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Licenciatura en Biotecnología

Informe Escrito Final

Identificación molecular de *Streptococcus* sp. y genes de resistencia a antibióticos presentes en granjas de tilapia (*Oreochromis niloticus*) de la región del Pacífico Norte, Costa Rica

Molecular identification of *Streptococcus* sp. and antibiotic resistance genes present in Tilapia farms (*Oreochromis niloticus*) from the Northern Pacific region, Costa Rica

Artículo científico presentado como requisito parcial para optar al grado de Licenciatura en Biotecnología

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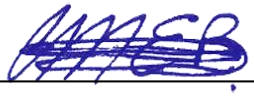

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Esta tesis la dedico a mis padres:
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Índice

Agradecimientos II
Dedicatoria III
Índice de Cuadros V
Índice de figuras VI
Índice de anexos VII
Abreviaturas o acrónimos VIII
Resumen IX
Abstract X
1. Molecular identification of *Streptococcus* sp. and antibiotic resistance genes present in Tilapia farms (*Oreochromis niloticus*) from the Northern Pacific region, Costa Rica 1
1.1. Introduction 2
1.2. Materials and methods 3
Sampling area 3
Isolation of total DNA from specific tissues 4
Detection of *S. agalactiae*, *S iniae* and antimicrobial resistance genes by endpoint PCR 4
Sequencing and taxonomic placement 5
Statistical analysis 6
1.3. Results 6
Clinical signs of tilapia 6
Isolation of total DNA in samples obtained from tilapia 7
Detection of *S. agalactiae* and *S. iniae* by endpoint PCR 7
Taxonomic sequencing and positioning of Streptococcus sp. 8
Detection of antimicrobial resistance genes by endpoint PCR and taxonomic placement analysis 9
Water quality and statistical analysis 10
1.4. Discussion 10
Clinical signs of tilapia individuals 10
Isolation of total DNA in samples obtained from tilapia 11
PCR detection of *S. agalactiae* and *S. iniae* 11
Taxonomic placement of *Streptococcus* sp. and detection of antimicrobial resistance genes 11
Water quality and statistical analysis 12
1.5. References 13
2. Conclusiones 19
3. Recomendaciones 20
4. Anexos 21

Índice de Cuadros

Cuadro 1..... 4

Cuadro 2..... 7

Cuadro 3..... 10

Índice de figuras

Figura 1 7

Figura 2 9

Figura 3 9

Figura 4 10

Índice de anexos

Anexo 1 21

Anexo 2 21

Anexo 3 22

Anexo 4 23

Abreviaturas o acrónimos

ARGs	Antibiotic Resistance-Genes
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
GBS	Group B Streptococcus
ATCC	American Type Culture Collection
TBE	Tris-Borate-Ethylenediaminetetraacetic acid
AIC	Akaike Information Criterion
ML	Maximun Likelihood
CDS	Coding Sequence
BLASTn	Basic Local Alignment

Resumen

La estreptococosis es una enfermedad bacteriana en tilapias que produce pérdidas económicas, causada principalmente por *Streptococcus agalactiae* y *S. iniae*. Se trata con oxitetraciclina y florfenicol, cuyo uso inadecuado favorece la selección de genes de resistencia a antibióticos (ARG). La enfermedad se ha asociado principalmente a eventos de estrés como variaciones de temperatura. Los objetivos del presente estudio fueron: (1) detectar por métodos moleculares dos especies de *Streptococcus* sp. en una granja de tilapia, (2) relacionar su presencia con parámetros físico-químicos en el sistema de cultivo, y (3) detectar la presencia de ARG en tejidos y / o estanques de tilapia. Se muestrearon estanques de crecimiento de tilapia (n = 30), recolectando 15 individuos por estanque. En cada estanque se midieron los parámetros físico-químicos del agua. Por estanque, se recolectaron órganos como el hígado, el bazo, el cerebro y los ojos de cada individuo. Luego, cada tipo de órgano se combinó con el órgano respectivo de los otros individuos, se procesó para la extracción de ADN y se utilizó para análisis de PCR para determinar la presencia de *S. agalactiae*, *S. iniae* y la detección de ARG (*tet*M, *tet*O, *fex*A y *erm*B). Se determinaron las correlaciones entre la presencia de *S. agalactiae* y los parámetros fisicoquímicos del agua. El sesenta por ciento de los estanques y el 46% de los grupos de órganos fueron positivos para *S. agalactiae*, mientras que *S. iniae* no se detectó. Las muestras positivas mostraron los siguientes genes de resistencia: *tet*(O) (29,1%), *tet*(M) (12,7%) y *erm*(B) (1,8%). Se encontró una correlación positiva moderada pero significativa entre la temperatura y la presencia de *S. agalactiae*. Este trabajo reportó la detección molecular de dos especies de Streptococcus y ARGs, brindando información que permite un control rápido y efectivo de estos patógenos en el cultivo de tilapia a nivel mundial. Además, futuros estudios complementarios sobre la distribución de serotipos de *Streptococcus* sp y su histopatología también podrían contribuir al conocimiento de estos patógenos.

Abstract

Streptococcosis is a bacterial disease in tilapia that produces economic losses, caused mainly by *Streptococcus agalactiae* and *S. iniae*. It is treated using oxytetracycline and florfenicol, which inappropriate use promotes the selection of antibiotic resistance-genes (ARGs). The disease has been mainly associated with stress events such as variations in temperature. The aims of the present study were: (1) to detect by molecular methods two species of *Streptococcus* sp. in a tilapia farm, (2) to relate their presence to physico-chemical parameters in the culture system, and (3) to detect the presence of ARGs in tilapia tissues and/or ponds. Tilapia grow-out ponds (n = 30) were sampled, collecting 15 individuals per pond. The physico-chemical parameters of water were measured in each pond. Per pond, organs such as liver, spleen, brain and eyes were collected from each individual. Then, each organ type was pooled with the respective organ of the other individuals, processed for DNA extraction and used for PCR analyses to determine the presence of *S. agalactiae*, *S. iniae* and for detection of ARGs (*tet*M, *tet*O, *fex*A and *erm*B). The correlations between the presence of *S. agalactiae* and water physicochemical parameters were determined. Sixty percent of the ponds and 46% of the organ pools were positive for *S. agalactiae*, whereas S. iniae was not detected. The positive samples showed the following resistance genes: *tet*(O) (29.1%), *tet*(M) (12.7%), and *erm*(B) (1.8%). A moderate but significant positive correlation was found between temperature and the presence of S. *agalactiae*. This work reported the molecular detection of two species of *Streptococcus* and ARGs, providing information that allows fast and effective control of this pathogens in tilapia farming worldwide. In addition, a future complementary studies on *Streptococcus* sp. serotype distribution and histopathology could also contribute to the knowledge of these pathogens.

