Genetic Monitoring by Denaturant Gel Electrophoresis of *Piscirickettsia salmonis*, a Bacterial Disease of Farmed Salmonids

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Introduction

Piscirickettsia salmonis is the agent of an aggressive transmissible bacterial disease affecting salmonid aquaculture in Chile (Almendras and Fuentealba et al. 1997, Fryer et al. 1992, Larenas et al. 1997). Commercial rearing of coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), and Atlantic salmon (*Salmo salar*) has been compromised in the aquafarms of southern Chile (Chiloé Island, Region Ten) since the emergence of this pathogen about 10 years ago. Efforts in various laboratories toward control of the disease include development of a vaccine. The success of this approach will depend in part on the degree of biological and genetic diversity within the *P. salmonis* lineage. For example, the effectiveness of a vaccine might be diminished by unpredictable temporal shifts of *P. salmonis* types invading salmon farms.

We have employed denaturing gradient gel electrophoresis (DGGE) and a simpler format, constant denaturing gel electrophoresis (CDGE), to monitor genetic diversity in *P. salmonis* obtained from salmon farms in southern Chile. Amplification and sequencing of a section of the internal transcribed spacer (ITS) between the 16S and 23S rRNA genes indicate that all reported strains of *P. salmonis* can be grouped into four sequence types. In this report, we demonstrate their distinction by CDGE. Initial surveys with this method show the presence of only one sequence type of *P. salmonis* in Chilean salmonid farms. The recurrent type groups with strain LF-89, the type strain (ATCC VR-1361) first described in the early 1990s (Fryer et al. 1990, Fryer et al. 1992), a few years after initial reports of the emergence of the salmonid rickettsial syndrome in Chile.

Methods

DNA was extracted by the Chelex® method from fish serum and tissue or infected cell lines (Marshall et al. 1998). Polymerase chain reaction (PCR) amplification was conducted as previously described with primers RTS1 and RTS4 positioned just inside the borders of the ITS with the 16S and 23S rRNA genes, to obtain a fragment 283 bp in length (Marshall et al. 1998). Amplification for subsequent electrophoresis on denaturant gels was done with the same primer pair, but with a 40-bp GC clamp (Murray et al. 1996) added at the 5'- end of primer RTS1, in order to produce a fragment suitable for detection of single-base changes (Sheffield et al. 1989). Sequences from fragments amplified without a GC clamp were obtained as previously described (Marshall et al. 1998).

The melting temperatures of representative PCR products were obtained on a perpendicular denaturing gradient gel cast with a Bio-Rad Model 475 gradient former in 16 cm long plates, which are part of the DCode™ universal mutation detection system (Bio-Rad Laboratories, Inc.). The gel was 6.0% 37.5:1 acrylamide/bis with a 10–40% urea-formamide gradient (Bio-Rad's Model 475 gradient delivery system instruction manual). Approximately 100 µl of the PCR product was distributed evenly along the top of the gel, and electrophoresis was conducted at 165 V and 56°C for 3.5 hr. Resolution of sequences was then assessed with parallel gels with a gradient from 22–25% denaturant. The CDGE format set at a denaturant level of 23.5% proved equally useful and was adopted as the method of choice for surveying natural isolates. Gels were stained with ethidium bromide, and separation patterns were revealed by ultraviolet (UV) fluorescence, digitized with the Gel Doc™ 1000 system (Bio-Rad), and analyzed with PC software provided with the system.

Results and Discussion

The migration behavior of amplified fragments from strains LF-89 and EM-90 during perpendicular DGGE at 56°C revealed two melting transitions (Figure 1), the first at inflection point 24% and a second at inflection point 27% denaturant (average of the two curves; Figure 1). The theoretical melting curves for the sequences in question obtained with WinMelt™ program (Bio-Rad) also displayed two melting domains, one evident at 62°C and a second between 62–63.5°C (Figure 2). The theoretical curve roughly corresponds to the 3% denaturant differential in the observed inflection points, should one adopt the relationship of 0.3°C/1% denaturant (Abrams and Stanton et al. 1992).

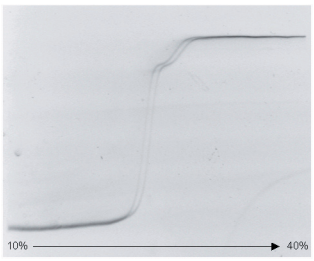

Fig. 1. Perpendicular denaturing gradient gel of amplified products obtained from strains LF-89 and EM-90. Melting theory predicts that LF-89 is the profile at left (see Figure 2).

