A COMPARATIVE INVESTIGATION OF *STREPTOCOCCUS AGALACTIAE* ISOLATES FROM FISH AND CATTLE

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Julio Carib García

Certificate of Approval:

______________________ Joyce J. Evans Affiliate Assistant Professor Fisheries and Allied Aquacultures	______________________ Phillip H. Klesius, Chair Affiliate Professor Fisheries and Allied Aquacultures
______________________ Craig A. Shoemaker Affiliate Assistant Professor Fisheries and Allied Aquacultures	______________________ Covadonga R. Arias Associate Professor Fisheries and Allied Aquacultures

____
Joe F. Pittman
Interim Dean
Graduate School

A COMPARATIVE INVESTIGATION OF *STREPTOCOCCUS AGALACTIAE* ISOLATES FROM FISH AND CATTLE

Julio Carib García

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A COMPARATIVE INVESTIGATION OF *STREPTOCOCCUS AGALACTIAE*

ISOLATES FROM FISH AND CATTLE

Julio Carib Garcia

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Signature of Author

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Date of Graduation

VITA

Julio Carib García Febres, son of Julio Ramón García Loubriel and Aida Teresa Febres Hernández was born June 23, 1976, in San Juan, Puerto Rico. He graduated with a Bachelor of Arts degree (Environmental Science) from Washington College at Chestertown, MD in 2000. Two years later he entered Graduate School, in the Department of Fisheries and Allied Aquaculture, College of Agriculture, Auburn University and graduated in December 2004 with a Master of Science degree (Fisheries and Aquaculture). Upon completion he continued his education in the Department of Fisheries and Allied Aquaculture, College of Agriculture, Auburn University.

DISSERTATION ABSTRACT

A COMPARATIVE INVESTIGATION OF *STREPTOCOCCUS AGALACTIAE* ISOLATES FROM FISH AND CATTLE

Julio Carib García

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Streptococcus agalactiae is the causative bacterium of streptococcosis and causes severe economic losses in wild and cultured fish and cattle, worldwide. In fish, infection can result in septicemia with hemorrhages on the body surface and in the external and internal organs. *Streptococcus agalactiae* may be isolated from brain, nares, head kidney and eye of infected fish. *Streptococcus agalactiae* also causes bovine mastitis and the organism can be isolated from milk samples. *Streptococcus agalactiae* is classified as Lancefield's group B *Streptococcus* (GBS). There are also nine GBS capsular

polysaccharide serotypes. Despite the significance of the disease, only limited information is available on the identification and characterization of the *S. agalactiae* isolates from fish and cattle. In the present study, GBS isolates from fish (n = 36), bottlenose dolphin (n=1) and cattle GBS isolates (n=10) were found to have a number of common phenotypic characteristics of the *S. agalactiae* reference strains (n=4). However, the phenotypic characteristics of these GBS isolates were different from the reference strains of *Streptococcus dysgalactiae* (n=2), *Streptococcus phocae* (n=1), *Streptococcus iniae* (n=1) and *Lactococcus garvieae* (n=1). The results showed that GBS isolates of fish and cattle origins could be distinguished from each other by capsular serotype, CAMP factor, D-lactose and D-trehalose fermentation, growth pattern in fluid medium and whole cell fatty acid profiles. Studies were performed to determine the pathogenicity of cattle isolates for Nile tilapia (*Oreochromis niloticus*) and channel catfish (*Ictalurus punctatus*). The cattle isolates were found not to be infectious for either fish species. No diseases signs or isolation of GBS cattle isolates from the brain or head kidney of fish at 24 and 48 hour post-injection were noted. Fish isolates were evaluated to determine whether a single clone or multi-clones of *S. agalactiae* were responsible for streptococcal disease in mullet and seabream during the Kuwait Bay, Kuwait epidemic by amplified fragment length polymorphism (AFLP). The results indicated that the infection was not caused by a single clone of *S. agalactiae.* The results suggest that five GBS *S. agalactiae* clones were involved in the epidemic that affected primarily mullet and seabream.

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TABLE OF CONTENTS

LIST OF TABLES X

LIST OF FIGURES XI

I. INTRODUCTION AND LITERATURE REVIEW 1

II. MATERIALS AND METHODS 10

III. RESULTS 16

IV. DISCUSSION 35

V. SUMMARY 41

VI. LITERATURE CITED 45

VII. APPENDIX Table A. *Streptococcus agalactiae* isolates, sources and origins from fish, bottlenose dolphin, cattle and reference strains of *S. agalactiae, S. difficile, S. dysgalactiae* and *S. phocae* 57

VIII. APPENDIX Table B. Target gene, oligonucleotide primers and target size for polymerase chain reaction (PCR) 59

IX. APPENDIX Table C. Phenotypic properties of *GBS Streptococcus agalactiae* isolates from fish, bottlenose dolphin, cattle and reference type strains *of S. agalactiae, S. difficile, S. dysgalactiae* and *S. phocae* 60

X. APPENDIX Table D. API-20 Strep results of α-galactosidase (α -Gal), β-glucosidase (β-Gluc), β-galactosidase (β-Gal), alkaline phosphatase (PAL), aminopepidase (LAP) properties for GBS *Streptococcus agalactiae* isolates from fish, bottlenose dolphin, cattle and reference strains of *S. agalactiae, S. difficile, S. dysgalactiae* and *S. phocae* 62

XI. APPENDIX Table E. Capsule properties, hyaluronidase activity and pigment production for GBS *Streptococcus agalactiae* isolates from fish, bottlenose dolphin, cattle and reference strains of *S. agalactiae, S. difficile, S. dysgalactiae* and *S. phocae*...64

XII. APPENDIX Table F. Growth properties in fluid medium and soft agar for GBS *Streptococcus agalactiae* isolates from fish, bottlenose dolphin, cattle and reference strains of *S. agalactiae*, *S. difficile*, *S. dysgalactiae* and *S. phocae* 66

XIII. APPENDIX Table G. Fatty acid methyl esters (FAME), Gram-positive identification system (GPI), API-20 Strep, polymerase chain reaction (PCR), for *Streptococcus agalactiae* isolates from fish, cattle, bottlenose dolphin and reference strains of *S. agalactiae*, *S. difficile*, *S. dysgalactiae* and *S. phocae* 68

XIV. APPENDIX Table H. Percent of whole-cell fatty acid composition of GBS *Streptococcus agalactiae* isolates from fish, cattle and reference strains determined by RCLIN 50 rapid method 72

XV. APPENDIX Table I. *Streptococcus agalactiae* isolates from marine fish used for amplified fragment length polymorphism (AFLP) fingerprinting 73

LIST OF TABLES

III.1. Phenotypic characteristics of GBS *Streptococcus agalactiae* isolates from fish and cattle 30

III. 2. Phenotypic and genotypic properties of GBS *Streptococcus agalactiae* isolates from mullet, seabream, Nile tilapia, Gulf killifish, hybrid striped bass, cattle and *S. agalactiae* reference strains 32

III. 3. Summary of cost, discrimination level, disadvantages and advantages of methods utilized for the comparison of *Streptococcus agalactiae* isolates from cattle and fish 43

LIST OF FIGURES

III. 1. Dendogram of fatty acid profiles of GBS *Streptococcus agalactiae* isolates from fish, cattle and references strains. Analysis of profiles based on Pearson's product-moment. The cutoff value of 94 % similarity 23

III. 2. The PCR product of GBS streptococcal species based on16S-23S rDNA intergenic sequence..25

III. 3. The PCR product of GBS streptococcal species based on 23S rDNA sequence...26

III. 4. The PCR product of GBS streptococcal species based on 16S-23S intergenic spacer region rDNA sequence 27

III. 5. Dendogram of amplified fragment length polymorphic (AFLP) profiles of GBS *S. agalactiae* isolates from mullet and seabream collected in Kuwait Bay, Kuwait...29

I. INTRODUCTION AND LITERATURE REVIEW

Streptococcus species are considered a diverse group, ranging from commensal organisms which occupy various niches of the human body to pathogens that have the capacity to infect a wide range of hosts (Fishcetti 2000). Further, *Streptococcus* spp. are considered among the most important pathogens affecting humans and mammals and have been reported to affect cold blooded animals including fish (Eldar et al. 1994). In fish, *Streptococcus* spp. have been reported to cause considerable fish morbidity and mortality worldwide, estimated to be $150 million annually (Klesius et al. 2000).

Streptococcus agalactiae is a group B *Streptococcus* (GBS) (Lancefield 1933). In addition, nine GBS capsular polysaccharide serotypes have been identified (Hickman et al. 1999; Kong et al. 2002). *Streptococcus agalactiae* is generally characterized as a Gram-positive coccus, catalase (-), oxidase (-), CAMP (+) and hippurate hydrolysis (-). The identification of *S. agalactiae* is partly based on its hemolytic reaction on blood agar and Lancefield grouping. The types of hemolysis are alpha (α-), beta (β-) and non-hemolytic. Beta hemolysis is characterized as a clear zone around the colony on blood agar. Alpha hemolysis is a partial lysis of blood cells recognized by greenish zone around the colony. Lancefield's grouping is based on the serological types of polysaccharide antigen that are extracted by acid from the bacterium. The identification of *S. agalactiae* is also based on phenotypic characterization using conventional and

rapid identification tests (Jayarao et al. 1991; MacFaddin 2000; Facklam 2002). Genotypic characterization has also been used to identify *S. agalactiae* (Berridge et al. 2001; Yildirim et al. 2002b; Duarte et al. 2004). Highly discriminative typing techniques, whole cell fatty acid composition and amplified fragment polymorphism fingerprinting, have been performed in Gram negative bacteria (Stead 1992; Arias et al. 2003; Arias et al. 2004; Shoemaker et al. 2005), but these tests have not been applied for characterization of *S. agalactiae* from fish and cattle. These highly discriminative typing techniques can reveal the possible polyclonal nature of *S. agalactiae*. Polymerase chain reactions has been used in identification schemes for *S. agalactiae* (Berridge et al. 2001; Mata et al. 2004; Kawamura et al. 2005). Vandamme et al. (1996) and Arias et al. (2003) emphasized the importance of a consensus approach to bacterial identification or a polyphasic approach using conventional and rapid phenotypic tests, molecular and genotypic identification schemes for the identification and discrimination of environmentally and medically important bacteria. Thus, a polyphasic approach was employed to identify and discriminate *S. agalactiae* from fish and cattle.

Streptococcus agalactiae is considered a pathogen responsible for causing infections in chicken, cattle, camels, dogs, bottlenose dolphin, horses, emerald monitors, cats, fishes, frogs, hamsters, humans, mice, monkeys and nutria (Wilkinson et al. 1973; Elliott et al. 1990; Wagner et al. 1997; Evans et al. 2002; Yildirim et al. 2002a; Yildirim et al. 2002b; Hetzel et al. 2003; Zappulli et al. 2005; Evans et al. 2006a; Evans et al. 2006b).

Wild and cultured saltwater and freshwater fish species that have been reported to be infected with GBS are golden shiner (*Notemigonus crysoleucas*) (Robinson and Meyer 1966), sea catfish (*Arius felis*), striped mullet (*Mugil cephalus*), pinfish (*Lagodon rhomboids*), Atlantic croaker (*Micropogon undulatus*), spot (*Leiostomus xanthrus*), string ray (*Dasyatis sabina*), Gulf menhaden (*Brevoortia patronus*) (Plumb et al. 1974), bullminnows (*Fundulus grandis*) (Rasheed and Plumb 1984), striped bass (*Morone saxatilis*), bluefish (*Pomatomus saltatrix*), sea trout (*Cynoscion regalis*) (Baya et al. 1990), hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) (Eldar et al. 1994; Eldar et al. 1995), mullet (*Liza klunzingeri*), seabream (*Sparus auratus*) (Evans et al. 2002), Nile tilapia (*O. niloticus*) (Evans et al. 2002; Suanyuk et al. 2005; Salvador et al. 2005) and silver pomfret (*Pampus argenteus*) (Duremdez et al. 2004). These infections have been documented in the United States, Japan, Kuwait, Colombia, Brazil, Thailand and Israel (Evans et al. 2006). Evans et al. (2006b; 2006c) have reviewed the geographical distribution of *S. agalactiae* in tilapia and other host. Hoshina (1958) reported the first case of *Streptococcus* sp. in fish. Robinson and Meyer (1966) reported what is believed to be the first case of GBS *S. agalactiae* in fish.

Plumb et al. (1974) isolated a nonhemolytic, group B, Type Ib *Streptococcus* sp. from 90 % of fish examined involving multiple fish species from a fish kill in Soldier Creek located in the gulf coast of Alabama and Florida. The phenotypic properties of the group B isolates were previously described by Wilkinson et al. (1973). The fish species affected reported by Plumb et al. (1974) were menhaden, sea catfish, mullet, pinfish, Atlantic croaker and stingray. Cook et al. (1975) conducted experimental fish infectivity

trials using a group B isolate from a moribund menhaden from Soldier Creek that had been isolated by Plumb et al. (1974). Five of the eight fish species involved in the Alabama-Florida fish kill were challenged and mortality recorded. Mortality was noted in Atlantic croaker, sea catfish, Gulf menhaden, striped mullet and spot.

