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NOTE

Bacterial species other than *Renibacterium salmoninarum* cross-react with antisera against *R. salmoninarum* but are negative for the p57 gene of *R. salmoninarum* as detected by the polymerase chain reaction (PCR)

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ABSTRACT: Genomic DNA was extracted from 4 strains of *Carnobacterium piscicola* and 2 strains of *Corynebacterium aquaticum* that had previously been reported to produce a 57 kDa protein that reacted with polyclonal antiserum against *Renibacterium salmoninarum*. Genomic DNA was also extracted from a Gram-negative bacterium isolated from the kidney tissue of a mature female coho salmon *Oncorhynchus kisutch*. The bacterium, tentatively identified as *Pseudomonas maltophila*, cross-reacts with 2 polyclonal antisera, one of which is used in an enzyme-linked immunosorbent assay and the other in a fluorescent antibody test to identify *R. salmoninarum*. The isolate of *P. maltophila*, and the *Carnobacterium piscicola* and *Corynebacterium aquaticum* strains, were negative by a polymerase chain reaction (PCR) that was designed to amplify a segment of the gene encoding p57, a major protein of *R. salmoninarum*. These results suggest that although antibodies directed against *R. salmoninarum* cross-react with antigens of bacterial species other than *R. salmoninarum*, the cross-reacting antigen(s) is clearly not the same protein, as the non-*R. salmoninarum* bacteria lacked the gene encoding p57. These findings highlight some of the shortcomings of immunodiagnostic tests for detecting *R. salmoninarum* and indicate the high degree of specificity associated with a PCR-based diagnostic technique.

KEY WORDS: PCR · DNA · BKD detection · Salmon eggs · Bacterial disease · Broodstock screening

The 2 immunodiagnostic techniques most commonly used to detect infections with *Renibacterium salmoninarum*, causative agent of bacterial kidney disease (BKD), are the fluorescent antibody test (FAT) and the enzyme-linked immunosorbent assay (ELISA) (Elliott et al. 1989). The antisera used in these tests are raised in mammals against whole, killed *R. salmoninarum* cells (Bullock et al. 1980, Evelyn et al. 1981, Elliott & Barila 1987), or against one of the dominant antigens of *R. salmoninarum*: a 57 kDa protein known as p57 (Turaga et al. 1987, Sakai et al. 1992). Fluorescein-conjugated antisera used in the FAT have been shown to react with bacterial species other than *R. salmoninarum* (Bullock et al. 1980, Austin & Rayment 1985, Yoshimizu et al. 1987, Barbash 1992, Foott et al. 1992, Brown et al. 1994). Similarly, false-positive *R. salmoninarum* reactions have been reported with the ELISA (Dixon 1985, Turaga et al. 1987). In attempts to avoid false-positive reactions, some workers have used monoclonal antibodies directed against selected epitopes of p57 (Wiens & Kaattari 1989, Hsu et al. 1991, Rockey et al. 1991). Others have used Western blot analysis to make the diagnosis less equivocal (Olivier et al. 1992). That technique is based on demonstrating that the serologically reactive antigen migrates in an SDS polyacrylamide electrophoretic gel to a position identical to that of p57. The Western blot technique, however, is insufficiently sensitive to detect very small amounts of the p57. Consequently Olivier et al. (1992) concluded that it was only suitable for diagnosing relatively active *R. salmoninarum* infections.

Recently Bandin et al. (1993) and Toranzo et al. (1993) detected a 57 kDa protein in 2 species of bacteria, *Carnobacterium piscicola* and *Corynebacterium aquaticum*, that reacted with antisera raised against *Renibacterium salmoninarum*. These authors did not indicate whether the presence of this protein also

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caused a cross-reaction in the FAT or ELISA, but the finding did raise the possibility that p57 might not be unique to *R. salmoninarum*. More recently, Brown et al. (1994) identified tissues from salmon that were positive for *R. salmoninarum* by the FAT but were negative for the pathogen by the more sensitive polymerase chain reaction (PCR). That discrepancy was caused by the presence of a cross-reacting bacterium, since tentatively identified as *Pseudomonas maltophila*.

The purposes of this study were, first, to determine whether strains of *Carnobacterium piscicola* and *Corynebacterium aquaticum* and *Pseudomonas maltophila* yielded false-positive reactions in both the FAT and ELISA, and second, to determine whether these organisms possess the gene encoding the p57 molecule as detected by the PCR.

Materials and methods. ***Salmonid tissue samples:*** Kidney tissues were aseptically removed from a female coho salmon from Robertson Creek Salmon Enhancement Hatchery (Port Alberni, BC, Canada), homogenized, and then assayed for the presence of *Renibacterium salmoninarum* by ELISA and FAT, as described previously (Brown et al. 1994).