1. Molecular identification of *Streptococcus* sp. and antibiotic resistance genes present in Tilapia farms (*Oreochromis niloticus*) from the Northern Pacific region, Costa Rica

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Abstract

Streptococcosis is a bacterial disease in tilapia that produces economic losses, caused mainly by *Streptococcus agalactiae* and *S. iniae*. It is treated using oxytetracycline and florfenicol, which inappropriate use promotes the selection of antibiotic resistance-genes (ARGs). The disease has been mainly associated with stress events such as variations in temperature. The aims of the present study were: (1) to detect by molecular methods two species of *Streptococcus* sp. in a tilapia farm, (2) to relate their presence to physico-chemical parameters in the culture system, and (3) to detect the presence of ARGs in tilapia tissues and/or ponds. Tilapia grow-out ponds (n = 30) were sampled, collecting 15 individuals per pond. The physico-chemical parameters of water were measured in each pond. Per pond, organs such as liver, spleen, brain and eyes were collected from each individual. Then, each organ type was pooled with the respective organ of the other individuals, processed for DNA extraction and used for PCR analyses to determine the presence of *S. agalactiae*, *S. iniae* and for detection of ARGs (*tet*M, *tet*O, *fex*A and *erm*B). The correlations between the presence of *S. agalactiae* and water physicochemical parameters were determined. Sixty percent of the ponds and 46% of the organ pools were positive for *S. agalactiae*, whereas *S. inia*e was not detected. The positive samples showed the following resistance genes: *tet*(O) (29.1%), *tet*(M) (12.7%), and *erm*(B) (1.8%). A moderate but significant positive correlation was found between temperature and the presence of *S. agalactiae*. This work reported the molecular detection of two species of Streptococcus and ARGs, providing information that allows fast and effective control of this pathogens in tilapia farming worldwide. In addition, a future complementary studies on Streptococcus sp. serotype distribution and histopathology could also contribute to the knowledge of these pathogens.

keywords: aquaculture, tilapia, streptococcosis, Antibiotic resistance genes (ARGs), endpoint-PCR, water quality.

Declarations

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Conflicts of interest

The authors declare no conflicts of interest.

Ethics approval

The animals used for this study were handled according to the Animal Welfare Law # 7451 of the Republic of Costa Rica.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

Not applicable

Author contributions

The authors contributed to the study as follows:
Karen Oviedo-Bolaños: Designed study, collected material, analysed data, and drafted paper.
Jorengeth Abad Rodríguez-Rodríguez: Collected material, analysed data, and drafted paper.
Carolina Sancho-Blanco: Obtained economic funds, collected material, analysed data, and drafted paper.
Juan Esteban Barquero-Chanto: Obtained economic funds, collected material, and drafted paper.
Nelson Peña Navarro: Obtained economic funds, analysed data, and drafted paper.
Cesar Marcial Escobedo-Bonilla: Designed study, analysed data, and drafted paper.
Rodolfo Umaña-Castro: Obtained economic funds, Designed study, analysed data, and drafted paper.
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1.1. Introduction

Streptococcosis is a bacterial disease in tilapia (*Oreochromis niloticus*) that has caused economic losses of around 400 million US dollars in China (Chen et al. 2012), one of the largest producers of tilapia in the world. In Costa Rica, tilapia is the farmed species with the greatest commercial importance (Sánchez and Cambronero 2016), which production was 16,667 metric tons in 2018, representing 80% of the total aquaculture production that year (Peña-Navarro and Chacón-Guzmán 2019).

Because of increased intensification of fish aquaculture, the stocking densities have increased, causing different types of stress and promoting the proliferation of a great diversity of microorganisms that cause diseases such as streptococcosis (Pradeep et al. 2016). In 2005, an outbreak caused losses of 2.5 million US dollars to farmers in the Northern Pacific region of Costa Rica. At that time, the causative agents were not determined with certainty (D. Salas, personal communication, 2018; MAG 2007). The greatest difficulty in combating this disease is that producers lack access to suitable diagnostic methods to accurate determine the pathogen species affecting their cultures. Overcoming this hurdle may help to apply specific and effective treatments.

Different *Streptococcus* species have been found as causative agents of streptococcosis, mainly S. *agalactiae* and *S. iniae* in Thailand (Kayansamruaj et al. 2014b; Suanyuk et al. 2008), China (Chen et al. 2012; Li et al. 2014; Ye et al. 2011), Indonesia (Anshary et al. 2014) and Malaysia (Najiah et al. 2012). In the Americas, these species have been reported in Brazil (Pretto-Giordano et al. 2010; Salvador et al. 2005) and Mexico (Huicab-Pechet et al. 2017; Ortega et al. 2018). In addition, the complete genome of an isolate of *S. agalactiae* obtained from tilapia grown in Costa Rica has been reported (Jaglarz et al. 2018) and in 2019, a molecular diagnostic analysis detected the presence of *Streptococcus* sp. in seven tilapia aquaculture farms in Costa Rica (López -Porras et al. 2019).

Both bacterial species are gram positive and have been characterized as β-hemolytic (Locke et al. 2007; Rahmatullah et al. 2017). In terms of serological classification, *S. agalactiae* belongs to Lancefield Group B (GBS) (Evans et al. 2002; Engelbrecht et al. 2017; Lancefield 1933), while *S. iniae* is not assigned to any of the groups.

Outbreaks of *Streptococcus* sp. have been associated with high stocking densities and sub-optimal water quality such as, extremely high temperatures, elevated levels of ammonium and nitrites, and low concentration of dissolved oxygen (Amal et al. 2015; Ismail et al. 2016). These factors cause stress and affect the immune response, increasing the susceptibility to opportunistic pathogens (Amal et al. 2015; Evans et al. 2003; Ndong et al. 2007).