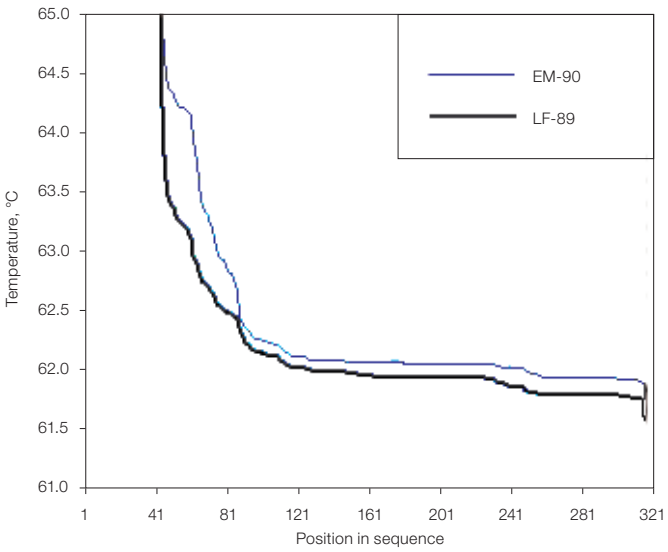

Fig. 2. Predicted melting profiles obtained with WinMelt software for strains LF-89 and EM-90 at 50% helicity with expanded y-axis. The x-axis covers the entire length of the amplified fragment, including the GC clamp at the left edge.

The predicted melting curves indicating higher melting values for EM-90 compared to LF-89 (Figure 2) were confirmed in the parallel gel format with a gradient ranging between 22–25% (results not shown) and also with the CDGE format at a set denaturant concentration of 23.5% (Figure 3). CDGE became the preferred format, given the reproducibility from run to run obtained by casting gels at a single urea-formamide concentration. CDGE revealed four migration classes: EM-90 as the sequence type having the highest melting value, and consequently the fastest migration; NOR-92; ATL-4-91; and the most slowly migrating class, including strains LF-89, C1-95, and SLGO-94.

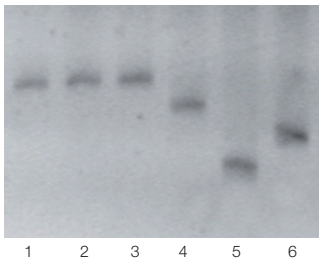

Fig. 3. CDGE of amplified fragments from *P. salmonis* strains. Lane 1, C1-95; lane 2, SLGO-94; lane 3, LF-89; lane 4, ATL-4-91; lane 5, EM-90; and lane 6, NOR-92.

Comparative analysis of sequences obtained from these strains confirmed the grouping of the fragments into four distinct melting classes on the denaturant gels. One sequence type was common to strains from farmed Chilean salmonids, including *P. salmonis* LF-89 (coho salmon), SLGO-94 (rainbow trout), and C1-95 (coho salmon), while strains EM-90 (isolated in Chile from Atlantic salmon), ATL-4-91 (Canada, Atlantic salmon), and NOR-92 (Norway, Atlantic salmon) each had unique sequences.

Surveys by CDGE of 44 samples (38 from coho salmon, 6 from rainbow trout) amplified from tissue biopsies obtained during 1997 and 1998 from five different salmon farms on Chiloé Island showed the presence only of *P. salmonis* that group with type strain LF-89. Earlier, Marshall et al. (1998) suggested, on the basis of DNA sequence data, that LF-89-like *P. salmonis* has been the prevalent type in the Chiloé region since the emergence of the disease during the 1980s.

The sequence data alluded to here differ at a few positions from those previously reported (GenBank accessions U36943-6 and U62103-4). This will be the subject of a future communication (Heath et al., unpublished). The congruence of melting behavior of the amplified fragments from the six *P. salmonis* strains and the corresponding DNA sequence data is consistent with our revisions.

The CDGE assay offers a rapid molecular approach to monitoring for bacterial types involved in the salmonid rickettsial syndrome. Conceivably, the appearance of a new *P. salmonis* type distinct from those already reported should be quickly evident from CDGE analysis. The CDGE protocol optimized here is sufficiently sensitive so that one base-pair substitution, as between NOR-92 and ATL-4-91 (Heath et al., unpublished), results in easily distinguishable differential mobility of the corresponding amplicons (Figure 3). The admittedly limited survey of salmon farms in Chile reported here is nevertheless reassuring in regard to the dependability of future vaccines against *P. salmonis*, given the observed constancy of type since the emergence of piscirickettsiosis about 10 years ago.

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