A streptococcal epidemic episode involving bluefish, striped bass and sea trout was reported in the Chesapeake Bay and some of the tributaries during the summer of 1988 (Baya et al. 1990). Similar signs to those reported by Rasheed et al. (1985) were observed in these fish. Yellow or reddish-yellow ascites fluid was found in the abdominal cavity of the majority of the diseased fish. Baya et al. (1990) compared their isolates with the isolate of Plumb et. al. (1974) and found them to be similar biochemically and serologically.

Elliot et al. (1990) examined a number of nonhemolytic GBS *Streptococcus* type Ib isolated from humans, mice, cattle, frogs and fish to determine whether these isolates were related. Using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), they compared the whole-cell protein profiles of these isolates from different animals. Elliot et al. (1990) concluded the PAGE profiles were very similar, suggesting that the isolates may have had a common ancestor.

In August and September 2001, Kuwait Bay, Kuwait experienced a massive fish kill involving wild mullet due to the bacterium *S. agalactiae* (Evans et al. 2002). This pathogen was isolated from cultured gilthead seabream (Evans et al. 2002) and bottlenose dolphin (*Tursiops truncatus*) (Evans et al. 2006a). Evans et al. (2002) reported the *Streptococcus* isolates were group B serogroup. Phenotypic characteristics of the isolates

compared favorably with those of American Type Culture Collection (ATCC) reference strains of *S. agalactiae*. The GBS *S. agalactiae* isolates were pathogenic for Nile tilapia by intraperitoneal (IP) injection (Evans et al. 2002).

Capsular polysaccharide serotypes and GBS surface protein antigens are believed to be important to the pathogenesis of GBS disease (Kong et al. 2002). Rasheed and Plumb (1984) isolated a non-hemolytic *Streptococcus* sp. from diseased Gulf killifish. Rasheed and Plumb (1984) used the isolate to determine the pathogenicity of the non-hemolytic *Streptococcus* sp. through different modes of infection, including IP injection, immersion and oral administration, to Gulf killifish. Mortality was produced by experimental IP injection, but not by immersion or oral administration. Furthermore, Rasheed et al. (1985) attributed the pathogenicity of streptococci to an exotoxin, but no evidence was provided to demonstrate the presence of an exotoxin. They noted that streptococcal infections in bullminnows were systemic infections localized in the eye, liver and spleen. Signs caused by *Streptococcus* sp. in fish species reported by Rasheed et al. (1985) included unilateral or bilateral exophthalmia, hemorrhage in the eye, dark coloration, erratic swimming and splenomegaly. Evans et al. (2002) reported that wild mullet had abnormal behavior including erratic swimming, swimming and whirling on the surface and "C" shaped body curvature while at the surface. Ocular abnormalities that included opacity, peri-orbital and intraocular hemorrhages, purulence and exophthalmia were reported. Reddening and hemorrhage were observed in the integumental and muscoskeletal system (Evans et al. 2002). Cranial and body surface

hemorrhages were observed. An atypical sign of a clear opercula "window to the gills" was also noted (Evans et al. 2002).

Gram positive cocci were isolated from tilapia and trout by Eldar et al. (1994). Two distinct phenotypic groups were found, including a nonhemolytic and α-hemolytic group. These isolates were tested with DNA-DNA hybridization and by DNA base composition. These two isolates belonged to two separate DNA groups. Eldar et al. (1994) proposed the nonhemolytic isolate be named *S. difficile* and the α-haemolytic be named *S. shiloi.*

Several studies have been conducted to correctly identify and name the nonhemolytic *Streptococcus* spp. Vandamme et al. (1997) using whole-cell protein SDS-PAGE analysis reported that the *S. difficile* strain (LMG 15799) was indistinguishable from a *S. agalactiae* strain. Moreover, Vandamme et al. (1997) was able to serologically type *S. difficile*. The capsular antigen was identified as Ib serotype (Vandamme et al. 1997).

Streptococcal isolates are often unspeciated (Robinson and Meyer, 1966, Plumb et al. 1974) or are improperly identified as other *Streptococcus* spp. (Wilkinson et al. 1973, Kusuda and Komatsu 1978, Elliott et al. 1990, Eldar et. al 1994). However, *S. agalactiae* is the only group B species of the genus. Thus, it is very important to determine both Lancefield group and serotype of the isolate to determine its identity. Molecular biological techniques such as ribotyping, oligonucleotide probes and polymerase chain reaction (PCR) have been proposed for the more accurate identification of the various species of *Streptococcus* (Kawata et al. 2004). Recently, studies performed by Berridge

et al. (2001), Kawata et al. (2004) and Mata et al. (2004) focused on developing species-specific primers that target the 16S-23S rDNA intergenic spacer region and the 23S rDNA PCR product specifically for *S. agalactiae*.

Berridge et al. (2001) developed species-specific PCR primers for the detection and identification of the 16S-23S rDNA intergenic spacer region of *S. agalactiae* and *S. difficile*. Berridge et al. (2001) demonstrated that the *S. difficile* fish isolates possess a common sequence found in the *S. agalactiae* type strain (ATCC-13813) and *S. difficile* type strain (ATCC-51487). However, the species-specific primers were unable to produce amplicons from related *Streptococcus* spp., further demonstrating the homology of *S. agalactiae* and *S. difficile.*

Kawata et al. (2004) targeted the 23S rDNA PCR sequence for the identification of eight streptococcal species, including *S. agalactiae*. Kawata et al. (2004) primers produced an amplicon of 875 bp. Their results showed that 100 % of the *S. agalactiae* isolates (N=35) used in their investigation were correctly identified. They also reported that the PCR method was able to identify biochemically typical GBS isolates as well as atypical GBS isolates. Mata et al. (2004) developed a multiplex PCR designed to simultaneously detect *Streptococcus* spp. associated with warmwater fish. These results showed that the species specific PCR primers successfully identified *S. agalactiae*, *S. difficile* isolates and were similar to the *S. agalactiae* reference strains by PCR.

Zlotkin et al. (1998) utilized restriction fragment length polymorphism (RFLP) to ribotype *S. iniae* strains associated with outbreaks in wild and cultured fish species in the Mediterranean Sea. The RFLP results confirmed that outbreaks of *S. iniae* were

produced by a single *S. iniae* clone. This allowed Zlotkin et al. (1998) to establish that, epidemiologically, all fish were infected by descendants of a single clone of *S. iniae*. Further, results suggest that cross-infection that occurred between wild and cultured fish was caused by a single clone.

Whole cell fatty acids have been used for identification of bacterial fish pathogens (Shoemaker et al. 2005). Further, growth medium, time and temperature of incubation differ among bacterial genus species and groups. Thus, the selection and the use of a standardized medium that produces good growth of the organisms is vital for optimal results (Shoemaker et al. 2005).

The pathogenesis of fish GBS isolates is poorly understood. Eldar et al. (1994) reported that *Streptococcus* spp. caused meningoencephalitis in fish. Pathogenicity studies conducted with GBS isolates from fish indicated that it is a highly virulent fish pathogen capable of infecting a wide variety of freshwater and saltwater fish (Evans et al. 2002). The infectivity of cattle GBS isolates for fish is unknown. No other information is available on cross-transmission of infection between the same fish species or among unrelated fish species.

The hypothesis of this investigation was that *S. agalactiae* isolates from fish and cattle are phenotypically and genetically distinct. To test this hypothesis, three objectives were proposed. The first objective was to compare GBS isolates from fish (n=36) and GBS from cattle (n=10) for phenotypic and genotypic characteristics. The second objective was to determine the infectivity of GBS isolates from cattle for Nile tilapia and channel catfish. The third objective was included in order to determine whether single or

multiple clones of GBS isolates were responsible for streptococcal disease in saltwater mullet and seabream during the epidemic event in Kuwait Bay, Kuwait by amplified fragment length polymorphism (AFLP) analysis.

II. MATERIALS AND METHODS

Experimental Design

Thirty six *S. agalactiae* isolates collected from infected mullet, seabream, tilapia, Gulf killifish and hybrid striped bass in Kuwait, United States of America, Israel, Honduras and Brazil were compared to 10 cattle isolates collected from Wisconsin, USA, for phenotypic and genotypic characteristics (Appendix VII. Table A). In addition, an isolate collected from a bottlenose dolphin was also investigated. American Type Culture Collection (ATCC) *S. agalactiae, S. difficile, S. dysgalactiae,* and *S. phocae* reference strains were included for comparison.

All cultures were grown on tryptic soy broth (TSB) or sheep blood agar (SBA) for 24 hours at 32 °C. Phenotypic characteristics were determined for hemolysin production (Evans et al. 2002), CAMP reaction (Darling 1975), Lancefield's grouping (Lancefield 1933), capsular serotyping (group B typing antisera, Denka Seiken, Tokyo, Japan) (Wilkinson et al. 1973), pyrrolidonylarylamidase reaction (PYR), Voges-Proskauer reaction (VP) by API Strep-20 (bioMérieux, Hazelwood, MO, USA); hippurate hydrolysis (HIP) (Jokipii and Jokipii 1976); urea hydrolysis (URE) (MacFaddin 2000); starch hydrolysis (Evans et al. 2004); arginine decarboxylase (ARG), and esculine hydrolysis (ESC) (Jokipii and Jokipii 1976); fermentation of D-sorbitol (SOR), D-trehalose (TRE), D-ribose (RIB), inulin (INU), D-mannitol (MAN), D-xylulose (XYL) and D-lactose (LAC) (Evans et al. 2002).

Hyaluronidase production (Smith and Willett 1968), pigment production (Merritt and Jacobs 1976), growth characteristics in soft agar and fluid medium (Kane et al. 1975) and encapsulation (Atlas et al. 1995) of GBS isolates were also evaluated.

Fatty Acid Methyl Ester (FAME)

Fatty acid methyl esters (FAME) were extracted according to the procedure described in the Microbial Identification System Operating Manual MIDI (2000) to generate a GBS library for the analysis of FAME profiles. GBS isolates were grown in SBA for 24 hours at 32 °C. Briefly, 20 ± 0.5 mg (wet wt.) of bacterial cells were harvested and placed in 13-mm X 100-mm glass screw cap test tubes (Stead 1992). The bacterial cells were treated with 1 ml of a strong methanolic base (45g sodium hydroxide, 250 ml methanol, 150 ml deionized water) and boiled in a water bath for 30 min to saponify the cells. The fatty acids were methylated (2 ml of 325 ml 6.0N hydrochloric acid, 275 ml of methanol) in a 100 °C water bath for 5 min. After methylation, the FAME were extracted in hexane and methyl tert-butyl ether. Residual fatty acids were removed from the organic phase by washing with dilute base for 5 min. After extraction, the FAME were injected into an Agilent 6850 gas chromatograph for analysis by the rapid RCLN50 method (MIDI, 2000). A library report was generated by MIDI Procedure (MIDI, 2000). The validation report uses comparative matrices to determine the number of correct matches to the genus and species levels in relation to other profiles available in the MIDI software (MIDI, 2000). The validation data were imported into the BioNumerics version 4 software package (Applied Maths, Sint-Martens-Latem, Belgium). A similarity matrix and the corresponding dendrogram using the Pearson's

product-moment correlation coefficient were generated. The reproducibility of the results was determined by results generated from three cultures grown and extracted on three different days. These results established a 94 % cutoff level.

API-20 Strep

GBS isolates grown on SBA plates for 24 hr at 32 °C were used in the API-20 Strep strip test. The cells were harvested in API GP medium (0.5 g L-cystine, 20 g tryptone, 5 g sodium chloride, 0.5 g sodium sulfite, 0.17 g phenol red and 1000 ml demineralized water) and the bacterial suspensions were adjusted to a turbidity ≥ 4 McFarland (bioMérieux, Durham, NC, USA). Each well of the API-20 Strep plate was inoculated with 100 µl of the bacterial suspension and incubated for 4 hr or 16 hr at 35 °C. The color changes in each individual well of the API-20 Strep plate were read and analyzed using the MINI API (bioMérieux) system. Results generated by the MINI API system were automatically compared against the system database for genus and species identification and the degree of identification.

Gram positive identification (GPI)

Streptococcus agalactiae isolates grown on SBA for 24 hr at 32 °C were utilized to inoculate the GPI 32 cards according to manufacture's directions (Vitek, bioMérieux). After inoculation, the GPI cards were incubated in the Vitek GPI system for 24 hours. The GPI validation reports were used to generate a GBS library. The validation reports provided the genus and species and probability for a correct identification.

Genotypic characterization.

