Identification of *Lactococcus garvieae* by PCR

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Lactococcus garvieae (junior synonym *Enterococcus seriolicida*) is an emerging zoonotic agent isolated from economically important fish (rainbow trout and yellowtail), from cattle, and from humans. Clindamycin susceptibility is the only phenotypic test which can differentiate *L. garvieae* from *Lactococcus lactis*, another emerging agent in humans. A PCR assay for the identification of *L. garvieae* was developed and resulted in an amplified fragment of 1,100 bp in size. The PCR assay was shown to be specific to *L. garvieae*. The PCR assay was positive for all the *L. garvieae* strains tested, which originated from three different continents (Asia, Australia, and Europe). The PCR assay was negative for the phenotypically similar *L. lactis* and for all the other fish pathogens tested, including *Streptococcus iniae* and *Aeromonas salmonicida*. The PCR assay was applied to plasma obtained from diseased animals and was found sensitive enough to detect bacteria from 1 µl of plasma. The PCR assay that was developed is the only practical test besides the clindamycin test which can specifically identify the zoonotic agent *L. garvieae* and which can differentiate it from *L. lactis*.

Lactococcus garvieae is an emerging zoonotic pathogen which has been isolated from cattle, from various species of fish, and from humans (2, 5, 6, 9, 13). With the development of intensive aquaculture, streptococcal infections of fish have become a major problem worldwide. The symptomatologies of fish infected by the various gram-positive cocci pathogenic for fish are similar and do not allow a rapid identification of the agent responsible for the disease (1). In addition, the isolation and the bacteriological diagnosis of these gram-positive bacteria are not simple and are time-consuming (8, 10, 11). *L. garvieae* has proven to be one of the major gram-positive coccus pathogens for fish (5, 9). Taxonomic studies based on DNA-DNA hybridization studies (5) and sequence analyses of 16S rRNA (4) indicate that another fish pathogen, *Enterococcus seriolicida* (12), is in fact synonymous with *Lactococcus garvieae* and should be classified as *L. garvieae* (5).

The published identification scheme of *L. garvieae* based on biochemical and antigenic characteristics (7, 10) can barely differentiate *L. garvieae* from *L. lactis* subsp. *lactis*, which has also been reported as an etiologic agent of infections in humans (6, 13). A clear test is necessary to differentiate the two species. In addition, a rapid bacteriological diagnostic method is necessary to initiate prompt therapeutic and prophylactic measures in order to limit the economic losses generated by these infections in aquaculture. In response to these needs, we have developed a new PCR assay based on primers deduced from the regions carrying the 16S rRNA genes of *L. garvieae* which specifically identifies *L. garvieae*, which differentiates *L. garvieae* from *L. lactis*, and which is sensitive enough to be applied to clinical specimens.

MATERIALS AND METHODS

Bacterial strains. The following bacterial strains were used to test the specificity of the PCR assay: *L. garvieae* ATCC 43921[T], *E. seriolicida* ATCC 49156[T], 28 *L. garvieae* strains isolated from diseased fish (rainbow trout) from different farms in northern Italy during a 4-year period (ITP 1371, ITP 1375, ITP 1457, ITP 1545, ITP 1547, ITP 1815, ITP 1833, ITP 1876, ITP 1877, ITP 1917, ITP 1920, ITP 1921, ITP 1957, ITP 1964, ITP 1991, ITP 1992, ITP 2001, ITP 2224, ITP 2451, ITP 2453, ITP 2454, ITP 2808, ITP 3365, ITP 3433, ITP 3620, ITP 3630, ITP 3631, and ITP 3995), two *L. garvieae* strains isolated from yellowtail fish in Japan (S1449 and S014), 1 *L. garvieae* strain isolated from rainbow trout in Australia (88/1400), 2 *L. garvieae* strains isolated from rainbow trout in Spain (Sp 1 and Sp 2), *L. lactis* NCFB 604[T], *Streptococcus iniae* ATCC 29178[T], *Streptococcus difficile* CIP 103769[T], *Lactococcus piscium* NCFB 2778[T], and *Vagococcus salmoninarum* NCFB 2777[T], as well as *Aeromonas salmonicida* 3173/86, a gram-negative bacterium pathogenic for fish. Bacteria were grown on blood agar base (Oxoid)–5% sheep blood plates for 24 h at 25°C. All the strains were identified by using either the API Strep system or the API-E system (BioMerieux, Marcy l'Etoile, France) in accordance with the manufacturer's instructions except that the incubation temperature used was 25°C. Susceptibility to clindamycin was determined for *L. garvieae* strains and for *L. lactis* by the Kirby-Bauer test by using 2-µg clindamycin disks (7). DNA-DNA hybridization (5) was used to confirm the speciation of all the *L. garvieae* strains described above.

