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Full Length Research Paper

Detailed analysis of *Streptococcus agalactiae* isolated from environmental samples and fish specimens during a massive fish kill in Kuwait Bay

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This study was undertaken to identify and characterize bacterial isolates obtained simultaneously from dead fish samples during a massive fish kill in Kuwait Bay and sewage water samples running into Kuwait Bay, using conventional and molecular techniques. Of the 71 bacterial isolates studied, 66 isolates were recovered from seven different fish species and five isolates were isolated from sewage samples. The species-specific identity of the isolates was established by phenotypic characteristics and by PCR amplification of the 16S rRNA gene, using *Streptococcus agalactiae*-specific primers. The genotyping of 12 isolates from fish samples and all five isolates from sewage samples was performed by random amplification of polymorphic DNA (RAPD) analyses. Culture methods identified 44 of 66 (67%) and 4 of 5 (80%) isolates obtained from fish and sewage samples, respectively, as *S. agalactiae.* The PCR amplification of 16S rRNA not only confirmed the results of conventional methods, but also resulted in additional identification of 14 of 66 (21%) isolates obtained from fish samples and the remaining isolate recovered from sewage sample as *S. agalactiae.* A total of nine RAPD patterns were observed among the 17 isolates studied and the RAPD patterns could be grouped into three clusters. Interestingly, four of the isolates recovered from sewage samples produced nearly identical RAPD banding patterns (85 - 100% similarity) with some of the *S. agalactiae* strains isolated from Mullet kidney and brain specimens, indicating the possibility of sewage being the source of infection.

Key words: *S. agalactiae,* fish infections, molecular characterization, RAPD

INTRODUCTION

Streptococcus spp. have emerged as important pathogens of a number of cultured fish species. Among *Streptococcus* species, *Streptococcus agalactiae* has been associated with numerous disease outbreaks in several fish species (Baya et al., 1990; Plumb, 1994; Evans et al., 2002), resulting in considerable economic losses. Because of the similarities in physiological and nutritional requirements, the available phenotypic methods are often unable to differentiate between different species/ strains.

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Likewise, serological typing methods also have limited discriminatory potential for strain differentiation among *S. agalactiae* isolates. In view of these limitations, many investigators prefer to use molecular methods in conjunction with phenotypic methods to characterize different strains of *S. agalactiae*. DNA-based typing techniques, such as pulsed field gel electrophoresis (PFGE) (Fasola et al., 1993), ribotyping (Chatellier et al., 1996), restriction enzyme analysis (REA) (Elis et al., 1996) and random amplification of polymorphic DNA (RAPD) (Chatellier et al., 1997) have been used to genotype *S. agalactiae* isolates originating from different sources. Among these, RAPD is a simple method that has sufficient discriminatory power to distinguish different strains of bacterial

Table 1. Comparative performance of phenotypic and molecular methods for *S. agalactiae*-specific identification of bacterial isolates recovered from fish and sewage samples

Method	No. of isolates identified as *S. agalactiae* from	
	Fish samples (*n*=66)	Sewage samples (*n*=5)
Phenotypic tests	44	4
16S rRNA PCR	58	5

species (Maslow et al., 1993).

The objective of this study was to use molecular methods for species-specific identification of bacterial isolates recovered from dead fish and sewage samples from Kuwait Bay. The typing of the isolates by RAPD analysis was performed to compare genetic relatedness between *S. agalactiae* isolates originating from infected fish and sewage water to trace the possible source of infection.

MATERIALS AND METHODS

Bacterial isolates

A total of 71 *Streptococcus* strains (isolated from the tissue of 66 different fish and five sewage samples) were obtained from the Mariculture and Fisheries Department of the Kuwait Institute for Scientific Research, Kuwait. Thirty-eight of the fish isolates were cultured from Mullet (*Liza klunzingeri*), 7 isolates were cultured from each Zobaidy (*Pampus argenteus*) and Cat fish (*Arius thalassinus*), while 11 isolates were cultured from Newaiby (*Orolithes orgenteus*) and 1 isolate was cultured from each European Seabream (*Sparus auratus*), Wahar *(Platycephalus indicus*) and Yemyam (*Pomad-dasys stidens*). The isolates were cultured from the moribund fishes during an outbreak that occurred in Kuwait Bay in August 2001. The cultures of the isolates were maintained in brain heart infusion broth, partially solidified with 0.8% agar, at 4°C. When required, the isolates were freshly subcultured and tested for purity on brain heart infusion agar supplemented with 0.5% lactose.

