Streptococcus ictaluri sp. nov., isolated from Channel Catfish *Ictalurus punctatus* broodstock

P. Lynn Shewmaker,¹ Alvin C. Camus,² Tim Bailiff,¹ Arnold G. Steigerwalt,¹ Roger E. Morey¹ and Maria da Glória S. Carvalho¹

Correspondence

P. Lynn Shewmaker
paw3@cdc.gov

¹Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

²National Warmwater Aquaculture Center, 127 Experiment Station Road, Stoneville, MS 38776, USA

A streptococcal-like organism was associated with diseased Channel Catfish *Ictalurus punctatus* broodstock on four commercial aquaculture operations in the Mississippi Delta. Conventional biochemical testing, 16S rRNA gene sequence analysis and DNA–DNA hybridization distinguished the isolates from these fish from previously published *Streptococcus* species. Comparative 16S rRNA gene sequencing studies revealed that the isolates were phylogenetically most similar to *Streptococcus iniae*, *Streptococcus uberis* and *Streptococcus parauberis* with divergence ranging from 2.0 to 2.3%. *Streptococcus pyogenes*, *Streptococcus urinalis*, *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus canis* were included in the analysis and showed even greater differences (2.5–3.2% divergence). DNA relatedness was 22% or less to the most phylogenetically related species at the optimal temperature. These data suggest that the isolates represent a novel species of *Streptococcus* for which the name *Streptococcus ictaluri* sp. nov. is proposed. The type strain is 707-05^T (=SO2-1108^T = ATCC BAA-1300^T = CCUG 52536^T).

During February 2002, September 2003, February 2004 and July 2004, four cases of suspected bacterial disease in Channel Catfish *Ictalurus punctatus* broodstock from four different commercial aquaculture operations in the Mississippi Delta were referred to the Aquatic Diagnostic Laboratory, Thad Cochran National Warmwater Aquaculture Center, Stoneville, Mississippi, USA. A streptococcal-like organism was isolated from three of the catfish. Compatible lesions and cocci were seen in the cytology smears from a fourth catfish; however, no viable bacteria could be cultured. The *Streptococcus* strains were associated with streptococcal arthritis, osteolysis, myositis and spinal meningitis in Channel Catfish broodstock. The isolates were initially identified as unidentified *Streptococcus* species and were referred to the *Streptococcus* laboratory in the Centers for Disease Control and Prevention (CDC) for further identification. Further evaluation of the strains (705-05, 706-05 and 707-05^T) by conventional biochemical testing, 16S rRNA sequencing and DNA–DNA hybridization studies revealed that these isolates represented a novel species of

Streptococcus for which the name *Streptococcus ictaluri* sp. nov. is proposed.

The isolates were characterized phenotypically using conventional biochemical tests as described by Facklam & Elliott (1995) and the Rapid ID 32 Strep system (bioMérieux) (Freney *et al.*, 1992). Growth temperature studies at 25, 30 and 35 °C showed that the optimal temperature for growth was 30 °C; therefore, conventional biochemical and rapid tests were incubated at 30 and 35 °C. The phenotypic characteristics for *S. ictaluri* are listed in the species description. The Rapid ID32 Strep system was used as described by the manufacturer. The profile numbers inaccurately identified all three isolates as 'good identification' for *Gemella haemolysans* with 96% confidence. This error in identification is somewhat expected, since the strains were pyroglutamylaminopeptidase (PYR)-positive and were relatively inert in both conventional and rapid testing, as are *Gemella* species. A Gram stain can readily distinguish the two genera.

Table 1 shows some of the biochemical tests that can be used to distinguish between *S. ictaluri* and phylogenetically related *Streptococcus* species. The majority of streptococci are negative for PYR; however, *S. ictaluri* and most of the species that are phylogenetically similar are PYR-positive. Two species, *Streptococcus canis* and *Streptococcus dysgalactiae* subsp. *dysgalactiae* are differentiated from *S. ictaluri* by a negative PYR reaction. *S. ictaluri* is readily distinguished

Abbreviation: PYR, pyroglutamylaminopeptidase.

The GenBank accession number for the 16S rRNA gene sequence of *Streptococcus ictaluri* 707-05^T is DQ462421.

A minimum-evolution phylogenetic tree based on 16S rRNA gene sequences including *Streptococcus ictaluri* strains and the most closely related full-length type strains is available with the online version of this paper.

Table 1. Conventional tests useful for distinguishing *S. ictaluri* from phylogenetically related species

Abbreviations: HEM, haemolytic reaction on trypticase soy agar plus 5% sheep blood; PYR, pyrrolidonylarylamidase; NaCl, growth in 6.5% NaCl broth; ESC, aesculin hydrolysis; MAL, MAN, SBL, SUC and TRE, acid production in maltose, mannitol, sorbitol, sucrose and trehalose broths; v, variable.