The most widely used antibiotics for the treatment of streptococcosis are florfenicol (de Oliveira et al. 2018; Zhang et al. 2020) and oxytetracycline (Sidhu et al. 2018; Younes et al. 2019). However, continuous exposure to low doses of antibiotics favors the selection of bacteria that are resistant to multiple drugs (Rodriguez-Mozaz et al. 2015; Wu et al. 2013). This is caused by the presence of genes that confer phenotypic resistance to antibiotics, commonly referred to as Antibiotic Resistance Genes (ARGs) (Pei et al. 2006). However, despite the importance of this phenomenon, no studies have been carried out in Costa Rica to characterize antimicrobial resistance in bacteria from aquaculture production systems and in diseased tilapia.

Currently, highly specific molecular techniques such as PCR make it possible to amplify specific fragments of the genome of pathogens of interest to determine their presence in commercial cultures (Asencios et al. 2016, Cui et al. 2019, Deng et al. 2019). The PCR analysis can be carried out with isolated strains, but the main advantage is that it allows direct molecular detection of infected tissues, water samples and sediments, among others (Ortega et al. 2018; Seyfried et al. 2010).

The aim of the present study was to detect two species of *Streptococcus* sp. and antimicrobial resistance genes present in tilapia (*O. niloticus*) cultures using molecular techniques, and to associate them to physicochemical parameters present in commercial tilapia production systems, providing information that allows effective control of the pathogen.

1.2. Materials and methods

Sampling area

A commercial tilapia farm with intensive culture ponds, located in the Northern Pacific region of Costa Rica was sampled. Here, 30 ponds were randomly selected to collect fish during the months of July, November and December of 2017, and February of 2018. In this farm, streptococcosis is treated with chemical antibiotics oxytetracycline and florfenicol (D. Salas, personal communication, 2018). In each pond, 15 tilapia individuals of 800 - 900 g were randomly collected and evaluated for health and presence of clinical signs of streptococcosis. All the collected fish were subsequently anesthetized with a clove oil solution poured in a bucket filled with pond water. Fish were sacrificed and dissected for organ collection. Per pond, pools of the following organs corresponding to 15 individuals were done: liver, spleen, brain, and eyes. The organ samples were accordingly stored in hermetically sealed bags containing 10-20 mL of SSS (Sterile Saline Solution 0.85% NaCl, pH 7). The organs were macerated to homogenize the sample. The bags were transported at 4° C and stored at -20° C at the Genomic Analysis Laboratory (LAGEN), Universidad Nacional, Costa Rica, for subsequent molecular analyses. Positive controls of *S. agalactiae* (ATCC® 12386 ™) and *S. iniae* (isolation E0188-17) were used. The *S. iniae* positive control was provided by the Laboratory of Medical Bacteriology of the School of Veterinary Medicine of the Universidad Nacional of Costa Rica.

Isolation of total DNA from specific tissues

Total DNA extraction was carried out using 25 mg of each of the organ pools (eyes, spleen, brain, liver) using the NucleoSpin® Tissue commercial kit (Macherey-Nagel) by manufacturer's recommendations; RNase A (100 µg/mL) treatment was used for the removal of RNA from genomic DNA samples. The concentration of DNA extracted from each tissue was determined using a UV-visible microvolume spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA). The purity of the nucleic acids was evaluated by A260/A280 and A260/A230 ratios. To assess the integrity of the DNA, a 1% w/v agarose gel electrophoresis was done. Electrophoretic mobility was performed at 80 V for 60 minutes in 0.5X TBE, and DNA staining was performed with the GelRed (Biotium). The gel was documented using a UV light transilluminator (ENDURO, Labnet).

Detection of S. agalactiae, S iniae and antimicrobial resistance genes by endpoint PCR

Detection and identification of *S. agalactiae* was carried out by endpoint PCR amplification of the 16S rRNA gene using F1 and R1 primers (Azad et al. 2012), generating amplicons with a size of 220 base pairs (bp). In the case of *S. iniae*, identification was performed by amplifying a region of the lactate oxidase enzyme gene (lctO gene) with LOX-1 and LOX-2 primers (Rahmatullah et al. 2017) with an expected size of 870 bp. The primers ACTB-S1 and ACTB-A1 were used as internal control, which recognize the tilapia β-actin gene by amplifying a fragment of 217 bp (Yang et al. 2013).

Presence of the *tet*(M) and *tet*(O) genes (genes with the highest prevalence in aquaculture), which confer resistance to tetracyclines, was molecularly detected (Dangwetngam et al. 2016; Higuera-Llantén et al. 2018; Nguyen et al. 2017; Seyfried et al. 2010). Additionally, presence of the erythromycin resistance gene *erm*(B) and the *fex*(A) gene that confers the florfenicol resistance phenotype (Kehrenberg and Schwarz, 2006), was analyzed (Table 1).

Table 1. Primer sequences used for molecular approach by endpoint PCR of *S. agalactiae*, *S. iniae* and antimicrobial resistance genes in different tissues (eyes, spleen, brain, liver) of tilapia.

Bacterial species or antibiotic	Target gene	Primer	Sequence	Target size (bp)	Reference
Streptococcus agalactiae	*16S rRNA*	F1	GAGTTTGATCATGGCTCAG	220	(Azad et al. 2012)
		R1	ACCAACATGTGTTAATTACTC		

Streptococcus iniae	*lox*	LOX-1	AAGGGGAAATCGCAAGTGCC	870	(Rahmatullah et al. 2017)
		LOX-2	ATATCTGATTGGGCCGTCTAA		
Oreochromis niloticus	*β-actin*	ACTB-S1	TGGTGGGTATGGGTCAGAAAG	217	(Yang et al., 2013)
		ACTB-A1	CTGTTGGCTTTGGGGTTCA		
Tetracyclines	*tet*(M)	F	GTGGACAAAGGTACAACGAG	406	(Warsa et al., 1996)
		R	CGGTAAAGTTCGTCACACAC		
Tetracyclines	*tet*(O)	F	AACTTAGGCATTCTGGCTCAC	515	(Levy et al., 1999)
		R	TCCCACTGTTCCATATCGTCA		
Florfenicol	*fex*(A)	fexA-fw	GTACTTGTAGGTGCAATTACGGCTGA	1272	(Kehrenberg & Schwarz, 2006)
		fexA-rv	CGCATCTGAGTAGGACATAGCGTC		
Macrolides (erythromycin)	*erm*(B)	Fw	GAAAAGGTACTCAACCAAATA	639	(Sutcliffe et al., 1996)
		Rv	AGTAACGGTACTTAAATTGTTTAC		