Phenotypic identification

The isolates were initially identified as *Streptococcus* species by positive Gram stain character, morphologic appearance, absence of motility, and negative oxidase and catalase tests. These isolates were further characterized using the API 20 Strep system (bio-Mérieux, France) and results were compared with the analytical profile index of the system. All the isolates reacted similarly in API 20 Strep. Using a commercial streptococcal grouping kit (Oxoid), based on the Lancefield grouping (Lancefield, 1933), all isolates reacted serologically with the group B antiserum.

Molecular characterization

DNA extraction and PCR amplification

The bacterial isolates were cultured in 5 ml of brain- heart infusion broth. The cells were harvested by centrifugation and suspended in 100 µl of TE buffer (10 mM Tris- HCl, 1 mM EDTA, pH 8.0). The genomic DNA was extracted with a commercial DNA extraction kit (Promega, Madison, WI, USA). Oligonucleotide primers, F1 (5'-GAGTTTGATCATGGCTCAG-3') and 1MOD capable of specifically amplifying a 220-bp amplicon from the 16S rRNA gene of *S. agalactiae* (Martinez et al., 2001) were obtained from Genosys Biotechnologies, UK. Amplification of target DNA was carried out in a thermal cycler (GeneAmp PCR system 7900, Perkin, Elmer, Cetus, USA). All DNA amplifications, including the appropriate positive and negative controls, were done according to the method of Telenti et al. (1993). Briefly, the reaction mixture contained 1 × AmpliTaq DNA polymerase buffer, genomic DNA, primers F1 and 1MOD (0.2 pM of each primer), 100 pM of each dNTP and 1.25 U of AmpliTaq DNA polymerase. The cycling conditions included an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min. The PCR products were resolved by agarose gel electrophoresis and photographed under UV light using standard procedures (Qasem et al., 2002).

Typing of *S. agalactiae* isolates by RAPD

Six different decamer oligonucleotide primers (P-1, P-2, P-3, P-4, P-5 and P-6) were tested by RAPD analyses (Duremdez et al., 2004). All the primers were purchased from Amersham-Pharmacia Biotech (Vienna, Austria). Amplification reactions were performed in 25- l reaction mixtures using the GeneAmp PCR system 7900 (Perkin, Elmer). Each reaction mixture contained 200 pM of each dNTP, 1.2 pM of primer, 50 ng of template DNA and 1.5 U of Ultima Taq DNA polymerase (Perkin Elmer USA) in 1 × PCR buffer. The cycling conditions included an initial denaturation at 95°C for 5 min, followed by 45 cycles of 1 min at 95°C, 1 min at 36°C, and 2 min at 72°C. The RAPD products were resolved by agarose gel electro-phoresis and photographed under UV light (Qasem et al., 2002). The computer analysis of RAPD patterns was performed with AlphaEaseFc software (Alpha Innotech, San Leandro, CA, USA). The resulting similarity matrix was used to construct a dendo-gram, employing the complete linkage method with arithmetic mean included in the molecular evolutionary genetics analysis software (Kumar et al., 1993).

RESULTS

Morphological and biochemical studies

All bacterial isolates were Gram-positive, coccoid and oxidase- and catalase-negative. Based on the API 20 Strep analytical profile index and on the results of the Lancefied grouping, these isolates were initially identified as *S. agalactiae*. Based on biochemical tests and carbon source utilization, 44 of 66 isolates recovered from fish samples and 4 of 5 sewage sample isolates exhibited patterns similar to the reference *S. agalactiae* strains