<i>Streptococcus</i> species	HEM	PYR	NaCl	ESC	MAL	MAN	SBL	SUC	TRE
<i>S. ictaluri</i> 707-05 ^T	γ	+	—	—	+	—	—	—	—
<i>S. iniae</i> ATCC 29178 ^T	β	+	+	+	+	+	—	+	+
<i>S. parauberis</i> CCUG 39954 ^T	γ	+	—	+	+	+	+	+	+
<i>S. canis</i> ATCC 43496 ^T	β	—	+	+	+	—	—	+	v
<i>S. pyogenes</i> ATCC 12344 ^T	β	+	+	+	+	—	—	+	+
<i>S. urinalis</i> CCUG 41590 ^T	γ	+	+	+	—	—	—	+	+
<i>S. uberis</i> ATCC 19436 ^T	γ	+	+	+	+	+	+	+	+
<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i> ATCC 43078 ^T	α	—	+	—	+	—	—	+	+

from the other phylogenetically similar streptococci based on its inactivity in most carbohydrate broths except for maltose and ribose. With the exception of *Streptococcus urinalis*, all the other *Streptococcus* strains tested in this study also produce acid from maltose, and all these species (including *S. urinalis*) produce acid from ribose. *Streptococcus iniae*, *Streptococcus uberis*, *Streptococcus parauberis*, *Streptococcus pyogenes*, and *S. dysgalactiae* subsp. *dysgalactiae* and *S. canis* are distinguished from *S. ictaluri* by the production of acid from sucrose and trehalose. *S. urinalis* produces acid from trehalose and *S. canis* produces acid from sucrose. *S. ictaluri* is negative for both these carbohydrates. Haemolytic reaction, growth in 6.5% NaCl, aesculin hydrolysis and acid production from mannitol and sorbitol are additional tests that are useful in differentiating *S. ictaluri* (Table 1).

The phylogenetic position of isolates 705-05, 706-05 and 707-05^T was determined by comparative 16S rRNA gene sequence analysis as described by Shewmaker *et al.* (2004). The identical sequences obtained from strains 705-05, 706-05 and 707-05^T were combined with related reference sequences from GenBank. These sequences were aligned with CLUSTAL in MEGA 3.1 (Kumar *et al.*, 2004), trimmed to a 1377 bp consensus and a neighbour-joining tree was created (Fig. 1). The same alignment was also used to create a minimum-evolution tree (Fig. S1, available as supplementary data with the online version of this paper). Reference strain 16S sequences from the CDC *Streptococcus* database were aligned in BioEdit with CLUSTAL and a distance matrix was created (data not shown). This analysis showed these isolates to be phylogenetically most similar to *S. iniae*, *S. uberis*, and *S. parauberis* with divergence ranging from 2.0 to 2.3%. *S. pyogenes*, *S. urinalis*, *S. dysgalactiae* subsp. *dysgalactiae* and *S. canis* were included in the analysis and showed even greater differences (2.5–3.2% divergence). While the focus of this report is not an epidemiologic investigation, the identical 16S rRNA sequence suggests that this could be the same clone, although the three isolates were separated by distance and time.

DNA–DNA relatedness studies were conducted as described by Shewmaker *et al.* (2004). Cultures were grown in 1 litre Todd–Hewitt broth and incubated for 24 h with the exception that *S. ictaluri* cultures were grown at 30 °C instead of 35 °C. Cells were harvested and lysed as described by Teixeira *et al.* (1995), and DNA extraction, purification and DNA–DNA reassociation using the hydroxyapatite method were performed as described by Brenner *et al.* (1982). DNA reassociation experiments were performed at the optimal temperature (55 °C) and at the stringent temperature (70 °C) (Table 2). The three catfish strains (705-05, 706-05 and 707-05^T) showed 92% DNA relatedness or greater at the optimal temperature, 89% or greater at the stringent temperature and a divergence of ≤0.5% when hybridized against the labelled type strain (707-05^T). The DNA relatedness of the type strain with *S. canis*, *S. dysgalactiae* subsp. *dysgalactiae*, *S. iniae*, *S. parauberis*, *S. pyogenes*, *S. uberis* and *S. urinalis* was 22% or less. These results confirm that the three *S. ictaluri* isolates are genetically distinct from these phylogenetically related species and clearly represent a novel species.

The DNA G+C content was determined by thermal denaturation as described by Mandel *et al.* (1970). The G+C content for strain 707-05^T was 38.5 mol%.

