# GENOMIC DIVERSITY OF *STREPTOCCOCUS AGALACTIAE* ISOLATES FROM MULTIPLE HOSTS AND THEIR INFECTIVITY IN NILE TILAPIA

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## *Abstract*

Our laboratory has conducted multiple studies to investigate the genomic diversity of GBS isolates from different phylogenetic hosts and geographical regions. We have examined fish and dolphin GBS strains using phenotypic, serological typing and multilocus sequence typing (MLST) techniques and compared these to bovine and human GBS isolates. Studies were also conducted on infectivity of fish, dolphin, bovine and human GBS isolates in Nile tilapia, Oreochromis niloticus. A previously unreported fish capsular serotype, Ia, was discovered for tilapia isolates originating from Brazil, Israel and the U.S.A, and mullet, seabream and dolphin isolates from Kuwait. Serotype Ib was noted for other tilapia isolates from Brazil and Honduras. Sequence typing of isolates produced six sequence types (ST-7, ST-257, ST-258, ST-259, ST 260 and ST-261), the latter five ST's representing allelic designations and allelic combinations unique to the S. agalactiae MLST database. Kuwait isolates (Ia, ST-7), although largely unrelated to the majority of bovine and human GBS strains, appear to share a common ancestry. Kuwait GBS isolates shared the same allelic profile, sequence type and capsular serotype as that reported from human GBS strains from Japan (Ia, ST-7). Genomic diversity existed between Kuwait GBS isolates and those from other geographical areas. Tilapia GBS isolates from Brazil, Israel, Honduras and the U.S.A. are part of a clonal complex and are unrelated to bovine and human GBS, thus representing a distinct genetic population. In experimental studies using fish isolates from different geographical regions, a Brazil GBS tilapia isolate was significantly more pathogenic to Nile tilapia than non tilapia isolates from Israel and Kuwait. However, all fish isolates caused mortalities at doses between $10^1$ and $10^6$ CFU/fish. Bovine serotype Ia, II and NT GBS isolates of unknown MLST type were not found to be infective to Nile tilapia although a human GBS isolate caused mortality in Nile tilapia. These experimental infectivity studies indicate enhanced virulence of fish GBS isolates to fish regardless of geographical origin. Here we summarize findings from GBS genomic and infectivity studies from our laboratories.

# INTRODUCTION

Treptococcus agalactiae, the Lancefield group B Streptococcus (GBS), has a broad host range and can be pathogenic to numerous animals, including fish. GBS is most recognized for causing cattle mastitis and human neonatal meningitis, although it also causes fatal meningo-encephalitis in fish. Natural tilapia infections resulting from GBS are increasing in occurrence. Despite the scant literature on GBS hosts and distribution, our laboratory has characterized GBS from various North, Latin and Central American and Asian tilapia farms. Three species of tilapia have been naturally infected with group B Streptococcus agalactiae in Israel, Thailand, and Brazil. Natural S. agalactiae infections in tilapia cultured in the U.S.A have not been published. Despite the initial reports of non-hemolytic GBS causing fish kills in cultured freshwater golden shiners in the U.S.A and later rainbow trout Oncorhynchus mykiss in Israel, the majority of natural infections of S. agalactiae in the U.S.A (Robinson and Meyer 1966; Plumb *et al.* 1974), Japan (Eldar *et al.* 1994), and Kuwait (Evans *et al.* 2002; Duremdez *et al.* 2004) have involved non-tilapine estuarine and marine fish. The reader is referred to Evans *et al.* (2006a; 2006c) for reviews of S. agalactiae in tilapia and non-tilapine species.