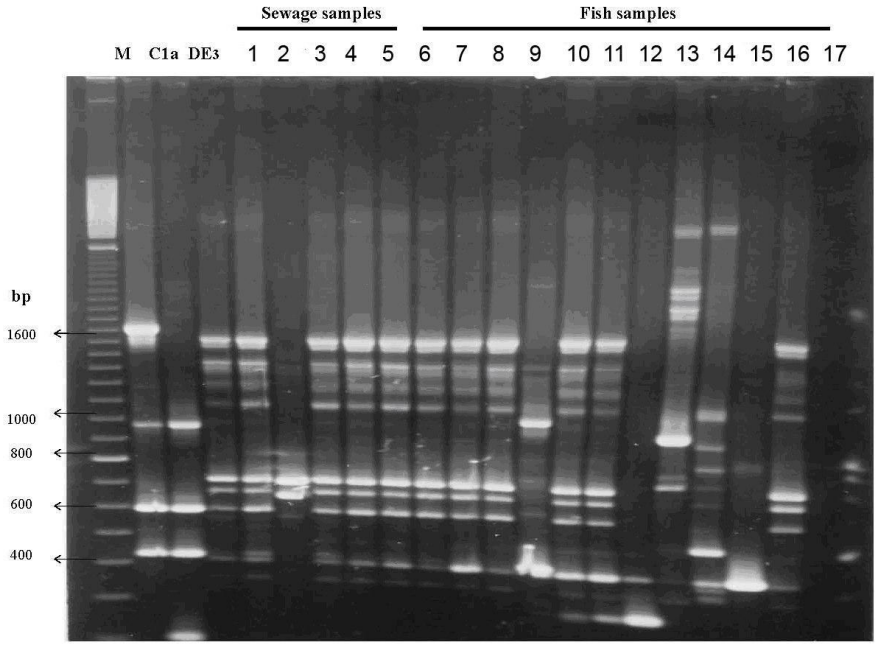

Figure 1. RAPD patterns for different isolates using primer P-2. Amplification products were resolved by electrophoresis in a 1.5% (w/v) agarose gel. The molecular weight marker (100-bp DNA ladder) is indicated in Lane M, *E. coli* strains C1a and DE3, used as references, are indicated in Lanes C1 and C2. Lanes 1-5 sewage isolates and Lanes 6-17 fish isolates.

(ATCC 13813 non-hemolytic and ATCC 27956 - hemolytic) (Table 1). The remaining isolates were only identified to the genus (*Streptococcus*) level.

Species-specific identification by PCR amplification of the 16S rRNA gene

When PCR amplification of the 16S rRNA gene was performed with *S. agalactiae*-specific primers, 58 of 66 isolates from fish samples and all five sewage sample isolates were identified as *S. agalactiae* (Table 1) . The species-specific identity of the remaining isolates from fish samples was not pursued further.

RAPD data analysis

Only primer P-2 (5'-GTTTCGCTCC-3'), but not any of the other RAPD primers, yielded a stable and reproducible number of bands, and provided maximum differentiation between the isolates. The results of RAPD amplification of *S. agalactiae* DNA recovered from 12 fish samples and from 5 sewage samples are shown in Figure 1. The isolates exhibited nine different patterns. The similarity matrices calculated from the RAPD data were used to generate a dendrogram by using the complete linkage (farthest neighbor) cluster method for frequency. The dendrogram depicting the relatedness of the isolates is shown in Figure 2. Three distinct clusters were apparent (Figure 2). The *S. agalactiae* isolates recovered from different organs of the same fish exhibited similar patterns (close relationship) and clustered together. Interestingly, four isolates recovered from sewage samples exhibited the same or very similar patterns obtained from the isolates recovered from the brain, kidney and spleen of Mullet and Zobaidy fish samples.

DISCUSSION

Conventional and molecular identification methods were used to investigate a panel of bacterial isolates recovered from tissue samples obtained from several fish species that died during a massive fish kill in Kuwait Bay in August 2001. Five isolates, recovered from sewage samples draining into Kuwait Bay, were also studied to trace the source of infection during the outbreak. The phenoltypic species-specific identification of several isolates was inconclusive. This is because of the close similarity in the biochemical characteristics of *Streptococcus iniae*, *Lactococcus graviae*, *Streptococcus dificile* and *Streptococcus shiloi* with *S. agalactiae.* Molecular identification was, therefore, carried out on the isolates culures. We applied PCR using species-specific primers for the identification of the isolates. Most of the isolates from the above specimens (63 of 71) were identified as *S. agalactiae.* These observations are consistent with earlier reports by Al-Marzouk et al. (2005) and Evans et al.

