Phylogenetic relationships among *Streptococcus agalactiae* isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan

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Streptococcus agalactiae, commonly known as group B streptococcus (GBS), is a cause of infectious disease in numerous animal species. This study examined the genetic relatedness of piscine, dolphin and human GBS isolates and bovine GBS reference strains from different geographical regions using serological and molecular serotyping and multilocus sequence typing (MLST) techniques. Piscine isolates originating from Kuwait, Brazil, Israel and the USA were capsular serotype Ia, a serotype previously unreported in GBS isolated from fish. Sequence typing of piscine isolates produced six sequence types (ST-7, ST-257, ST-258, ST-259, ST-260 and ST-261), the latter five representing allelic designations and allelic combinations not previously reported in the *S. agalactiae* MLST database. Genomic diversity existed between dolphin and piscine GBS isolates from Kuwait and other geographical areas. Piscine GBS isolates from Brazil, Israel, Honduras and the USA appeared to represent a distinct genetic population of strains that were largely unrelated to human and bovine GBS. The Kuwait dolphin and piscine lineage (ST-7, Ia) was also associated with human neonatal infections in Japan. Comparative genomics of piscine, human and bovine GBS could help clarify those genes important for host tropism, the emergence of unique pathogenic clones and whether these hosts act as reservoirs of one another's pathogenic lineages.

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INTRODUCTION

Streptococcus agalactiae, commonly known as group B streptococcus (GBS), emerged as an important infectious agent in humans during the 1960s–1970s (Schuchat, 1998; Wilkinson, 1978) following its initial description by Brown (1939). GBS has been isolated from a variety of non-human sources and is well recognized as an important causative agent of bovine mastitis (Pattison *et al.*, 1955). The potential for human GBS to be a zoonosis has been investigated in bovine isolates, and human GBS isolates have been shown largely to be genetically distinct from

bovine GBS isolates based on phenotypic properties, PFGE and randomly amplified polymorphic DNA analysis (Bohnsack *et al.*, 2004; Elliott *et al.*, 1990; Finch & Martin, 1984; Martinez *et al.*, 2000; Sukhnanand *et al.*, 2005; Vandamme *et al.*, 1997; Wilkinson *et al.*, 1973).

More recently, multilocus sequence typing (MLST) has helped define the phylogenetic relationship between human and bovine strains. In MLST, GBS isolates are classified as sequence types (STs) based on their composite allele numbers, and phylogenetic relationships are inferred from the similarities between the allele numbers comprising each ST or between the concatenated sequences of the individual amplicons (Bisharat *et al.*, 2004; Davies *et al.*, 2004; Jones *et al.*, 2003). MLST has demonstrated that

Abbreviations: CAMP, Christie–Atkins–Munch–Petersen; GBS, group B streptococcus; MLST, multilocus sequence typing; ST, sequence type.

many bovine GBS isolates do not share STs with human GBS isolates, confirming the largely divergent nature of human and bovine GBS. However, two lineages of bovine GBS (ST-23 and ST-61) do appear to have a genetic relationship with human GBS. Human serotype Ia strains in the ST-23 lineage and human ST-17 strains, which are almost entirely serotype III GBS and are related to bovine ST-61 strains, are frequently associated with neonatal infections. The latter finding has led to the hypothesis that human ST-17 strains arose from a bovine GBS ancestor (Bisharat *et al.*, 2004; Bohnsack *et al.*, 2004; Jones *et al.*, 2006).

GBS also sporadically causes epizootic infections in fish. First recognized as a piscine pathogen in captive freshwater shiners, *Notemigonus crysoleucas* (Robinson & Meyer, 1966), GBS has subsequently been reported from wild and captive fish involved in epizootics in the USA, Israel, Kuwait, Thailand and Brazil (Baya *et al.*, 1990; Duremdaz *et al.*, 2004; Eldar *et al.*, 1994; Evans *et al.*, 2002; Plumb *et al.*, 1974; Salvador *et al.*, 2005; Suanyuk *et al.*, 2005). GBS has also been reported from aquatic mammals, both captive and wild bottlenose dolphins, *Tursiops truncatus* (Evans *et al.*, 2006; Zappulli *et al.*, 2005). The phylogenetic relationship between fish, dolphin, bovine and human GBS has not been studied. Therefore, we examined fish GBS isolates from various geographical locations using phenotypic, serotypic and molecular techniques, and used MLST to help elucidate their phylogenetic similarity with and differences from non-fish GBS isolates.

