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# First identification and characterization of *Streptococcus iniae* obtained from tilapia (*Oreochromis aureus*) farmed in Mexico

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## Abstract

This is the first study to isolate, identify and characterize *Streptococcus iniae* as the causative disease agent in two tilapia (*Oreochromis aureus*) populations. The populations were geographically isolated, of distinct origins, and did not share water sources. Affected fish showed various external (e.g., exophthalmia and cachexia, among others) and internal (e.g., granulomatous septicaemia and interstitial nephritis, among others) signs. All internal organ samples produced pure cultures, two of which (one from each farm, termed S-1 and S-2) were subjected to biochemical, PCR and 16S rRNA sequencing (99.5% similarity) analyses, confirming *S. iniae* identification. The two isolates presented genetic homogeneity regardless of technique (i.e., RAPD, REP-PCR and ERIC-PCR analyses). Pathogenic potentials were assessed through intraperitoneal injection challenges in rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*). Rainbow trout mortalities were respectively 40% and 70% at $10^4$ and $10^6$ CFU per fish with the S-1 isolate, while 100% mortality rates were recorded in zebrafish at $10^2$ and $10^4$ CFU per fish with the S-2 isolate. The obtained data clearly indicate a relationship between intensified aquaculture activities in Mexico and new disease appearances. Future studies should establish clinical significances for the tilapia industry.

**KEYWORDS**
*Oreochromis*, outbreak diseases, *Streptococcus iniae*, Tilapia

## 1 | INTRODUCTION

Fish from the *Cichlidae* family, commonly known as tilapia (*Oreochromis* spp.), grow rapidly and are highly adaptable. These traits have made tilapia species the development focus of the worldwide aquaculture industry in recent years (Fitzsimmons, 2016). Tilapia are considered disease resistant; however, intensified farming practices have favoured the expression of emerging pathologies, especially of streptococcosis (Amal & Zamri-Saad, 2011), a systemic disease caused by *Streptococcus* bacteria. While these bacteria are mostly known for affecting terrestrial species (Evans, Klesius, & Shoemaker, 2006; Garcia, Klesius, Evans, & Shoemaker, 2008), a number of

  

different freshwater and saltwater fish are also affected globally (Rahimi-Kia & Mehrabi, 2013).

Tilapia production for 2006 suffered approximate economic losses of USD$250 MM as a result of *Streptococcus* spp. infections (Amal & Zamri-Saad, 2011). While *Streptococcus iniae* and *Streptococcus agalactiae* were established as the primary species responsible (Evans et al., 2006; Wang et al., 2013; Zamri-Saad, Amal, & Siti-Zahrah, 2010), other species able to induce infection include *S. dysgalactiae*, *S. milleri*, *S. ictaluri* and *S. parauberis*. Some biotypes even present zoonotic potential (Rahimi-Kia & Mehrabi, 2013; Yanong & Francis-Floyd, 2002).

*Streptococcus* spp. are Gram-positive, spherical- or ovoid-shaped (≈0.5–2.0 μm diameter) microorganisms that, in liquid media, exist in pairs or as part of chains. Furthermore, *Streptococcus* spp. are immobile, do not form spores, produce gas-free lactic acid during fermentation metabolism, are oxidase and catalase negative, are facultative anaerobic organisms and require enriching media for growth (Amal & Zamri-Saad, 2011; Lau et al., 2006). The diagnosis and identification of *Streptococcus* spp. via biochemical profiles are difficult as not all bacteria within this genus are included in databases, thus complicating discrimination (Lau et al., 2006; Rahimi-Kia & Mehrabi, 2013). In turn, molecular diagnostics using markers, such as the 16S ribosomal RNA (rRNA) and 16S-23S rRNA intergenic spacer genes, can be used to identify distinct *Streptococcus* species (Lau et al., 2003; Pourgholam et al., 2013).

In the Mexican fishing industry, tilapia ranks fifth by volume and third by economic income (CONAPESCA, 2013). Intensified farming of this fish is provoking the appearance of diseases, such as the recently confirmed francisellosis (Ortega, Mancera, et al., 2016) and rainbow trout fry syndrome, as caused by *Flavobacterium psychrophilum* (Castillo et al., 2017). Furthermore, there are informal reports on the isolation of *S. agalactiae* biotype 2 (Sheehan, 2009) and *Streptococcus* sp. (Conroy, 2009), and also isolates of *Streptococcus* spp. are even commonly obtained from apparently healthy fish (Ortega & Valladares, 2015).

This study is the first to report on septicaemic processes associated with *Streptococcus* spp. infection in tilapia. *Streptococcus iniae* was isolated and identified as the causative agent in two disease outbreaks among distinct tilapia populations from geographically separated farms in Mexico. Isolates were obtained from different organs and wounds, with identity confirmed through conventional biochemical tests and sequencing analysis of the 16S rRNA gene. Furthermore, observations are provided for the clinical and histological pathologies of affected fish.