Genomic DNA templates were prepared using the High Pure PCR preparation Kit (Roche Diagnostic, Indianapolis, USA). The GBS isolates were incubated in TSB at 32 °C for 24 hr. Samples were then added to a sterile 2 ml microcentrifuge tube and centrifuged for 10 min at 5,000 x *g*. Following centrifugation, the sample supernatant was discarded. The bacterial samples were prepared according to the manufacturer's instructions. After centrifugation, the eluted DNA was stored at 20 °C until PCR analysis. The methods described to amplify *S. agalactiae* 16S -23S rDNA (Mata et al. 2004), 23S rDNA (Kawata et al. 2004) and 16S-23S rDNA intergenic spacer region (Berridge et al. 2001) were used to amplify species-specific targets by PCR to identify the forty seven *S. agalactiae* isolates (VIII. Appendix Table B). The PCR reagent mixture contained 1 X reaction buffer, 2.5 mM MgCl, 0.8 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM each oligonucleotide primer, 0.5 μL of bovine serum albumin, 1.25 units of *Taq* DNA polymerase, 50-100 ng template DNA, and sterile water (Berridge et al. 2001). Briefly, PCR mixture were cycled 35 times at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min with a final extension for 5 min at 72 °C. The annealing temperature was adjusted to 60 °C for the specific *S. agalactiae* primers. The PCR products were analyzed by 1.0 % agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Samples were considered positive when a clear band of the expected molecular weight was observed under UV light.

Amplified Fragment Length Polymorphism (AFLP)

Twelve isolates of *S. agalactiae* collected from 6 wild mullet and 6 cultured seabream were tested for interspecific diversity by AFLP analysis. These fish were collected from a *Streptococcus* epidemic in Kuwait Bay, Kuwait. The GBS isolates were incubated in TSB at 32 °C for 24 hr and the cells harvested by centrifugation. Briefly, genomic DNA templates were prepared using the DNeasy Tissue Kit (Qiagen Science, Maryland, USA). The kit was used according to manufacturer's instructions and Arias et al. (2004). One hundred nanograms of total DNA was digested with *Hin*dIII and *Taq*I (Promega, Madison, WI, USA). Next, the adapters were ligated to the restricted fragments using T4 DNA lipase (Promega). The *Hin*dIII primer was labeled with IR700 Fluorochrome (LICOR, Lincoln, NE, USA). The presence of PCR products was determined by electrophoresis in a NEN Global Edition IR2 DNA analyzer (LICOR) according the manufacture's directions. The AFLP data were processed with BioNumerics and levels of similarity between GBS isolates were calculated with the Pearson's product moment correlation coefficient and a dendrogram of the GBS isolates was generated.

Experimental infectivity trials – Nile tilapia and channel catfish exposed to GBS isolates from cattle

Ten *S. agalactiae* isolates from cattle were grown in TSB for 24 hr at 32 °C. The cattle isolates were taken from original frozen stock cultures stored at -80 °C. The freshly prepared broth cultures of each isolate were adjusted to an optical density (OD) of 1.0 at 540 nm using a SmartSpec™3000 (Bio Rad, Foster City, CA) and then adjusted to give final concentrations of 1 x 10^9 and 1 x 10^{10} colony forming unit (CFU)/mL. Two

hundred noninfected Nile tilapia (15.23 ± 1.03 g mean weight) and two hundred channel catfish (15.0 ± 1.20 g mean weight) were IP injected with 0.1 mL at 1 x 10^9 CFU/mL and 1 x 10^{10} CFU/mL of each of the GBS cattle isolates. Two separate tanks containing ten control fish each were inoculated with 0.1 mL of TSB. Two separate tanks with ten fish each served as positive controls and were inoculated with 0.1 mL of a mullet isolate (KU-MU-11-Br) at 1 x 10^9 CFU/mL and 1 x 10^{10} CFU/mL, respectively. This mullet isolate (KU-MU-11-Br) had previously been shown to be virulent for Nile tilapia (Evans et al. 2002), even after repeated passages in TSB. Mean water temperature was 32 ± 0.6 °C. Fish were exposed to a light and dark period of 12:12 hours and flow- through water (0.5 L/hour). Aeration was supplied through air stones. The fish were fed daily to satiation with Aquamax Grower 400 (Brentwood, MO). The inoculated fish were then held and observed in individual 57 L aquaria per group for 14 days. Moribund fish were removed and head kidneys and brains were cultured for *S. agalactiae*.

III. RESULTS

Serotypes

Differences in capsular serotypes between fish and cattle isolates were found (Tables 1 and 2, IX. Appendix Table C). Mullet (n=11), seabream (n=6) and bottlenose dolphin (n=1) isolates collected from the Kuwait Bay epidemic and Nile tilapia from Brazil and Mississippi were serotypes Ia. One hybrid striped bass GBS isolate (Israel) was Ia serotype. In contrast, the cattle isolates (n=10) were non-typable (NT). The other Nile tilapia GBS isolates from Brazil and Honduras were NT. The GBS isolates from Gulf killifish (n=3) were also NT. Further, *S. agalactiae* references strains ATCC-27956 (n=1) and CECT-183 (n=1) were NT. The *S. agalactiae* reference strain ATCC-13813 (n=1) was serotype II. The *S. difficile* reference strain ATCC-51487 (n=1) was serotype Ib.

Hemolytic characteristics

The results of hemolytic activity showed variations between fish and cattle GBS isolates (Tables 1 and 2, IX. Appendix Table C). All of the mullet, seabream and bottlenose dolphin GBS isolates from the Kuwait Bay epizootic were β-hemolytic, while five of the cattle isolates were β-hemolytic and the other five were α-hemolytic. The GBS Nile tilapia isolates from Larvas, Brazil were non-hemolytic while the GBS Nile tilapia isolates from Parana State, Brazil were β-hemolytic. The GBS hybrid striped bass isolate (Israel) was also non-hemolytic and the GBS isolates (Louisiana Veterinary

Diagnostic Laboratory, LVDL) from tilapia, Gulf killifish and hybrid striped bass were non-hemolytic.

CAMP characteristics

The results of CAMP factor production revealed that all of the mullet, bottlenose dolphin, and cattle isolates were positive. Three seabream isolates also yielded a positive CAMP test (Tables 1 and 2, IX. Appendix Table C). Nile tilapia GBS isolates from both regions of Brazil and LVDL were found to be CAMP factor negative. The killifish and hybrid striped bass isolates were CAMP negative. Seventy five percent of GBS reference strains were shown to be CAMP factor positive while one of the GBS reference strain was CAMP factor negative.

Biochemical characteristics

Phenotypic similarities and differences between the GBS fish and cattle isolates were observed (Tables 1 and 2). The mullet, seabream, bottlenose dolphin and cattle isolates were positive for Voges-Proskauer (VP) activity by API-20 Strep and negative for pyrrolidonylarylamidase (PYR) activity by conventional tests. Sixty four percent of the GBS isolates hydrolyzed hippurate (HIP). Of these, 27 % of the mullet isolates were positive. All of the seabream isolates hydrolyzed hippurate. The bottlenose dolphin GBS isolate was negative for hippurate hydrolysis. Hippurate was hydrolyzed by 86 % of the Nile tilapia isolates. Thirty three percent of killifish isolates hydrolyzed hippurate, whereas one hundred percent of the hybrid striped bass were hydrolytic. Hippurate hydrolysis was observed for 50 % of the cattle GBS isolates. The GBS reference strains

hydrolyzed hippurate. None of the fish, cattle GBS and reference strains hydrolyzed starch (results not shown).

Arginine decarboxylation (ARG) varied between mullet, seabream, bottlenose dolphin and cattle GBS isolates. Mullet and bottlenose dolphin isolates hydrolyzed arginine. Fifty percent of seabream GBS isolates were arginine positive. Sixty seven percent of the Nile tilapia GBS isolates was found not to decarboxylate arginine. Sixty seven percent of the killifish and fifty percent of the hybrid striped bass isolates were arginine positive. Only 50 % of cattle isolates were positive. GBS reference strains decarboxylated arginine. None of the GBS isolates hydrolyzed esculin (ESC) and urea (results not shown). Two GBS reference strains were positive for ESC.

Fermentation of D-lactose (LAC) also varied between mullet, seabream and cattle GBS isolates. Mullet, seabream (93 %) and bottlenose dolphin isolates were negative. Only one GBS isolate from Larvas, Brazil were positive for D-lactose. None of the killifish and hybrid striped bass isolates were positive. Ninety percent of cattle GBS isolates fermented D-lactose. Seventy five percent of the GBS reference strains fermented D-lactose. The mullet, seabream and bottlenose dolphin isolates fermented D-trehalose (TRE). One of 14 Nile tilapia was positive. None of the killifish and hybrid striped bass isolates was D-trehalose positive. The *S. agalactiae* reference strains were positive. All GBS isolates and reference strains fermented ribose. Negative results were noted for sorbitol, inulin and xylulose (results not presented). Mannitol (MAN) was also negative.

Rapid enzyme characteristics

The results of API-20 Strep determination for α-galactosidase (α-Gal), β-glucouronidase (β-Glu), β-galactosidase (β-Gal) enzyme activities, (PAL) and (LAP) reactions are presented in Tables 1 and 2 and X. Appendix Table D. The seabream and bottlenose dolphin isolates were positive for α-Gal while about half of the mullet isolates were positive. Only two GBS Nile tilapia isolates from Brazil were α-Gal positive. None of Nile tilapia GBS isolates from LVDL were positive. The killifish and hybrid striped bass isolates were α-Gal negative. All of the GBS isolates from cattle were negative. One GBS reference strain was α-Gal positive. Seabream GBS isolates were negative for β-Gluc while four mullet GBS isolates were negative (n=4) and six mullet were positive (n=6) for β-Gluc activity. The Nile tilapia GBS isolates from Larvas, Brazil were β-Gluc positive while the GBS isolates from Parana State, Brazil were β-Gluc negative except for one positive isolate. The LVDL tilapia, killifish and hybrid striped bass and Israel hybrid striped bass GBS isolates were observed to be β-Gluc negative. The cattle isolates were positive. Fifty percent of the GBS reference isolates were β-Gluc negative.

The assays for β-Gal activity revealed that none of GBS isolates from fish were positive while 2 cattle isolates were positive. The GBS dolphin isolate and reference strains were β-Gal negative. All GBS fish, cattle isolates and reference strains were PAL and LAP positive.

Capsule and hyaluronidase characteristics

All of the GBS isolates were encapsulated. The mullet, seabream, bottlenose dolphin and cattle produced hyaluronidase (Tables 1 and 2, XI. Appendix Table E). GBS isolates of Nile tilapia from Brazil did not produced hyaluronidase. Two killifish isolates produced hyaluronidase in contrast to the hybrid striped bass isolates that were negative. One half of the GBS reference strains did not produce this enzyme.

Pigment production

Pigment production was variable among fish and cattle isolates (Tables 1 and 2, XI. Appendix Table E). The mullet, seabream and bottlenose dolphin isolates produced pigment. None of the Nile tilapia, Gulf killifish, hybrid striped bass or cattle isolates produced pigment. All of the GBS reference strains produced pigment.

The growth patterns of GBS isolates in fluid medium and soft agar are presented in Tables 1 and 2 and XII. Appendix Table F. The growth patterns of the GBS isolates from mullet and cattle in fluid medium were sedimentary while the growth pattern of seabream, bottlenose dolphin and the majority Nile tilapia isolates were turbid. In soft agar, GBS displayed generally diffused colony growth (XII. Appendix Table F).

FAME characteristics

The results for FAME profiles of GBS *S. agalactiae* from fish and cattle produced by the Microbial Identification System (MIS) and RCLIN50 method are presented in XIII. Appendix Table G. The FAME similarity indexes showed that fish and cattle isolates were a correct match at genus and species levels for *S. agalactiae*. The FAME similarity indexes (SI) for the GBS *S. agalactiae* isolates from fish, bottlenose dolphin

and reference strain were very good to excellent (0.3 – 0.9 SI). Lower discrimination at the genus level was noted for one mullet isolate (0.27 SI) and for two cattle isolates (0.18 and 0.12 SI). The SI for ATCC -51487, *S. difficile* was 0.61 and was correctly matched at the genus and species levels for *S. agalactiae*.

Cattle and fish GBS isolates differed with regard to percent whole cell fatty acid composition (XIV. Appendix Table H). Cattle isolates had 17:iso w5c, 18:1 w5c and 19:1 iso1 whole cell fatty acids while fish isolates and reference strains lacked these whole cell fatty acids. The four GBS *S. agalactiae* reference strains lacked the 17:0 fatty acid. GBS *S. agalactiae* isolates from fish were higher in 12:0 and 14:0 whole cell fatty acid percent than found in cattle (16:0, followed by 18:1 w9c, 18:1 w7c and 18:0).

FAME data were exported from the GBS library to BioNumerics version 3.0 software package (Applied Maths,) using Pearson's product-moment correlation coefficients to generate a dendrogram for grouping of the GBS isolates and reference strains with a cut off level of 94 % similarity (Figure 1). The GBS *S. agalactiae* fish, cattle and reference strains belonged to one of nine groups based on their similarities in whole cell fatty acids. The majority of mullet isolates were grouped together with the *S. difficile* ATCC-51973 reference strain. The majority of cattle isolates also grouped together in the next cluster. The ATCC-12388 and -12394, *S. dysgalactiae* reference strains were noted to be similar to the whole cell fatty acid profiles of a Brazil tilapia isolate (04ARS-BZ-TN-005) and a cattle (MOS-59998-17) isolate. Another group of GBS *S. agalactiae* was represented by the Brazil and Israel striped bass isolates. One Brazil tilapia isolate (03ARS-BZ-TN-01) clustered separately. The largest group

consisted of the majority of the tilapia, seabream, killifish, hybrid striped bass, bottlenose dolphin isolates and *S. agalactiae* reference strains. At 85 % similarity, two major clusters could be observed that consisted of mullet and cattle isolates and isolates from tilapia and other fish species. The FAME analysis appeared to discriminate between the GBS *S. agalactiae* isolates at the host species and/or origin levels.

