NOTE

Identification of *Streptococcus phocae* strains associated with mortality of Atlantic salmon (*Salmo salar*) farmed at low temperature in Chile

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Abstract

This work describes the identification of the causal agent of disease outbreaks that occurred in Atlantic salmon (700–750g) cage-cultured at water temperature of 5±0.5ºC in a farm located around the city of Puerto Natales in the Antarctic region of Chile, reaching a cumulative mortality lower than 1% of the affected population. Infections most often were characterized by exophthalmia with accumulation of purulent and haemorrhagic fluid around eyes and ventral petechial haemorrhages. Abundant pure growth cultures from kidney samples and mixed cultures from external lesions were obtained. Using biochemical, sequencing techniques six different isolates were studied and two bacterial species were identified, corresponding to *Streptococcus phocae* and *Aliivibrio* sp. Challenge tests by intraperitoneal injection showed that a representative *S. phocae* isolate 151 is pathogenic for Atlantic salmon. No mortalities or morphological alterations were observed in fish injected with *Aliivibrio* sp. (isolate 222). Infections of Atlantic salmon with *S. phocae* have been restricted to temperatures higher than 15ºC; however our data represent the first report of *S. phocae* associated with mortality of salmonids farmed in cold water (5ºC). Additional studies are necessary to evaluate the risk for salmonid cultured below 10ºC, particularly because the prevalence of infections by *S. phocae* could be underrated due to their possible confusion with other bacterial infections.

Streptococcus phocae, a beta-haemolytic streptococcal species member of the pyogenic streptococcal group (Köhler, 2007), was first isolated in Norway from clinical specimens of harbor seal (*Phoca vitulina*) affected by respiratory infection (Skaar et al., 1994).

A few years later, the pathogen was isolated from other marine mammals, including Cape fur seal (*Arctocephalus pusillus pusillus*), ringed seal (*Phoca hispida*), harbor porpoise (*Phocoena phocoena*), gray seal (*Halichoerus grypus*), spotted seal (*Phoca largha*) and cetaceans (Henton et al.,

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1999; Lawson et al., 2004; Raverty and Fiessel, 2001; Raverty et al., 2004; Vossen et al., 2004; Kuiken et al., 2006; Hueffer et al. 2011), in several countries of north-western Europe, Africa, the Caspian Sea and north America.

Since 1999, *S. phocae* has also been repeatedly isolated during the summer months (temperatures higher than 15ºC) from diseased Atlantic salmon (*Salmo salar*), causing serious economic losses in the salmon industry in Chile (Romalde et al., 2008). Based on the biochemical, antigenic and genetic characteristics of the *S. phocae* isolates obtained from salmon, these are considered as a homogeneous taxon (Valdés et al., 2009), which constitute a different group from the seal isolates, including the type strain ATCC 51973^T.

In July-August 2010 mortalities occurred in Atlantic salmon (700–750g) cage-cultured in a farm located around the city of Puerto Natales at the Antarctic region of Chile. During the outbreak, affected fish was farmed at water temperature of 5±0.5ºC with cumulative mortality lower than 1% of the affected population. The external gross finding noted in affected fish was panophthalmitis and ventral petechial haemorrhages. Internally, pale liver with petechiae as well as haemorrhaging in the abdominal fat and muscular tissues, in some cases, muscle liquefaction with formation of deep ulcerated areas were also detected (Figure 1).

A total of 5 moribund fish from two separated cage-net were used for microbiological analysis onto Columbia blood agar (CBA) and tryptone soy agar supplemented with 1% (w/v) sodium chloride (TSA-1) and samples were taken from

Figure 1. Clinical signs observed in the diseased Atlantic salmon: (a) ventral petechiae hemorrhage, (b) exophthalmia and hemorrhage around the eye and (c) hemorrhage in the abdominal cavity and pale liver.

external lesions and kidney of moribund fish. In addition, fish samples (i.e. kidney, gills and heart) were analyzed for the detection of other fish pathogen such as *Renibacterium salmoninarum* and infectious salmon anaemia virus (ISAV) using indirect fluorescent antibody test (IFAT, BKD Fluoro test, GrupoBios S.A.) and by TaqMan® real-time RT-PCR as described Godoy et al. (2010), respectively. The results of the IFAT examination together with the real-time RT-PCR analyses on these samples obtained from Atlantic salmon cage-cultured, regardless of the organs analyzed, were all positive.

The results of the microbiological analysis showed that in all cases, abundant pure growth cultures from kidney samples and mixed cultures from external lesions were also obtained. Single colonies were streaked from mixed cultures on the same media to obtain pure isolates. A total of six different isolates were obtained and maintained frozen at –80°C in Criobilles tubes (AES Laboratory, France). All isolates were identified using standard microscopic techniques, phenotypical properties, standard bacteriological test (MacFaddin, 1984), other biochemical tests (Romalde et al., 2008) and API 20E (bioMérieux) strips according to the manufacturer's instruction with the exception of the incubation temperature, which was fixed at 20°C.