Clinical specimens. Blood specimens were obtained from trout farmed in northern Italy. Ten rainbow trout (*Oncorhynchus mykiss* Walbaum) from a pond with a high level of mortality due to *L. garvieae* as well as 10 antibiotic-treated rainbow trout from the same farm but another raceway were sampled randomly. In addition, 10 rainbow trout from a farm without disease where the fish were vaccinated against *L. garvieae* and 10 rainbow trout from a different farm with no reported disease or mortality but where the disease had occurred a previous year were also sampled randomly. Half a milliliter of blood was taken from each of the anesthetized rainbow trout. Heparin at a final concentration of 10 U/ml (Kamada, Beit Kama, Israel) was added. The blood specimens were centrifuged at 2,000 × g for 10 min to obtain the plasma. Then, 1 µl of the plasma was added to a PCR tube and the DNA was extracted by adding 20 µl of Genereleaser (Bioventures, Murfreesboro, Tenn.) in accordance with the manufacturer's protocol. PCR mix (see below) was added to a final volume of 100 µl. The PCR assay was done under the conditions described below.

Primers. Specific primers to identify *L. garvieae*, pLG-1 (5′-CATAACAATG AGAATCGC-3′) and pLG-2 (5′-GCACCCTCGCGGGTTG-3′), were selected by comparing the 16S rDNA sequence of *L. garvieae* (EMBL accession no. X54262) (3) to other bacterial sequences by using the GCG (version 9.0-UNIX) FastA program.

PCR sensitivity was evaluated by testing 10-fold dilutions of *L. garvieae* suspensions in saline that were simultaneously plated to determine the number of CFU.

Amplification by PCR. Typical PCRs were carried out in a final volume of 50 or 100 µl containing 1 U of Vent DNA polymerase (New England Biolabs) per 50 µl, 1× buffer (New England Biolabs), nucleotides (0.25 mmol/liter, final concentration for each nucleotide), bovine serum albumin (0.1 mg/µl), and primers (8 ng/µl each). Bacterial DNA was obtained from cells by touching colonies grown on blood-agar medium with a sterile syringe needle and then dipping the needle directly into the PCR mix. Typical cycling parameters were 1 min of denaturation (94°C), 1 min of annealing (55°C), and 1.5 min of extension (72°C), for 35 cycles. The reaction was started by a denaturation step (3 min at

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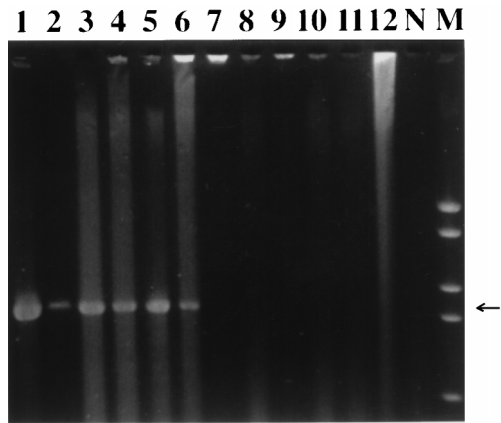
FIG. 1. Specificity of the *L. garvieae* PCR assay. Agarose gel (2%)-resolved ethidium bromide-stained PCR products from template DNA of various needle-touched bacterial colonies are shown. Lanes: 1, *L. garvieae* ATCC 43921^T; 2, *E. seriolicida* ATCC 49156^T; 3, *L. garvieae* ITP 2001 (Italy); 4, *L. garvieae* S1449 (Japan); 5, *L. garvieae* 88/1400 (Australia); 6, *L. garvieae* Sp 1 (Spain); 7, *L. lactis* NCFB 604^T; 8, *L. piscium* NCFB 2778^T; 9, *V. salmoninarum* NCFB 2777^T; 10, *S. iniae* ATCC 29178^T; 11, *S. difficile* CIP 103769^T; 12, *A. salmonicida* 3173/86; N, negative control (no DNA); M, DNA molecular weight marker VI. The arrow indicates the band of the expected length of 1,100 bp.