<u>Gram Positive Identification (GPI) characteristics</u>

The results of the GPI analyses showed the genus and species identification was acceptable to very good ranging from 77-99 % probability for the mullet and seabream GBS *S. agalactiae* isolates (XIII. Appendix Table G). However, the identification of GBS *S. agalactiae* isolates from cattle was generally unacceptable. Four of the ten cattle isolates were not identified as *S. agalactiae*. The Israeli hybrid striped bass isolate was not identified as *S. agalactiae* and had the lowest identification probability at 21 %. GPI analyses correctly identified the reference strains.

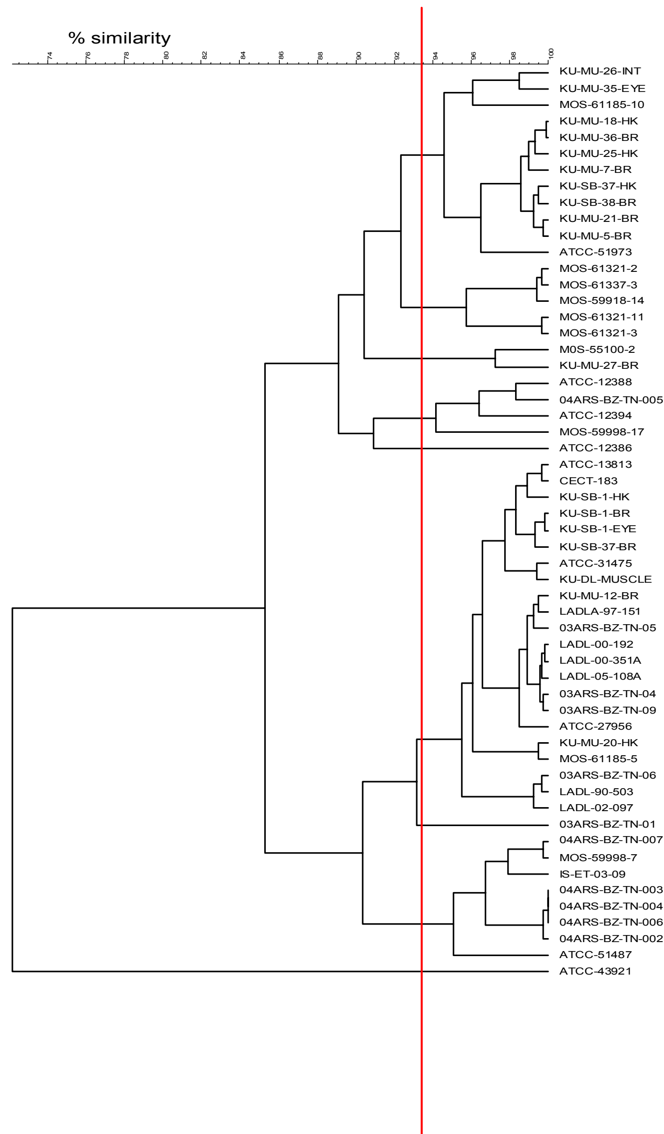

Figure 1. Dendogram of fatty acid profiles of GBS *Streptococcus agalactiae* from fish, cattle and references strains. Analysis of profile based on Pearson's product-moment. Cutoff value is 94 % similarity.

API-20 Characteristics

The API-20 Strep system provided accurate identification of GBS *S. agalactiae* fish and cattle isolates that ranged from excellent to good identification (X. Appendix Table D). The bottlenose dolphin GBS isolate and the hybrid striped bass isolate from Israel gave a good identification similar to *S. agalactiae*. The majority of Nile tilapia GBS isolates and cattle isolates varied from good to very good identification.

Genotypic characterization

Molecular characterization of the GBS isolates was conducted by PCR with specific primers designed against the 23S rRNA gene and 16S-23S intergenic spacer region (ISR). Two different sets of primers were used against the ISR yielding a 190 bp and 130 bp product respectively (XIII. Appendix Table I). The PCR results with 16S-23S (190 bp) specific primer did not yield the specific amplicon for one seabream and nine Nile tilapia GBS isolates. Positive PCR tests were observed for all GBS cattle and dolphin isolates. The 16S-23S (190 bp) primers did not amplify one seabream isolate, hybrid striped bass isolates, one Gulf killifish isolate, and 11 of the Nile tilapia isolates. A representative PCR pattern for four *S. agalactiae, S. dysgalactiae, S. phocae* and *S. iniae* isolate is presented in Figure 2. The 190 bp reactions were observed for all *S. agalactiae* isolates, but not for the other streptococcal isolates. The 23S specific primer (866 bp) did not amplify 3 seabream, 12 Nile tilapia and 9 cattle isolates. The 23S PCR failed to amplify three reference strains. Figure 3 shows that PCR pattern for the 23S PCR primer. The 866 bp reactions were observed in all *S. agalactiae* isolates, but not for the other streptococcal isolates. The specific intergenic region 16S-23S (130 bp) primers

amplified specific DNA fragments from all fish, cattle, dolphin GBS isolates and reference strains. Figure 4 shows an acceptable PCR pattern for 130 bp reactions.

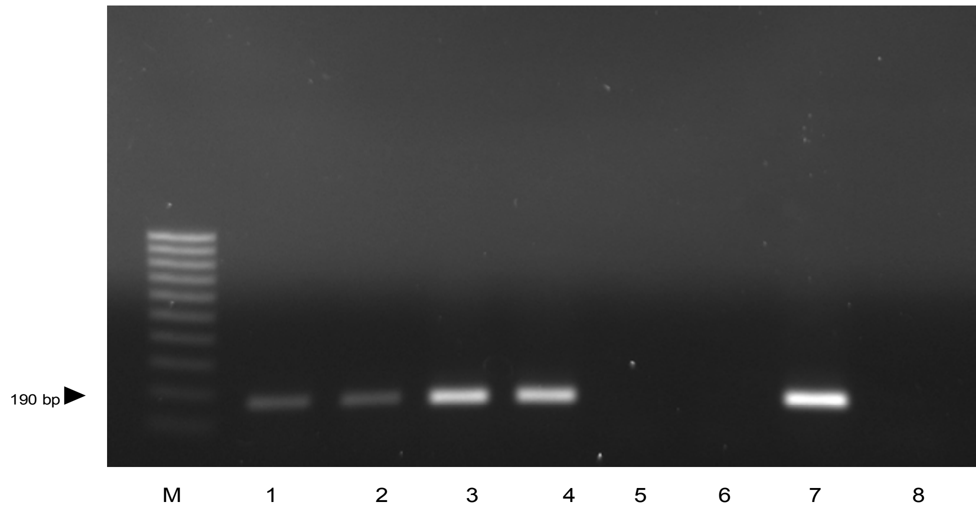

Figure 2. The PCR products of streptococcal species based on 16S-23S intergenic rDNA[1] sequence.

Lanes: 1, *S. agalactiae* KU-MU-5-BR; 2, *S. agalactiae* KU-DL-MUSCLE; 3, *S. agalactiae* Cattle-55100-2; 4, *S. agalactiae* type strain ATCC-12386; 5, *S. dysgalactiae* type strain ATCC-12388; 6, *S. phocae* type strain ATCC-51973; 7, *S. agalactiae* type strain ATCC-51487; 8, *S. iniae*; M, molecular DNA size markers (Bio-Rad, Hercules, CA, USA). [1] Targeted sequence used for analysis of 16S-23S intergenic rDNA obtained from Mata et al. 2004.

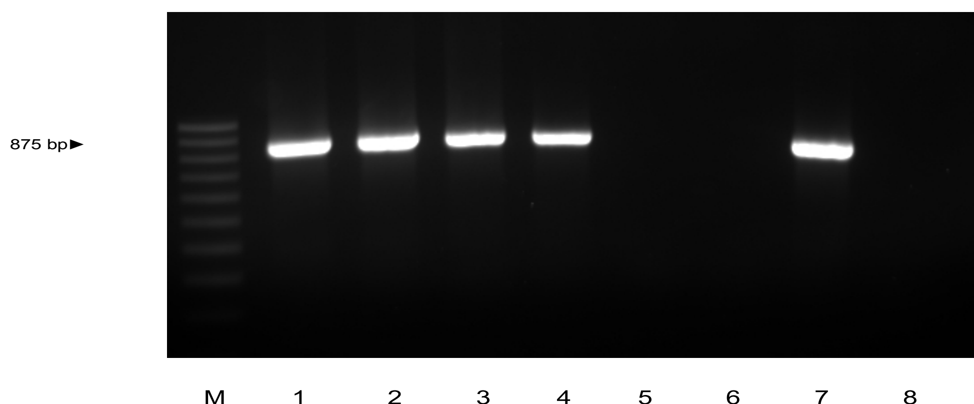

Figure 3. The PCR products of streptococcal species based on 23S rDNA[1] sequence.

Lanes: 1, *S. agalactiae* KU-MU-5-BR; 2, *S. agalactiae* KU-DL-MUSCLE; 3, *S. agalactiae* Cattle-55100-2; 4, *S. agalactiae* type strain ATCC-12386; 5, *S. dysgalactiae* type strain ATCC-12388; 6, *S. phocae* type strain ATCC-51973; 7, *S. agalactiae* type strain ATCC-51487; 8, *S. iniae*; M, molecular DNA size markers(Bio-Rad, Hercules, CA, USA). [1] Targeted sequence used for analysis of 16S-23S rDNA obtained from Kawata et al. 2004.

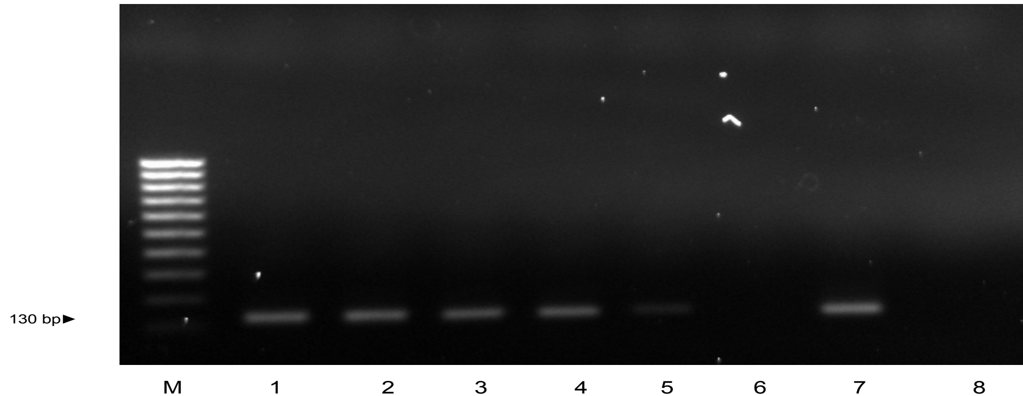

Figure 4. The PCR products of streptococcal species based on 16S-23S intergenic rDNA[1] sequence.

Lanes: 1, *S. agalactiae* KU-MU-5-BR; 2, *S. agalactiae* KU-DL-MUSCLE; 3, S. *agalactiae* Cattle-55100-2; 4, *S. agalactiae* type strain ATCC-12386; 5, *S. dysgalactiae* type strain ATCC-12388; 6, *S. phocae* type strain ATCC-51973; 7, *S. agalactiae* type strain ATCC-51487; 8 *S. iniae;* M, molecular DNA size markers (Bio-Rad, Hercules, CA, USA). [1] Targeted sequence used for analysis of 16S-23S rDNA obtained from Berridge et al. 2001.

Experimental virulence trials

The cattle isolates were not infectious (0 % mortality) for Nile tilapia and channel catfish. GBS cattle isolates were also not recovered from *S. agalactiae* inoculated fish at 24 and 48 hr post-injection. Clinical signs and/or mortality did not occur in the challenged or control fish. Tilapia IP injected with mullet isolate (KU-MU-11-Br) had 60 and 90 % mortality at 1 x 10^9 CFU/mL and 1 x 10^{10} CFU/mL, respectively (positive control).

AFLP characteristics

DNA samples from mullet and seabream isolates from Kuwait Bay epizootic were analyzed by AFLP for genetic similarity between the mullet and seabream isolates (XV. Appendix Table I). Seabream isolates had AFLP profiles similar to the mullet isolates (Figure 5). At 90 % similarity, five distinct clonal groups were identified among these isolates. Clones I contained isolates from mullet and clone II contained isolates from seabream. Clone III contained four mullet and one seabream isolate. A single seabream isolate was observed in clone IV. Clone V contained two seabream isolates.

No particular clone could be correlated with a particular fish species. Clone IV contained one seabream isolates that presented the most dissimilar AFLP profile. Interestingly, two seabream isolates recovered from the same fish (SB-37-HK and SB-37-BR) belonged to different AFLP clones. The results suggested that infection was not caused by a single clone of *S. agalactiae* that originated from either seabream or mullet. The results seem to suggest that at least five different *S. agalactiae* clones were responsible for the GBS *S. agalactiae* epidemic.