## 2 | MATERIAL AND METHODS

### 2.1 | Fish

A comprehensive sanitary diagnosis was conducted for 150 tilapia (*Oreochromis aureus*) farms in the central region of Mexico. Fish with signs of septicaemia, including corneal opacity and exophthalmia, inactivity, cachexia, and incoordination, were detected at two distinct farms located in the Mexican states of San Luis Potosí (February 2013; 68% mortality rate) and Querétaro (November 2013; 80% mortality rate). San Luis Potosí samples originated from ten diseased fish belonging to a lot of 13,000 animals; fish within the lot were irregularly sized, weighed 150–250 g, and were maintained in circular concrete tanks (8 kg/m$^3$ density) supplied with spring water (100% daily water turnover; 27–29°C; 4.5 mg/L oxygen concentration). For Querétaro, ten clinically disease fish were obtained from a lot of 6,000 through semi-guided sampling and transported live in plastic bags with oxygen supplementation to the Aquatic Animal Health Laboratory (Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Toluca, Mexico) for complete pathological and bacteriological workups. Querétaro tilapia weighed 150–200 g and were maintained in geomembrane tanks (7 kg/m$^3$) supplied with well water from a greenhouse system (30% daily water turnover; 28°C; 4.5 mg/L oxygen concentration).

### 2.2 | Macroscopic, histological and bacteriological analyses

All fish were killed via anaesthetic overdose (240 mg/L tricaine methanesulfonate 222; Sigma) and immediately subjected to post-mortem examination. Initial external observations were conducted, and the wounds of each specimen were recorded. Examinations were then conducted for the presence of external parasites, and scrapings were obtained from the gills and skin for microscopic observation at 10× and 40× magnifications.

Internally, the aspect, colour and contents of the body cavity were also reviewed. For histological analysis, liver, spleen, kidney, heart, gill, brain and intestine samples were taken from each fish, fixed in vials containing 10% buffered formalin, dehydrated and embedded in paraffin wax following standard procedures. Each tissue was sectioned at 5 μm and stained with haematoxylin and eosin to describe histopathological alterations as in Ortega, Mancera, et al. (2016). Sections were observed at different magnifications under an Olympus BH2 light microscope.

Samples for bacterial isolation were aseptically taken from the external skin lesions and from the kidney, spleen and brain of each specimen. The samples were streaked onto Columbia sheep blood (AES Laboratories). All plates were aerobically incubated at 28°C and examined every 24 hr for 10 days. A representative isolate colony from each group (i.e., San Luis Potosí and Querétaro) was selected from the Columbia sheep blood plate, streaked onto a new Columbia sheep blood plate to obtain pure cultures and stored at −80°C in Criobille tubes (AES Laboratories).

### 2.3 | Phenotypical characterization of isolate

For phenotypical characterization, isolates were grown on tryptone soya agar (TSA, Oxoid), incubated at 22°C for 24 hr. Gram staining, cell and colony morphology, motility, catalase (3% $H_2O_2$) and cytochrome oxidase (Liofilchem) were analysed. Growth at different

temperatures (4, 15, 28 and 37°C) and salt tolerance (0, 1, 1.5, 3, 6 and 10% NaCl) were tested on TSA and tryptone soya broth (Oxoid), respectively. Growth was also tested on the following culture media, all of which are routinely used in the Aquatic Animal Health Laboratory: MacConkey agar (Oxoid); Sabouraud agar (Oxoid); Marine Agar 2216 (Oxoid); nutrient agar (Oxoid), thiosulfate citrate bile salts sucrose agar (Oxoid); TSA supplemented with 5% sheep blood (Liofilchem); brain–heart infusion broth; chocolate agar (Liofilchem); Luria-Bertani (Oxoid); De Man, Rogosa, and Sharpe agar (Oxoid); Mueller–Hinton agar with and without 1% NaCl supplement (Oxoid); Reasoner's 2A agar (Oxoid); cysteine agar with and without 5% foetal serum bovine (Gibco); and tryptone yeast extracts. Additional biochemical features were determined through the bile-esculin test (Liofilchem), DNAse test agar (Liofilm), and starch (0.4% v/w) and gelatine (1% v/w) hydrolysis tests, all of which employed TSA as the basal medium. Furthermore, the API Rapid ID 32 Strep system (bioMérieux) was employed according to manufacturer instruction, excepting incubation temperature, which was set at 22°C.

Isolate drug sensitivities were determined by the disc diffusion method on Mueller-Hinton agar as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006) for fish pathogens. *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658$^T$ was employed as the quality-control type strain. Commercial discs were tested with ampicillin (10 μg; BD), bacitracin (10 μg; BD), gentamicin (10 μg; Oxoid), erythromycin (15 μg; Oxoid), florfenicol (30 μg; Oxoid), nalidixic acid (30 μg; Oxoid), oxolinic acid (2 μg; Oxoid) and oxytetracycline (30 μg; Oxoid). After incubation at 22°C for 44–48 hr, the diameter of each inhibition zone (i.e., where visible growth ended) was read twice at right angles by measuring to the nearest millimetre.

## 2.4 | PCR analysis

Heart, kidney and spleen samples were aseptically removed from ten symptomatic, diseased fish and divided into 50–500 mg pieces. The samples were then homogenized with phosphate-buffered saline (100 μL, pH 7.4) by repeated pipetting. DNA extraction was performed with the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek) following manufacturer instructions. From pure bacterial isolates, DNA was extracted using the InstaGene DNA Purification Matrix (Bio-Rad) according to manufacturer instructions. From each extracted DNA sample, 2 μL were immediately used for single PCR amplifications. Remaining DNA extracts were stored at −20°C.