METHODS

Strain collection. Fish isolates ($n=21$) originated from infections in Klunzinger's mullet (*Liza klunzinger*) and seabream (*Sparus auratus*) from Kuwait (Evans *et al.*, 2002), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) from Israel and Nile tilapia (*Oreochromis niloticus*) from the USA, Brazil and Honduras (Table 1). Nile tilapia GBS isolates were provided by Dr John Hawke (Louisiana State University, LA, USA) and Dr Salvador (Parana State, Brazil). Dr Dina Zilberg (Ben-Gurion University of the Negev, Israel) provided the hybrid striped bass isolate. The bottlenose dolphin isolate (Kuwait) was obtained as described by Evans *et al.* (2006). Six ST-7, serotype Ia GBS isolates from humans in Japan were provided by S. Takahashi. Reference strains were obtained from the ATCC (ATCC 13813 and ATCC 27956 GBS from bovine species and ATCC 51487 deposited as *Streptococcus diffcilis* from diseased tilapia).

Identification and characterization of GBS strains. Isolates were grown on 5% sheep blood agar (Remel) as described previously (Evans *et al.*, 2002). Conventional biochemical tests [haemolysis, Christie-Atkins-Munch-Petersen (CAMP) test, pyrrolidonylarylamidase reaction and hippurate, urea, starch, arginine and aesculin hydrolysis] were performed as described by MacFaddin (2000). The Voges-Proskauer reaction and fermentation of sorbitol, trehalose, ribose, inulin, mannose, xylulose and lactose were derived from the API 20 Strep Multi-test System (bioMérieux), following the manufacturer's instructions. Lancefield grouping was performed with type B antisera (Oxoid). Capsular serotyping was performed twice on all strains in the laboratories of J. F. Bohnsack and repeated in a second laboratory by P. H. Klesius with GBS typing antisera directed against capsular types Ia, Ib, II, III, IV and V (Denka Seiken).

Serotypes were additionally identified using a multiplex PCR assay performed twice on all isolates. The protocol was modified from that of Poyart *et al.* (2007) and the serotypes was determined as Ia, Ib or II according to size in base pairs of the capsular polysaccharide (*cps*) gene amplicons. Briefly, a cocktail of type-specific primers was created for the PCR; cycling conditions were 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Banding patterns were determined by electrophoresis on 2% agarose gels and serotypes corresponded to 521 bp (Ia), 770 bp (Ib) or 397 bp (II).

Identification of species-specific 23S rRNA genes. One colony of each GBS isolate was incubated in tryptic soy broth at 32 °C for 24 h. Genomic DNA templates were prepared using a DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. Species-specific target DNA amplification of the *S. agalactiae* 23S rRNA gene was carried out as described by Kawata *et al.* (2004). The primer sequences were 5'-AACAGCCTCGTATTTAAATGATAGATTAAC-3' (forward) and 5'-TCCTACCATGACACTAATGTGTC-3' (reverse). The PCR products were analysed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

MLST. In MLST, 500 bp fragments of seven housekeeping genes are amplified by PCR and the amplicons sequenced. Allele numbers are then assigned to each sequence. Individual isolates are assigned STs based on their composite allele numbers. MLST, allelic assignment and sequence typing were performed as described previously (Jones *et al.*, 2003). Allele sequences can be found at <http://pubmlst.org/sagalactiae/>. MEGA software (Kumar *et al.*, 2004) was used to construct an unrooted dendrogram showing the relationships among fish, dolphin, bovine and human STs from this study and from a collection of human and bovine isolates from North America, Japan and Brazil. The origin and serotypes of these strains have been reported previously and the dendrogram was constructed using the STs from this collection of isolates, all of which have appeared in previous publications (Bohnsack *et al.*, 2008; Jones *et al.*, 2003; Lin *et al.*, 2006; Oliveira *et al.*, 2006; Seifert *et al.*, 2006; Takahashi *et al.*, 2002). The eBurst program (Feil *et al.*, 2004) was used to identify clonal complexes among these isolates. A total of 1150 strains were used to perform the eBurst analysis.

RESULTS AND DISCUSSION

Identification of strains as GBS

All isolates were Lancefield group B, catalase-negative, Gram-positive cocci, appearing in pairs and chains. A 23S rRNA gene fragment specific for GBS was amplified by PCR from the genomic DNA of all isolates.

Serogroup typing

The dolphin GBS isolates and 18 of the 21 piscine GBS isolates were serotype Ia (Table 1). The capsular serotypes of three piscine isolates could not be determined with the available antisera but were confirmed as serotype Ib by molecular multiplex PCR typing. Serotype Ia is previously unrecognized as a serotype among piscine GBS isolates. Wilkinson *et al.* (1973) serotyped non-haemolytic piscine GBS strains obtained from Alabama (one isolate) and Arkansas (three isolates), USA, and invasive neonatal GBS strains (four isolates) using agar gel diffusion techniques

Fig. 1. Dendrogram illustrating the phylogenetic relationship of the piscine isolates to human and bovine isolates. Clonal complexes (CC) are indicated, as are the STs of the piscine isolates.