Each PCR reaction was performed using Dream Taq PCR Master Mix 2X (Thermo Scientific), 0.2 µM (16S, *tetM*), 0.3 µM (*tetO*) or 0.4 µM (LOX, *ermB*) of each primer, and approximately 30 ng (bacterial detection) or 100 ng (ARGs detection) of template DNA. PCR amplification was carried out in a ProFlex thermocycler (Applied Biosystems®). The amplification profile consisted of an initial denaturation of 4 min at 94° C, followed by 35 alternating cycles of 94° C for 1 minute, primer alignment for 1 minute, and extension at 72 ° C for 2 minutes, and a final extension at 72° C for 10 minutes. Duplex PCR was also optimized for the simultaneous detection of *S. agalactiae and S. iniae*. PCR reactions were carried out maintaining the concentration of recipe and thermal profile was performed with a thermal gradient, in order to determine the optimal annealing temperature. The PCR products were resolved by 1.5% w/v agarose gel electrophoresis and staining with GelRed (Biotium). Bands with an expected molecular size were considered positive, taking as a reference the positive controls and GeneRuler 1 kb DNA ladder molecular weight marker (Thermo Scientific).

Sequencing and taxonomic placement

The identity of the amplicons obtained by PCR was confirmed by Sanger sequencing in two directions (forward and reverse) on a 3500 Genetic Analyzer, using BigDye Terminator ™ V3.1 chemistry (Applied Biosystems). For purification of the amplicons obtained by end-point PCR was carried out by precipitation with absolute ethanol and sodium acetate 3M. Subsequently, PCR reactions for sequencing were purified to remove unincorporated dye terminators, following the manufacturer's recommendations using the BigDye XTerminator Purification Kit. The sequences obtained were edited in Geneious v.R9 program (Biomatters), and were then analyzed using the BLASTn online program (Altschul et al. 1990) available from the NCBI and compared with sequences obtained from Genbank database (http://www.ncbi.nlm.nih.gov/genbank) to achieve their preliminary identity. A molecular taxonomy analysis was performed by multiple sequence alignment using the MAFFT 7.0 program (http://mafft.cbrc.jp/alignment/server), with the iterative refinement method (FFT-NS-i) and parameter 1PAM k = 2. Finally, blocks conserved with GBlocks v0.91b were selected (Castresana 2000).

The best-fit nucleotide substitution model was determined using the jModelTest v2.1.10 program (Darriba et al. 2012) by Akaike information criterion (AIC). Taxonomic placement trees were created based on the maximum likelihood (ML) algorithm of the raxmlGUI v.1.3 program (Stamatakis at al.

2005), using the general time reversible model (GTR-GAMMA). The positioning topology were obtained by editing with the FigTree v1.4 program (Rambaut 2009).

Water quality

For all ponds were determined: temperature (°C), turbidity (cm), pH, dissolved oxygen (mg/L), alkalinity (mg/L), ammonia (mg/L), nitrates (mg/L), nitrites (mg/mL), potassium (mg/L), iron (mg/L), calcium (mg/L), magnesium (mg/L) and hardness (mg/L). A portable multi-parameter device (Hanna instruments HI9828) was used to determine oxygen, temperature, and pH, while the remaining parameters were measured by a photometer (LoviBond MD 200). All parameters were measured each time organ samples were obtained. The water samples were collected at a depth of 0-20 cm below the surface in sterile 600 mL bottles (APHA 1989). The determination of all the physicochemical parameters was carried out *in situ*.

Statistical analysis

A linear correlation was performed at a confidence level of 95% using the R statistical program, to determine if there was a relationship between the presence of *S. agalactiae* per pond determined by molecular detection, and physicochemical parameters (temperature, pH and dissolved oxygen). In addition, the Spearman correlation coefficient (r) was used to determine the strength of the correlation, since the data analyzed were not normally distributed. The criteria for interpreting the strength of correlations were: 0.00-0.39 no correlation to weak correlation; 0.40-0.70 moderate correlation; and 0.70 -1.00 strong correlation (Abdullah et al. 2017; Amal et al. 2015).

To extract highly correlated variables from the data of physical-chemical parameters (temperature, pH and dissolved oxygen) and presence of *S. agalactiae*, a principal component analysis (PCA) was performed using the R statistical program and plots were generated using the same program.

1.3. Results

Clinical signs of tilapia

During sampling, external clinical signs related to streptococcosis were observed in some individuals, including erratic swimming, lethargy, unilateral and bilateral exophthalmia, melanosis of the skin, hemorrhagic lesions on the body and fins, granulomas in the brain and fibrinous layer covering internal organs such as the heart. The signs varied in severity, depending on the stage of the disease in the fish collected (Figure 1).

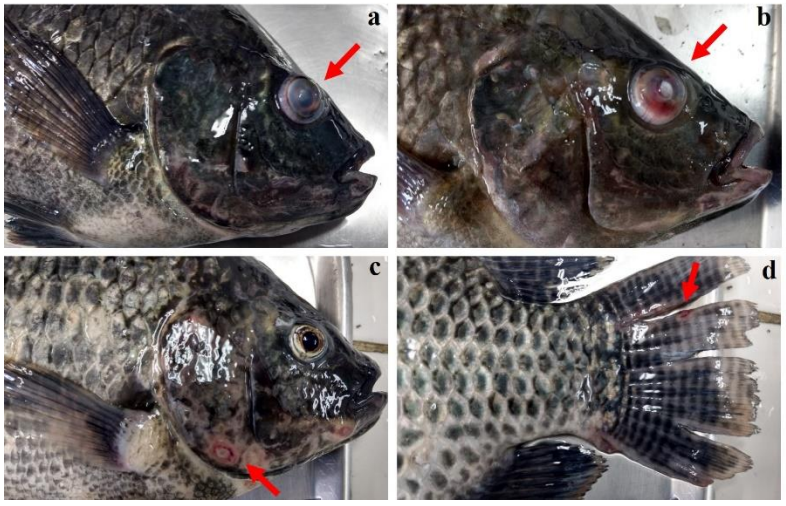

Fig. 1 The most common clinical signs compatible with streptococcosis observed in tilapia sampled: a) and b) Exophthalmia, c) Hemorrhagic lesions on skin, d) lesions and hemorrhages on fins. Arrows point to the specific injuries.

