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Lactococcus garvieae, an emerging pathogen for the Portuguese trout culture

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Abstract

We report here the first description in North Portugal of epizootic episodes of lactococcosis causing important mortalities in cultured rainbow trout associated with high water temperatures. The outbreaks that occurred during the warm seasons of 2002 and 2003 affected all size fish, reaching up to 90% mortality in some farms. The taxonomic studies of the isolates obtained indicated that they belonged to *Lactococcus garvieae*. This identification was confirmed by PCR amplification using specific primers. The isolates proved to be highly pathogenic for rainbow trout ($LD_{50} \leq 10^2$ cells), and belonged to the same genetic group as revealed by RAPD analysis. All these findings indicate that lactococcosis by *L. garvieae* is an emerging disease for the Portuguese trout culture and that appropriate measures must be taken in order to avoid the spread of this disease to other areas of the country.

Lactococcus garvieae, one of the major Gram positive cocci pathogenic for fish, is considered a serious problem in cultured marine and freshwater fish species such as yellowtail (*Seriola quinqueradiata*) in Japan and rainbow trout (*Oncorhynchus mykiss*) in Europe and Australia (Diler et al., 2002; Eldar & Guittino, 1999; Eldar et al., 1999; Kusuda et al., 1991). In Spain, also within the Iberic peninsula, this disease has appeared in rainbow trout farms from 1991 onward (Doménech et al., 1993; Ravelo et al., 2001) and, at present, is considered one of the most important risk factors in the trout industry during the summer months. To our knowledge, and regardless of the inclusion in some works of Portuguese isolates of *L. garvieae* (Doménech et al., 1993; Ravelo et al., 2003), there are no reports of lactococcosis outbreaks in Portugal. One reason for this lack of descriptions in Portugal can be the difficulty in the identification of *L. garvieae* on the basis of phenotypical tests, which can barely differentiate this bacterium from other Gram positive cocci such as the human pathogen *L. lactis* subsp. *lactis* (Elliot et al., 1991; Fefer et al., 1998) or the *Enterococcus* – like strains isolated from diseased fish (Eldar et al., 1999; Toranzo et al., 1994).

Besides fish, *L. garvieae* has been isolated from cows and buffalos (Teixeira et al., 1996) and it has also been recovered from human sources (Elliot et al., 1991; Fefer et al., 1998). In addition, this bacterium has been isolated recently in Taiwan from diseased freshwater prawn *Macrobrachium rosembergii* (Chen et al., 2001). All these facts indicate the expanding importance of *L. garvieae*.

Between June and October of 2002, several disease outbreaks causing high mortalities occurred in four rainbow trout farms located in the north of Portugal. Epizootics were associated with a rise in the water temperatures, which in some cases were higher than 21°C for more than two months. An initial treatment with oxytetracycline was applied in two of the farms, but although at first it seemed to control the outbreaks, after three weeks the mortalities started again with higher virulence. Therapy was subsequently changed to enrofloxacin and erythromycin with similar results. Total fish losses at the end of September amounted from 20 to 90% depending on the farm. It is interesting that fish of all sizes were affected, from 5 g to market-size (>200 g), although mortalities were heavier in bigger fish (>100 g). A similar situation occurred during 2003, where cumulative mortalities between 25 and 30% were observed in the farms at the end of the warm season. The use of vaccination as preventive measure during 2004 avoided the appearance of epizootic outbreaks in most of the affected farms.

In all cases, diseased fish showed a pronounced uni- or bilateral exophthalmia with periocular haemorrhages and, in some cases, loss of the eye. Internally, clinical signs consisted of an accumulation of ascitic fluid in the peritoneal cavity and haemorrhages in the liver and muscle. These signs are in concordance with those previously described for lactococcosis outbreaks (Austin & Austin, 1999).

