Discrimination of streptococcosis agents in olive flounder (*Paralichthys olivaceus*)

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Abstract

The olive flounder (*Paralichthys olivaceus*) is an important mariculture fish in Korea, but its farming has been negatively impacted by bacterial diseases such as streptococcosis. Here, we sought new methods for identifying and characterizing the *S. iniae* and *L. garvieae* populations present in fish farms located along the southern coast of Korea. Consistent with the type strains *S. iniae* ATCC29178 and *L. garvieae* KG9408, the bacterial isolates from olive flounder were all found to be catalase and oxidase test-negative and non-motile. Although the bacterial isolates were phenotypically similar, differences were noted in the results of the Voges-Proskauer (VP), alkaline phosphatase (PAL) and hemolysis tests. PCR using specific primers was used to confirm that the isolates were *S. iniae* or *L. garvieae*, and SDS-PAGE and immunoblot assays were used to compare the cell lysate and antigenic protein profiles of each isolate with that of the appropriate type strain. The results of the biochemical tests and PCR were highly consistent with the findings of our SDS-PAGE and immunoblot assay, indicating that the SDS-PAGE and immunoblot protocols could be used for identification and tentative classification of the causative agents of olive flounder streptococcosis.

Introduction

In recent years, the aquaculture industry in Korea has been negatively impacted by serious losses due to various bacterial, viral, fungal and parasitic diseases. The bacterial “fish streptococcosis” is a major threat against the sustainable production and development of marine and freshwater aquacultures.

Fish streptococcosis is most common during the warmer months, and has been associated with other component causes such as poor water quality (Roberts, 2001; Seng & Colorni, 2002). Clinically, this fish disease is characterized by a darkened body color, unilateral or bilateral exophthalmia, hemorrhages on the opercula and the base of the fins, dropsy, meningitis, septicemia and eventual mortality (Eldar & Ghittino, 1999; Roberts, 2001; Woo et al., 2002). *Streptococcus iniae* and *Lactococcus garvieae* have been identified as major causative agents of fish streptococcosis (Kusuda et al., 1976; Nakatsugawa, 1983; Eldar et al., 1999) in many species worldwide, including olive flounder (*Paralichthys olivaceus*) (Nakatsugawa 1983;...

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Heo et al., 2001), rainbow trout (Oncorhynchus mykiss) (Chang et al., 2002; Diler et al., 2002), red drum (Sciaenops ocellatus) (Eldar et al., 1999), rabbitfish (Siganus canaliculatus) (Yuasa et al., 1999), and yellowtail (Seriola quinqueradiata) (Kusuda et al., 1976). These bacteria have also been isolated from human clinical samples, suggesting that they may be important emerging zoonotic agents (Weinstein et al., 1997; Fefer et al., 1998). According to Bergey's Manual of Systematic Bacteriology (1984), the genus Streptococcus belongs to the family Streptococcaceae, but it was recently reclassified into three genera (Streptococcus, Enterococcus and Lactococcus) based on DNA-ribosomal RNA hybridization and rRNA sequencing (Facklam, 2002). The genus streptococcus is characterized by the coccoid shape of the bacteria, the formation of chains, and biochemical characteristics such as the ability to hemolyze blood agar, negative tests for catalase and oxidase ability, and characteristic antigenic structures of the cell wall (Facklam & Elliott, 1995; Bascomb & Manafi, 1998; Facklam, 2002). Several attempts have been made to isolate, identify and classify these bacteria using biochemical tests, but these efforts were unsuccessful due to differences in the growth parameters, inoculum sizes and incubation periods among the possible isolates (Facklam & Elliott, 1995; Bascomb & Manafi, 1998). Previous studies have shown that parallel genomic and proteomic studies can be used to identify and characterize bacteria (Hantula et al., 1990; Niemi et al., 1993; Eldar et al., 1996; Dodson et al., 1997; Zlotkin et al., 1998; Eldar et al., 1999), but these strategies have been limited by the fact that they are expensive and/or time consuming, often requiring advanced equipment and training.