Four isolates were obtained from kidney from the different moribund fish, showing colonies with pinpoint white colour (0.5mm diameter) and beta-hemolytic properties on CBA plates. All isolates were Gram-positive chain forming cocci (0.6–0.9mm diameter), negative for catalase and aesculin hydrolysis tests also indicating that the bacteria could be assigned presumptively to the genus *Streptococcus*.

DNA was extracted from each pure bacterial culture employing the Insta-Gene Matrix commercial system (Bio-Rad), according to the instructions of the manufacturer. When the PCR-analysis for specific identification of the genes encoding the 16S ribosomal RNA and 16S-23S rDNA intergenic spacer of *S. phocae* proposed by Avendaño-Herrera (2008) were employed, amplification of a unique and clear PCR product of the expected 900 and 180 bp lengths were obtained, respectively; which are identical to the type strain ATCC 51973^T, allowing the confirmation of all isolates as *S. phocae* species (Figure 2). Sequencing analysis of the nearly complete (1361 bp) 16S rDNA gene revealed that all the isolates studied were identical, and that the sequence obtained showed 99.79% similarity with the type strain *S. phocae* strain CCUG 35103 (accession number AJ621053). The relationship between a representative *S. phocae* (isolate 151) and other streptococci species are presented as a phylogenetic tree in Figure 3.

Until now, *S. phocae* obtained from Atlantic salmon cage-farmed in estuary and marine waters has been isolated exclusively during the summer months at temperatures higher than 15ºC. Based on this, Romalde et al. (2008) included this pathogen as a member of the warm water streptococcosis. Therefore, an inconsistency between the temperature ranges reported by Skaar et al. (1994) and Romalde et al. (2008), whom described that *S. phocae* species does not grow below 10ºC and our study were observed. We can speculate that this difference might be due to the influence of the solid nature of the culture medium as well as the incubation time used by these authors, which seem to affect to *S. phocae* species. In our study, the ability of the *S. phocae* to grow at different temperatures

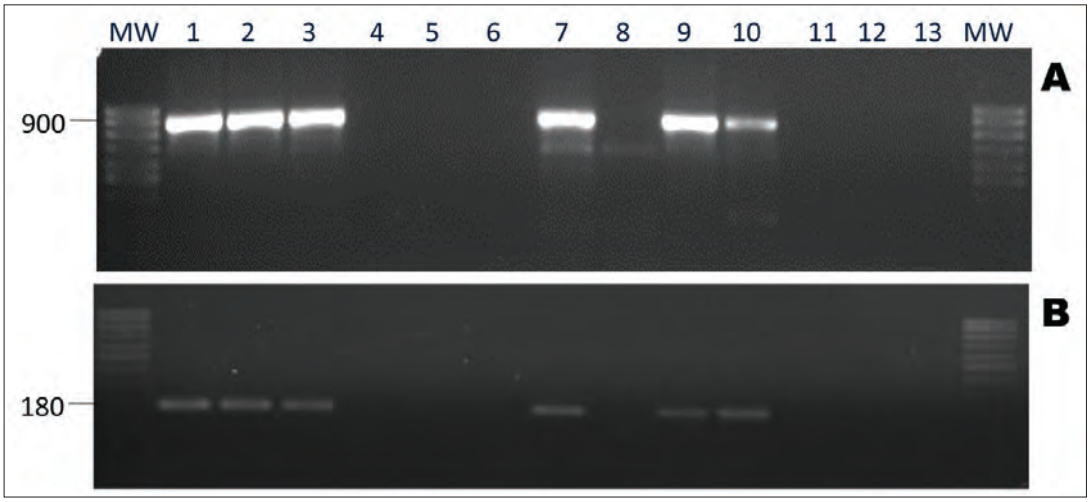

Figure 2. Specific PCR products amplified with PX1–PXVQ2 (A) and cae1–caeVQ2 (B) primer sets. Lanes: MW: molecular ruler (100 bp DNA ladder, Bioron); 1 and 10, DNA extracted from *Streptococcus phocae* ATCC 51973T; 2 to 9, DNAs extracted from bacterial samples and 11 to 13, negative control (no DNA). Numbers on the left indicate the size of the specific amplified products in bp.