Polyphasic characteristics

The results of the comparative investigation of GBS *S. agalactiae* isolates indicate that phenotypic characteristic were different between fish and cattle origins (Table 10). The differences between fish and cattle isolates were noted in capsule serotype, growth pattern in fluid medium, pigment production, production of hyaluronidase, CAMP factor production, fermentation of D-trehalose and D-lactose and FAME. The results indicated that fish and cattle isolates can be differentiated from each other by the use of a polyphasic approach.

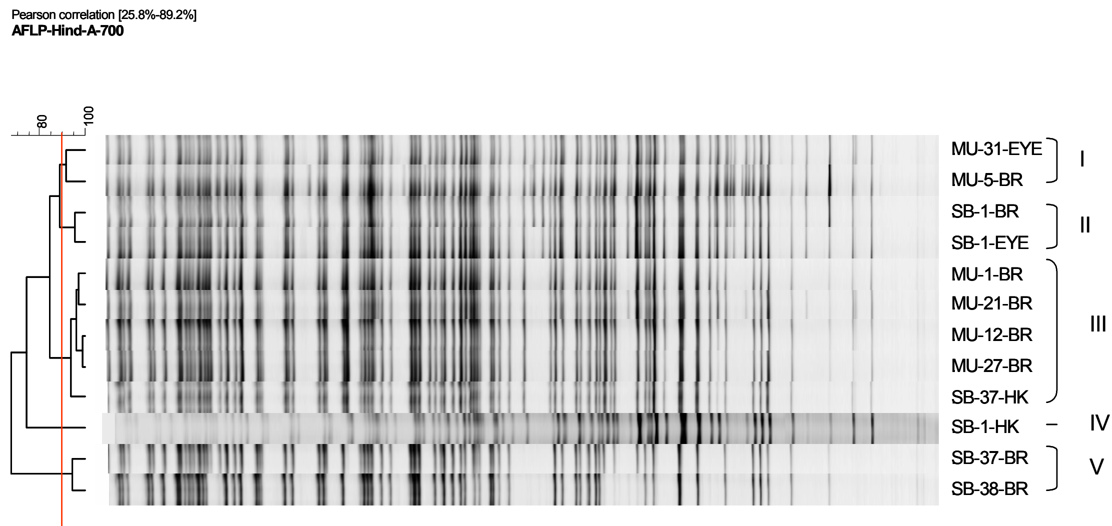

Figure 5. Dendogram of amplified fragment length polymorphic (AFLP) profiles of twelve *Streptococcus agalactiae* isolates from marine fish. The tracks show the processed band patterns after conversion, normalization, and background subtractions. Pearson product-momen**t** similarity coefficients were used to determine linkage levels.

Table 1. Phenotypic characteristics of GBS *S. agalactiae* isolates of fish and cattle and percent of isolates that expressed that characteristic. (NT=non-typable)

	Percent of isolates (%)	
Characteristics	Fish n=36	Cattle n=10
Catalase production Oxidase production Lancefield group B	- - + (100)	- - + (100)
Serotype	Ia (56)	NT (100)
Capsule	+ (100)	+ (100)
Turbidity pattern in fluid medium	Sedimentary (41)	Sedimentary (100)
Colony morphology in soft agar	Diffuse colonies (97)	Diffuse colonies (100)
Pigment	+ (50)	- (100)
Production of hyaluronidase	+ (53)	+ (100)
VP test	+ (100)	+ (100)
Hemolysis	β (67)	β (50)
CAMP factor	- (61)	+ (100)
Hippurate hydrolysis	+ (61)	+ (50)
Esculine hydrolysis	- (100)	- (100)
Arginine Decarboxylase	+ (47)	+ (50)
Starch hydrolysis	- (100)	- (100)
Urea hydrolysis	- (100)	- (100)
Pyrolidonylarylamidase	- (100)	+ (10)

Table 1. Continued

	% isolates	
α-Galactosidase	+ (38)	- (100)
β-Glucoronidase	+ (22)	+ (100)
ß-Galactosidase	- (100)	+ (20)
Acid production from:		
D-Sorbitol	- (100)	- (100)
D-Trehalose	+ (50)	- (100)
D-Ribose	+ (100)	+ (100)
Inulin	- (100)	- (100)
D-Mannitol	- (100)	- (100)
D-Xyulose	- (100)	- (100)
D-Lactose	+ (6)	+ (90)

[1]The symbols denote: n= number of isolates; (-) = negative; (+) = positive; percent of isolates = (%).

Phenotypic variations were observed among fish GBS isolates from different species and origins (Table 2). The mullet, seabream and a minority of the Nile tilapia isolates were Ia serotypes, while the Gulf killifish, hybrid striped bass and cattle were NT. A similar pattern of differences between these species was observed for the growth pattern in fluid medium. Nile tilapia, Gulf killifish and hybrid striped bass isolates did not produce pigment. β-hemolysis was observed in the mullet, seabream, cattle and in a minority of Nile tilapia isolates. CAMP factor production was absent for the Nile tilapia, Gulf killifish and hybrid striped bass isolates.

Table 2. Phenotypic and genotypic properties of GBS *Streptococcus agalactiae* isolates from mullet, seabream, Nile tilapia, Gulf killifish, hybrid striped bass, and *S. agalactiae* reference strains[1]

Phenotypic and genotypic properties	Mullet n=11	Seabream n=6	Tilapia n=14	Gulf killifish n=3	Hybrid striped bass n=2	*S. agalactiae* reference strains n=4
	(Percent of isolates)					
Serotype	Ia (100)	Ia (100)	NT (97)	NT (100)	NT (100)	NT (50)
Capsule	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)
Growth in fluid medium	Sed[2] (100)	Tur (100)	Tur (85)	Sed (100)	Sed (50)	Sed (50)
Colony morphology in soft agar	Diffuse colonies (100)	Diffuse colonies (100)	Diffuse colonies (93)	Diffuse colonies (100)	Diffuse colonies (50)	Diffuse colonies (100)
Pigment production	+ (100)	+ (100)	- (100)	- (100)	- (50)	+ (50)
Production of hyaluronidase	+ (100)	+ (100)	- (100)	+ (66)	- (100)	+ (14)
VP test	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)
β-Hemolysis	+ (100)	+ (100)	+ (43)	- (100)	- (100)	+ (50)
CAMP reaction	+ (100)	+ (50)	- (100)	- (100)	- (100)	+ (75)
Hippurate	+ (27)	+ (100)	+ (86)	+ (33)	+ (100)	+ (50)
Esculine	- (100)	- (100)	- (100)	- (100)	- (100)	+ (50)
Arginine	+ (100)	+ (50)	- (31)	+ (67)	+ (50)	+ (100)
Starch	- (100)	- (100)	- (100)	- (100)	- (100)	- (100)
Urea	- (100)	- (100)	- (100)	- (100)	- (100)	- (100)
α-Gal	+ (45)	+ (100)	+ (14)	- (100)	- (100)	+ (50)

Table 2. Continued

Phenotypic and genotypic properties	Mullet n=11	Seabream n=6	Tilapia n=14	Gulf killifish n=3	Hybrid striped bass n=2	*S. agalactiae* reference Strains n=4
β-Gluc	+ (64)	- (100)	- (92)	- (100)	- (100)	+ (75)
β-Gal	- (100)	- (100)	- (93)	- (100)	- (100)	- (100)
D-Sorbitol	- (100)	- (100)	- (100)	- (100)	- (100)	- (100)
D-Trehalose	+ (100)	+ (100)	+ (7)	- (100)	- (100)	+ (100)
D-Ribose	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)
Inulin	- (100)	- (100)	- (100)	- (100)	- (100)	- (100)
D-Mannitol	- (100)	- (100)	- (100)	- (100)	- (100)	- (100)
D-Xylulose	- (100)	- (100)	- (100)	- (100)	- (100)	- (100)
D-Lactose	- (100)	+ (17)	+ (7)	- (100)	- (100)	+ (100)
Specific PCR 16S-23S rDNA 130bp	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)	+ (100)
Specific PCR 16S-23S rDNA 190bp	+ (100)	+ (83)	+ (31)	- (67)	+ (50)	+ (100)
Specific PCR -23S rDNA 866 bp	+ (100)	+ (67)	+ (15)	- (100)	- (100)	+ (75)

[1] The symbols denote:n = number of isolates; (-) = negative; (+) = positive; (% isolates).

[2] The following symbols denote: sed = sediment; tur = turbid.

Hydrolysis of hippurate was more pronounced for cattle isolates. Fifty percent of the cattle isolates deaminate arginine while fish isolates did not. The majority of Nile tilapia did not ferment D-trehalose while cattle, mullet and seabream did ferment this sugar to acid. The majority of the cattle isolates fermented D-lactose and other fish isolates were non-fermentative. In addition, 16S-23S (190 bp) and 23S (866 bp) primers did not identify all the fish isolates by PCR.

The phenotypic characteristics and 16S-23S intergenic spacer region primer (130 bp) for *S. agalactiae* by PCR clearly revealed that the *S. difficile* was *S. agalactiae* (IX. and XIII. Appendix C, Appendix G). *S. agalactiae* isolates and reference strains were differentiated from the *S. dysgalactiae, S. phocae*, *S. iniae* and *Lactococcus garviae* by bacteriological properties and specific primers for 16S-23S intergenic region (130 bp) by PCR.

IV. DISCUSSION

GBS *S. agalactiae* is the cause of severe disease affecting fish and cattle. However, the possibility of transmission of GBS fish isolates infecting cattle or vice versa has not been established. Based on phenotypic, biological, molecular characteristics and mortality results from infectivity studies using cattle GBS isolates to infect tilapia and catfish, the potential likelihood for cattle GBS isolates to infect fish appears to be limited or nonexistent. The hypothesis that phenotypic and genotypic characteristics of GBS fish isolates were different from GBS cattle isolate was verified. The phenotypic characteristics of GBS *S. agalactiae* isolates from fish were similar to the properties of the ATCC *S. agalactiae* reference strains and different from ATCC *S. dysgalactiae, S. iniae, S. phocae* and *L. garviae* reference strains. The ATCC *S. difficile* reference strain was found to be identical to *S. agalactiae* from fish and cattle and to the ATCC *S. agalactiae* reference strains.

Finch and Martin (1984) demonstrated that cattle and human isolates were separate populations that shared the common group B antigen. In a comparative molecular study between GBS *S. agalactiae* of bovine mastitis and human origins, Bohnsack et al. (2004) reported that bovine and human GBS isolates were largely unrelated. Yildirim et al. (2002a) reported that cattle GBS isolates were nonpigmented, utilized lactose and hemagglutinated rabbit erythrocytes. The results also showed that GBS isolates from dogs, cats and humans were generally lactose negative, pigmented and

did not hemagglutinate rabbit erythrocytes. In the present study, we observed that GBS isolates from cattle were not pigmented and generally fermented lactose. Yildirim et al. (2002a), also reported that the capsular serotype of GBS cattle isolates was generally NT with a few IV and Ia serotypes.

In the present study, GBS isolates from cattle were NT serotype. The results of this study also agree with those of Yidirim et al. (2002a) for the growth patterns of GBS cattle isolates in fluid medium and soft agar, sedimentary and diffuse colony morphology, respectively. Yildirim et al. (2002a) reported that serotype, growth properties in fluid medium, soft agar and fermentation of lactose may be generally used to distinguish between GBS isolates from cattle, humans, dogs and cats. A similar conclusion that fish and cattle isolates may be generally distinguished based on these bacteriological characteristics was derived here.

Merl et al. (2003) observed that GBS cattle isolates were generally nontypable, pigmented, expressed sedimentary growth in fluid medium and diffuse colony morphology on soft agar. The only bacteriological characteristic difference between their study and those of the present study was pigmentation. McDonald et al. (2005) reported that the bacteriological characteristics of GBS *S. agalactiae* isolates from cattle were group B, CAMP factor positive, Voges-Proskauer and esculin negative, hippurate positive, lactose, ribose and trehalose positive. A small number of isolates were mannitol and sorbitol positive. The principal differences in bacteriological characteristics between results of McDonald et al. (2005) and the present study using cattle isolates from Wisconsin were hippurate hydrolysis, sorbitol and trehalose fermentation.

Phenotypic characteristics of GBS *S. agalactiae* from mullet and seabream were reported using conventional and API Rapid ID 32 tests by Evans et al. (2002). The results of Evans et al. (2002) and those of the present study were generally identical for mullet and seabream GBS isolates with regard to hemolysis, oxidase, catalase, leucine aminopeptidase, Voges-Proskauer, hydrolysis of hippurate, arginine deamination , α-galactosidase, β-galactosidase, β-glucuronidase, alkaline phosphatase, fermentation of ribose, trehalose and lactose. The phenotypical results of GBS *S. agalactiae* isolates from silver pomfret (*Pampus argenteus*) using the API -20 Strep system reported by Duremdez et al. (2004) were generally similar to those of the present study and Evans et al. (2002) for mullet and seabream GBS isolates. A major difference was that Evans et al. (2002) reported that GBS isolates were β-hemolytic and Duremdez et al. (2004) reported that the GBS isolates were α-hemolytic.