*Streptococcus iniae* was confirmed using the species-specific SP1 5′-GAAAATAGGAAAGAGACGCAGTGTC-3′ and SP2 5′-CCTTATT TCCAGTCTTTCGACCTTC-3′, which amplify an expected 377 bp fragment of the 16S-23S internal transcribed spacer (ITS) region (Zhou, Fan, Zhu, Xie, & Li, 2011). Samples were run in a Multigene Optimax thermocycler (Labnet International Inc.), and sterile phosphate-buffered saline and tissue samples obtained from healthy tilapia (i.e., farm with no disease history) were employed as negative controls.

The PCR products were electrophoresed on a 1.5% (w/v) agarose gel visualized with 1/10,000 GelRed Nucleic Acid Gel Staining (Biotium) and photographed under UV light. An AccuRuler 100 bp Plus DNA Ready-to-Use Ladder (Maestrogen) was employed as a molecular mass marker. A single 377 bp band positively identified *S. iniae*.

## 2.5 | 16S rRNA sequencing

Genomic DNA was extracted from two colonies of each isolate using the InstaGene DNA Purification Matrix (Bio-Rad) following manufacturer instructions. The 16S rDNA was PCR-amplified using the universal primer pair pA and pH (Edwards, Rogall, Blöcker, Emde, & Böttger, 1989). 16S rDNA sequences were aligned with ClustalW algorithm against phylogenetically related organisms available in GenBank, and manual adjustments were made in Aliview v1.19 (Larsson, 2014). Phylogenetic relationships among the samples were assessed via Bayesian inference using the MrBayes v3.1 software (Ronquist & Huelsenbeck, 2003). The best-fit evolutionary model was established with jModelTest v2.1.6 (Posada, 2008).

## 2.6 | Genetic characterization

To determine if the two isolates belonged to different clonal lineages, three PCR-based genetic typing methods were employed (i.e., RAPD-PCR, ERIC-PCR, and REP-PCR), the procedures of which were previously described and validated in other Gram-positive fish pathogens, including *Streptococcus phocae* (Valdés et al., 2009). RAPD amplifications were performed using Ready-To-GoTM RAPD analysis beads with the P6 (CCCGTCAGCA) primer. In turn, ERIC-PCR and REP-PCR amplifications were performed using Ready-To-Go™ PCR beads and previously described primer pairs (Versalovic, Koeuth, & Lupski, 1991). All gels were scanned, and images were captured with the Gel Doc-2000 Gel Documentation System (Bio-Rad). Data were analysed using the Diversity Database software (Bio-Rad).

## 2.7 | Virulence tests

The isolates recovered from tilapia (termed S-1 and S-2) were used to conduct respective challenges in rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) specimens. Thirty healthy rainbow trout (4–6 g) were obtained from a hatchery with no recent disease records. Similarly, 45 zebrafish (average 4 cm and 0.4 g) were obtained from an ornamental fish farm that has not reported any infectious events. To ensure that fish were uninfected by *S. iniae* or other pathogens, samples were subjected to standard microscopic and bacteriological examinations, as well as PCR analysis (Zhou et al., 2011). Fish specimens were randomly allocated among three groups —two experimental and one control, with 10 (rainbow trout) or 15 (zebrafish) individuals in each group. All groups were acclimated for 7 days in plastic tanks (8 L) containing aerated, dechlorinated water.

Challenges were conducted using inoculums prepared from tryptone soya broth cultures (22°C, overnight, 120 rpm). The experimental rainbow trout groups received 0.1 ml intraperitoneal injections of the S-1 isolate at either a dose of $1.6 \times 10^4$ or $1.6 \times 10^6$ CFU per fish. The experimental zebrafish groups received 0.01 ml intraperitoneal injections of the S-2 isolate at either a dose of $1.2 \times 10^2$ or

$1.2 \times 10^4$ CFU per fish. The control group of each fish species received a 0.1 or 0.01 ml intraperitoneal injection of tryptone soya broth.

The challenge period lasted up to 21 days, during which time, each group was maintained in a closed system, with a 14:10 hr light:day regime, and with dechlorinated water kept at either 16°C (rainbow trout) or 28°C (zebrafish). All fish were fed in proportion to 1.5% body weight, and the water of each tank was changed one every other day to remove faecal waste.

Daily during the challenge period, clinical signs were recorded and dead fish were removed. Dead specimens were microbiologically analysed to confirm *S. iniae*-caused mortality. Kidney, brain and external lesions were directly streaked onto tryptone soya broth plates and incubated at 22°C for 4 days. If pure colonies were obtained, biochemical and PCR methods were used for identification.

## 3 | RESULTS AND DISCUSSION

Streptococcosis is one of the most important bacterial infections affecting tilapia (Ramesh, Johnson, Matthew, & Donald, 1994; Wang et al., 2013; Zamri-Saad et al., 2010). This disease has significant economic impacts, especially for larger fish. In particular, streptococcosis can cause mortality rates >50% during acute infection (e.g., in a 3- to 7-day period) or low, yet daily, mortalities during chronic infection (Yanong & Francis-Floyd, 2002; Zamri-Saad et al., 2010). A similar situation was evidenced in the two currently assessed fish lots, with affected individuals weighing between 150 and 250 g, presenting high mortalities and showing a tendency towards chronicity. It is worth mentioning that the evaluated farms were separated by approximately 160 km, with one in the Mexican state of Querétaro and the other in the state of San Luis Potosí. Furthermore, each fish lot was of distinct origin, and water sources were not shared between the farms. This information highlights that the outbreaks occurred independently, even being recorded during different seasons of the year. In relation to this, *Streptococcus* spp. are opportunistic pathogens that are widely disseminated in aquatic environments, with pathogenicity associated with stress processes and environmental fluctuations (Pourgholam et al., 2013).