GBS worldwide incidence and epidemiological and zoonotic considerations necessitate an undertaking of a comparison of GBS isolates from different phylogenetic hosts. The application of molecular and genetic techniques to human and bovine GBS have resulted in better discrimination between their genetic relatedness geo-spatially (Jones *et al.* 2003; Bohnsack *et al.* 2008). Genetic variations in fish GBS isolates and differences between fish, human and bovine GBS isolates have only recently been explored. Piscine and dolphin GBS isolates have recently been reported to share the same serotype (Ia) and sequence type (ST-7) as that reported from human GBS adult and neonatal invasive strains from Japan and North America (Evans *et al.* 2008a in press). This study (Evans *et al.* 2008a in press) examined geo-spatial relationships among GBS fish isolates using phenotypic, serological and multilocus sequence typing (MLST) molecular techniques and compared these to previously-characterized human and bovine GBS sequence types (STs) (Bohnsack *et al.* 2008) to help elucidate their phylogenetic similarity and differences. Here we summarize results from GBS genomic and infectivity studies from our laboratories.

# MATERIALS AND METHODS

### Identification and characterization of GBS strains

The genomic study employed 30 geographically diverse isolates from fish, bottlenose dolphin, bovine and human sources (Table 1) (Evans *et al.* 2008a in press).

Isolates were grown on 5% sheep blood agar (SBA) (Remel, Lexena, KS). Conventional biochemical tests (hemolysis, CAMP, pyrrolidonylarylamidase (PYR) reaction, and hippurate, urea, starch, arginine and esculin hydrolysis) were performed as described by MacFaddin (2000). The Voges-Proskauer (VP) reaction and fermentation of sorbitol, trehalose, ribose, inulin, mannose, xylulose and lactose were derived from the API 20 multi-test system following manufacturers' instructions. Lancefield grouping was performed with type B antisera (Oxoid, Basingstoke, United Kingdom).

Capsular serotyping was performed twice on all strains in the laboratories of John Bohnsack and repeated in a second laboratory by Phillip Klesius (USDA, ARS Aquatic Animal Health Laboratory, Auburn, AL 36830 USA). Capsular serotype was determined by group B streptococcal typing antisera, directed against capsular type Ia, Ib, II, III, IV, and V from Denka Seiken, Tokyo, Japan. Serotypes were additionally identified by a multiplex PCR assay performed twice on some isolates. The protocol was modified from Poyart *et al.* (2007) and determined serotypes Ia, Ib, or II according to capsular polysaccharide gene (cps) amplicon base pair size.

Table 1. Collection of GBS strains

| Isolates (No.) | Country | Host | Serotype | Sequence type |
|---|---|---|---|---|
| 01-ARS-KU-MU-2 NA, 3 BR, 9 BR, 15 NA, 24 BL, 24 BR, 34 E, KU-SB-37 HK, 38 BR,1 NA, 9 NA, 11 NA, 11 BR, 19 HK (14) | Kuwait | Mullet [1] | Ia | 7 |
| 01-ARS-KU-SB-1HK (1) | Kuwait | Seabream | Ia | 7 |
| 03-ARS-BZ-TN-05 (1) | Brazil | Nile tilapia | Ib | 257 |
| 03-ARS-BZ-TN-06 (1) | Brazil | Nile tilapia | Ia | 257 |
| LADL05-108A (1) | Honduras | Nile tilapia | Ib | 260 |
| LADL00-351A (1) | USA | Nile tilapia | Ia | 259 |
| IS-ET-09-03 (1) | Israel | Hybrid striped bass | Ib | 258 |
| ATCC 51487 (1) | Israel | Hybrid tilapia | Ia | 261 |
| 01-ARS-KU-BD-MU (1) | Kuwait | Dolphin [2] | Ia | 7 |
| 841254, 510036, 560249, 630635, 510012, 510029 (6) | Japan | Human [3] | Ia | 7 |
| ATCC 13813 (1) | United Kingdom | Bovine [4] | II | 61 |
| ATCC 27956 (1) | USA | Bovine | NT | 23 |