Isolation of total DNA in samples obtained from tilapia

The concentration of extracted DNA ranged from 2.9 to 591.8 ng/µL; the eyes were the organs with the lowest concentrations followed by the liver. In contrast, the spleen was the organ with the highest DNA concentrations obtained. The values of the two ratios obtained for the samples were homogeneous, with purity values of 1.8 (260/280) and 2.0 (260/230) for brain and spleen tissues, as well as values close to the standards (1.7-2.0 for 260/280 and 1.7-2.2 for 260/230) for eyes and liver. By carrying out electrophoresis of the total DNA extracted of the tissues, in organs such as the brain and spleen, high molecular weight bands without degradation were visualized, representing complete and highly concentrated DNA. Likewise, the presence of degraded DNA was observed in the cases of the eyes and liver.

Detection of S. agalactiae and S. iniae by endpoint PCR

The endpoint PCR assay was found to be highly sensitive, with a detection limit of 0.001 ng of *S. agalactiae* DNA (positive control ATCC® 12386 ™ added to total DNA from uninfected tilapia tissue). The detection limit in tilapia organs (liver, spleen, brain, and eyes) was approximately 0.01 ng of total DNA from samples infected with *S. agalactiae* (supplementary material: Figures S1 and S2), with the liver showing the clearest positive amplification products.

The detection of *S. agalactiae* was achieved by a partial amplification of the 16S rRNA region by PCR, showing amplicons with an approximate size of 220 bp. The optimal annealing temperature for duplex PCR detection was 53.2-56.8 °C, because both products were strong and clear (supplementary material, Figure S3). Of all the ponds analyzed, 60% (18/30) were positive, of which 33.3% (10/30) were found to have the bacteria in all four organs sampled, and the remaining 26.6% (8/30) were positive for at least one of the organs (Table 2).

All the 120 tissue samples obtained were analyzed, showing that 45.8% (55/120) were positive for *S. agalactiae* DNA. Of these, 30.9% (17/55) corresponded to eyes, 23.6% (13/55) to liver, 23.6% (13/55) to brain and 21.8% (12/55) to spleen. In contrast, all the samples were negative for *S. iniae*, since no amplicons of the expected molecular weight were visualized, only amplified the positive control.
The house-keeping tilapia gene (β-actin), used as an internal control, verified the integrity of the total DNA obtained. It was visualized in all the samples with the expected molecular size (217 bp).

Table 2. Endpoint PCR evaluation of the presence of *S. agalactiae* by amplifying a partial region of the 16S rRNA gene in samples from different organs of tilapia (*O. niloticus*).

Pond number	Organ			
	Eyes	Spleen	Brain	Liver
49	+	+	+	+
51	+	-	+	+
53	+	-	-	+
56	-	-	-	-
57	-	-	-	-
129	+	+	+	+
133	+	+	+	+
134	-	-	-	-
135A	+	+	+	+
135B	+	-	+	-
136A	+	-	-	-
136B	+	-	-	-

137	+	+	-	-
138A	+	+	+	+
138B	-	-	-	-
140	-	-	-	-
141A	+	+	+	+
141B	-	-	-	-
142	-	-	-	-
143A	+	-	+	+
143B	-	-	-	-
144	-	-	-	-
145A	+	+	+	+
145B	-	-	-	-
146A	+	+	+	+
146B	-	+	-	-
147A	+	+	+	+
147B	-	-	-	-
148A	+	+	+	+
148B	-	-	-	-

***Taxonomic sequencing and positioning of Streptococcus* sp.**

The BLASTn analysis revealed that sequences derived from the PCR products obtained from the molecular detection of the partial gene 16S rRNA showed a 99% similarity with the *S. agalactiae* strains accessed in the GenBank (for example: accession MH628242), while the strain used as a positive control for the detection of *S. iniae* showed a 99% similarity with a sequence of the *S. iniae* lctO gene (accession EU086702). The sequences obtained were deposited in the Genbank: 16S ribosomal RNA, partial sequence (MT560220 to MT560224), tetracycline resistance *tet*(O) gene, partial cds (MT563160 to MT563163), and *tet*(M) gene, partial cds (MT569432).

The identity of the specific amplified products of the partial 16S rRNA gene was confirmed by taxonomic placement analysis, which grouped the sequences of this study with the partial sequences of *S. agalactiae*: MF113269, MH497587, and MH628242. The topology showed that the strains analyzed are in the cluster containing the *S. agalactiae* sequences, with a branch support value of 74% (Figure 2).

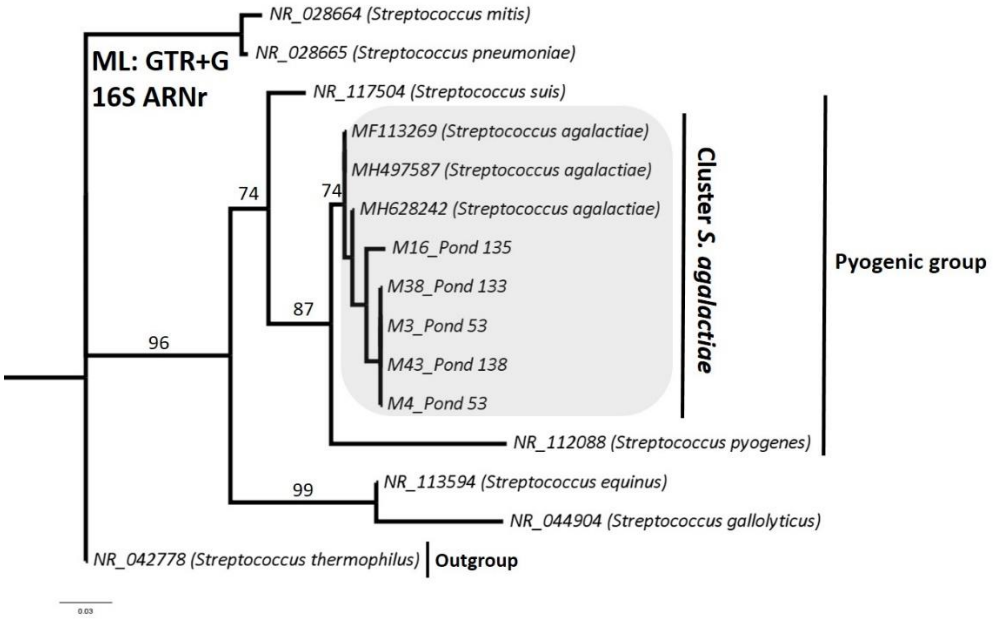

Fig. 2 Taxonomic placement topology using maximum likelihood (ML) between sequences of this study and partial sequences of the 16S ribosomal RNA gene obtained from the Genbank. The number on branches is the bootstrap value (5000 repetitions). *Streptococcus thermophilus* is defined as an external group. Accession numbers of the database sequences are shown in the terminal nodes of each branch.