Bacteria: A bacterium was isolated from the salmonid kidney tissue (Table 1) by streaking the tissue homogenate onto Tryptic Soy Agar (TSA; Difco, Detroit, MI, USA) and selective Kidney Disease Medium (SKDM-C) (Austin et al. 1983, Daly & Stevenson 1985). The plates were then incubated at 15°C (24 to 48 h for TSA and up to 8 wk for SKDM-C). Bacterial growth from the TSA plates was subcultured at least 3 times, after which the cells were harvested from the plates, suspended in sterile saline (0.85%), and subjected to the ELISA, FAT, and the Gram stain. Biochemical tests on the bacterium were also done using an API kit (API Analytab Products, Plainview, NY, USA) according to the manufacturer's instructions. These tests yielded a profile consistent with that of *Pseudomonas maltophila*: the bacterium was positive in the features β-galactosidase, lysine decarboxylase, citrate utilization, gelatin liquefaction, glucose utilization, NO_2 reduction; and negative in the reactions arginine dihydrolase, ornithine decarboxylase, H_2S production, urease, tryptophane deaminase, indole, Voges-Proskauer, mannose, inositol, sorbitol, rhamnose, sucrose, melibiose, and arabinose utilization, amylase, oxidase, and CO_2 production.

Carnobacterium piscicola strains ATCC 35586, PT-31, HB-245, and HB-246, and *Corynebacterium aquaticum* strains ATCC 14665, and 968BA were kindly donated by Dr A. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain) (Table 1). They were grown at room temperature on brain heart infusion agar (BHIA) supplemented with 1% (v/v) bovine serum. The bacterial growth was aseptically scraped off the plates and suspended in sterile saline. Aliquots of the suspensions were kept at −80°C until required for DNA extraction. Additional aliquots of the suspensions were used in the ELISA.

ELISA and FAT: An ELISA was performed on the suspensions of the *Pseudomonas maltophila*, *Carnobacterium piscicola* and *Corynebacterium aquaticum* strains using a commercially available antiserum raised against *Renibacterium salmoninarum* cells in goats (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), as described by Brown et al. (1994). The FAT, also as described by Brown et al. (1994), was performed on all bacterial strains. Cells of *R. salmoninarum* (strain 384) from the Pacific Biological Station in Nanaimo, BC (Table 1), were used as positive controls for these assays. In order to ensure that any differences observed in these immunoassays were qualitative and not quantitative, all cell suspensions were diluted to give an absorbance of 2.5 at 420 nm corresponding to ca 10^8 cells ml^{-1}.

DNA extraction: DNA for the PCR tests was extracted from the *Pseudomonas maltophila* isolate, *Renibacterium salmoninarum* (ATCC strain 33209), the 4 *Carnobacterium piscicola* strains, and from the 2 *Corynebacterium aquaticum* strains by a phenol/chloroform method (Sambrook et al. 1989).

PCR: The PCR was performed according to a previously described protocol (Brown et al. 1994) using primers designed to amplify a 501 base-pair segment

Table 1. Provenance of bacterial strains examined by PCR

Species	Strain	Provenance
Renibacterium salmoninarum	ATCC 33209 Rs 384	*Oncorhynchus tshawytscha*, USA *O. tshawytscha*, Canada
Unknown Gram-negative, tentatively identified as *Pseudomonas maltophila*		*O. kisutch*, Robertson Creek, British Columbia, Canada
Carnobacterium piscicola	ATCC 35586 PT-31 HB-245 HB-246	*O. clarki*, USA *Morone saxatilis*, USA *M. saxatilis*, USA *O. mykiss*, Spain
Corynebacterium aquaticum	ATCC 14665 968 BA	Distilled water, USA *M. saxatilis*, USA

of the *Renibacterium salmoninarum* gene encoding p57.

Results. In the FAT, fluorescing bacterial cells were observed in the kidney homogenates obtained from the Robertson Creek female coho salmon. These cells were too long (5 to 10 µm long) to be *Renibacterium salmoninarum*, as reported previously (Brown et al. 1994). The Gram-negative *Pseudomonas maltophila*, harvested from the TSA plates after subculturing, were also positive by the FAT, and were the same large size as the cells seen in the kidney homogenates. In contrast, no fluorescence was observed in the *Corynebacterium aquaticum* and *Carnobacterium piscicola* strains in the FAT. The kidney homogenates from the Robertson Creek female coho salmon also yielded a positive result in the ELISA: the optical density (OD) values observed were higher than the positive threshold established by Meyers et al. (1993) for salmonid kidney tissues (Brown et al. 1994). Similarly, the ELISA performed on the *P. maltophila* cells subcultured on TSA and suspended in saline also yielded positive OD values (Table 2). The ELISA performed on the *Carnobacterium piscicola* and *Corynebacterium aquaticum* isolates yielded negative results in 5 strains. One strain of *Carnobacterium piscicola* (strain PT-31) yielded moderately positive results (Table 2). When the PCR was performed on DNA extracted from the *P. maltophila* cells harvested from the TSA plates, and on DNA extracted from *Carnobacterium piscicola* and *Corynebacterium aquaticum* strains no bands were observed. However, the expected 500 bp band was observed in the control samples of DNA extracted from *R. salmoninarum* cells (Figs. 1 & 2).

