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Molecular Investigation of *Streptococcus agalactiae* Isolates from Environmental Samples and Fish Specimens During a Massive Fish Kill in Kuwait Bay

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Abstract: 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 were recovered from 7 different fish species and 5 strains were isolated from sewage samples. The species-specific identity of the isolates was established by phenotypic characteristics and by PCR amplification of 16S rRNA by using *Streptococcus agalactiae*-specific primers. The genotyping of 12 isolates from fish samples and all 5 isolates from sewage samples was performed by random amplification of polymorphic DNA (RAPD) analysis. 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 9 RAPD patterns were observed among the 17 isolates studied; these RAPD patterns were grouped into three clusters. Interestingly, four of the isolates recovered from sewage samples produced nearly identical RAPD band patterns (85-100% similarity) with some of the *S. agalactiae* strains isolated from Mullet kidney and brain indicting the possibility of sewage being the source of infection.

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

INTRODUCTION

Streptococcus sp. have emerged as important pathogens of a number of cultured fish species. Among *Streptococcus* species, *S. 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. 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) (Ellis *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 species (Maslow *et al.*, 1993).

The objective of this study was to use molecular methods for specie-specific identification of bacterial isolates recovered from dead fish and sewage samples from Kuwait Bay during an outbreak. The typing of the

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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 66 different fish tissues and 5 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*), while 7, 7, 11, 1, 1 and 1 were cultured from Zobaidy (*Pampus argenteus*), Cat fish (*Arius thalassinus*), Newaiby (*Orolithes orgenteus*), European Seabream (*Sparus auratus*), Wahar (*Platycephalus indicus*) and Yemyam (*Pomaddasys stidens*) fish types, respectively. 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 on brain heart infusion broth at 4°C partially solidified with 0.8% agar. 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-Merieux, 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, cells were harvested by centrifugation and resuspended in 100 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The genomic DNA was extracted by using a commercial DNA extraction kit (Promega, Madison, WI, USA).

Oligonucleotide primers, F1:5'-GAGTTTGATC ATGGCTCAG-3' and 1MOD 5'-ACCAACATG TGTTAATTACTC-3', capable of specifically amplifying 220 bp amplicon from 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 methods of Telenti *et al.* (1993). In brief, the reaction mixture contained 1x AmpliTaq DNA polymerase buffer, genomic DNA, primers F1 and IMOD (0.2 pM of each primer), 100 pM of each dNTP and 1.25 U 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 analysis (Duremdez *et al.*, 2004). All the primers were purchased from Amersham-Pharmacia Biotech (Vienna, Austria). Amplification reactions were performed in 25 µL reaction mixture using GeneAmp PCR system 7900 (Perkin, Elmer). Each reaction mixture contained 200 pM of each dNTP, 1.2 pM primer, 50 ng template DNA and 1.5 U Ultima Taq DNA polymerase (Perkin Elmer USA) in 1x PCR buffer (10 mm Tris-HCl, pH 8.0, 50 mm KC1, 1.5 mM $MgCl_2$ and 0.1% Triton x-100). 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 electrophoresis and photographed under UV light (Qasem *et al.*, 2002). The computer analysis of RAPD patterns was performed by using software AlphaEaseFc stand alone (Alpha Innotech, San Leandro, CA, USA). The resulting similarity matrix was used to construct a dendogram employing the complete linkage method with arithmetic mean included in the molecular evolutionary genetics analysis software (Kumar *et al.*, 1994).

RESULTS AND DISCUSSION

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, theses isolates were initially identified as *Streptococcus 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* strain (ATCC 13813 non – hemolytic and ATCC 27956 β- hemolytic) (Table 1). The remaining isolates were only identified to the genus (*Streptococcus*) level.

Species-specific identification by PCR amplification of 16S rRNA gene: When PCR amplification of 16S rRNA gene was performed with *S. agalactiae*-specific primers, 58 of 66 isolates from fish samples and all the 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 other RAPD primers yielded stable and reproducible number of bands and provided maximum differentiation between isolates. The results of RAPD amplification of *S. agalactiae* DNA recovered from 12 fish samples and 5 sewage-samples are shown in Fig. 1. The