Epidemiology studies have reported strong correlations between whole cell protein profiles of bacteria and DNA-DNA hybridization. Bacterial isolates from the environment and clinical specimens can be differentiated by one-dimensional SDS-PAGE (Hantula et al., 1990; Niemi et al., 1993; Vandamme et al., 1998). On the other hand, the comparison of antigenic profiles using immunoblotting provides useful information about similarities and differences among bacteria isolates or strains (Schade et al., 2000). Therefore, the classification of bacteria based on protein and antigenic profiles may play a role in tentative identification of bacterial isolates and can be used in parallel with other methods for epidemiological studies.

A number of epidemiological studies (Lee and Ha, 1991; Oh et al., 1998; Heo et al., 2001) have reported that the frequent occurrence of streptococcosis in fish farms has led to great economic losses, which are currently threatening the sustainability of aquaculture off the southern coast of Korea. However, these studies did not examine whole cell protein or antigenic profiles for isolates tentatively identified as S. iniae and L. garvieae in biochemical tests. Here, we sought to identify and characterize the pathogenic S. iniae and L. garvieae isolates found in Korean fish farms.

**Materials and methods**

**Bacteria**

Bacterial strains were isolated from diseased olive flounder and named according to their...
Table 1. Biochemical characteristics of type strains and bacteria isolated from diseased olive flounder.

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**Legend:**
- : Present
- : Absent

**Note:**
- The table continues with similar entries for other genera and strains, with columns for different years.
place of origin. All isolates were gram-positive cocci, and their details are summarized in Table 1. As positive controls, the *Streptococcus iniae* type strain was obtained from the ATCC (USA), and *L. garvieae* KG9408 was kindly provided by Dr. Yoshida (Ooyama et al., 2002).

**Bacterial culture and biochemical tests**
All isolates were stored at -70°C in tryptic soy broth (TSB) containing 10% glycerol until use. For amplification, the stored isolates were inoculated to Todd-Hewitt broth (THB), incubated at 30°C for 12 hr, and then incubated at 30°C for 24 hr on blood agar. The resulting colonies were used for general and biochemical tests. A single colony was subcultured to an OD 610nm of 1.0 (4 × 10^9 CFU ml⁻¹) in THB for use in the PCR, SDS-PAGE and immunoblot assays. Other colonies were picked from the blood agar, suspended in PBS (3 mM KCl, 137 mM NaCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄, pH 7.4) and used for the catalase, oxidase and motility tests. The biochemical tests were performed with the API 20 Strep kit (BioMerieux, Inc., USA), according to the manufacturer’s instructions. The biochemical results of each isolate were compared with those of *S. iniae* ATCC29178 and *L. garvieae* KG9408.

**Polymerase Chain Reaction (PCR)**
PCR-based assays were used to identify whether the bacteria isolated from olive flounder were *S. iniae* or *L. garvieae*. The *S. iniae*-specific primers (NSI-F 5’-CAT GAC ACT AGA GTA C-3’ and NSI-R 5’-GCT TGC CGT CAC CG-3’) were designed by comparing the 16S-23S ribosomal DNA intergenic spacer sequences of *S. iniae* ATCC29178 with those of isolates Jeju-13 and Jeju-45. The *L. garvieae* primers were as previously reported by Zlotkin et al. (1998) (pLG-F 5’-CAT AAC AAT GAG AAT CGC-3’ and pLG-R 5’-GCA CCC TCG CGG GTT G-3’). Bacterial DNA was extracted from each isolate using the Accuprep® genomic DNA extraction kit (Bioneer, Korea) according to manufacturer’s instructions. PCR was carried out by adding 1 µl of each primer and template DNA to Accupower® PCR premixes (Bioneer, Korea) containing dNTPs, DNA polymerase, and reaction buffer, then adjusting the final volume to 20 µl with DNase- and RNase-free double distilled water. Amplification was performed in a PTC-100™ programmable thermal controller (MJ Research, Inc. USA), and consisted of 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 54°C for 45 sec and 72°C for 1 min, followed by a final extension at 72°C for 5 min. In screening for *S. iniae*, type strain *S. iniae* ATCC29178 was used as the positive control and *L. garvieae* KG9408 was used as the negative control, while the reverse was true when we screened for *L. garvieae*. The PCR products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized under UV light and photographed (Olympus, USA).