Detection of antimicrobial resistance genes by endpoint PCR and taxonomic placement analysis

Of the 55 samples positive for *S. agalactiae*, 29.1% (16/55) showed the presence of the *tet*(O) gene, 12.7% (7/55) for *tet*(M) (supplementary material: Figure S4, Tables S1 and S2), and 1.8% (1/55) for the gene *erm*(B), while *fex*(A) was not detected in any sample. The sequences obtained from the detection of the ARG *tet*(O) were compared with reference sequences in the genebank, finding 97.45% to 100% identity with sequences from tetracycline resistance protein (*tet*O) gene, complete cds (for example: accessions MT383669, MH837959, MT176412). On the other hand, the amplicon generated from molecular detection of partial gene *tet*(M) shows 100% identity with the accession MT383666, corresponding to tetracycline resistance protein (*tet*M) gene, complete cds.

Because the analysis for the detection of ARGs was carried out directly on tilapia tissues, a taxonomic positioning tree was constructed to confirm the identity and bacterial source of the sequenced *tet*(O) and *tet*(M) genes. The topology of the tree showed that the M25 sequence, obtained from pond 49 for *tet*(M), was grouped in the clade that includes *S. agalactiae*, with a branch support value of 59%. However, the identity of the *tet*(M) gene is more related to other species of the genus *Streptococcus*. This also occurred with the sequences obtained for *tet*(M), which were grouped with different species of the *Streptococcus* genus including *S. agalactiae*, with a bootstrap support value of 71%. The partial *tet*(O) and *tet*(M) sequences of sample M25 and the *tet*(M) sequences of samples M16, M24, and M37 revealed the molecular identity of traces of antimicrobial resistance genes (ARGs), which suggests their presence within tilapia culture ponds identified as 49, 133, 135 and 147 (Figure 3).

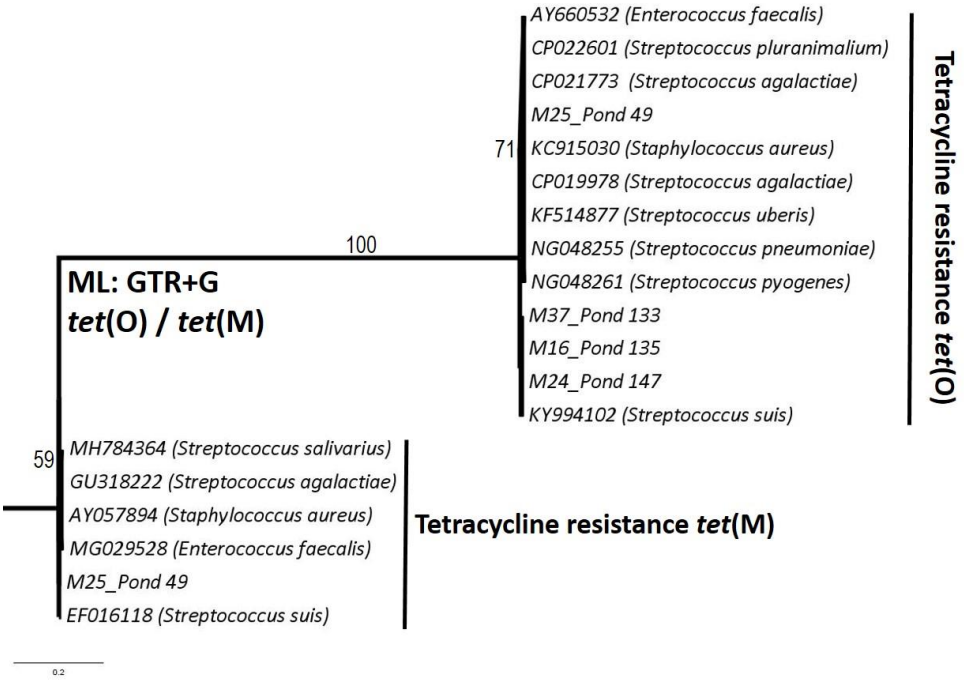

Fig. 3 Taxonomic placement topology using maximum likelihood (ML), between sequences of this study and Genbank partial sequences of the antimicrobial resistance genes *tet*(O) and *tet*(M) of different bacterial sources. The number in the branches is the bootstrap value (5000 repetitions). Accession numbers of the database sequences are shown in the terminal nodes of each branch.

Water quality and statistical analysis

The average values of temperature, dissolved oxygen and median pH are shown in Table 3. A moderate but significant positive correlation was obtained at a 95% confidence level (r = 0.6291, n = 30, P = 0.0002) between temperature and the presence of *S. agalactiae*, whereas a weak and non-significant negative correlation was found between the presence of *S. agalactiae* and pH and dissolved oxygen (Table 3). The mean values and standard deviations of the remaining parameters were as follows: ammonia (0.20 ± 0.06 mg/L), alkalinity (35.61 ± 18.04 mg/L), potassium (1.75 ± 1.20 mg/L), nitrite (0.01 ± 0.01 mg/L), hardness (28.17 ± 2.06 mg/L), phosphate (0.17 ± 0.05 mg/L) and nitrate (1.56 ± 0.61 mg/L).

Table 3. Mean and standard deviation (SD) values for the water quality parameters analyzed and their correlation with the presence of *S. agalactiae*.

Parameter	Value			Spearman's correlation coefficient	Correlation strength	P-value
	Mean ±SD	Min.	Max.			
Temperature	25.54 ±1.28 °C	23.01	27.26	0.6291	Moderate	0.0002***
pH	7.40 ±0.11	7.19	7.66	-0.0275	Weak	0.8851
Oxygen	6.39 ±1.79 mg/L	3.83	10.16	-0.2477	Weak	0.1870

Asterisks (*) indicate significant correlation: *p<0.05, ** p<0.01, *** p<0.001

The PCA results showed that the first component (dissolved oxygen) explained 50.61% of the total variance in the data set, while the second component (pH) explained a further 32.65%. Altogether, both components explained 83.27% of the variance. The eigenvalues were 2.02 and 1.30 for components 1 and 2, respectively. Figure 4 graphically shows the correlation between the vectors corresponding to temperature and the presence of bacteria (r = 0.6291).