Fish isolates (n=21) originated from infections in Klunzingeri mullet, Liza klunzingeri and seabream, Sparus auratus (Kuwait) (Evans *et al.* 2002), hybrid striped

bass, Morone chrysops x Morone saxatilis (Israel), Nile tilapia, Oreochromis niloticus (U.S.A, Brazil, Honduras), and hybrid tilapia, Oreochromis spp. (Israel) (Eldar *et al.* 1994). Nile tilapia GBS isolates were provided by Dr. John Hawke (Louisiana Veterinary Diagnostic Laboratory, Louisiana State University, Baton Rouge, Louisiana, U.S.A. and Dr. Rogério Salvador (Parana State, Brazil).   Dr. Dina Zilberg (The Albert Katz Department of Dryland Biotechnologies, Ben-Gurion University of the Negev, Israel) provided the hybrid striped bass isolate.

[2] The bottlenose dolphin isolate (Kuwait) was obtained as described in Evans *et al.* (2006b).

[3] Six ST-7, serotype Ia GBS isolated from humans in Japan were provided by Dr. Shinji Takahashi (Division of Microbiology, Joshi-Eiyoh University, Chiyoda, Sakado, Saitama 350-0288, Japan).

[4] References strains were obtained from the American Type Culture Collection (ATCC), Washington, DC, USA. (ATCC 13813 and 27956 GBS from bovine species and ATCC 51487 deposited as S. difficilis, but later identified as S. agalactiae, from diseased tilapia).

**Multilocus sequence typing (MLST)**

MLST, allelic assignment and sequence typing was performed as previously described (Jones *et al.* 2003).   Allele sequences can be found at http://sagalactiae.mlst.net . MEGA softwar (Kumar *et al.* 2004) was used to construct an unrooted dendrogram showing the relationships among fish, dolphin, bovine and human STs as compared to an extensive collection of human (n = 899) and bovine isolates (n = 63) from North America (Bohnsack *et al.* 2008).  The eBurst program (Feil *et al.*, 2004) was used to look for evidence of clonal complexes among these isolates (Evans *et al.* 2008a in press).

**Infectivity of GBS isolates**

Experimental infectivity trials were performed on Nile tilapia in USDA laboratories as using Kuwait mullet and seabream GBS isolates (Evans *et al.* 2002), an Israeli hybrid striped bass isolate and a Brazilian Nile tilapia isolate (ARS unpublished), Kuwait bottlenose dolphin isolate *(Evans et al. 2006b)*, bovine isolates (Garcia *et al.* 2008 in press) and a Japanese human GBS isolate (2008b in press) and compared to other published reports (Table 2).  Briefly, Nile tilapia (n > 500; weight ranging from 3 to 100 g) were injected intraperitoneally with doses ranging from $10^1$ to $10^9$ colony-forming units (cfu) S. agalactiae/fish and maintained at approximately 30°C.  Control fish were injected with TSB.  Fish were monitored for up to 14 days post-challenge, and culture samples were aseptically obtained from the nares, brain, anterior kidney, and posterior intestine of dead fish to confirm the presence of S. agalactiae.

# RESULTS AND DISCUSSION

## Phenotypic characterization

Phenotypically, all isolates were Lancefield group B, catalase-negative, Gram-positive cocci, appearing in pairs and chains. Kuwait mullet, seabream and bottlenose dolphin isolates, human isolates and one bovine reference isolate (ATCC 27956) were ∃-hemolytic. Tilapia isolates from Brazil, Honduras, U.S.A, Israel and one bovine reference isolate from the United Kingdom were non-hemolytic. All isolates were PYR negative. None of the isolates hydrolyzed urea or starch or fermented sorbitol, mannose, or xylose. All isolates hydrolyzed hippurate, fermented ribose and were positive by the VP reaction. Non-hemolytic tilapia and hybrid striped bass isolates phenotypically varied from ∃-hemolytic fish and human isolates in that these isolates did not ferment trehalose and were CAMP negative. In contrast, the non-hemolytic bovine reference isolate was CAMP positive while the ∃-hemolytic bovine isolate was CAMP negative although both fermented trehalose. The majority of isolates hydrolyzed arginine. Esculin hydrolysis was primarily negative with the exception of two Kuwait fish isolates and one bovine reference isolate (ATCC 27956). Likewise, the majority of isolates did not ferment inulin, with the exception of two Kuwait fish isolates, or lactose. Lactose fermentaion was noted in one Kuwait mullet and one Brazil tilapia isolate and in both bovine isolates.