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

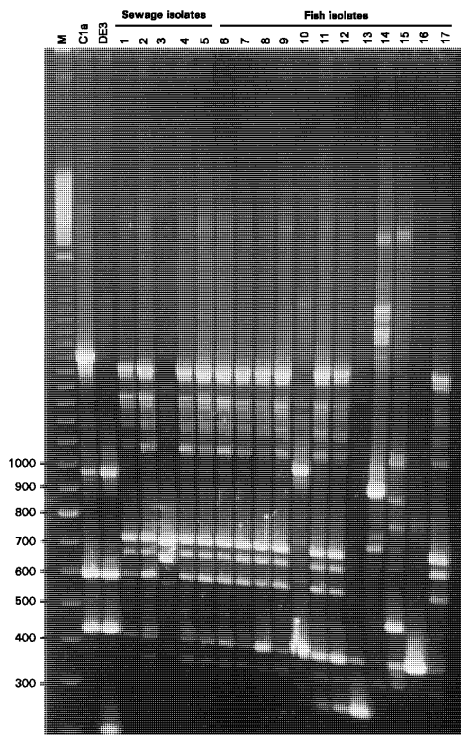|	No. of isolates identified as *S. agalactiae*	
Method	Fish samples (n = 66)	Sewage samples (n = 5)
Phenotypic tests	44	4
16S rRNA PCR	58	5

Fig. 1: RAPD patterns for different isolates using primer P2. Amplification products were resolved by electrophoresis in 1.5% agarose gel. Molecular weight markers (100 bp DNA ladder) are as indicated in Lane M, *E. coli* strains C1a and DE3, used as reference, are indicated in Lanes C1 and C2. Lanes 1-5 sewage isolates and Lanes 6-17 fish isolates

isolates exhibited nine different patterns. The similarity matrices calculated from the RAPD data were used to generate a dendrogram by using complete linkage (farthest neighbor) cluster method for frequency. The dendrogram depicting the relatedness of the isolates is shown in Fig. 2. Three distinct clusters were apparent (Fig. 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 same or very similar patterns obtained from the isolates recovered from the brain, kidney and spleen of Mullet and Zobaidy fish samples.

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 phenotypic species-specific identification of several isolates was inconclusive. This is because of the close similarity in the biochemical characteristics among *S. iniae*, *Lactococcus graviae*, *S. dificile*, *S. shiloi* with *S. agalactiae*. Molecular identification was, therefore, carried out on the isolates cultures. We applied PCR using species-specific primers for the identification of the isolates. Most (63 of 71) of the isolates from the above specimens were identified as *S. agalactiae*. These observations are consistent with earlier reports by Al-Marzouk *et al.* (2005) and Evans *et al.* (2002) showing association of β-hemolytic group B *Streptococci* with infections in fish species. Majority (38 of 66) of the fish isolates were recovered from Mullet which is known to be highly susceptible to *S. agalactiae* infection at 33°C following intraperitoneal 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). The 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 study, RAPD analysis were used to study a large 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 earlier study carried out on isolates of *Legionella* species, primer P-2 was also found to be discriminatory in RAPD analysis (Qasem *et al.*, 2008). The RAPD
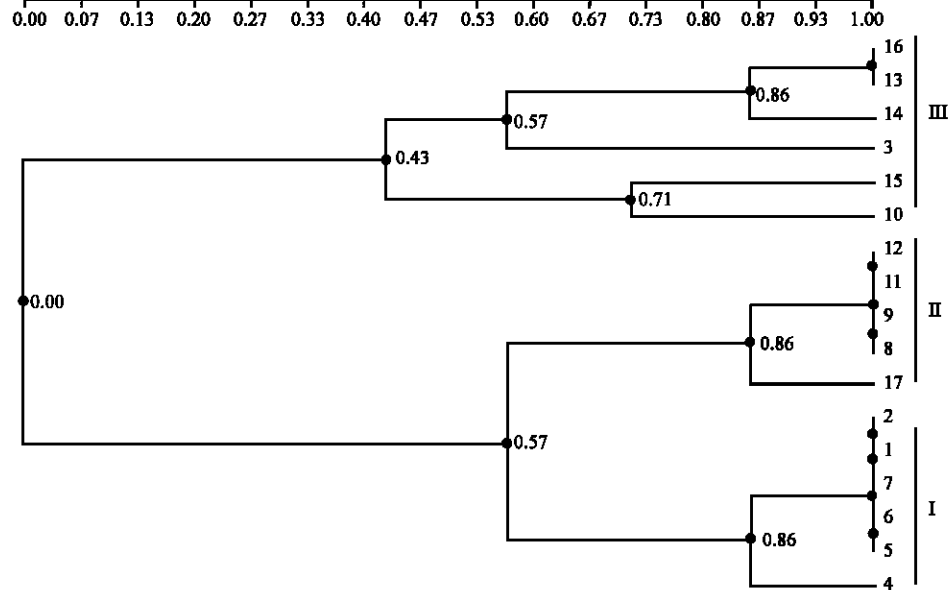
Fig. 2: Dendrogram generated from the RAPD profiles of the 17 *Streptococcus agalactiae* isolates. The ruler indicates the similarity percentages using complete linkage (furthest neighbor) cluster method

analysis showed that 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 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). According to the latest available data about *Streptococcus agalactiae* in Kuwait, this is the first study describing the isolation, identification and genotypic relatedness of *S. agalactiae* isolates recovered from fish samples and sewage water from Kuwait Bay.

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