Eight of ten San Luis Potosí specimens and ten of ten Querétaro specimens evidenced macroscopic lesions typical of septicaemia. Notable among these were corneal opacity and exophthalmia (Figure 1a) and a poor body condition, symptoms in line with the pathological processes caused by bacteria of the *Streptococcus* genus (Baums et al., 2013; Chen, Chao, & Bowser, 2007; Zamri-Saad et al., 2010). When cases tended towards chronicity, as in the San Luis Potosí sample group, the affected animals presented a poor body

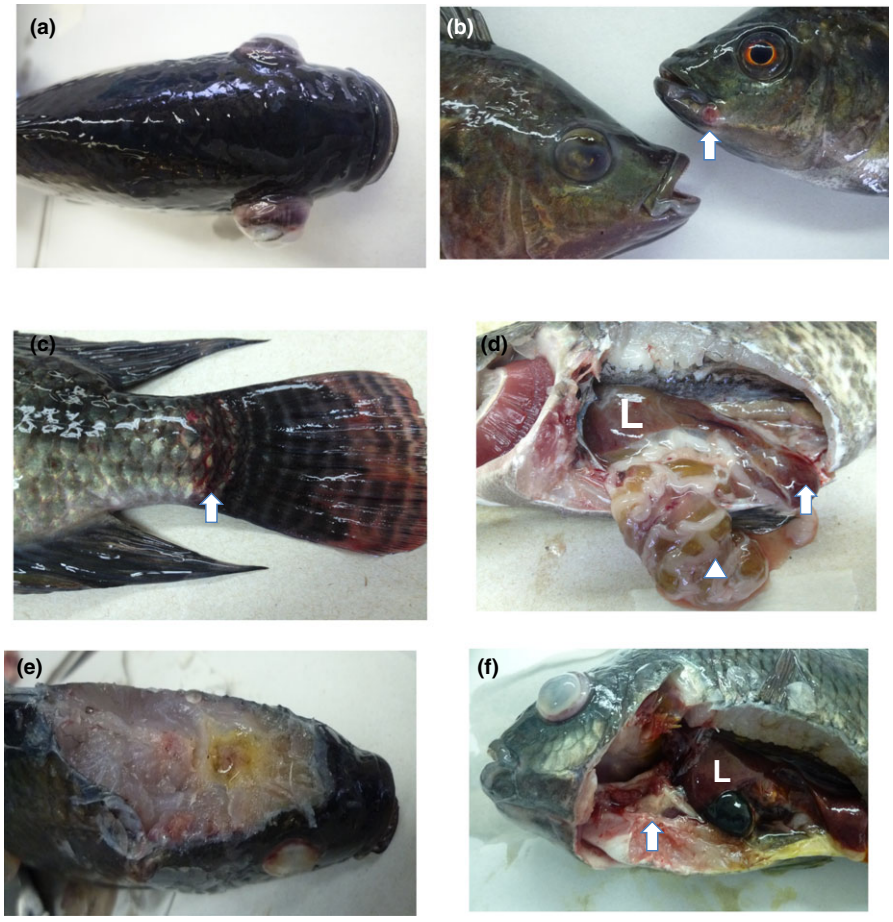


**FIGURE 1** Internal and external lesions recorded on tilapia (*Oreochromis* spp.) specimens affected by a systemic disease for *Streptococcus iniae*. (a) Corneal opacity, exophthalmia and darkening of the body; (b) Mandibular abscess and ulcerations (arrow); (c) Haemorrhaging at the base of the caudal peduncle (arrow); (d) Liver (L) with haemorrhaging (arrow) and irregular colouring and aspect, adherences (arrowhead); (e) Cerebral oedema and haemorrhaging; and (f) Heart surrounded by white-greyish, purulent-mass membranes (arrow) and adherences, note the severe exophthalmia and corneal opacity

condition and, even, endoftalmitis. Importantly, streptococcosis in tilapia is primarily attributed to *S. agalactiae* (Li et al., 2013, 2014; Zamri-Saad et al., 2010), including in Latin America (Conroy, 2009; Ortega, Barreiro, et al., 2016; Sheehan, 2009). However, the majority of *S. iniae* reports also originate from infections in tilapia (Hossain, Ehsan, Rahman, Haq, & Chowdhury, 2014; Sheehan, 2009; Shoemaker, Evans, & Klesius, 2000; Weinstein et al., 1997; Zhou et al., 2011).

The following external observations were made for San Luis Potosí specimens: five swam erratically at the moment of capture; eight evidenced uni- or bilateral corneal opacity and exophthalmia (Figure 1a); three presented mandibular abscess and ulcerations (Figure 1b) and haemorrhaging at the base of the caudal peduncle (Figure 1c); and all manifested cachexia, pale gills and darkening of the body. Furthermore, external parasite analysis revealed that the skin and gills of all San Luis Potosí specimens were moderately infested by monospecific genus parasites such as *Gyrodactylus* sp., *Dactylogyrus* sp. and the protozoan *Trichodina* sp. Importantly, parasitic infestations act a predisposing factor for bacterial infections (Evans, Klesius, Pasnik, & Shoemaker, 2007; Xu, Shoemaker, & Klesius, 2007). In turn, the following external observations were made for Querétaro specimens, which were collected via semi-directed sampling: two presented unilateral ulcerations on the mandible; and all showed inactivity, uni- or bilateral corneal opacity and exophthalmia, body darkening and cachexia.