## Relatedness between ST and capsular serotype

The nine capsular serotypes of GBS (Ia, Ib, II-VII) have been the gold standard in differentiation between GBS isolates. Two capsular serotypes, Ia and Ib, were determined for the fish isolates (Table 1). Serotype Ia, a previously unrecognized serotype for fish GBS isolates, was the most common serotype occurring in all of the Kuwait fish isolates, dolphin isolate and Japan human isolates and in one tilapia species isolate each from Israel (ATCC 51487), Brazil (03-BZ-TN-05) and U.S.A (LADL00-351A). Three fish isolates were serotype Ib (one isolate each from Israel (IS-ET-09-03), Brazil (03-BZ-TN-06) and Honduras (LADL05-108A). Capsular serotype was not necessarily restricted to a specific ST. One ST (ST-257) contained isolates with different serotypes, Ia and Ib. Reference bovine isolates were serotype II (ATCC 13813-United Kingdom) and nontypable (ATCC 27956-U.S.A.). Serotype Ib and no serotype (NT or nontypable) have been previously reported for piscine GBS isolates. Wilkinson *et al.* (1973) serotyped non-hemolytic piscine GBS strains obtained from Alabama, U.S.A (1 isolate) and Arkansas, U.S.A (3 isolates) (Robinson and Meyer 1966) and invasive neonatal GBS (4 isolates) using agar gel diffusion techniques and found these to be type Ib. Eldar *et al.* (1994) found the Israeli S. difficile isolate to be

non-typable. Vandamme *et al.* (1997), using a coagglutination assay (DARO group B Streptococcus serotyping test; DAKO A/S Glostrup, Denmark) later reported the capsular serotype of Eldars' S. difficile isolate (LMG 15799) as type Ib.  Piscine non-hemolytic GBS isolates from Brazil (Salvador *et al.* 2005) and beta-hemolytic GBS isolates from Kuwait (Evans *et al* 2002; Duremdez *et al.* 2004) and Thailand (Suanyuk *et al.* 2005) were not previously serotyped.  Evans *et al.* (2008a in press), in replicate capsular serotyping of the Israeli ATCC type isolate (51487) revealed the isolate to be Ia.  These results are inconsistent with those reported by Eldar *et al.* (1994) and Vandamme *et al.* (1997).  It appears this isolate may have been mistyped or differences in typing antiserum or technique employed play a role in disparate capsular serotype results.

## Relatedness of GBS isolates and variations in the MLST

The 21 fish isolates were categorized into six sequence types (ST's), four of which were only identified once (ST-258, ST-259, ST-260, ST-261) (Table 1) in fish isolates.  All of the Kuwait fish isolates and dolphin isolate and human isolates were represented by ST-7.  Six fish isolates were represented in four newly recognized ST's. Both Brazilian Nile tilapia isolates were assigned to ST-257.  One Israeli hybrid striped bass isolate was represented by ST-258 while another Israeli tilapia isolate (ATCC 51487) was represented as ST-261.  The U.S.A. and Honduras Nile tilapia isolates were assigned to ST-259 and ST-260, respectively.  Bovine reference isolates were ST-23 and ST-61.  A dendrogram shows genetic relatedness between human, bovine and fish isolate STs (Figure 1).

Figure 1.  Dendrogram illustrating the phylogenetic relationship of the piscine isolates to previously characterized human and bovine GBS ST's (Bohnsack *et al.* 2008).  Clonal Complexes (CC) are outlined with brackets, as are the ST's of piscine isolates.