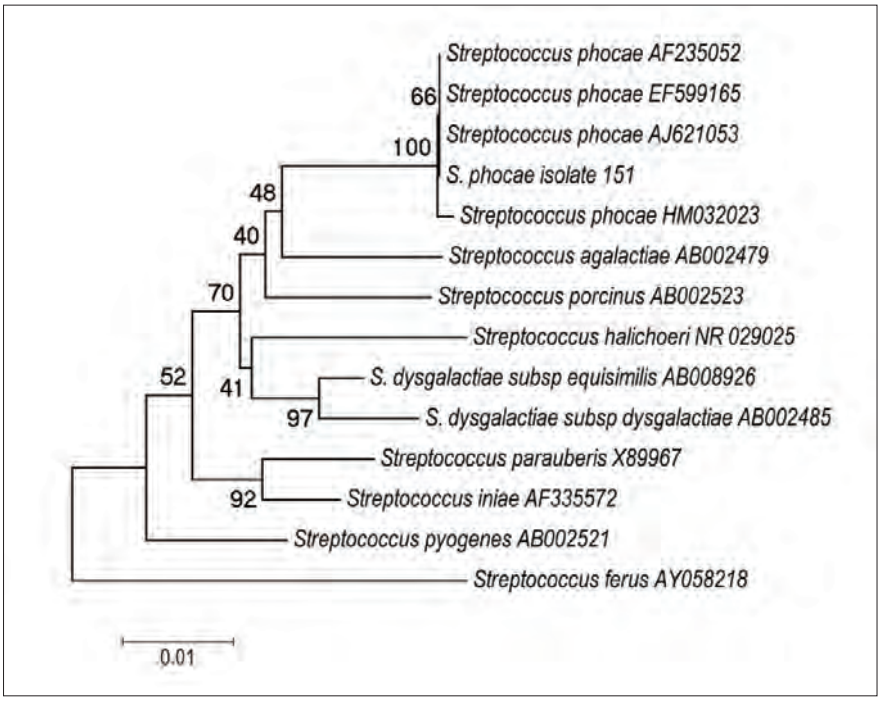

Figure 3. Phylogenetic tree based on 16S rRNA sequences from *Streptococcus phocae* as well as the most closely related Streptococcus type strains with our isolate 151 (1361 base pairs) using the neighbour-joining method and the ClustalX. *Streptococcus ferus* (AY058218) was employed as out-group. Horizontal branch lengths are proportional to evolutionary divergence. Significant brootstrap values of 1000 replicates appear next to the corresponding branch. Sequences from relative species were obtained from GeneBank Database, and their accession numbers are indicated after the species name.

(4 to 37ºC) was tested in tryptone soy broth supplemented with 1% sodium chloride over a period of 7 days, being growth observed in the first 60 h.

It is important to note that the diseased fish were not moved from farms located in other geographical areas or farms with streptococcosis, factor that as suggested by Romalde et al. (2008) plays an important role in the spreading of the disease in Chile. However, in this outbreak, the origin of the *S. phocae* in marine environment as well as the possible route of infection in Atlantic salmon is not yet clarified.

Biochemical homogeneity was seen among the other two isolates recovered from the external lesion in diseased Atlantic salmon. They were Gram-negative, motile, rod-shaped cells, catalase and cytochrome oxidase positive, presented glucose fermentative metabolism without gas production and were sensitive to the vibriostatic compound O/129. These results indicated that the bacteria could be identified as *Vibrio* species. The utilization of the miniaturized API 20E kit rendered for all the isolates the same profile 6716024, showing a positive reaction for arginine, lysine and ornithine tests, but the isolates did not produce acetoin (Voges–Proskauer test). Sequencing analysis of the 16S rRNA gene (1383 bp) revealed that the two isolates studied were identical, and that the sequences obtained showed similarities higher than 99.02% with *Aliivibrio logei* (Figure 4). Although this microorganism (formerly *Vibrio logei*; Urbanczyk et al., 2007) has been previously reported to be associated with lesions on crab and Atlantic salmon (Bang et al., 1978; Benediktsdóttir et