94°C) and ended by a 10-min extension step at 72°C. The PCR-amplified samples were subjected to electrophoresis (90 min, 90 V) in a 2% agarose gel (FMC) with 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8]) and stained with ethidium bromide after the run. The DNA molecular weight marker VI (Boehringer Mannheim) was used to estimate the size of the amplified fragment.

RESULTS AND DISCUSSION

The PCR assay resulted in the amplification of a band of 1,100 bp in size that was detected for all 35 *L. garvieae* strains tested, including the *L. garvieae* ATCC 43921^T and the *E. seriolicida* ATCC 49156^T reference strains. Examples of the amplified band can be seen in Fig. 1 (lanes 1 to 6). The *L. garvieae* strains tested by PCR originated from diseased fish from three different continents (Asia, Australia, and Europe), proving the universality of the primers selected for the PCR assay. The specificity of the PCR assay was demonstrated by the fact that no specific band was amplified when *L. lactis* or any other fish pathogen was used as the DNA template (Fig. 1, lanes 7 to 12). The amplification of the specific 1,100-bp band from both *L. garvieae* and *E. seriolicida* reference strains (Fig. 1, lanes 1 and 2) confirms once more the genetic identity of these two bacterial species.

The PCR assay was applied to 10-fold dilutions of *L. garvieae* ITP 1992. The PCR assay was positive (presence of a specific 1,100-bp band) down to the dilution corresponding to 4 bacteria (4 CFU) per reaction tube (Fig. 2b, lane 4) but not for the next dilution (0.4 CFU) (Fig. 2b, lane 5). These data showed that the primers that were selected allowed a highly sensitive detection of *L. garvieae* cells suspended in saline. In fact, we have also tested the sensitivity of the PCR assay for *L. garvieae* cells suspended in trout plasma and found a similar level of detection (data not shown).

Seven of the 10 plasma specimens obtained from trout sampled from a pond with active *L. garvieae* disease as well as 1 of 10 of the antibiotic-treated trout originating from the same fish farm with active clinical *L. garvieae* disease were found positive by PCR (Fig. 2a). More animals might have been found PCR positive in the pond stricken by *L. garvieae* mortalities if a larger volume of plasma from each fish had been used or more

than one PCR had been performed. The PCR assay was negative for the plasma samples obtained from 10 vaccinated trout originating from a farm without active disease. However, the PCR assay was positive for 3 of 10 plasma samples from non-vaccinated trout reared at another farm without current active disease but where the disease was active the previous year. This observation may reflect the presence of *L. garvieae* carriers among fish that will develop the disease only under detrimental environmental conditions, as already suggested (1). The data presented above show that the PCR assay performed on a very limited amount of plasma (1 µl) is sensitive enough to detect specifically *L. garvieae* in fish originating from a pond where an active epizootic is occurring (Fig. 2a, lanes 1 to 4). In addition, the PCR assay could detect carriers of *L. garvieae* in a pond where the disease had occurred a previous year (Fig. 2a, lane 4). From these data, it can be concluded that the proposed PCR assay can be useful not only for diagnostic but also for epidemiological surveys and could be an efficient tool to establish preventive measures on time (antibiotic prophylaxis, vaccination).

Finally, all the *L. garvieae* strains, including the *E. seriolicida* ATCC 49156^T strain, were contact resistant to clindamycin, whereas the *L. lactis* strain presented a 22-mm-diameter inhibition zone around the clindamycin disk. Our results confirm those of Elliot and Facklam (7) in that all the *L. garvieae* strains tested were resistant to clindamycin whereas the *L. lactis* strain tested was susceptible to clindamycin.

Besides determination of clindamycin susceptibility, only comparisons of whole-cell protein patterns (6) and DNA, RNA, and restriction fragment length polymorphism data (5, 7) can distinguish between *L. lactis* and *L. garvieae*, which are phenotypically similar. These techniques are useless for routine identification. The clindamycin susceptibility test has been the only practical phenotypic test which differentiates *L. garvieae* from *L. lactis*. However, the occurrence of clindamycin-resistant *L. lactis* strains would result in an erroneous diagnosis. The data presented here show that *L. garvieae* and *L. lactis* subsp. *lactis*, which are both recently recognized pathogens, can be easily differentiated by the proposed PCR assay. The PCR assay that we have developed is a convenient, fast, and simple technique that can be accomplished in 5 h and that can be applied not only to the specific identification of *L. garvieae* but also to the surveillance of *L. garvieae* infections in aquaculture.
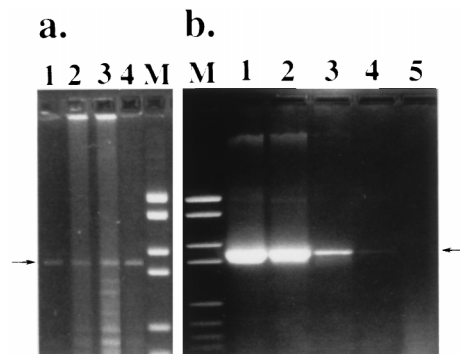