Table 1. Piscine, dolphin, bovine and human GBS phenotype properties

PYR, pyrrolidonylarylamidase reaction; VP, Voges–Proskauer reaction; +, positive reaction; –, negative reaction; NT, non-typable. Shaded areas denote differences between isolates.

Isolates (<i>n</i>)	Serotype	ST	Haemolysis	CAMP	PYR	Hippurate*	Urea*	Starch*	Arginine*	Aesculin*	Sorbitol†	Trehalose†	Ribose†	Inulin†	Mannose†	Xylulose†	Lactose†	VP
Fish																		
KU-MU-2 NA, -3 BR, -9 BR, -15 NA, -24 BL, -24 BR, -34 E, KU-SB-37 HK, -38 BR (9)	Ia	7	β	+	–	+	–	–	+	–	–	+	+	–	–	–	–	+
KU-MU-1 NA, -9 NA (2)	Ia	7	β	+	–	+	–	–	+	–	–	+	+	+	–	–	–	+
KU-MU-11 NA (1)	Ia	7	β	+	–	+	–	–	–	–	–	+	+	–	–	–	–	+
KU-MU-11 BR, 19 HK (2)	Ia	7	β	+	–	+	–	–	+	+	–	+	+	–	–	–	–	+
KU-SB-1HK (1)	Ia	7	β	+	–	+	–	–	–	–	–	+	+	–	–	–	+	+
03ARS-BZ-TN-05 (1)	Ia	257	–	–	–	+	–	–	–	–	–	+	+	–	–	–	–	+
03ARS-BZ-TN-06 (1)	Ib	257	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	+
LADL05-108A (1)	Ib	260	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	+
LADL00-351A (1)	Ia	259	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	+
IS-ET-09-03 (1)	Ib	258	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	+
ATCC 51487 (1)	Ia	261	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	+
Dolphin																		
KU-BD-Muscle (1)	Ia	7	β	+	–	–	–	–	+	–	–	+	+	–	–	–	–	+
Human																		
841254, 510036, 560249, 630653, 510012 (5)	Ia	7	β	+	–	+	–	–	+	–	–	+	+	–	–	–	–	+
510029 (1)	Ia	7	β	+	–	+	–	–	–	–	–	+	+	–	–	–	–	+
Bovine																		
ATCC 13813 (1)	II	61	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	+
ATCC 27956 (1)	NT	23	β	–	–	+	–	–	+	+	–	+	+	–	–	–	+	+

*Hydrolysis.

†Fermentation.

and found these to be type Ib. Eldar *et al.* (1994) originally reported the ATCC 51487 isolate to be a novel species, *S. difficile*, and to be non-typable. Later, Vandamme *et al.* (1997) and Brochet *et al.* (2006) provided evidence that this isolate is actually *S. agalactiae* and reported the isolate to be serotype Ib. In the study reported here, replicate capsular serotyping of the ATCC 51487 isolate using GBS antisera and multiplex PCR revealed the isolate to be serotype Ia, a result inconsistent with those of Eldar *et al.* (1994), Vandamme *et al.* (1997) and Brochet *et al.* (2006).

MLST

The fish isolates were found to have six different STs (ST-7, ST-257, ST-258, ST-259, ST-260 and ST-261). The 16 isolates from Kuwait were collected from a bottlenose dolphin, three seabream and 12 mullet following a disease outbreak in wild mullet in Kuwait Bay beginning in August 2001 (Evans *et al.*, 2002). All of the Kuwait isolates were ST-7, whilst the six remaining fish isolates (03ARS-BZ-TN-05, 03ARS-BZ-TN-06, LADL05-108A, LADL00-351A, IS-ET-09-03 and ATCC 51487) were found to contain novel alleles and STs (Table 2). The novel STs were found in GBS strains isolated from diverse geographical areas and were clearly distinguishable from ST-7, as all of the novel STs shared two or fewer alleles with ST-7 (Table 2). Each ST was confined to a single geographical location.

The ST-7 and non-ST-7 fish isolates (ST-257, ST-258, ST-259, ST-260 and ST-261) appeared in separate branches of the dendrogram (Fig. 1), which was constructed using the STs of a collection of 1150 human, bovine and piscine GBS isolates (see Methods). The Kuwait dolphin and piscine GBS isolates were part of a clone of serotype Ia ST-7 GBS that has previously been reported to be associated with bloodstream infections in human neonates in Japan (Jones *et al.*, 2003). This clone, which was originally recognized by

analysis of restriction digest patterns and designated two closely related restriction digest pattern types, Ia-3 and Ia-4, by Nagano *et al.* (1991), appears to be extremely rare among GBS isolates associated with human infections or with maternal colonization in Sweden, Israel, the UK, Portugal, Italy and the USA (Bisharat *et al.*, 2005; Gherardi *et al.*, 2007; Jones *et al.*, 2003, 2006; Luan *et al.*, 2005; Martins *et al.*, 2007; Nagano *et al.*, 2002). Despite the rarity of serotype Ia ST-7 GBS among human isolates from Western countries, a serotype Ia strain from this clone, called A909, was isolated by R. Lancefield from a human source many years ago and its genome was recently sequenced (Tettelin *et al.*, 2005).