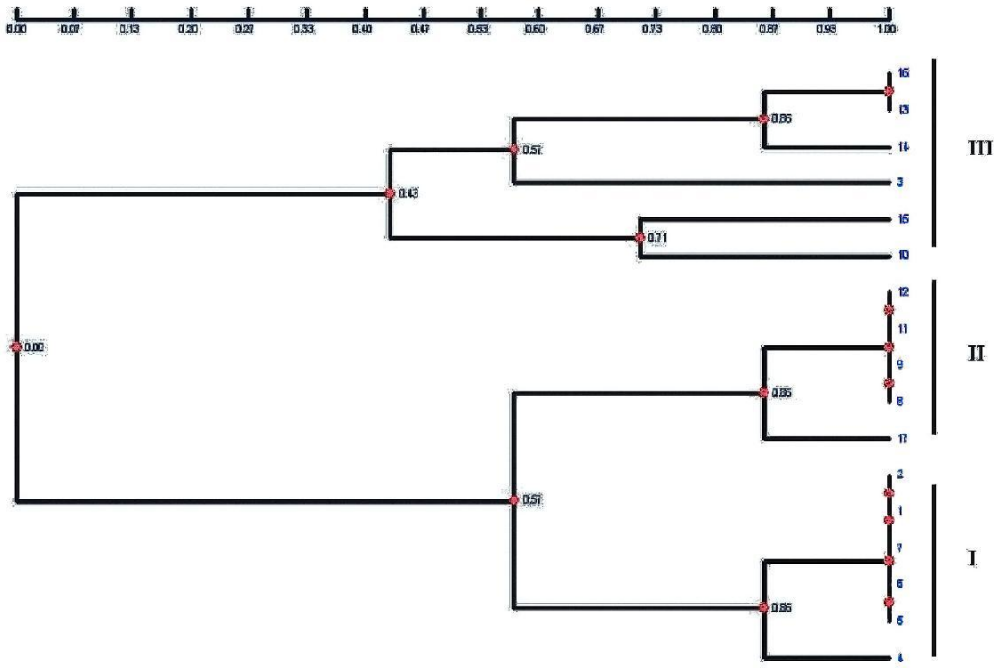

Figure 2. Dendrogram generated from the RAPD profiles of the 17 *S. agalactiae* isolates. The ruler indicates the similarity percentages, using complete linkage (furthest neighbor) cluster method.

(2002) showing association of -hemolytic group B Streptococci with infections in fish species. The majority of the fish isolates were recovered from Mullet (38 of 66), which is known to be highly susceptible to *S. agalactiae* infection at 33°C, following intraperitonal infection (Al-Marzouk et al., 2005).

There is limited information on the epidemiology of *S. agalactiae* infections in different fish species found in Kuwait Bay (Olivares -Fuster et al*.,* 2008; Duremdez et al*.,* 2004). RAPD is a simple and reliable molecular method for genotyping of *S. agalactiae* isolates recovered from the environment and infected fish and for tracking the origin of a possible infection. In the present work, RAPD analyses were used to study a collection of *S. agalactiae* isolates recovered from dead fish and all the isolates collected from sewage water running into Kuwait Bay that were collected at nearly the same time. Although, six different primers with varying G+C content were used, only primer P-2 yielded discriminatory results (data not shown). In a previous study carried out on isolates of *Legionella* species, primer P-2 was also found to be discriminatory in RAPD analyses (Qasem et al*.,* 2008). The RAPD analyses showed that the majority of the isolates recovered from Mullet exhibited RAPD patterns that were highly similar to those obtained from *S. agalactiae* recovered from sewage water, thereby implying their common origin. This is consistent with a few other reports that have also shown the association of *S. agalactiae* isolates recovered from fish samples and sewage water in United States (Rivas et al*.,* 1997). To the best of our knowledge, this is the first study describing the isolation, identification and geneotypic relatedness of *S. agalactiae* isolates recovered from fish samples and sewage water from Kuwait Bay.