Internally, 50% of fish from both groups (San Luis Potosí and Querétaro) evidenced serosanguineous ascites fluid, adherences, and a liver with irregular colouring and aspect (Figure 1d and f). The majority of specimens also presented an enlarged spleen, kidney and liver, while the ten Querétaro specimens had cerebral oedema and haemorrhaging (Figure 1e). Furthermore, the hearts of five San Luis Potosí fish were surrounded by white-greyish, purulent-mass membranes (Figure 1f). The pathological picture for San Luis Potosí and Querétaro fish, for both internal and external wounds, clearly corresponded to the description of a typical streptococcosis process in tilapia (Baums et al., 2013; Chen et al., 2007; Ramesh et al., 1994; Zamri-Saad et al., 2010), which was apparently more chronic at the San Luis Potosí farm.

Subsequent histological analysis revealed the primary anomaly in damaged tissues to be a granulomatous reaction between mononuclear macrophage cells, with a scarce lymphocytary presence, and granular eosinophilic cells with evident bacterial bodies. The liver showed severe multifocal perivascular hepatitis; multifocal granulomatous necrosis and severe granulomatous serositis (data not shown). Heart tissue evidenced severe granulomatous epicarditis and pericarditis (Figure 2a); the degeneration and necrosis of myocardial fibres; and mononuclear myocarditis. In turn, the kidney presented severe, multifocal interstitial nephritis (Figure 2b), while the spleen-evidenced depletion; severe multifocal granulomatous splenitis; degeneration; and multifocal necrosis with granulomatous, fibrinous serositis and a bacterial presence (Figure 2c). This is in addition to showing severe, diffuse, mononuclear meningoencephalitis (Figure 2d). Finally, eye tissue presented severe granulomatous endophthalmitis (Figure 2e), choroiditis and corneal oedema, as well as

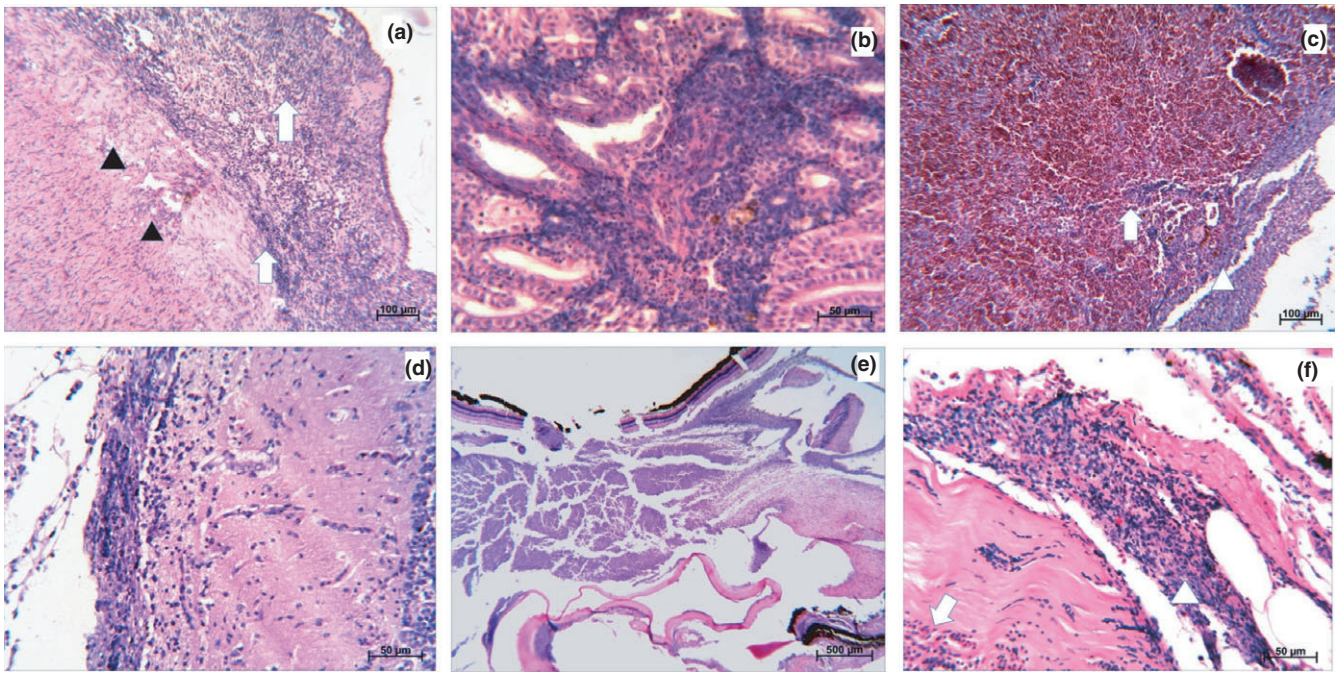


**FIGURE 2** Lesions recorded on primary organs of tilapia (*Oreochromis* spp.) specimens affected by a systemic disease for *Streptococcus iniae*. (a) Heart with severe granulomatous epicarditis (arrowhead) and pericarditis (arrow); (b) Kidney with severe interstitial nephritis; (c) Spleen with depletion severe, multifocal splenitis (arrow); multifocal degeneration with granulomatous, fibrinous serositis (arrowhead); (d) Severe, diffuse, granulomatous leptomeningitis; perivascular oedema; (e) Eye with severe granulomatous endophthalmitis and (f) Severe haemorrhagic (arrow) and necrotic granulomatous multifocal myositis (arrow head). Bar scale = 50, 100 or 500 µm