The phenotypic properties of the dolphin and fish isolates were characterized and compared with those of six ST-7, serotype Ia human GBS strains and two bovine mastitis reference strains (Table 1). All of the human GBS strains were isolated in Japan: two from the genitourinary tract of women (560249 and 841254), one from the gastric aspirate from a neonate (510036) and three from the bloodstream or cerebrospinal fluid of neonates (630653, 510029 and 510012). All of the Kuwait ST-7 isolates (designated KU) and human ST-7 isolates were β -haemolytic and CAMP factor positive, and shared a similar biochemical profile.

Analysis of the human, bovine and piscine GBS isolates in the larger sample revealed that ST-7 clustered with human isolates with ST-6 and ST-84 into a single clonal complex (Fig. 2), here designated CC 7 but which has previously been designated CC 6 (Bisharat *et al.*, 2005; Jones *et al.*, 2006). All of the human ST-7 isolates that we have characterized have been serotype Ia, as has the sole ST-84 isolate (Bohnsack *et al.*, 2008). Two ST-7, serotype V strains were isolated from neonates in a recent series of human isolates from Sweden (Luan *et al.*, 2005), and a single non-typable ST-7 was reported in a series of isolates

Table 2. Piscine and dolphin GBS isolates: serotype, ST, source and country of origin

Bold-formatted STs and allelic designations are new to the MLST database.

Serotype (n)*	ST	Allelic profile†							Host	Source‡	Country
		<i>adhP</i>	<i>pheS</i>	<i>atr</i> ₋	<i>glnA</i>	<i>sdhA</i>	<i>glcK</i>	<i>tk</i>			
Ia (16)	7	10	1	2	1	3	2	2	Seabream (3), mullet (12), dolphin (1)	N (5), Br (5), K (3), B (1), E (1), M (1)	Kuwait
Ia (1), Ib (1)	257	52	17	31	4	26	2	19	Nile tilapia (2)	Br (2)	Brazil
Ib (1)	258	10	17	2	2	26	26	2	Hybrid striped bass (1)	Br (1)	Israel
Ia (1)	259	52	17	31	28	26	26	2	Nile tilapia (1)	Br (1)	USA
Ib (1)	260	52	17	31	28	26	26	19	Nile tilapia (1)	Br (1)	Honduras
Ia (1)	261	54	17	31	4	26	25	19	<i>Tilapia</i> sp. (1)	Br (1)	Israel\$

*The number of isolates studied is given in parentheses.

†Seven loci selected for MLST from Jones *et al.* (2003): *adhP*, alcohol dehydrogenase; *pheS*, phenylalanyl tRNA synthetase; *atr*₋, amino acid transporter; *glnA*, glutamine synthetase; *sdhA*, serine dehydratase; *glcK*, glucose kinase; *tk*, transketolase.

‡N, Nares; Br, brain; K, kidney; B, blood; E, eye; M, muscle.

\$ATCC 51487 (Eldar *et al.*, 1994).

All of the other piscine isolates causing epizootics from four different geographical regions (Brazil, Israel, USA and Honduras) had STs not previously deposited in the MLST database. The STs of these isolates were quite dissimilar from the STs of human and bovine isolates, including ST-7, and eBurst did not cluster these five novel STs with any of the STs from the human or bovine sample. The five novel piscine STs also did not cluster into a single clonal

1374 *Journal of Medical Microbiology* 57

isolates belong to a broadly related group of GBS that is distributed worldwide and is capable of causing fish epidemics, but the available evidence indicates that strains from the non-ST-7 piscine isolates rarely, if ever, colonize or infect humans.

Whilst fish have previously been identified as a host for GBS, the population structure of piscine GBS has not been investigated. The data presented here demonstrate that at least two divergent populations of GBS are capable of causing epizootics in fish, and that one of these populations is also capable of causing human infections, although rarely and apparently in restricted geographical areas. These findings raise the possibility that humans or dolphins were the source of the Kuwait epizootic and, conversely, that ST-7, serotype Ia GBS may be a zoonosis. Comparative genomics of piscine, human and bovine GBS could help clarify those genes important for host tropism and for the emergence of unique pathogenic clones, and whether these hosts act as reservoirs for one another's pathogenic lineages.

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