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REFERENCES

Al-Marzouk A, Duremdez R, Yuasa K, Al-Zenki S, Al-Gharabally H, Munday B (2005). Fish kill of mullet Liza klunzingeri in Kuwait Bay: The role of *Streptococcus agalactiae* and the influence of temperature.. In: Walker P, Lester R and Bondad-Reantaso MG (eds) Diseases in Asian Aquaculture V. Fish Health Section, Asian Fisheries Society, Manila. pp. 143-153.

Baya AM, Lipiani B, Hetrick FM, Robertson BS, Lukacovic R, May E, Poukish C (1990). Association of *Streptococcus* sp. with fish mortalities in the Chesapeake Bay and its tributes. J. Fish Dis. 13: 251-253.

Chatellier S, Huet H, Kenji S, Rosenau A, Geslin P, Quentin R (1996). Genetic diversity of rRNA operons of unrelated *Streptococcus agalactiae* strains isolated from cerebrospinal fluid of neonates suffering from meningitis. J. Clin. Microbiol. 34: 2741-2747.

Chatellier S, Ramanantsoa C, Harriau P, Rolland K, Rosenau A, Quentin R (1997). Characterization of *Streptococcus agalactiae* strains by random amplified polymorphic DNA analysis. J. Clin. Microbiol. 35: 2573-2579.

Duremdez R, Al-Marzouk A, Qasem JA, Al-Harbi A, Gharabally H

(2004). Isolation of *Streptococcus agalactiae* from cultured silver pomfret, *Pampus argenteus* (Euphrasen), in Kuwait. J. Fish Dis. 27: 307-310.
Elis S, Kotiw M, Garland SM (1996). Restriction endonuclease analysis of group B streptococcal isolates from two distinct geographical regions. J. Hosp. Infect. 35: 279-287.
Evans JJ, Klesius PH, Gilbert PM, Shoemaker C, Al -Sarawi M, Duremdez J, Landsberg R, Al-Marzouk A, Al-Zenki S (2002). Charecterization of -haemolytic Group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus* L and wild mullet, *Liza Klunzingeri* (Day), in Kuwait. J. Fish Dis. 25: 505-513.
Fasola E, Livdahl C, Ferrieri P (1993). Molecular analysis of multiple isolates of the major serotypes of group B streptococci. J. Clin. Microbiol. 31: 2616-2620.
Kumar S, Tamara K, Nei M (1993). MEGA-Molecular Evolutionary Genetics Analysis, version 1.02. University Park, Pennsylvania State University.
Lancefield RC (1933). A serological differentiation of human and other groups of haemolytic streptococci. J. Experimental Med. 59: 571-591.

Martinez G, Harel J, Gottschalk M (2001). Specific detection by PCR of *Streptococcus agalactiae* in milk. Can. J. Vet. Res. 65: 68-72.
Maslow JM, Arimi SM, Arbeit RD (1993). Molecular epidemiology : application of contemporary techniques to the typing of microorganisms. Clin. Infect. Dis. 17: 153-164.
Olivares -Fuster O, Klesius PH, Evans J, Arias CR (2008). Molecular typing of *Streptococcus agalactiae* isolates from fish. J. Fish Dis. 31 (4): 277-283.
Plumb JA (1994). Health Maintenance of Cultured Fishes: Principal Microbial Diseases. CRC Press, Boca Raton, FL, pp. 231-238.
Qasem JA, Khan ZU, Shiji G, Mustafa AS (2002). Polymerase chain reaction as a sensitive and rapid method for specific detection of Mycoplasma pneumoniae in clinical samples. Microbiol. Res. 157: 77-82
Qasem JA, Mustafa AS, Khan ZU (2008). *Legionella* in clinical specimens and hospital water supply facilities: molecular detection and genotyping of the isolates. Med. Princ. Pract. 17(1): 49-55.
Rivas AL, Gonzalez RN, Wiedmann M, Bruce JL, Cole EM, Bennett GJ, Schulte III HF, Wilson DJ, Mohammed HO, Batt CA (1997). Diversity of *Streptococcus agalactiae* and *Staphylococcus aureus* ribotypes recovered from New York dairy herds. Am. J. Vet. Res. 58: 482-487.

Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T (1993). Rapid identification of *Mycobacteria* to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. 31: 175-178.