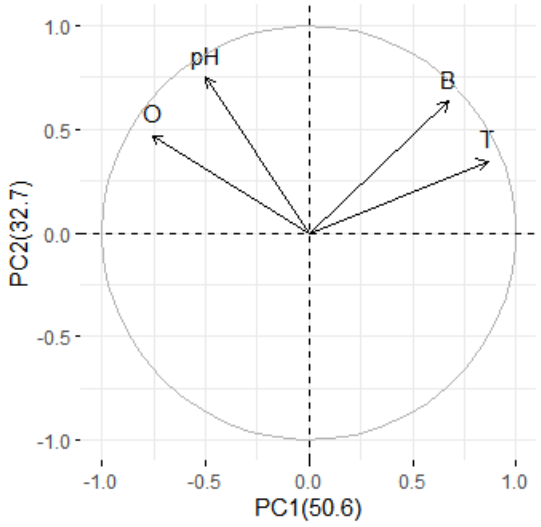

Fig. 4 Principal Component Analysis of the correlations between physico-chemical variables and presence of *S. agalactiae*. Graphical representation of the main components (PC1): dissolved oxygen and (PC2): pH, which explain 83.27% of the variance. T = temperature, B = presence of *S. agalactiae*, pH = power of hydrogen, O = dissolved oxygen.

1.4. Discussion

Clinical signs of tilapia individuals

The clinical signs displayed by the tilapia used in this study correspond to those caused by streptococcosis. These signs included: lethargy, anorexia, curvature of the body, distended abdomen, corneal opacity (unilateral or bilateral), exophthalmia, petechial hemorrhage of the skin, discolored liver, enlarged spleen and pallored gills (Abuseliana et al 2011; Al-Harbi, 2016; Anshary et al. 2014).

Isolation of total DNA in samples obtained from tilapia

The DNA extraction method successfully provided optimal DNA purity, except for some samples taken from the eyes and liver. These variations can be attributed to the composition of the organs. The eye has a large amount of pigmented epithelium – a tissue with high content of melanin granules (King-Smith et al. 2014). Also, teleost eyes have been reported to contain significant amounts of lipids such as triacylglycerols and phospholipids, which can interfere with the DNA purity (Stoknes et al. 2004). These components in the eye may have contributed to the decreased quality of DNA obtained in some samples. In the liver, nucleic acids are exposed to rapid degradation due to high levels of nucleases and other circulating enzymes, and are susceptible to contamination by bile salts, affecting DNA stability and purity (Wong et al. 2012). Some degradation of liver DNA may have occurred during dissection, nonetheless for most of the samples, an adequate DNA isolation procedure and lack of inhibitors was observed since the amplification of the fish internal control gene was successful.

PCR detection of S. agalactiae and S. iniae

The PCR technique for the detection of *S. agalactiae* was highly sensitive to amplify a partial region of the 16S rRNA gene, to a concentration of 0.01 ng of infected tilapia DNA and 0.001 ng of bacterial DNA mixed with total DNA from a healthy tilapia (Supplemental Figure S1 and S2). A lower detection limit (0.0001 ng) was reported by Rodkhum et al. (2012) for the diagnosis of *S. iniae* and *S. agalactiae* in tilapia tissues by duplex endpoint PCR.

The bacterium *S. agalactiae* DNA was efficiently detected in tissues of the eyes, spleen, brain, and liver through amplification of the 16S rRNA gene. This agrees with other studies that successfully identified *S. agalactiae* in the brain, kidney and spleen from farmed red tilapia (*Oreochromis* sp.) (Abuseliana et al. 2011). Further, diagnosis of *S. iniae* and *S. agalactiae* isolated from the liver, spleen, brain, eyes and kidney of tilapia has been reported through the use of multiplex PCR, by amplification of the *lox* gene and the 16S rRNA gene, respectively (Dangwetngam et al. 2016). The *lox* gene has also been used to directly detect the presence of *S. iniae* in infected tilapia tissues (Rahmatullah et al. 2017). Strains of *S. iniae* have also been identified and characterized directly from heart, kidney, and spleen tissues in infected tilapia by PCR amplification of the 16S rRNA gene, followed by sequencing (Ortega et al. 2018). The 16S rRNA gene has also been used for the identification and molecular characterization of both species (Jantrakajorn et al. 2014).

Hernández et al. (2009) showed that *S. agalactiae* has preference for infecting certain organs, such as the brain, eyes and heart (71.2%, 43.7% and 37.1%, respectively). The brain and eyes have been reported as target tissues, with the optic nerve identified as a transport route between these organs (Su et al. 2017). This agrees with the present results, where the highest percentage of positives (30.9%) was obtained from the eyes, followed by the brain and liver (23.6%), and, to a lesser extent, the spleen (21.8%). That is, it was detected in all sampled organs.

Taxonomic placement of Streptococcus sp. *and detection of antimicrobial resistance genes*

The genus *Streptococcus* has been divided into six taxonomic groups, based on the similarity of the 16S rRNA gene: pyogenic, anginosus, mitis, mutans, salivarius and bovis. The species *S. agalactiae* is classified within the pyogenic group (Kawamura et al. 1995; Lal et al. 2011). The same result was found with the taxonomic positioning topology of this study, in which *S. agalactiae* and *S. pyogenes* were found to be phylogenetically closer to each other than to any of the remaining species.

The taxonomic placement tree based on the 16S ribosomal RNA gene are very useful to distinguish the different species of the genus *Streptococcus*, positioning them in well-supported taxonomic clusters, except for *S. pneumoniae* and *S. mitis* species, which both have a high similarity for this gene and are consistently grouped together (Al-Harbi, 2016; Figueiredo et al. 2012; Lal et al. 2011; Piamsomboon et al. 2020). The findings of the present study agree with those of other works, taxonomically locating the 16S rRNA gene of *S. agalactiae* by means of tissues directed analyses. The present study made the taxonomic placement analysis of *S. agalactiae* from tissues of farmed tilapia using molecular techniques in Costa Rica.