**SDS-PAGE**
SDS-PAGE analysis of bacterial cell lysates was used to compare the protein profiles of *S. iniae* and *L. garvieae* isolates. The bacterial isolates were cultured in THB at 30°C for 12 hr, centrifuged at 2,000 ×g for 30 min and washed three times with PBS. The resulting bacterial pellets were resuspended in 200 µl of PBS and mixed with 50 µl of 5 x SDS-PAGE sample buffer (5:1; 60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol
and 0.1% bromophenol blue). The samples were placed on an ice slurry, sonicated five times (XL-2020, Misonix Inc., USA) at 5.5 watts for 10 sec each, and then boiled for 10 min. The boiled samples were cooled to room temperature (RT) and centrifuged at 16,000 x g for 20 min at 4°C. The supernatants were transferred to fresh tubes and stored at -20°C until use. The proteins were resolved by 12.5% SDS-PAGE at 50 V for the first 15 min and then at 100 V until the front line reached the gel bottom. Gels were stained with Coomassie Brilliant Blue (CBB) G-250 at RT. The stained gel was digitalized with an Agfa Arcus 1200™ image scanner (Agfa-Gevaert, Belgium) and the acquired images were analyzed using the Phoretix 2D software (Ver. 5.01; NonLinear Dynamics, UK).

Production of chicken anti-L. garvieae IgY
Lactococcus garvieae KG9408 grown in THB was harvested by centrifugation, resuspended in PBS, and inactivated with 10% formalin for 12 hr at 4°C. The formalin was removed by repeated washes with PBS, and the cells were stored at -20°C until use. Chickens were initially immunized with 10^8 cells ml^-1 of formalin-killed bacteria (FKB) emulsified with an equal volume of Freund’s complete adjuvant. Subsequently, the birds were injected with 10^9 cells ml^-1 of FKB emulsified with Freund’s incomplete adjuvant three times at intervals of 2 weeks. One week after the last immunization, chicken eggs were collected, and chicken IgY was purified from the eggs using the GGstract® IgY purification kit (Promega, USA) according to the manufacturer’s instructions. Briefly, each egg yolk was transferred into a collecting tube and ruptured with a needle. Three volumes of Precipitation Solution A were mixed with the ruptured yolk and thoroughly stirred for 5 min. The lipid portion was removed by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant was collected and mixed with 1/3 volume of Precipitation Solution B and centrifuged for precipitation of IgY. The purified IgY pellet was resuspended in PBS and stored at -20°C until use.

Immunoblot assay
Immunoblotting was used to compare the antigenic profiles of the isolated S. iniae and L. garvieae strains. Cellular proteins were isolated and resolved as described above, the protein bands were transferred to a PVDF membrane, and the membrane was soaked in 100% methanol for 20 sec and dried at RT. The membrane was blocked with 5% skim milk in PBS-T (3 mM KCl, 137 mM NaCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.2 and 0.05% Tween-20) for 60 min at RT, and then incubated with chicken anti-KG9408 IgY (1:200) for 60 min at RT. The membrane was then washed 3 times with PBS-T and incubated with rabbit anti-chicken IgG-HRP (1:4000) for 90 min at RT. After three washes with PBS-T for 15 min, the membrane was developed with an Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, UK) and exposed to x-ray film for visualization of the antigenic proteins. The results were digitalized with an Agfa Arcus 1200™ image scanner (Agfa-Gevaert, Belgium) and the acquired image was analyzed using the Phoretix 2D software (Ver. 5.01; NonLinear Dynamics, UK).
Results