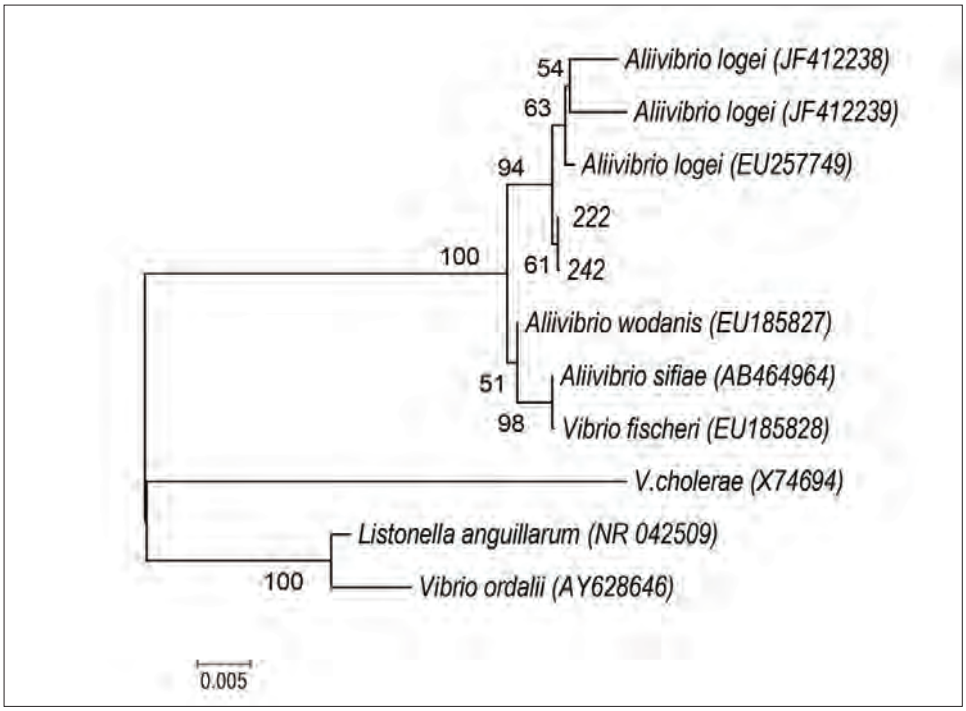

Figure 4. Phylogenetic tree of the 16S rRNA sequence, showing the relationships between the two Atlantic salmon isolates, other members of the closest *Aliivibrio logei* and representatives of other related genera in the family *Vibrionaceae*. Sequences from relative species were obtained from GeneBank Database, and their accession numbers are indicated after the species name.

al., 1998), sequencing of the 16S rRNA gene does not distinguish among *Vibrio* nor *Aliivibrio* species at the species level; further studies using molecular techniques (i.e. multilocus sequence analysis, DNA–DNA hybridization and others) would be needed to establish if these two isolates belong to *A. logei*. To avoid confusion with the taxonomic status, we will use *Aliivibrio* sp. Antimicrobial tests were applied by disc diffusion method on Mueller–Hinton agar (MHA, Oxoid) containing 5% sheep blood for *S. phocae* (Avendaño-Herrera et al., 2011) and Mueller–Hinton agar supplemented with 1% NaCl (MHA-1, Oxoid) as suggested by CLSI (2006) for Group 2 (obligate halophilic strains). Florfenicol (30µg), oxytetracycline (30µg) and erythromycin (15µg) were selected because they are routinely used for the treatment of streptococcosis as well as for other bacteria affecting Chilean salmonid aquaculture. All *S. phocae* isolates did not show inhibition zones (<7 mm), regardless of the drug tested and were classified as resistant (Avendaño-Herrera et al., 2011); while for the two *Aliivibrio* spp. an identical antibiotic susceptibility pattern was observed, ranging from 27 to 34 mm. The susceptibility pattern of the control strain *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, grown on Mueller–Hinton agar under standard growth conditions, was within the limits given in M42-A (CLSI 2006).

In order to investigate the virulence capacities of the *S. phocae* and *Aliivibrio* sp., one representative isolate from each bacterium species was chosen. Experimental infection trials were conducted in a closed system with sea water up to 21 days at 10ºC using unvaccinated Atlantic salmon (average weight 60g) obtained from a commercial farm, with no history of problems related to this disease, located at central Chile. Fish were divided into four groups of 5 each and were intraperitoneally inoculated with a bacterial dose of 10^7 cells (0.1 ml/fish). Control fish received 0.1ml of saline solution (0.85% NaCl) and another fish group stayed without treatment.

The results of the virulence assays showed that *S. phocae* isolate 151 was able to induce mortality in 4 out of 5 fish after 2 days of the exposure to the bacterium. Inoculated *S. phocae* was recovered from dead fish which showed in all cases the typical internal lesions observed in the natural outbreaks. None of the fish injected with *Aliivibrio* sp. isolate 222 died or presented clinical signs after 21 days. Similar results were observed in the control groups (without bacterium). Given that the isolate 222 was recovered from mixed culture from external lesions, we speculate that *S. phocae* was one of the main pathogens associated with the disease and that *Aliivibrio* sp. was likely to be an opportunistic microorganism.

In summary, the results presented here confirmed that *S. phocae* provoked mortality of the Atlantic salmon farmed below 10ºC, allowing us extend the known range of this fish pathogen into a member of the cold water streptococcal. Additional studies are necessary to evaluate the risk for salmonid cultured below 10ºC, particularly because the prevalence of infections by this bacterium could be underrated due to their possible confusion with other bacterial infections.

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