**TABLE 1** Phenotypic and biochemical results obtained for the two studied Mexican *Streptococcus iniae* isolates. Isolates were routinely grown on tryptone soya agar and incubated at 22°C. +, positive; −, negative; nd, not determined. Data for the type strain were obtained from Pier and Madin (1976)

| Characteristics | S-1 | S-2 | Perera, Johnson, Collins, and Lewis (1994) | Yuasa, Kitancharoen, Kataoka, and Al-Murbaty (1999) | Russo, Mitchell, and Yanong (2006) | ATCC 29178 |
|---|---|---|---|---|---|---|
| Source | Tilapia | Tilapia | Tilapia | Rabbitfish | Rainbow shark | Dolphin |
| Gram | + | + | + | + | + | + |
| Morphology | Cocci | Cocci | Cocci | Cocci | Cocci | Cocci |
| Motility | − | − | | | | − |
| Catalase | − | − | − | − | − | − |
| Oxidase | − | − | | | | |
| Aesculin | − | − | nd | − | − | |
| Gelatinase | − | − | | | | |
| Haemolysis | β | β | β | β | α, β | β |
| API Rapid ID 32 STREP | | | | | | |
| α-galactosidase | − | − | | − | | − |
| β-galactosidase | − | − | | − | | − |
| β-glucuronidase | + | + | | + | | + |
| Alkaline phosphatase | + | + | | + | | + |
| Arginine dihydrolase | − | − | | | | |
| Acid production from | | | | | | |
| Arabinose | − | − | + | − | + | − |
| Glycogen | − | − | | + | | + |
| Lactose | − | − | − | − | − | − |
| Mannitol | + | − | + | + | − | + |
| Raffinose | − | − | − | − | − | − |
| Ribose | + | + | | + | | + |
| Sorbitol | − | − | − | − | − | − |
| Starch | + | + | | + | | + |
| Trehalose | + | + | + | + | + | + |
| Glucose | + | + | + | | + | |

severe haemorrhagic and necrotic granulomatous myositis (Figure 2f). These findings are consistent with the development process of bacterial septicaemia, with similar histological descriptions existing for *Streptococcus* bacteria in tilapia (Baums et al., 2013; Chen et al., 2007; Zamri-Saad et al., 2010).

Although prior reports have characterized the histopathological lesions caused by infection with *S. agalactiae* (Laith et al., 2017; Ortega, Barreiro, et al., 2016; Zamri-Saad et al., 2010) and *S. iniae* (Baums et al., 2013; Chen et al., 2007; Ramesh et al., 1994), it is very difficult, histologically speaking, to obtain a differential diagnosis between these microorganisms and other *Streptococcus* species (Akhlaghi & Mahjor, 2004; Fawzy et al., 2014; Laith et al., 2017). As such, bacterial isolates were obtained from the observed wounds in tilapia specimens, the aim of which being to determine the aetiology and species of the involved *Streptococcus* bacterium. All internal organs from both groups (San Luis Potosí and Querétaro) produced pure cultures on Columbia sheep blood plates. However, of the sampled organs, only two (one from each farm) were stored for posterior identification analyses. The S-1 isolate was obtained from a San Luis Potosí kidney, while the S-2 isolate was recovered from a Querétaro brain.

The genus of most bacteria can be identified through a limited number of important fundamental reactions, such as motility, catalase and oxidation production, and carbohydrate breakdown (Barrow & Feltham, 1993). Both the S-1 and S-2 isolates were found Gram-positive. Colonies grown on Columbia sheep blood presented the following traits: 1- to 2-mm diameter; white-greyish; convex with entire edges; presented β-haemolysis; oxidase and catalase negative; non-motile; and growth at 4–28°C, but not at 37°C. Interestingly, growth and colony size decreased at lower temperatures (<15°C) and with a 3% NaCl supplement. In turn, neither isolate grew on MacConkey agar; De Man, Rogosa and Sharpe agar; or thiosulfate citrate bile salts sucrose agar. Other biochemical properties are shown in Table 1. These properties indicated biochemical homogeneity among the recovered isolates, regardless of the farm, thus supporting initial bacterial assignment to the *Streptococcus* genus (Table 1).