Genetic Distance
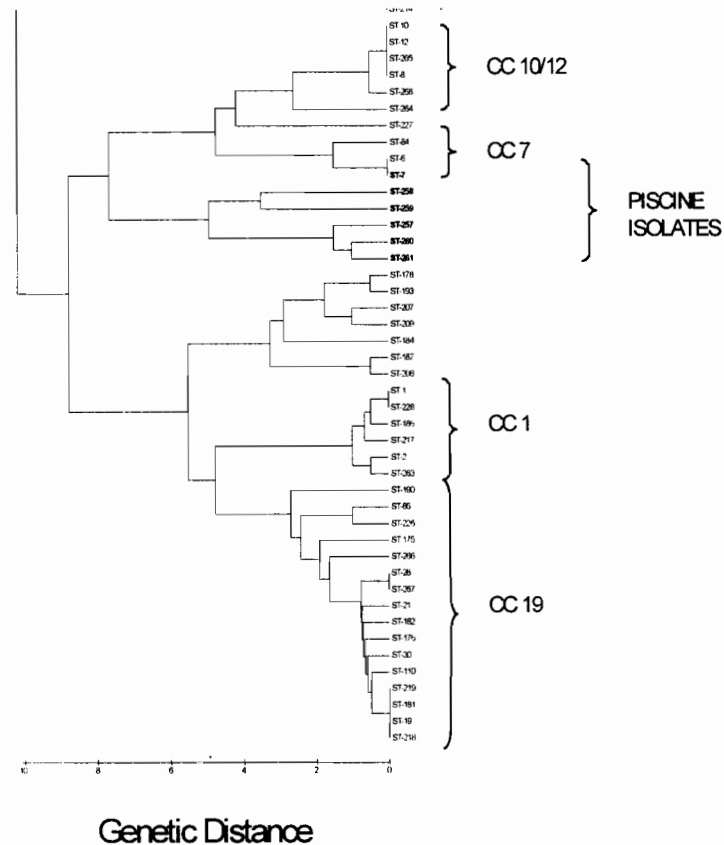
MLST demonstrated the unrelatedness of the different geographical fish GBS isolates to each other or to human or bovine STs. Kuwait fish and dolphin isolates appear to be from a single clone and largely unrelated to the majority of human GBS strains. However, Kuwait GBS fish isolates shared the same allelic profile, sequence type and capsular serotype as that reported from one human GBS carried strain and two neonatal invasive strains from Japan (Ia, ST-7) (Jones *et al.* 2003). None of the other piscine isolates examined conformed to ST's or allelic profiles reported from humans. Five of the piscine isolates causing epizootics from 4 different geographical regions (Brazil, Israel, U.S.A and Honduras) were more diverse and had alleles and allelic sequences not previously deposited in the MLST database. These isolates required assignment of numbers to the alleles and assignment of an ST to the combination of alleles. Based on dendrogram results, these isolates appear to be part of a clonal complex. An examination of the GBS population structure demonstrate that at least two divergent populations of GBS are capable of causing epizootics in fish, and that one of these populations (ST-7) is capable of also causing human infections, although rarely and apparently in restricted geographical areas.

## Infectivity of GBS isolates

Experimental infectivity studies indicate enhanced virulence of fish GBS isolates to fish regardless of geographical origin. Regardless of the fish isolate used, some fish in each experimental challenge study exhibited clinical signs such as: going off feed, lethargy, exophthalmia, C-shaped body posturing, erratic swimming, and fecal string formation. The Kuwait mullet and seabream GBS isolates caused 90-100% mortality in Nile tilapia and had a Lethal Dose ($LD_{50}$) of 1.9 X $10^3$ cfu/fish (Evans *et al.* 2002) (Table 2). In subsequent infectivity studies, a Brazil GBS tilapia isolate (03-ARS-BZ-TN-05) was significantly more pathogenic to Nile tilapia than non tilapia isolates from either Israel (03-ARS-IS-ET-09) and Kuwait. However, all fish isolates caused mortalities at doses between $10^1$ and $10^6$ CFU/fish. Figueiredo *et al.* (2007)also indicated increased virulence with Brazilian GBS tilapia isolates. In other studies, the Israeli S. difficile tilapia isolate (Eldar *et al.* 1995) increased in virulence after a series of in vivo passages decreasing the $LD_{50}$ to $10^2$ cfu/fish. Chang and Plumb ( 1996) noted increased susceptibility of Nile tilapia to GBS isolates from non tilapia fish species with elevated salinities and temperature suggesting environmental conditions are important to GBS susceptibility.