Streptococcal species have been documented to cause disease in fish for decades (Hoshina *et al.*, 1958; Robinson & Meyer, 1966; Plumb *et al.*, 1974; Baya *et al.*, 1990; Perera *et al.*, 1994, 1998; Romalde *et al.*, 1999; Nomoto *et al.*, 2004; Baeck *et al.*, 2006); however, identification and taxonomic placement of the offending organisms have often been confused or incorrect. Although Chang & Plumb (1996) investigated a β-haemolytic *Streptococcus* species isolated from Channel Catfish in 1991, streptococcosis has not been previously recognized as a disease of *I. punctatus*. While this study confirms the presence of a streptococcal disease in Channel Catfish, the potential significance of this emerging pathogen to the commercial catfish farming industry is currently unknown. Similarly, the zoonotic potential of

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, including *S. ictaluri* strains and the most closely related type strains. Distances were estimated using the Kimura two-parameter model. Bootstrap values from 1000 replicates are displayed as percentages. *Streptococcus thoralensis* S69^T was used to root the tree. Bar, 0.5 nt substitutions per 100 nt.

Table 2. DNA–DNA relatedness between *S. ictaluri* strains and related type strains

Source of unlabelled DNA	Results with labelled DNA from strain 707-05 ^T *		
	RBR at 55 °C	D	RBR at 70 °C
<i>S. ictaluri</i> 707-05 ^T	100	0.0	100
<i>S. ictaluri</i> 705-05	96	0.0	92
<i>S. ictaluri</i> 706-05	98	0.0	96
<i>S. uberis</i> ATCC 19436 ^T	12	TNP	TNP
<i>S. iniae</i> ATCC 29178 ^T	15	TNP	TNP
<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i> ATCC 43078 ^T	20	TNP	TNP
<i>S. urinalis</i> CCUG 41590 ^T	10	TNP	TNP
<i>S. pyogenes</i> ATCC 12344 ^T	21	TNP	TNP
<i>S. parauberis</i> CCUG 39954 ^T	13	TNP	TNP
<i>S. canis</i> ATCC 43496 ^T	22	TNP	TNP

*RBR (relative binding ratio) was determined by the following equation (percentage DNA bound to hydroxyapatite in heterologous reactions)/(percentage DNA bound in homologous reactions) × 100. Calculation of D (percentage divergence) assumed that a 1% decrease in thermal stability of a heterologous DNA duplex compared with that of the homologous duplex was caused by 1% unpaired bases; TNP, test not performed.

S. ictaluri is also unknown, but as other fish-pathogenic streptococci have been transmitted to humans (Lehane & Rawlin, 2000; Facklam *et al.*, 2005), there is cause for concern that this organism could pose a potential health threat to aquaculture workers and recreational fisherman. Food safety is another potential human health concern (Ghittino *et al.*, 2003). However, these questions can only be answered by accurate methods for identification of the species.

Description of *Streptococcus ictaluri* sp. nov.

Streptococcus ictaluri (ic.ta.lur'i. N.L. gen. masc. n. *ictaluri* of/from *Ictalurus*, the catfish from which the organism was isolated.)

Cells are Gram-positive cocci that occur in pairs or short chains, catalase-negative, do not produce haemolysis or pigment when grown on tryptic soy agar supplemented with 5% defibrinated sheep blood, and are susceptible to vancomycin. Strains are positive for PYR and leucine aminopeptidase activity, and produce acid from maltose and ribose. Strains are negative for arginine, hippurate, aesculin and urea hydrolysis, growth at 45 °C, growth in 6.5% NaCl broth and pyruvate utilization. Acetoin is not produced and tellurite is not tolerated. Using the rapid ID32 Strep test system, alkaline phosphatase, alanine-phenylalanine-proline arylamidase and pyroglutamic acid arylamidase are produced. Arginine dihydrolase, β -glucosidase, β -galactosidase, β -glucuronidase, α -galactosidase, *N*-acetyl- β -glucosaminidase, glycine-L-tryptophan arylamidase, β -mannosidase and urease are not produced. Hippurate is not hydrolysed. Acid is not produced from ribose, mannitol, sorbitol, lactose, trehalose, raffinose, sucrose, L-arabinose, D-arabitol, cyclodextrin, glycogen, pullulan, maltose, melibiose, melezitose, methyl- β -D-glucopyranoside and tagatose. The natural habitat is unknown.

The type strain is 707-05^T (=S02-1108^T=ATCC BAA-1300^T=CCUG 52536^T); G+C content is 38.5 mol%. Strains 705-05 (=S04-782-1), 706-05 (=S04-045) and 707-05^T are distinguished by 16S rRNA gene sequencing.

Acknowledgements

We are grateful to H. G. Trüper for assisting with the species epithet.

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