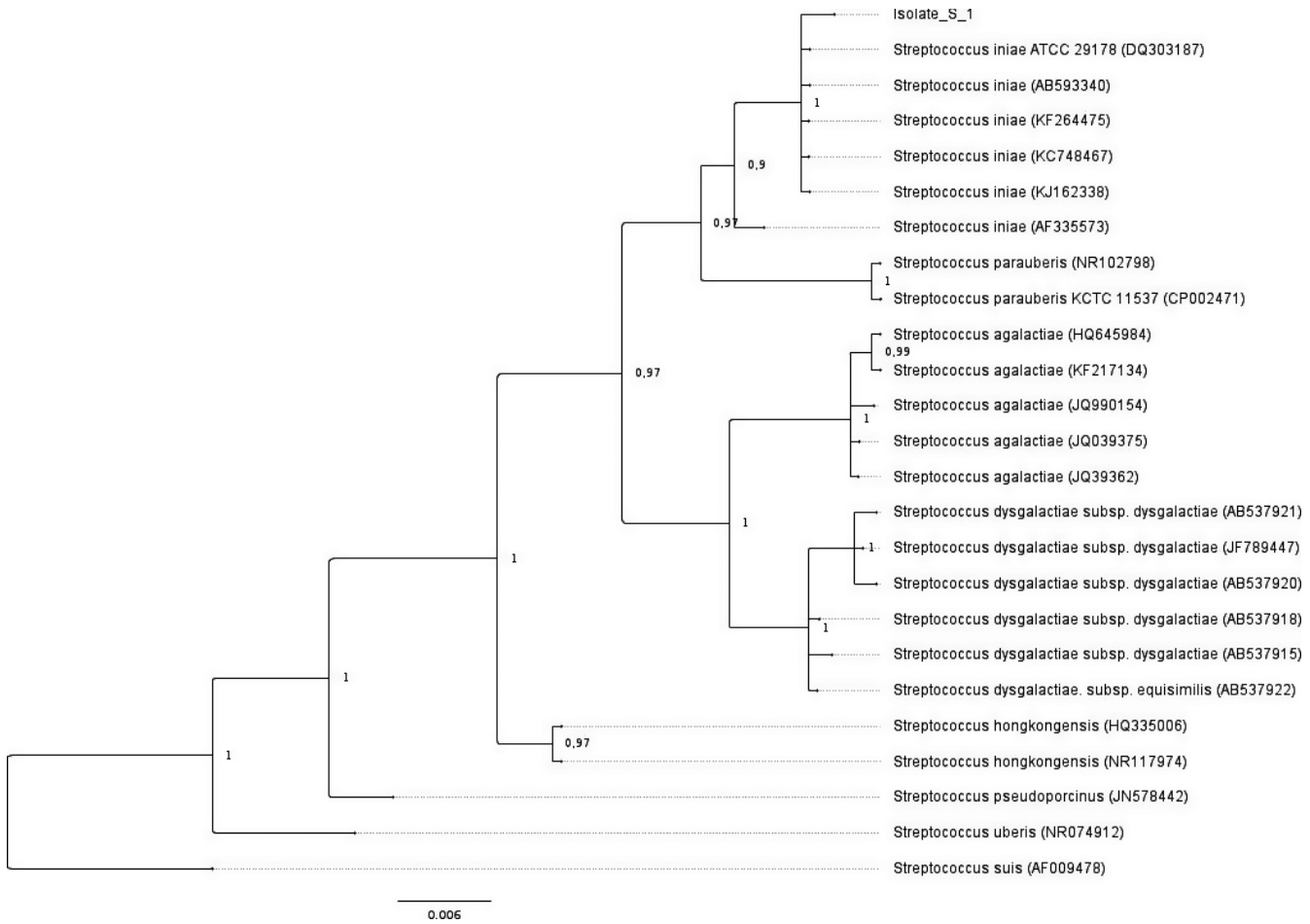


**FIGURE 3** Inferred species relationships between the Mexican isolate S-1 and other *Streptococcus* species, as based on Bayesian analysis of partial 16S rRNA gene sequences (1,261 bp). Phylogenetically, the S-1 isolate was placed in the same cluster as *Streptococcus iniae* ATCC 29178$^T$ (accession number DQ303187) and was nearest to *Streptococcus parauberis* NCFD 2020 (accession number AEUT02000001) and *Streptococcus hongkongensis* HKU30 (accession number HQ335006). The posterior probabilities recovered by Bayesian analysis are indicated in the nodes and values <50% are not shown

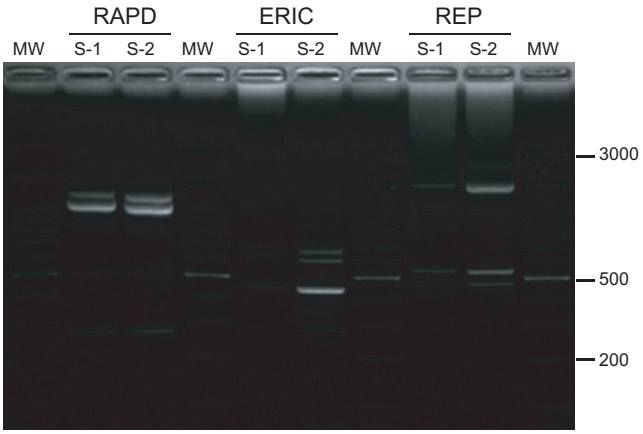


**FIGURE 4** Amplification fingerprints obtained for the Mexican *Streptococcus iniae* isolates S-1 and S-2 using RAPD with primer 6, ERIC-PCR and REP-PCR analyses. Lanes: molecular weights (MW) measured with the AccuRuler 100 bp DNA Plus DNA RTU Ladder (Maestrogen). Numbers to the left of the image indicate the position of the molecular size marker in bp

*Streptococcus* spp. are opportunistic pathogens that have been commonly isolated from the external surfaces of clinically healthy fish, including tilapia, farmed across distinct geographical areas in Mexico (Ortega & Valladares, 2015). The lack of prior reports on clinical cases is likely due to an absence of disease-triggering factors. Nevertheless, *S. agalactiae* biotype 2 has been reported in tilapia farmed in Mexico (Sheehan, 2009), although it was not stated if the samples originated from clinically diseased fish or not. Likewise, septicaemia caused by *Streptococcus* spp. can be erroneously diagnosed as related to other pathogens. However, with the exception of francisellosis (Ortega, Mancera, et al., 2016), there are no other reports of septicaemia diseases in tilapia in Mexico.