Table 2. Results of enzyme-linked immunosorbent assay (ELISA) performed on isolates of *Renibacterium salmoninarum*, *Pseudomonas maltophila*, *Carnobacterium piscicola* and *Corynebacterium aquaticum*, using a commercially available antiserum raised against *R. salmoninarum* in goats. Suspensions of bacteria used in the tests were standardized [2.5 optical density (OD) at 420 nm]. OD values at 410 nm are given as the means (± SD) of triplicates. Negative control was a homogenate of kidney tissue from a coho salmon *Oncorhynchus kisutch* that gave OD values in the ELISA below the threshold value (0.095) established for *R. salmoninarum*-positive salmon (Meyers et al. 1993)

Species	Strain	OD at 410 nm
Negative control		0.083 ± 0.004
Renibacterium salmoninarum (positive control)	384	0.304 ± 0.015
Pseudomonas maltophila		0.171 ± 0.004
Carnobacterium piscicola	ATCC 35586	0.096 ± 0.001
	PT-31	0.106 ± 0.004
	HB-425	0.087 ± 0.004
	HB-426	0.095 ± 0.003
Corynebacterium aquaticum	ATCC 14665	0.092 ± 0.003
	968BA	0.084 ± 0.002

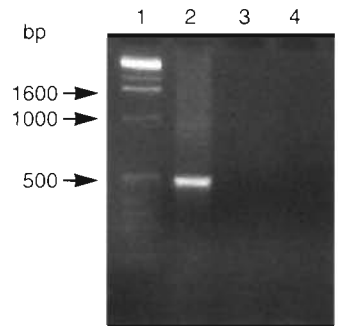

Fig. 1 Agarose gel electrophoresis of amplification products from DNA extracted from *Renibacterium salmoninarum* and the bacterial isolate tentatively identified as *Pseudomonas maltophila*. Lane 1: molecular weight markers (base pairs, bp); Lane 2: 3.72 µg ml^{-1} *R. salmoninarum* template DNA; Lane 3: 6.15 µg ml^{-1} bacterial isolate *P. maltophila* template DNA; Lane 4: DNA omitted

Discussion. The first question addressed in this study was whether organisms possessing cross-reacting antigens, such as the 57 kDa protein, would yield cross-

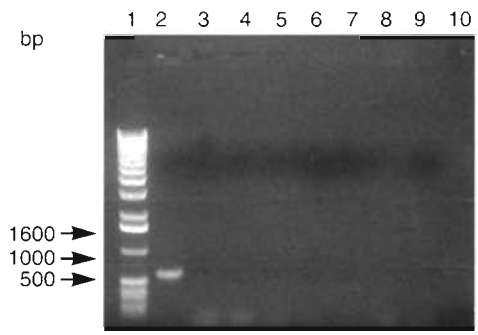

Fig. 2. Agarose gel electrophoresis of amplification products from DNA extracted from *Renibacterium salmoninarum*, *Carnobacterium piscicola* and *Corynebacterium aquaticum* isolates. Lane 1: molecular weight markers (base pairs, bp); Lane 2: 6.15 µg ml^{-1} *R. salmoninarum* template DNA; Lane 3: DNA omitted; Lane 4: 7.44 µg ml^{-1} *Carnobacterium pisicola* ATCC 35586 template DNA; Lane 5: 7.44 µg ml^{-1} *Carnobacterium piscicola* PT-31 template DNA; Lane 6: 7.44 µg ml^{-1} *Carnobacterium piscicola* HB 425 template DNA; Lane 7: 7.44 µg ml^{-1} *Carnobacterium piscicola* HB 426 template DNA; Lane 8: 7.50 µg ml^{-1} *Corynebacterium aquaticum* ATCC 14665 template DNA; Lane 9: 7.50 µg ml^{-1} *Corynebacterium aquaticum* 968 BA template DNA; Lane 10: empty

reactions in the FAT and ELISA designed to detect *Renibacterium salmoninarum*. Our results show that none of the *Carnobacterium piscicola* and *Corynebacterium aquaticum* strains possessing this protein gave positive reactions in the FAT. This result suggests that if the protein is a cell-surface protein, it must occur in amounts insufficient to obtain detectable cross reactions in the FAT. In the more sensitive ELISA, however, 1 of the 6 *Carnobacterium piscicola/Corynebacterium aquaticum* strains tested (*Carnobacterium piscicola*, strain PT-31) yielded a moderately strong cross reaction. In addition, our *Pseudomonas maltophila* isolate also yielded positive reactions in both the FAT and ELISA. These false-positive reactions provide further evidence for the limitations of the immunodiagnostic techniques currently in use for detecting *R. salmoninarum* infections. Also, they highlight the advantages of the specificity of the PCR for the detection of a gene that has been shown to encode a specific protein that is apparently unique to *R. salmoninarum*. None of the bacteria other than *R. salmoninarum* were found to possess the p57 gene. Therefore, the 57 kDa proteins expressed in *Carnobacterium piscicola* and *Corynebacterium aquaticum* are not p57; rather they are proteins of like size that share 1 or more epitopes with p57. Similarly, the uncharacterized but serologically reactive antigen present on the surface of *P. maltophila* cells, which caused a positive reaction in the FAT, is not p57 but rather an antigen also having epitopes in common with that protein.

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