Isolate	Farm	Date of isolation	Cumulative mortality in the farm	LD_{50} (cells/fish)
PT 2.1	A	July, 2002	90%	2×10^1
PT 4.3	B	August, 2002	80%	4×10^1
PT 5.1	B	August, 2002		NT[a]
PT 7.1	C	July, 2002	40%	2×10^2
PT 8.3	C	August, 2002		NT
PT 26.1	C	July, 2003	30%	3×10^1
PT 29.1	C	August, 2003		NT
PT 33.2	C	September, 2003		NT
PT 15.1	D	June, 2002	20%	6.5×10^2
PT 18.3	D	August, 2002		NT
PT 20.2	D	October, 2002		NT
PT 20.3	D	October, 2002		NT
PT28.1	D	July, 2003	25%	2×10^1
PT 32.1	D	August, 2003		NT

[a]NT, not tested

Table 1. Origin of *Lactococcus garvieae* isolates used in this study.

Samples of spleen, kidney, liver, brain, and eye were subjected to microbiological examination by inoculation on Tryptic Soy Agar supplemented with 1% (wt/vol) NaCl (TSA-1)(Difco Laboratories, Detroit, Mich.) and on Columbia sheep blood agar (CBA)(Oxoid Ltd., Madrid, Spain), and incubated at 25°C for 48 h. Pure cultures of the different colony types obtained were subjected to taxonomic analysis by standard morphological, physiological, and biochemical plate and tube tests, as well as the commercial RAPID ID 32 Strep system (Biomeriux, Madrid, Spain) following the recommendations of Ravelo et al. (2001). A total of 14 isolates were obtained from the different mortality episodes in the four farms (Table 1). The features of the isolates obtained from the sampled fish, regardless of their origin, were similar, all of them being Gram-positive facultatively anaerobic, non-motile, α-haemolytic, non-spore-forming cocci that occurred singly or in short chains. They were Voges-Proskauer positive but did not produce oxidase, catalase, or indole. In addition, isolates possessed the enzyme arginine dihydrolase but not lysine or ornithine decarboxilase. These characteristics allowed their presumptive identification as *Lactococcus garvieae*.

The use of RAPID ID 32 Strep system showed that the isolates were distributed within two identifiable profiles, 30333111111 (isolates from farms B and C) and 30337131111 (isolates from farms A and D). According to the API database, the profiles obtained corresponded to a low discriminative identification (between *L. garvieae* and *L. lactis* subsp. *lactis*) and a non-acceptable profile (with *Enterococcus faecium* and *L. lactis* subsp. *lactis* as significant taxa) respectively. However, these profiles have been described by Ravelo et al. (2001) as belonging to *L. garvieae*. The test responsible of the failure of the RAPID ID 32 Strep database to identify the isolates as *L. garvieae* is the hydrolysis of hippurate, which has been described as uncommon for this microorganism. However, several authors (Cheng & Chen, 1998; Ravelo et al., 2001) reported some strains positive for this character. In addition, variability in the utilization of carbohydrates or in the presence of enzymes have been widely described elsewhere (Eldar et al.,1999; Ravelo et al., 2001; Vela et al., 2000). In our study, variability between profiles were determined by the different results observed among strains not only for hydrolysis of hippurate, but also for β-galactosidase activity, and acid production from L-arabinose and glycogen. On the other hand, the majority of the Portuguese isolates were susceptible "in vitro" to all the chemotherapeutic agents tested. Only in farm D were resistance observed to some compounds including oxolinic acid, trimethoprim-sulfamethoxazole and enrofloxacin in two *L. garvieae* strains (PT 18.3 and PT 20.3) isolated at the end of the 2002 summer period, as well as in the 2003 isolates.

Two serotypes associated with the presence (serotype KG^-) or absence (serotype KG^+) of capsular material have been described within *L. garvieae*, with the capsulated serotype being associated with greater virulence (Romalde & Toranzo, 2002). For the serological characterization of the Portuguese isolates, slide agglutination tests were conducted as described by Toranzo et al. (1987) using antisera against representative strains of capsulated and non-capsulated phenotypes

within *L. garvieae*, the Japanese isolate YT-3 (non-capsulated) and the Spanish reference strain CECT 5800 (capsulated)(Romalde et al., 2003). All the Portuguese isolates belonged to the capsulated serotype KG^-. The presence of a capsule was also highlighted by the glutaraldehyde-lysine-fuchsine staining method (data not shown).