Biochemical tests

*S. iniae* ATCC29178, 9 isolates from Jeju Island and isolate Yosu-10 (collectively called Group 1) were positive for β-hemolysis, whereas *L. garvieae* KG9408, isolate Jeju-21 and all 4 isolates from Namhae County (collectively called Group 2) were negative for hemolysis on blood agar. All 18 bacteria examined in this study were negative for catalase and oxidase activity, and were non-motile. The results of the API 20 Strep kit tests are summarized in Table 1. All isolates were positive for esculin, pyrrolidonyl arylamidase (PYRA) and leucine arylamidase (LAP) but negative for hippurate, α-galactosidase (αGAL), β-glucuronidase (βGUR) and β-galactosidase (βGAL). All isolates except Jeju-13, Jeju-45 and Yosu-10 could hydrolyze arginine. All members of Group 1 were negative for VP but positive for alkaline phosphatase (PAL), whereas the members of Group 2 were positive for VP but negative for PAL. Group 1 showed various patterns in terms of carbohydrate utilization. Acid production from trehalose, glycogen and starch was observed only in *S. iniae* ATCC29178. In contrast, all members of Group 2 were found to utilize carbohydrates such as ribose, mannitol, sorbitol, lactose and trehalose.

PCR assay

We first confirmed that the specific primers newly designed from 16S-23S rDNA intergenic spacer sequences could amplify a 1,100 bp product from *S. iniae* ATCC29178 but could not amplify other type strains, such as *S. difficile*, *L. garvieae* KG9408, *L. piscium* KCTC3639 and *E. faecalis* KCTC3206 (Figure 1A). We then tested the various isolates and found that the *S. iniae*-specific primers amplified the expected 1,100 bp fragment from all Group 1 isolates (Figure 1B), but not from any of the Group 2 isolates. The isolates that were negative for the *S. iniae*-specific primers were subjected to PCR with *L. garvieae*-specific primers, which successfully amplified a 1,100 bp product from *L. garvieae* KG9408, Jeju-21 and the 4 Namhae County isolates.

![Figure 1. Detection of *S. iniae* by PCR.](image-url)
The gel protein patterns of the *S. iniae* and *L. garvieae* isolates are shown in Figure 2. Although the profiles were quite similar, differences were noted in the 20 to 62 kDa range, where *S. iniae* isolates showed 11 major protein bands (~ molecular weights of 69, 65, 62, 60, 45, 42, 35, 32, 27, 24 and 23 kDa) and *L. garvieae* isolates showed only 5 major bands (42, 40, 38, 36, and 28 kDa).

**Immunoblot assay**

As shown in Figure 3, chicken IgY raised against *L. garvieae* could strongly recognize antigens of both *L. garvieae* and *S. iniae* isolates, but the patterns differed between the two. Shared bands were found at 54 and 39 kDa, while unique bands appeared at 80, 48, 28 and 24 kDa in *S. iniae*, and at 45, 37, 34, 29 and 27 kDa in *L. garvieae*.

**Discussion**

The genera *Streptococcus*, *Lactococcus* and *Enterococcus* are generally characterized as Gram-positive coccoid bacteria that form chains and small colonies, test negative for catalase and oxidase activity, and are non-motile (Facklam & Elliott, 1995). These characteristics were found in all 16 bacterial isolates examined in this study, making it difficult to distinguish between *S. iniae* and *L. garvieae* strains based on these biochemical tests. Thus, we sought to identify molecular tests that could be used to easily distinguish between these organisms.
Previous studies showed that β-hemolytic Streptococcus sp. isolated from diseased olive flounder found at farms in Korea and Japan, and S. iniae isolated from tilapia grown in the USA, were negative for VP and hippurate but positive for esculin and arginine (Nakatsugawa, 1983; Lee et al., 1991; Ramesh et al., 1994; Hoe et al., 2001). Isolates from rabbitfish in Bahrain and barramundi in Australia were found to be β-hemolytic and positive for esculin, PYRA, αGUR, PAL, LAP and ADH (Yuasa et al., 1999; Bromage et al., 1999), while ADH-negative strains of S. iniae have been isolated from rainbow trout vaccinated with ADH-positive strains in Israel and striped bass in the USA (Bachrach et al., 2001; Barnes et al., 2003). In this work, 9 isolates from Jeju and isolate Yosu-10 showed β-hemolysis on blood agar, negativity for VP, hippurate, αGAL and βGAL, and positivity for esculin, PYRA, PAL and LAP; these characteristics were consistent with the type strain of S. iniae, ATCC29178, and also with previously reported fish isolates of S. iniae (Ramesh et al., 1994; Yuasa et al., 1999; Bromage et al., 1999). Of these, isolates Jeju-13, Jeju-45 and Yosu-10 were negative for ADH, which is consistent with previous reports in S. iniae isolates from rainbow trout and striped bass (Bachrach et al., 2001; Barnes et al., 2003). Based on these characteristics, the 9 isolates from Jeju Island and isolate Yosu-10 were tentatively identified as strains of S. iniae.

