garvieae* strains evolved from mammals and that they are hypothetically implicated in the epidemiology of fish lactococcosis. However, our results cannot substantiate this hypothesis. In order to do that, it will be necessary to carry out larger molecular epidemiological studies including environmental, human, and animal *L. garvieae* isolates, as well as experiments to assess the pathogenicity of animal or human isolates from different fish species, mainly for trout.

In summary, the analysis of phenotypic and genetic results indicated that trout lactococcosis outbreaks in Spain and Portugal and those in France and Italy were produced by genetically unrelated clones, suggesting the existence of diverse infection sources for the different lactococcosis outbreaks.

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