Hetzel et al. (2003) reported that emerald monitors (*Varanus prasinus*) became septicemic when infected by *S. agalactiae* that was acquired from wild mice. Genotypic analysis of DNA from the *S. agalactiae* isolates from monitors and mice revealed that they were identical. However, identification of the zoonotic species of cattle streptococci and their relevance to fish is likely to be unimportant. Cross-species transmission of GBS cattle isolates is unlikely to occur. The result of the present study indicated that cattle GBS *S. agalactiae* isolates were not virulent for experimentally challenged Nile tilapia and channel catfish (Hypothesis II). Sukhnanand et al. (2005) concluded from the results of molecular and biochemical characterization of GBS *S. agalactiae* isolates from cattle and humans that they appeared different with regard to

host specificity and pathogenesis. Sukhnanand et al. (2005) suggested that the potential for transmission of *S. agalactiae* from a bovine host to a human host or vice versa was low. Transmission of *S. agalactiae* within a fish species and between fish species is probable. Evans et al. (2002) demonstrated that tilapia were susceptible to mullet and seabream *S. agalactiae*, even after culture There is a limited possibility that the single passage of the cattle isolate in culture immediately before the experimental challenge trials affected virulence.

In the present study, the GBS disease in mullet and seabream was caused by five clones of *S. agalactiae,* but it was not possible to determine whether the disease was transmitted from seabream to mullet or vice versa (objective III). The virulence and evolutionary adaptation of GBS *S. agalactiae* for fish makes *S. agalactiae* a source of infection for susceptible fish from dead diseased fish. The duration of *S. agalactiae* survival and pathogenicity was investigated by Garcia (2004). He suggested that *S. agalactiae* can survive for long periods of time in fresh, brackish and marine waters and remains pathogenic for Nile tilapia.

The *S. difficile* reference strain was identified as a *S. agalactiae* isolate from tilapia as previously reported by Vandamme et al. (1997), Berridge et al. (2001) and Kawamura et al. (2005). In our studies, bacteriological properties and 16S-23S intergenic region (130 bp) specific primer for *S. agalactiae* by PCR clearly revealed that the *S. difficile* was undistinguishable from *S. agalactiae*. *Streptococcus agalactiae* isolates and reference strains were differentiated from the *S. dysgalactiae* and *S. phocae* by bacteriological properties and specific primers for 16S-23S intergenic region by PCR.

The phenotypic characteristics used in this study were able to identify and differentiate GBS *S. agalactiae* from fish and cattle origins. The phenotypic characteristics of *S. agalactiae* reference strains were similar to those of the fish and cattle isolates. Each fish isolate possessed the group B antigen, turbidity growth pattern in fluid medium and produced hyaluronidase and fermented D-trehalose. In contrast, the cattle isolates possessed the group B antigen, produced sediment growth pattern in fluid medium and pigment, were CAMP positive, hydrolyzed hippurate, decarboxylated β-glucoronidase, fermented D-lactose and had a generally defined FAME profile. The results suggest that these phenotypic characteristics can be used to differentiate between fish and cattle GBS *S. agalactiae*.

However, CAMP test and hydrolysis of hippurate were noted not to be presumptive identification tests for GBS *S. agalactiae* isolates as suggested by Facklam (2002). Fuchs et al. (1978) demonstrated that presumptive tests of CAMP, hippurate hydrolysis and pigment production correlated with human clinical GBS cases. Our results do not support the use of CAMP, hippurate hydrolysis and pigment production for the presumptive identification of GBS *S. agalactiae* isolates from fish. The PCR tests with specific primer, API-32 Strep and Gram positive identification system (GPI) did not differentiate GBS *S. agalactiae* isolated from fish from those of cattle origin. On the other hand, FAME did produce a good degree of correlation with the isolate cluster and the host species.

Jayarao et al. (1991) reported that GPI and API Rapid Strep systems gave comparable results for the identification of GBS *S. agalactiae* from cattle. The majority

of cattle isolates were identified at the excellent level. In the present study, we found the GPI system to be less accurate than API-20 Strep system in the identification of GBS *S. agalactiae* from cattle. The API-20 Strep system gave very good to excellent identification of fish and cattle isolates.

Molecular genetic methods such as AFLP may be useful to distinguish *S. agalactiae* isolates between host species at the clonal level. Rapid and accurate PCR tests using specific 16S-23S intergenic region primers can identify *S. agalactiae* isolates from fish and cattle. Future identification and strain differentiation tests may be based on specific monoclonal antibody and/or molecular genetic technologies.

V. SUMMARY

The collection of GBS *S. agalactiae* cattle isolates used in the present study was heterogeneous in regards to phenotypic characteristics, and GBS fish isolates could be distinguished from GBS cattle isolates by selected phenotypic properties. The hypothesis that the GBS fish and cattle populations were distinct was validated. FAME profiles were able to distinguish between the major clusters of fish and cattle isolates that had similar profiles. Mullet and seabream *S. agalactiae* isolates were distinguishable from Nile tilapia isolates, both phenotypically and with regard to whole cell fatty acid profiles. The cattle *S. agalactiae* isolates were found not to be infectious for experimentally inoculated Nile tilapia. The twelve *S. agalactiae* isolates from mullet and seabream isolated during the Kuwait Bay epidemic were revealed to belong to five distinct clonal groups by AFLP. Two seabream isolates recovered from the brain and head kidney from the same fish belonged to separate AFLP clones. Therefore, the GBS epidemic involved the dissemination of multi clones within the fish population rather than the dissemination of a single clone of *S. agalactiae*. This finding validated the final objective that infection between the two fish populations was not due to a single clone of *S. agalactiae*.

To our knowledge, this is the first report to describe phenotypic, genotypic characteristics, API-20 Strep analysis, GPI and FAME characterization of GBS *S. agalactiae* isolates from fish and cattle. A polyphasic approach should be used to identify and distinguish isolates of GBS *S. agalactiae* similar to that proposed by Arias et

al. (2003, 2004). Genotypic, bacteriological and specific monoclonal antibody characterization may contribute to a more complete understanding of the epidemiological aspects of GBS isolates from fish from a regional and global standpoint.

Table 3: Summary of cost, discrimination level, disadvantages and advantages of methods utilized for the comparison of *Streptococcus agalactiae* isolates from cattle and fish.

Method	Advantage	Disadvantages	Discrimination Level[1]	Estimated Cost[2]
Phenotypic Methods	Important for delineation of taxa. Easy to perform test. Proven methods.	Laborious, time consuming, often difficult to standardized and interpret.	Effective for delineation of taxa.	$ - $$$$$*
API 20 Strep strip	Multi biochemical test, time efficient, semi automated systems.	Results dependent on color reaction. Variable results dependent on reader.	Effective for identification of bacterial species already identified by the API software. However, it can be misleading when used for the identification of a new species.	$$$$
Gram positive identification (GPI)	Time efficient, fully automated and computerized system.	High equipment cost. Will not identify or will misidentify organisms not present in the software database.	Effective for species level identification.	$$$$

Table 3. Continued

Method	Advantage	Disadvantages	Discrimination Level[1]	Estimated Cost[2]
Gram positive identification (GPI)		Can be affected by growth phase, temperature and growth medium.		
Polymerase chain reaction (PCR)	Time efficient, reproducible, semi automated system. Can be very sensitive. Good for identification and detection of bacterial samples.	High equipment cost, expensive reagents, technologically complex. Prone to contamination.	Depending on application, species or strains can be identified.	$$$$
Amplified fragment length polymorphism (AFLP)	Excellent discrimination level. Semi-automated.	High equipment cost, time consuming, specific analyzing software required, success of technique relies in expert operator.	Effective for discrimination between species of a genus or different strains of a single species.	$$$$$

[1] Discrimination power of each of the methods utilized for the phenotypic and genotypic identification of *Streptococcus agalactiae* isolates from cattle and fish. Family, Genus, Single clone.

[2] Estimated cost of methods used for identification of *Streptococcus agalactiae* isolates from cattle and fish. Estimated cost of individual sample; $ = $1-2, $$ = $3-4, $$$ = $5-6, $$$$ = $7-8, $$$$$ = $9-10.

Table 3. Continued

* Estimated cost of phenotypic methods will vary depending on bacterial species targeted for identification.

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VII. APPENDIX Table A. *Streptococcus agalactiae* isolates, sources and origins from species of marine and fresh water fish, bottlenose dolphin, cattle and references strains of *S. difficile*, *S. dysgalactiae* and *S. phocae*.

Isolate	Fish species, organ of isolation, state and country of origin
KU-MU-5-BR[1]	Mullet, brain, Kuwait Bay, Kuwait
KU-MU-7-BR	Mullet, brain, Kuwait Bay, Kuwait
KU-MU-12-BR	Mullet, brain, Kuwait Bay, Kuwait
KU-MU-18-HK	Mullet, head kidney, Kuwait Bay, Kuwait
KU-MU-20-HK	Mullet, head kidney, Kuwait Bay, Kuwait
KU-MU-21-BR	Mullet, brain, Kuwait Bay, Kuwait
KU-MU-25-HK	Mullet, head kidney, Kuwait Bay, Kuwait
KU-MU-26-INT	Mullet, intestine, Kuwait Bay, Kuwait
KU-MU-27-BR	Mullet, brain, Kuwait Bay, Kuwait
KU-MU-35-EYE	Mullet, eye, Kuwait Bay, Kuwait
KU-MU-36-BR	Mullet, brain, Kuwait Bay, Kuwait
KU-SB-1-BR	Seabream, brain, Kuwait Bay, Kuwait
KU-SB-1-HK	Seabream, head kidney
KU-SB-1-EYE	Seabream, eye, Kuwait Bay, Kuwait
KU-SB-37-BR	Seabream, brain, Kuwait Bay, Kuwait
KU-SB-37-HK	Seabream, head kidney, Kuwait Bay, Kuwait
KU-SB-38-BR	Seabream, brain, Kuwait Bay, Kuwait
KU-DL-MUSCLE	Bottlenose dolphin, muscle, Kuwait bay, Kuwait
03ARS-BZ-TN-01[2]	Nile tilapia, brain, Larvas, Brazil
03ARS-BZ-TN-03	Nile tilapia, brain, Larvas, Brazil
03ARS-BZ-TN-04	Nile tilapia, brain, Larvas, Brazil
03ARS-BZ-TN-05	Nile tilapia, brain, Larvas, Brazil
03ARS-BZ-TN-06	Nile tilapia, brain, Larvas, Brazil
03ARS-BZ-TN-09	Nile tilapia, brain, Larvas, Brazil
LADL-90-50[3]	Hybrid striped bass, brain, Louisiana, USA
LADL-97-151	Gulf killifish, brain, Louisiana, USA
LADL-00-192	Gulf killifish, brain, Louisiana, USA
LADL-00-351a	Nile tilapia, brain, Mississippi, USA
LADL-02-097	Gulf killifish, brain, Louisiana, USA
LADL-05-108a	Nile tilapia, brain, Honduras
IS-ET-09-03[4]	Hybrid striped bass, brain, Israel
04ARS-BZ-TN-002[5]	Nile tilapia, brain, Parana State, Brazil
04ARS-BZ-TN-003	Nile tilapia, brain, Parana State, Brazil
04ARS-BZ-TN-004	Nile tilapia, brain, Parana State, Brazil
04ARS-BZ-TN-005	Nile tilapia, brain, Parana State, Brazil
04ARS-BZ-TN-006	Nile tilapia, brain, Parana State, Brazil
04ARS-BZ-TN-007	Nile tilapia, brain, Parana State, Brazil
Cattle-55100-2[6]	Bovine mastitis, milk sample, Wisconsin, USA
Cattle-59918-14	Bovine mastitis, milk sample, Wisconsin, USA
Cattle-59998-7	Bovine mastitis, milk sample, Wisconsin, USA

VII. Appendix Table A. Continued

Isolate	Fish species, organ of isolation, state and country of origin
Cattle-59998-1	Bovine mastitis, milk sample, Wisconsin, USA
Cattle-61185-5	Bovine mastitis, milk sample, Wisconsin, USA
Cattle-61185-10	Bovine mastitis, milk sample, Wisconsin, USA
Cattle-61321-2	Bovine mastitis, milk sample, Wisconsin, USA
Cattle-61321-3	Bovine mastitis, milk sample, Wisconsin, USA
Cattle-61321-11	Bovine mastitis, milk sample, Wisconsin, USA
Cattle-61337-3	Bovine mastitis, milk sample, Wisconsin, USA
ATCC-12386[7]	*Streptococcus agalactiae,* grouping strain, American Type Culture Collection (ATCC)
ATCC-13813	*Streptococcus agalactiae*, bovine mastitis, milk samples United Kingdom, ATCC
ATCC-27956	*Streptococcus agalactiae*, bovine mastitis, milk samples Wisconsin, USA, ATCC
ATCC-31475	*Streptococcus agalactiae*, human meningitis, Texas, USA, ATCC
ATCC-51487	*Streptococcus difficile* (*S. agalactiae*), tilapia sp., brain, Israel, ATCC
ATCC-51973	*Streptococcus phocae*, group strain, USA, seal
ATCC-12388	*Streptococcus dysagalactiae*, grouping strain, ATCC
ATCC-12394	*Streptococcus dysagalactiae,* grouping strain, ATCC
ATCC-43921	*Lactococcus garvieae*, bovine mastitis, milk, USA, ATCC
CECT-138[8]	*Streptococcus agalactiae,* bovine mastitis, Spain, Colección, Española De Cultivos

[1] *Streptococcus agalactiae* isolates from Evans et al. 2002.
[2] *Streptococcus agalactiae* isolates from Nile tilapia (*Oreochromis niloticus*), reared in cage culture in lakes and ponds in the region of Larvas, Brazil.
[3] *Streptococcus agalactiae* isolates from Dr. John Hawke, Louisiana Veterinary Diagnostic Laboratory, Louisiana State University, Baton Rouge, Louisiana.
[4] Dr. Dina Zilberg, The Albert Katz Department of Dryland Biotechnologies, Ben-Gurion, University of the Negev, Israel.
[5] *Streptococcus agalactiae* isolates from Nile tilapia, (*Oreochromis niloticus*) reared in cage culture in lakes and ponds in the region of Parana State, Brazil.
[6] *Streptococcus agalactiae* cattle mastitis milk isolates from Department of Pathobiology, Diagnostic Laboratory, College of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin
[7] References strains from American Type Culture Collection, Washington, DC, USA.
[8] References strains from Colección Española De Cultivos, Universidad de Valencia y Unidad Asociada al Consejo Superior de Investigaciones Científicas, Spain

VIII. Appendix Table B. Target gene, oligonucleotide sequence and target size for polymerase chain reaction (PCR) used in the present study.