Bovine serotype Ia, II and NT GBS isolates of unknown MLST type were not found to be infective to Nile tilapia, although a serotype Ia ST-7 human GBS isolate (ID# 510012) obtained from a human neonatal meningitis clinical case in Japan, caused mortality in Nile tilapia (Evans et al 2008b in press). The human isolate was virulent in tilapia at $10^2$, $10^3$ and $10^7$ cfu/fish and morbid fish were culture positive for GBS. In tilapia infectivity studies using a dolphin GBS isolate, disease signs and 90% mortality were noted within six days post-inoculation with $10^7$ cfu/fish. These latter experimental findings suggest the possibility of mammalian GBS infectivity to fish and origination of GBS-induced fish epizootics from mammalian sources. While only one human and dolphin isolate was studied here, it is possible that other isolates with different ST's or isolates repeatedly passed through fish may display increased virulence. The relationship between capsular serotype, ST and infectivity of GBS isolates to different hosts is an area deserving more research.

Table 2. Experimental infectivity of S. agalactiae from multiple hosts to Nile tilapia[1]

| Isolate (s) | Host Origin | Country | Dose cfu/fish | % Mortality | Reference |
|---|---|---|---|---|---|
| 01-ARS-KU-MU-11BR | Mullet | Kuwait | $1 \times 10^7$ | 100 | Evans *et al.* 2002 |
| 01-ARS-KU-SB-37BR | Seabream | Kuwait | $1 \times 10^7$ | 90 | Evans *et al.* 2002 |
| 03-ARS-IS-ET-09 | Striped bass | Israel | $10^1$-$10^6$ | 20-90 | USDA unpublished |
| 03-ARS-BZ-TN-05 | Nile tilapia | Brazil | $10^1$-$10^6$ | 20-100 | USDA unpublished |
| Lake | Speckled trout | USA | $2 \times 10^6$ | 27-100 | Chang and Plumb 1996 |
| DL 805 | Gulf killifish | USA | $2 \times 10^6$ | 33-100 | Chang and Plumb 1996 |
| MS91-452 | Channel catfish | USA | $2 \times 10^6$ | 3-63 | Chang and Plumb 1996 |
| Not reported | Nile tilapia | Thailand | $10^1$-$10^8$ | 20-90 | Suanyuk *et al.* 2005 |
| ND 2-22 (ATCC 51487) | Hybrid tilapia | Israel | $10^7$ | 50 | Eldar *et al.* 1995 |
| ST 20-06 | Hybrid tilapia | Brazil | $6.4 \times 10^1$ | 50 | Figueiredo *et al.* 2007 |
| MO-55100, 59918, 59998, 61185, 61321 | Bovine | USA | $10^9$-$10^{10}$ | 0 | Garcia *et al.* 2008 in press |
| ATCC 27956 | Bovine | USA | $10^5$ , $10^7$ | 0 | USDA unpublished |
| 01-ARS-KU-BD-MU | Bottlenose dolphin | Kuwait | $1 \times 10^7$ | 90 | Evans *et al.* 2006c |
| 510012 | Human | Japan | $10^2$-$10^7$ | 11.7 | Evans et al 2008b in press |

Fish were challenged by intraperitoneal injection with the specified isolate at he indicated doses and observed for mortalities for up to 14 days post-challengè.

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