When a bacterial infection occurs in the aquaculture industry, effective chemotherapeutic treatments are essential for reducing mortality and mitigating impacts to production. The results obtained from in vitro disc diffusion assays showed that both Mexican isolates had identical antibiotic susceptibility patterns (inhibition zones: 28–46 mm [ampicillin]; 34 mm [bacitracin]; 16 mm [gentamicin]; 31–39 mm [erythromycin]; 30 mm [florfenicol]; 28–46 mm

[oxytetracycline]; and <7 mm [nalidixic acid and oxolinic acid]). Additionally, the disc susceptibility patterns of ATCC 33658$^T$ were within the accepted limits for florfenicol and oxytetracycline (CLSI, 2006). Unfortunately, information was non-existent regarding treatment applications for San Luis Potosí fish. In turn, facility records indicated that symptomatic Querétaro fish received treatment with oxytetracycline, resulting in decreased daily mortalities. However, this therapy was applied late, with 50% of the lot already having died and the remaining individuals already severely affected by infection.

Specific PCR analyses, following Zhou et al. (2011), on DNA extracted from the recovered Mexican isolates resulted in the expected 377-bp fragment (data not shown), corresponding to ITS 16S rRNA gene, thus supporting *S. iniae* identification. Similar findings were obtained for the ten samples collected from tested symptomatic fish, regardless of organ. Negative controls did not yield any amplification product. Sequencing analysis of the nearly complete (1261 bp) 16S rDNA gene revealed that all the Mexican isolates studied were identical, with the obtained sequence showing 99.5% similarity with *S. iniae* ATCC 29178$^T$ (GenBank accession number DQ303187) (Figure 3). The next nearest species was *Streptococcus parauberis* NCFD 2020$^T$ (accession number AEUT02000001), *Streptococcus hongkongensis* HKU30$^T$ (accession number HQ335006), with a similarity of 98.1% and 97.9%, respectively.

Typing processes are essential for understanding pathogen outbreaks, cross-transmission, geographical and host distributions, virulence, and vaccination programmes (Olive & Bean, 1999). RAPD analysis and PCR amplification with primers specific to the repetitive genetic elements REP and ERIC are fast, simple and frequently used for the genomic fingerprinting of fish and shellfish pathogens (Romalde, 2005). In this study, apart from some variations in band intensity, no differences were observed between the two profiles for the Mexican *S. iniae* isolates, regardless of the typing method (Figure 4). These results indicate the existence of some common genetic components among tilapia *S. iniae* isolates, even though the tilapia eggs were of different origins. However, this genetic homogeneity might be the result of juvenile/adult tilapia being moved within Mexico, a practice associated with production processes at the San Luis farm. Future *S. iniae* isolates should be obtained from fish across distinct geographical areas and farms to genetically and antigenically characterize the population of this pathogen in the Mexican aquaculture industry.

Both *S. iniae* isolates were pathogenic to rainbow trout and zebrafish. The S-1 isolate respectively caused accumulated mortalities of 40% and 70% with the lowest and highest inoculum doses at 4 and 6 days post-inoculation. Regarding zebrafish, the S-2 challenge period lasted only 7 days, at which point a 100% mortality rate was recorded with the $10^2$ CFU per fish dose. Likewise, 100% zebrafish mortality was recorded at just 4 days post-inoculation with the $10^4$ CFU per fish dose. For both species, fish manifested less activity or slower swimming, colour loss during early infection and moderate body darkening during late infection. Furthermore, the majority dead rainbow trout evidenced exophthalmia. Internally, fish presented abdominal congestion and haemorrhaging. Importantly, no mortalities were recorded in either control group, which were inoculated with the culture medium.

*Streptococcus iniae* S-1 and S-2 isolates were recovered on TSA-1 plates from the brain and internal organs of dead fish for each respective species. Isolates were confirmed *S. iniae* through biochemical and PCR analyses (data not shown). The obtained findings indicate no relationship between virulence degree and *S. iniae* origin, as supported by the observed pathogenicity in both species regardless of dose. Indeed, the similarity of the zebrafish immune system to that of humans and other vertebrates has made this fish a model for evaluating various pathogenic infections, including *S. iniae* (Neely, Pfeifer, & Caparon, 2002; Saralahti & Rämet, 2015).

The obtained data clearly indicate that intensified aquaculture activities in Mexico are being accompanied by the appearance of new diseases. Notably, this is the first study to identify and characterize *S. iniae* as a causative outbreak agent in Mexican tilapia aquaculture, and future studies should establish the clinical significance for the tilapia industry.

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