Target Gene		Sequence	PCR amplicon base pair (bp)
16S-23S rDNA intergenic spacer	Forward	CCACGATCTAGAAATAGATTG	130
	Reverse	TGCCAAGGCATCCACC	
16S-23S rDNA intergenic spacer[2]	Forward	AGGAAACCTGCCATTTGCG	190
	Reverse	CAATCTATTTCTAGATCGTGG	
23s rDNA[3]	Forward	AACAGCCTCGTATTTAAAATGATAGATTAAC	875
	Reverse	TCCTACCATGACACTAATGTGTC	

[1]Berridge et al. 2001, [2]Mata et al. 2004, and [3]Kawata et al. 2004. The amplicons for each set of primers were sequenced by these authors.

IX Appendix Table C. Phenotypic properties of GBS *Streptococcus agalactiae* isolates from fish, bottlenose dolphin, cattle isolates and reference strains of *S. agalactiae*, *S. dysagalactiae*, *S. difficile and S. phocae*

Isolate	Ser[1]	He	Ca	Py	Hip	Arg	Es	Tre	Man	Lac
KU-MU-5-BR	Ia	β	+	-	-	+	-	+	-	-
KU-MU-7-BR	Ia	β	+	-	-	+	-	+	-	-
KU-MU-12-BR	Ia	β	+	-	+	+	-	+	-	-
KU-MU-18-HK	Ia	β	+	-	+	+	-	+	-	-
KU-MU-20-HK	Ia	β	+	-	+	+	-	+	-	-
KU-MU-21-BR	Ia	β	+	-	-	+	-	+	-	-
KU-MU-25-HK	Ia	β	+	-	-	+	-	+	-	-
KU-MU-26-INT	Ia	β	+	-	-	+	-	+	-	-
KU-MU-27-BR	Ia	β	+	-	-	+	-	+	-	-
KU-MU-35-EYE	Ia	β	+	-	-	+	-	+	-	-
KU-MU-36-BR	Ia	β	+	-	-	+	-	+	-	-
KU-SB-1HK	Ia	β	+	-	+	+	-	+	-	+
KU-SB-37HK	Ia	β	+	-	+	+	-	+	-	-
KU-SB-38BR	Ia	β	+	-	+	+	-	+	-	-
KU-SB-1-EYE	Ia	β	-	-	+	-	-	+	-	-
KU-SB-37-Br	Ia	β	-	-	+	-	-	+	-	-
KU-SB-1-Br	Ia	β	-	-	+	-	-	+	-	-
KU-DL-MUSCLE	Ia	β	+	-	-	+	-	+	-	-
03ARS-BZ-TN-01	NT	-	-	-	+	-	-	-	-	-
03ARS-BZ-TN-04	NT	-	-	-	-	-	-	-	-	-
03ARS-BZ-TN-05	Ia	-	-	-	+	-	-	+	-	+
03ARS-BZ-TN-06	NT	-	-	-	+	+	-	-	-	-
03ARS-BZ-TN-09	NT	-	-	-	+	-	-	-	-	-
LADL-90-503	NT	-	-	-	+	+	-	-	-	-
LADL-97-151	NT	-	-	-	+	+	-	-	-	-
LADL-00-192	NT	-	-	-	+	+	-	-	-	-
LADL-00-351A	Ia	-	-	-	+	+	-	-	-	-
LADL-02- 097	NT	-	-	-	-	-	-	-	-	-
LADL-05-108A	NT	-	-	-	+	+	-	-	-	-
IS-TN-09-03	NT	-	-	-	+	-	-	-	-	-

IX. Appendix Table C. Continued

Isolate	Ser[1]	He	Ca	Py	Hip	Arg	Es	Tre	Man	Lac
04ARS-BZ-TN-003	NT	β	-	-	+	-	-	-	-	-
04ARS-BZ-TN-004	NT	β	-	-	+	-	-	-	-	-
04ARS-BZ-TN-006	NT	ß	-	-	+	-	-	-	-	-
04ARS-BZ-TN-005	NT	β	-	-	+	-	-	-	-	-
04ARS-BZ-TN-007	NT	β	-	-	+	-	-	-	-	-
Cattle-55100-2	NT	β	+	-	+	-	-	-	-	-
Cattle -59918-14	NT	α	+	-	+	-	-	-	-	+
Cattle -59998-7	NT	α	+	-	-	-	-	-	-	+
Cattle -59998-17	NT	α	+	-	+	-	-	-	-	+
Cattle -61185-5	NT	β	+	-	-	+	-	-	-	+
Cattle -61185-10	NT	β	+	+	+	+	-	-	-	+
Cattle -61321-2	NT	α	+	-	-	+	-	-	-	+
Cattle -61321-3	NT	β	+	-	+	+	-	-	-	+
Cattle -61321-11	NT	β	+	-	-	+	-	-	-	+
Cattle -61337-3	NT	α	+	-	-	-	-	-	-	+
ATCC-12386	NT	β	+	+	+	+	+	+	-	+
ATCC-13813	II	α	+	-	+	+	-	+	-	+
ATCC-27956	NT	β	-	-	+	+	+	+	-	+
ATCC-31475	III	β	+	+	+	+	-	+	-	-
ATCC-51487	lb	-	-	-	+		-	-	-	-
CECT-183	NT	-	+	-	+	-	-	+	-	+
ATCC-12388	NT	β	-	-	-	-	+	+	-	-
ATCC-12394	NT	β	-	-	-	+	+	+	-	+
ATCC-51973	NT	β	+	-	-	-	-	+	+	-

[1]Capsule serotype determined by typing antisera (Ia, Ib, II and III), 270023, Denka Seiken, Tokyo, Japan. The following symbols denote: He = type of hemolysis on 5% sheep blood agar; Ca = CAMP reaction; Py = Pyrrolidonylarylamidase reaction;

IX. Appendix Table C. Continued

Vp = Voges-Prokauers reaction, all isolates negative, not shown; Hip = Hippurate hydrolysis; Arg= Arginine hydrolysis; Es = Esculin hydrolysis; Fermentation of Tre, D-trehalose; Man, D-mannitol; Lac, D-lactose; and (+) = positive reaction and (-) = negative reaction.

X. Appendix Table D. API-20 Strep results of α-galactosidase (α-gal), β-glucosidase (β-Gluc), β-galactosidase, (β-Gal), alkaline phosphase (PAL), aminopepidase (LAP). Properties for GBS *Streptococcus agalactiae* isolates from fish, bottlenose dolphin, cattle isolates and reference strains of *S. agalactiae*, *S. difficile*, *S. dysagalactiae* and *S. phocae*

Isolates	α-Gal	β-Gluc	β-Gal	PAL	LAP
KU-MU-5-BR	-[1]	+	-	+	+
KU-MU-7-BR	-	+	-	+	+
KU-MU-12-BR	+	-	-	+	+
KU-MU-18-HK	+	-	-	+	+
KU-MU-20-HK	+	-	-	+	+
KU-MU-21-BR	-	+	-	+	+
KU-MU-25-BR	-	+	-	+	+
KU-MU-26-INT	-	+	-	+	+
KU-MU-27-BR	-	+	-	+	+
KU-MU-35-EYE	+	-	-	+	+
KU-MU-36-BR	+	-	-	+	+
KU-SB-1-HK	+	-	-	+	+
KU-SB-37-HK	+	-	-	+	+
KU-SB-38-BR	+	-	-	+	+
KU-SB-1-EYE	+	-	-	+	+
KU-SB-37-BR	+	-	-	+	+
KU-SB-1-BR	+	-	-	+	+
KU-DL-MUS	+	+	-	+	+
03ARS-BZ-TN-01	-	+	-	+	+
03ARS-BZ-TN-04	-	+	-	+	+
03ARS-BZ-TN-05	-	+	-	+	+
03ARS-BZ-TN-06	-	+	-	+	+
03ARS-BZ-TN-09	-	+	-	+	+
LADL-90-503	-	-	-	+	+
LADL-97-151	-	-	-	+	+
LADL-00-192	-	-	-	+	+
LADL-00-351A	-	-	-	+	+
LADL-02-097	-	-	-	+	+
LADL-05-108A	-	-	-	+	+
IS-TN-09-03	-	-	-	+	+
04ARS-BZ-TN-002	+	-	-	+	+

X. Appendix Table D. Continued

Isolates	α-Gal	β-Gluc	β-Gal	PAL	LAP
04ARS-BZ-TN-003	-	-	-	+	+
04ARS-BZ-TN-004	-	-	-	+	+
04ARS-BZ-TN-005	+	+	-	+	+
04ARS-BZ-TN-006	-	-	-	+	+
04ARS-BZ-TN-007	-	-	-	+	+
Cattle-55100-2	-	+	-	+	+
Cattle-59918-14	-	+	-	+	+
Cattle-59998-7	-	+	+	+	+
Cattle-59998-17	-	+	-	+	+
Cattle-61185-5	-	+	-	+	+
Cattle-61185-10	-	+	-	+	+
Cattle-61321-2	-	+	-	+	+
Cattle-61321-3	-	+	+	+	+
Cattle-61321-11	-	+	-	+	+
Cattle-61337-3	-	+	-	+	+
ATCC-12386	-	+	-	+	+
ATCC-27956	+	-	-	+	+
ATCC-31475	-	+	-	+	+
ATCC-12394	-	+	-	+	+
ATCC-51487	-	+	-	+	+
CECT-183	-	+	-	+	+
ATCC-12388	-	+	-	+	+
ATCC-13813	+	-	-	+	+
ATCC-51973	-	-	-	+	+

[1] The symbols denote: (+) = positive and (-) = negative reactions.

XI. Appendix Table E. Capsule properties, hyaluronidase activity and pigment production for GBS *Streptococcus agalactiae* isolates from fish, bottlenose dolphin, cattle isolates and reference strains of *S. agalactiae*, *S. difficile*, *S. dysagalactiae* and *S. phocae*.

Isolate	Capsule Property[1]	Hyaluronidase activity[2]	Pigment production[3]
KU-MU-5-BR	+	+	+
KU-MU-7-BR	+	+	+
KU-MU-12-BR	+	+	+
KU-MU-18-HK	+	+	+
KU-MU-20-HK	+	+	+
KU-MU-21-BR	+	+	+
KU-MU-25-BR	+	+	+
KU-MU-26-INT	+	+	+
KU-MU-27-BR	+	+	+
KU-MU-35-EYE	+	+	+
KU-MU-36-BR	+	+	+
KU-SB-1-HK	+	+	+
KU-SB-37-HK	+	+	+
KU-SB-38-BR	+	+	+
KU-SB-1-EYE	+	+	+
KU-SB-37-BR	+	+	+
KU-SB-1-BR	+	+	+
KU-DL-MUSCLE	+	+	+
03ARS-BZ-TN-01	+	-	-
03ARS-BZ-TN-04	+	-	-
03ARS-BZ-TN-05	+	-	-
03ARS-BZ-TN-06	+	-	-
03ARS-BZ-TN-09	+	-	-
LADL-90-503	+	-	-
LADL-97-151	+	+	-
LADL-00-192	+	-	-
LADL-00-351A	+	-	-
LADL-02-097	+	+	-
LADL-05-108A	+	-	-
IS-TN-09-03	+	-	+
04ARS-BZ-TN-002	+	-	-
04ARS-BZ-TN-003	+	-	-
04ARS-BZ-TN-004	+	-	-

XI. Appendix Table E. Continued

Isolate	Capsule Property[1]	Hyaluronidase activity[2]	Pigment production[3]
04ARS-BZ-TN-005	+	-	-
04ARS-BZ-TN-006	+	-	-
04ARS-BZ-TN-007	+	-	-
Cattle-55100-2	+	+	-
Cattle-59918-14	+	+	-
Cattle-59998-7	+	+	-
Cattle-59998-17	+	+	-
Cattle-61185-5	+	+	-
CattleS-61185-10	+	+	-
Cattle-61321-2	+	+	-
Cattle-61321-3	+	+	-
Cattle-61321-11	+	+	-
Cattle-61337-3	+	+	-
ATCC-12386	+	+	+
ATCC-13813	+	-	+
ATCC-27956	+	-	+
ATCC-31475	+	+	+
ATCC-51487	+	-	+
CECT-183	+	-	-
ATCC-12388	+	+	-
ATCC-12394	+	+	-
ATCC-51973	+	+	-

The symbols denote: (+) = positive and (-) = negative reactions.
[1] Atlas et al. 1995. The negative capsule stain test were denoted by present of capsule (+) and absence of capsule (-).
[2] Smith and Willett 1968. The hyaluronidase plate tests were denoted by present enzymes (+) and absence of enzyme (-).
[3] Merritt and Jacobs 1976.