FIG. 2. Field application and sensitivity of the *L. garvieae* PCR assay. (a) PCR product from 1 µl of fish plasma. Lanes: 1 to 3, diseased fish; 4, antibiotic-treated fish. (b) PCR assay sensitivity evaluated with a serially diluted bacterial suspension. Lanes: 1, 3,600 CFU; 2, 360 CFU; 3, 36 CFU; 4, 4 CFU; 5, 0.4 CFU. The arrows indicate the band of the expected length of 1,100 bp. Lane M contains DNA molecular weight marker VI.

REFERENCES

1. **Bercovier, H., C. Ghittino, and A. Eldar.** 1997. Immunization with bacterial antigens: infections with streptococci and related organisms. Dev. Biol. Stand. **90:**153–160.
2. **Carson, J., N. Gudkovs, and B. Austin.** 1993. Characteristics of an *Enterococcus*-like bacterium from Australia and South Africa, pathogenic for rainbow trout (*Oncorhynchus mykiss* Walbaum). J. Fish Dis. **16:**381–388.
3. **Collins, M. D., C. Ash, J. A. E. Farrow, S. Wallbanks, and A. M. Williams.** 1989. 16S ribosomal ribonucleic acid sequence analyses of lactococci and related taxa. Description of *Vagococcus fluvialis* gen. nov., sp. nov. J. Appl. Bacteriol. **67:**453–460.
4. **Domenech, A., J. Prieta, J. F. Fernandez-Garayzabal, M. D. Collins, D. Jones, and L. Dominguez.** 1993. Phenotypic and phylogenetic evidence for a close relationship between *Lactococcus garvieae* and *Enterococcus seriolicida*. Microbiologica **9:**63–68.
5. **Eldar, A., C. Ghittino, L. Asanta, E. Bozzetta, M. Goria, M. Prearo, and H. Bercovier.** 1996. *Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningoencephalitis in fish. Curr. Microbiol. **32:**85–88.
6. **Elliott, J. A., M. D. Collins, N. E. Pigott, and R. R. Facklam.** 1991. Differentiation of *Lactococcus lactis* and *Lactococcus garvieae* from humans by comparison of whole-cell protein patterns. J. Clin. Microbiol. **29:**2731–2734.
7. **Elliot, J. A., and R. R. Facklam.** 1996. Antimicrobial susceptibilities of *Lactococcus lactis* and *Lactococcus garvieae* and a proposed method to discriminate between them. J. Clin. Microbiol. **34:**1296–1298.
8. **Facklam, R. R., D. Hollis, and M. D. Collins.** 1989. Identification of gram-positive coccal and coccobacillary vancomycin-resistant bacteria. J. Clin. Microbiol. **27:**724–730.
9. **Ghittino, C., and M. Prearo.** 1993. Comparison of some strains isolated from rainbow trout affected by streptococcosis. Boll. Soc. Ital. Patol. Ittica. **11:**30–43.
10. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, and S. T. Williams (ed.).** 1994. Bergey's manual of determinative bacteriology, 9th ed., p. 527–558. The Williams & Wilkins Co. Baltimore, Md.
11. **Kitao, T.** 1993. Streptococcal infections, p. 196–210. *In* V. Inglis, R. J. Roberts, and N. R. Bromage (ed.), Bacterial diseases of fish. Blackwell Scientific Publications, Oxford, United Kingdom.
12. **Kusuda, K., K. Kawai, F. Salati, C. R. Banner, and J. L. Freyer.** 1991. *Enterococcus seriolicida* sp. nov., a fish pathogen. Int. J. Syst. Bacteriol. **41:**406–409.
13. **Mannion, P. T., and M. M. Rothburn.** 1990. Diagnosis of bacterial endocarditis caused by *Streptococcus lactis* and assisted by immunoblotting of serum antibodies. J. Infect. **21:**317–318.