DNA isolation from the isolates was performed using the Insta-Gene Matrix (Bio-Rad, Madrid, Spain). Identification by PCR and random amplified polymorphic DNA (RAPD) typing were carried out following the protocols and conditions of Ravelo et al. (2003), using a T Gradient thermocycler (Biometra, Goettingen, Germany). In the RAPD assays, representive strains of the different genetic groups of Ravelo et al. (2003) were included for comparison. Identification of isolates as *L. garvieae* was confirmed by PCR, since all of them gave the specific 1,100 bp long amplification product, which was not observed in the negative control (data not shown). In addition, all the Portuguese isolates yielded the same RAPD profile (100% similarity) which, on the other hand, was identical to that obtained for the Spanish reference strain CECT 5800 (Figure 1). RAPD has recently been described as a good tool for epidemiological studies in *L. garvieae* (Ravelo et al., 2003). By using this technique, three genetic groups were described within this bacterial species, composed by the Spanish, English and Turkish strains (Group A), the French and Italian strains (Group B) and the Japanese strains (Group C)(Ravelo et al., 2003). On the basis of the results obtained in this study, all the Portuguese isolates were included within the genetic Group A.

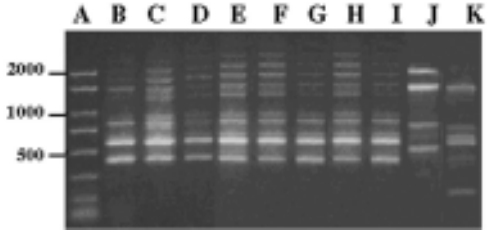

Figure 1. RAPD fingerprints obtained for thePotuguese *L. garvieae* isolates in comparison with representative strains from the genetic groups described by Ravelo et al. (2003). Lanes: A, AmpliSize™ Molecular Ruler (50-2000 bp ladder; Bio-Rad); B, reference strain CECT 5800 (genetic group A); C, PT 2.1; D, PT 4.3; E, PT 7.1; F, PT 18.3; G, PT 20.2; H, PT 29.1; I, PT 32.1; J, Italian isolate 657-2 (genetic group B); K, reference strain NCDO 2155 (genetic group C). The molecular sizes (in bp) are indicated on the left.

Representative isolates from the different farms were tested for pathogenicity in fingerling rainbow trout (10 g) maintained at 19 ± 1°C in freshwater aquaria with aeration. After fish stocks were determined by culture to be free of *L. garvieae* or other bacterial species, challenges were performed by intraperitoneal (i.p.) injection as previously reported (Toranzo et al., 1983). The degree of virulence (50% lethal dose: LD_{50}) was calculated by the Reed and Müench method (1938). Virulence tests indicated that the isolates were highly pathogenic for rainbow trout, with LD_{50} values ranging from 2×10^1 to 6.5×10^2 cells (Table 1). Mortalities occurred within 3 to 9 days after challenge. It is noteworthy that all moribund fish exhibited most of the external and internal clinical signs of the natural disease. In addition, haemorrhages in the brain were also observed. In all cases, the challenge strains were recovered in pure culture from kidney, spleen and brain. This elevated pathogenicity is in accordance with the high mortalities observed

in the farms during the diverse outbreaks.

It is interesting to point out that the affected Portuguese farms do not possess their own trout production and all the eggs and/or fingerlings originate from imports from other European countries. However, the specific origin of the Portuguese isolates could not be positively traced since the majority of the European strains belong to the same genetic group (Ravelo et al., 2003). On the other hand, we cannot rule out the increased aquaculture operations in the Northern Portugal area (Sousa et al., 2001) or the presence of this microorganism in the natural environment of the farms as the origins of the lactococcosis outbreaks.

In summary, we report here the first description of lactococcosis outbreaks in Portugal. The important mortalities observed in rainbow trout of all sizes (from fingerlings to market-size fish), as well as the number of farms affected suggested that this *L. garvieae* can become one of the main limiting factors of the trout culture in Portugal, as has occurred in other European Countries such as Spain. Therefore, the implementation of preventive measures, like vaccination with appropriate vaccines, can be of critical importance in order to avoid the spreading of this disease to other areas of the country.

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