Figure 3. Immunoblot assay using chicken anti-L. garvieae KG9408 IgY.
L. garvieae isolates have been shown to be negative for hemolysis on blood agar but positive for VP, esculin, PYRA, LAP and ADH (Eldar et al., 1996; Eldar et al., 1999). However, various L. garvieae isolates have been shown to differ in terms of metabolic enzyme reactions. For example, trout isolates from Italy did not react with PYRA (Vela et al., 2000), rainbow trout isolates from Spain were positive for βGAL reaction and hydrolysis of hippurate, yellowtail isolates from Japan were positive for βGAL reaction and catfish isolates from Italy were positive for hydrolysis of hippurate (Ravelo et al., 2001). In this study, isolate Jeju-21, 4 isolates from Namhae County and type strain L. garvieae KG9408 showed no hemolysis on blood agar, positive reactions for VP, esculin, PYRA, LAP and ADH, but negative reactions for hippurate, αGAL, βGUL, βGAL and PAL. These findings are consistent with those previously reported in L. garvieae isolates (Eldar et al., 1996; Eldar et al., 1999; Vela et al., 2000), allowing us to identify isolate Jeju-21 and the 4 isolates from Namhae County as strains of L. garvieae.

Previous reports have shown strong correlations between bacterial whole cell protein profiles and the results of DNA-DNA hybridization (Hantula et al., 1990; Niemi et al., 1993; Vandamme et al., 1998). Elliott et al. (1991) differentiated L. garvieae and L. lactis isolates from human clinical specimens based on one-dimensional SDS-PAGE protein patterns. In this work, we found that S. iniae isolates and type strain ATCC29178 had unique protein bands of 69, 65, 62, 60, 45, 42, 35, 32, 27, 24, and 23 kDa, while the L. garvieae isolates and type strain KG9408 showed unique protein bands of 42, 40, 38, 36 and 28 kDa. We also observed strong correlations among the results of the biochemical, PCR and protein profile assays, indicating that SDS-PAGE and protein profile comparisons might be effective tools for tentative identification of the causative agents of olive flounder streptococcosis.

We next performed immunoblot assays using chicken IgY for L. garvieae KG9408 to compare antigen profiles of bacterial whole cells. Chicken IgY has several advantages over rabbit antisera: production of IgY does not require sacrifice of the experimental animal, immunized chickens may produce specific antibodies for long periods of time, large amounts of IgY are produced and may readily be isolated (Schade et al., 2000). Immunoblot assays using IgY raised against L. garvieae KG9408 revealed several common antigenic protein bands, as well as specific bands that could be used to distinguish between the bacteria. These findings indicate that immunoblotting might also be a useful tool for grouping isolates.

In terms of epidemiology, bacterial pathogens previously isolated from diseased olive flounder yielded a Streptococcus sp. infection rate of 25.8% in Jeju Island from 1991 to 1997 (Oh et al., 1998), and 4 and 5 Jeju isolates from diseased flounder were identified as ß-hemolytic Streptococcus sp. in 1991 and 2000, respectively (Lee et al., 1991; Heo et al., 2001). In the present work, 9 isolates from Jeju Island (all but one from this island) were identified as S. iniae. This finding along with the previous reports indicate that S. iniae might be an important causative agent of streptococcosis on Jeju Island, and is likely responsible for at least a portion of the
economic losses suffered by fish farmers in this part of Korea. Thus, it is vitally important that we develop additional methods for identifying infective strains, as an early step toward producing effective vaccines.

In sum, the olive flounder is a very important mariculture fish species in Korea. Here, we show that \textit{S. iniae} is highly prevalent in Korea, and used biochemical tests, PCR, SDS-PAGE and immunoblot assay to examine the occurrence of \textit{L. garvieae} and \textit{S. iniae} in diseased fish.

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