XII. Appendix Table F. Growth properties in broth and soft agar for GBS Streptococcus agalactiae isolates from fish, bottlenose dolphin, cattle isolates and reference strains of *S. agalactiae, S. difficile*, *S. dysagalactiae*, *S. phocae*[1].

Isolate	Fluid medium[2]	Soft agar medium[3]
KU-MU-5-BR	S	D
KU-MU-7-BR	S	D
KU-MU-12-BR	S	D
KU-MU-18-HK	S	D
KU-MU-20-HK	S	D
KU-MU-21-BR	S	D
KU-MU-25-BR	S	D
KU-MU-26-INT	S	D
KU-MU-27-BR	S	D
KU-MU-35-EYE	S	D
KU-MU-36-BR	S	D
KU-SB-1-HK	T	D
KU-SB-37-HK	T	D
KU-SB-38-BR	T	D
KU-SB-1-EYE	T	D
KU-SB-37-BR	T	D
KU-SB-1-BR	T	D
KU-DL-MUSCLE	T	D
03ARS-BZ-TN-01	T	D
03ARS-BZ-TN-04	T	D
03ARS-BZ-TN-05	T	D
03ARS-BZ-TN-06	T	C
03ARS-BZ-TN-09	T	D
LADL-90-503	S	D
LADL-97-151	S	D
LADL-00-192	S	D
LADL-00-351A	S	D
LADL-02-097	S	D
LADL-05-108A	S	D
IS-TN-09-03	T	D
04ARS-BZ-TN-002	T	D
04ARS-BZ-TN-003	T	D
04ARS-BZ-TN-004	T	D
04ARS-BZ-TN-005	T	D
04ARS-BZ-TN-006	T	D
04ARS-BZ-TN-007	T	D
Cattle-55100-2	S	D

XII. Appendix Table F. Continued

Isolate	Fluid medium	Soft agar medium
Cattle-59918-14	S	D
Cattle-59998-7	S	D
Cattle-59998-17	S	D
Cattle-61185-5	S	D
CattleS-61185-10	S	D
Cattle-61321-2	S	D
Cattle-61321-3	S	D
Cattle-61321-11	S	D
Cattle-61337-3	S	D
ATCC-12386	T	D
ATCC-13813	S	D
ATCC-31475	S	D
ATCC-27956	S	C
ATCC-51487	S	D
CECT-183	T	D
ATCC-12388	T	D
ATCC-12394	S	D
ATCC-51973	S	D

[1] Merl et al. 2003.

The following symbols denote the results of growth characteristics in broth cultures;

[2]S=sediment and clear supernatant and T = turbidity.

[3]The results in soft agar were denoted by C = compact colonies and D = diffuse colonies.

XIII. Appendix Table G. Fatty acid methyl esters, Gram positive identification (GPI), API-20 Strep, polymerase chain reaction (PCR), *Streptococcus agalactiae* isolates from fish, Bottlenose dolphin, cattle and reference strains of *S. agalactiae*, *S. dysagalactiae* and *S. phocae*

Isolates and species obtained from	FAME[1]	GPI Profile[2]	API-20 Strep Profile[3]	PCR 16S-23S rDNA[4] 190 bp	PCR 23S rDNA[5] 875 bp	PCR 16S-23S intergenic rDNA[6] 130 bp
KU-MU-5-BR	0.863	99.0%	3463014 Ex	+	+	+
KU-MU-7-BR	0.798	89.0%	3463014 Ex	+	+	+
KU-MU-12-BR	0.725	99.0%	3263010 VG	+	+	+
KU-MU-18-HK	0.724	92.0%	3262010 G	+	+	+
KU-MU-20-HK	0.833	99.0%	3463014 Ex	+	+	+
KU-MU-21-BR	0.905	99.0%	3463014 Ex	+	+	+
KU-MU-25-HK	0.827	99.0%	3463014 Ex	+	+	+
KU-MU-26-INT	0.657	89.0%	3063014 Ex	+	+	+
KU-MU-27-BR	0.270	79.0%	3063014 Ex	+	+	+
KU-MU-35-EYE	0.539	99.0%	3463014 Ex	+	+	+
KU-MU-36-BR	0.856	92.0%	3463014 Ex	+	+	+
KU-SB-1-HK	0.878	99.0%	3263014 VG	-	+	+
KU-SB-37-HK	0.831	99.0%	3263014 VG	+	+	+
KU-SB-38-Br	0.871	99.0%	3263014 VG	+	+	+
KU-SB-1-EYE	0.861	77.0%	3263014 VG	+	-	+

XIII. Appendix Table G. Continued

Isolates and species obtained from	FAME[1]	GPI Profile[2]	API-20 Strep Profile[3]	PCR 16S-23S rDNA[4] 190 bp	PCR 23S rDNA[5] 875 bp	PCR 16S-23S intergenic rDNA[6] 130 bp
KU-SB-37-Br	0.921	88.0%	3263014 VG	+	-	+
KU-SB-1-Br	0.885	77.0%	3263014 VG	+	-	+
KU-DL-MUS	0.704	71.0%	1663014 G	+	+	+
03-ARS-BZ-TN-01	0.633	94.0%	3061000 G	+	+	+
03-ARS-BZ-TN-04	0.815	94.0%	3061000 G	+	-	+
03-ARS-BZ-TN-05	0.887	94.0%	3061000 G	+	+	+
03-ARS-BZ-TN-06	0.825	94.0%	3061000 G	-	-	+
03-ARS-BZ-TN-09	0.840	94.0%	3061000 G	+	-	+
LVDL-90-503	0.784	94.0%	3023000 G	-	-	+
LVDL-97-151	0.835	94.0%	3023000 G	+	-	+
LVDL-00-192	0.695	94.0%	3023000 G	+		+
LVDL-00-351a	0.843	94.0%	3023000 G	-	-	+
LVDL-02-097	0.587	94.0%	3023000 G	-	-	+
LVDL-05-108a	0.658	94.0%	3023000 G	-	-	+
IS-TN-09-03	0.860	21.0%	3463000 G	-	-	+
04ARS-BZ-TN-002	0.735	94.0%	3063000 VG	-	-	+
04ARS-BZ-TN-003	0.795	94.0%	3063000 VG	-	-	+

XIII. Appendix Table G. Continued

Isolates and species obtained from	FAME[1]	GPI Profile[2]	API-20 Strep Profile[3]	PCR 16S-23S rDNA[4] 190 bp	PCR 23S rDNA[5] 875 bp	PCR 16S-23S intergenic rDNA[6] 130 bp
04ARS-BZ-TN-004	0.792	94.0%	3063000 VG	-	-	+
04ARS-BZ-TN-007	0.560	94.0%	3063000 VG	-	-	+
Cattle-55100-26	0.510	UN	3463410 G	+	+	+
Cattle-59918-14	0.645	93.0%	3463404 Ex	+	+	+
Cattle - 59998-7	0.584	UN	3472400 G	+	+	+
Cattle - 59998-17	0.353	82.0%	3463404 Ex	+	+	+
Cattle - 61185-5	0.752	99.0%	3563400 G	+	-	+
Cattle - 61185-10	0.746	94.0%	3563400 G	+	-	+
Cattle - 61321-2	0.269	88.0%	563400 G	+	+	+
Cattle - 61321-3	0.178	71.%	3563404 G	+	+	+
Cattle - 61321-11	0.134	UN	563410 Ex	+	+	+
Cattle - 61337-3	0.321	48.0%	3463404 VG	+	+	+
ATCC-12386	0.529	73.0%	3563415 VG	+	-	+
ATCC-13813	0.754	91.0%	3263414 VG	+	+	+
ATCC-27956	0.557	53.0%	3261415 VG	+	+	+
ATCC-31475	0.624	91.0%	3563014 VG	+	-	+

XIII. Appendix Table G. Continued

Isolates and species obtained from	FAME[1]	GPI Profile[2]	API-20 Strep Profile[3]	PCR 16S-23S rDNA[4] 190 bp	PCR 23S rDNA[5] 875 bp	PCR 16S-23S intergenic rDNA[6] 130 bp
CECT-183	0.759	91.0%	3463410 Ex	+	-	+
ATCC-12394	0.380	99.0%	0463015 VG	-	-	-
ATCC-12388	0.070	99.0%	5143110 D	-	-	-

The symbols denote: (+) = positive and (-) = negative reactions.

[1]Shoemaker et al. 2005. Results were excellent identification of *Streptococcus agalactiae* at similarity indexes < 0.500, very good at 0.300- 0.499; acceptable at 0.200-0.299; low discrimination at 0.100-0.199 and unidentified (UN) at 0.000-099.

[2] Jayarao et al. 1991. Results were excellent identification of S. agalactiae at 98-99.9%; very good at 90-97%; acceptable at 70-89%; low discrimination at 50-69% and unidentified (UN) at 0-49%.

[3]API-20 Strep system was evaluated according to manufacturer's instructions. The results were read after 4-4.5 h and/or 24 h* incubation at 35°C. The generated profile number was recorded and then referenced to API-20 Rapid Strep Profile Index (SPI) for excellent (Ex), very good (VG), good (G), doubtful (D) and unidentified (UN) results.

[4] Mata et al. 2004. The PCR reactions were denoted by present of specific PCR product (+) and absence of specific PCR product (-).

[5]Kawata et al. 2004. The PCR reactions were denoted by present of specific PCR product (+) and absence of specific PCR product (-).

[6]Berridge et al. 2001. The PCR reactions were denoted by present of specific PCR product (+) and absence of specific PCR product (-).

XIV. Appendix Table H. Percent of whole-cell fatty acid composition of GBS S*treptococcus agalactiae* isolates from fish, cattle and reference strains determined by RCLIN50 rapid method[1].

	Percent in profile		
Fatty acid[2]	Fish (n = 35)	Cattle (n = 10)	Reference (n = 4)
12:0	4.0	2.3	1.9
14:0	11.8	6.1	7.7
16:1 w9c	3.7	7.1	5.4
16:1 w5c	1.6	2.4	2.0
16:0	35.8	30.0	42.0
17:1 iso w5c	-	1.9	-
17:0	1.5	1.7	-
18:1 w9c	16.3	16.6	13.3
18:1 w7c	10.4	15.7	15.6
18:1 w5c	-	1.5	-
18:0	14.9	12.0	12.0
19:1 iso 1	-	2.8	-
Total	77.29	77.34	77.13

[1] The rapid method for the RCLIN 50 was used to obtain fatty acid methyl esters (FAME) for use in library generation (MIDI, 2002a) and analysis of fatty acid.

[2] Percent of the total fatty acids for fish, cattle and reference strains.

XV. Appendix Table I. *Streptococcus agalactiae* isolates from marine fish used for amplified fragment length polymorphism (AFLP) fingerprinting.

Isolate	Fish species	Organ of isolation	Geographic origin
KU-MU-5-BR[1]	Mullet	Brain	Kuwait Bay, Kuwait
KU-MU-7-BR	Mullet	Brain	Kuwait Bay, Kuwait
KU-MU-12-BR	Mullet	Brain	Kuwait Bay, Kuwait
KU-MU-21-BR	Mullet	Brain	Kuwait Bay, Kuwait
KU-MU-27-BR	Mullet	Brain	Kuwait Bay, Kuwait
KU-MU-35-EYE	Mullet	Eye	Kuwait Bay, Kuwait
KU-SB-1-BR	Seabream	Brain	Kuwait Bay, Kuwait
KU-SB-1-HK	Seabream	Head kidney	Kuwait Bay, Kuwait
KU-SB-1-EYE	Seabream	Eye	Kuwait Bay, Kuwait
KU-SB-37-BR	Seabream	Brain	Kuwait Bay, Kuwait
KU-SB-37-HK	Seabream	Head kidney	Kuwait Bay, Kuwait
KU-SB-38-BR	Seabream	Brain	Kuwait Bay, Kuwait

[1] *Streptococcus agalactiae* isolates from: Evans et al. 2002.