On the other hand, the presence of genes that confer tetracycline resistance were found in ponds that have been treated with the antibiotics oxytetracycline and florfenicol. The proportion of samples showing the presence of the *tet*(O) gene (29.1%) was higher than the proportion of samples (12.7%) containing the *tet*(M) gene. In contrast to the results obtained in this study, Zeng et al. (2006) found a considerably higher percentage, 80.7% (413/512) of *S. agalactiae* isolates with the *tet*(M) gene, while only 4.9% (25/512) contained the *tet*(O) gene. Likewise, various authors have confirmed the presence of tetracycline resistance genes in aquaculture farms, with a higher prevalence of *tet*(M) (Dangwetngam et al. 2016; Higuera-Llantén et al. 2018; Liang et al. 2018; Nguyen et al. 2017; Suanyuk et al. 2008; Tamminen et al. 2011; Zeng et al. 2006). It is possible that this gene has global distribution since it is frequently associated with mobile elements such as transposons and plasmids (Higuera-Llantén et al. 2018). It is worth to notice that tetracycline resistance genes persist in aquaculture environments and do not disappear in the long term, long after use of antibiotics was stopped (Tamminen et al. 2011).
Similarly, studies have identified the presence of other antibiotic resistance genes such as erythromycin (*erm* genes) and macrolides (*mef* genes) in aquaculture (Zeng et al. 2006). Nguyen et al. (2017) reported 6.3% (5/79) of isolates carrying the *erm* gene (B), while Zeng et al. 2006 reported a similar proportion, 6.6% (25/512). The present study detected the presence of *erm* (B) in only one sample (1.8%), a lower proportion with respect to the *tet* genes found. This was probably due to the selection of these genes by the application of oxytetracycline, while erythromycin was not used. However, it is possible to find bacteria carrying resistance genes despite they have not been exposed to antibiotics, by acquiring plasmids or other extrachromosomal elements with ARGs (Allen et al. 2010). Also, a direct positive correlation has been shown between the concentration of residues of applied antibiotics such as oxytetracycline, tetracline and florfenicol in tissues of tilapia and resistance to multiple drugs (Monteiro et al. 2016; Rodriguez-Mozaz et al. 2015). This shows that the continuous use of antibiotics reduces their efficacy and promotes the selection of ARGs (Rodriguez-Mozaz et al. 2015). Also, reports of the presence of florfenicol resistance genes (*fex* genes) have been done in aquaculture farms (Zhang et al. 2020). Nonetheless in the present study, resistance genes for that antibiotic were not detected in the analyzed samples. In the present study, it was not possible to confirm by molecular methods that the tetracycline resistance genes detected in tilapia tissues infected with *S. agalactiae* were identical to partial fragments of ARGs from *S. agalactiae*. This could be explained because aquaculture environments have been described as reservoirs of ARGs, due to the constant application of antibiotics (Harnisz et al. 2015). Have been reported other relevant opportunistic pathogens in tilapia, in addition to *S. agalactiae and S. iniae* as carriers of antibiotics resistance (Preena et al. 2020; Wamala et al. 2018), such as: *Flavobacterium columnare*, *Edwardsiella* sp. (Huicab-Pech et al. 2017), *Aeromonas* sp. (Liu et al. 2020), *Francisella* sp. (Mauel et al. 2007), *Mycobacterium* sp. (Wamala et al. 2018). These pathogens can be detected in internal organs of infected tilapia (brain, heart, liver, reproductive organs, spleen, kidney) (Huicab-Pech et al. 2017; Preena et al. 2020), some of them cause symptoms similar to streptococcosis, such as exophthalmia and granulomas (Mauel et al. 2007; Wamala et al. 2018). In addition, other frequent bacterial communities that promote the dispersal of ARGs, through mobile genetic elements available in aquaculture environments are *Bacillus* sp. (Yuan et al. 2019), *Acinetobacter* sp., *Enterobacter* sp., *Escherichia coli* (Harnisz et al. 2015) and *Staphylococcus* sp. (Mahmoud and Abdel-Mohsein 2019). The most frequent ARGs subtypes in aquaculture are tetracycline resistance genes (*tet*) (Harnisz et al. 2015; Mahmoud and Abdel-Mohsein 2019).

Water quality and statistical analysis

The results showed a positive correlation between the presence of *S. agalactiae* and water temperature, which is consistent with reports that has shown that temperature is the main factor positively correlated with the presence and virulence of *Streptococcus* sp. (Al-Harbi, 2016; Amal et al. 2015; Suanyuk et al. 2008).

Water temperature affects the metabolic profile of the fish, reducing their capacity to survive infection (Zhao et al. 2015), and induces an increase in hemolytic activity and viability of group B *Streptococcus* (GBS), which are determining factors during the infection process. It has also been reported that many virulence factors are over-expressed at high temperatures (Kayansamruaj et al. 2014a). Another factor positively correlated with temperature is antibiotic multi-resistance. However, the cause of this correlation has not yet been established and it may also be influenced by multiple factors (Reverter et al. 2020).

A similar study determined the association between water quality in tilapia culture ponds and the presence of *S. agalactiae*. It found a significant positive correlation between the temperature, clarity and pH of the water, and presence of bacteria in cultured fish (Amal et al. 2015). In the present study, only temperature was strongly correlated with bacterial presence, while for the other two parameters the correlation was moderate.

In conclusion, the present study determined the presence of *S. agalactiae* and the absence of *S. iniae* in samples of tilapia in the Northern Pacific region of Costa Rica. Based on the analysis of various tissues (liver, spleen, brain and eyes), *S. agalactiae* was most frequent in ocular tissue. The statistical analyses showed that temperature was the main physicochemical parameter positively correlated with the presence of *S. agalactiae* in tilapia culture ponds. Molecular analyses from infected tissues taxonomically placed *S. agalactiae* within the pyogenic group, providing molecular identity. The presence of tetracycline resistance genes from tilapia tissues identified with *S. agalactiae* DNA were recorded in Costa Rica. However, as future prospects, complementary studies on *Streptococcus* sp. serotype distribution and antibiotic resistance-genes from tilapia cultured in Costa Rica could contribute to the knowledge of *S. agalactiae* and *S. iniae* infections in tilapia farming worldwide.

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2. Conclusiones

El presente estudio determinó la presencia de *S. agalactiae* y la ausencia de *S. iniae* en muestras de tilapia en la región del Pacífico Norte de Costa Rica. Según el análisis de varios tejidos (hígado, bazo, cerebro y ojos), *S. agalactiae* fue más frecuente en el tejido ocular. Los análisis moleculares de tejidos infectados colocaron taxonómicamente a *S. agalactiae* dentro del grupo piogénico, proporcionando identidad molecular.

Los análisis estadísticos mostraron que la temperatura fue el principal parámetro fisicoquímico correlacionado positivamente con la presencia de *S. agalactiae* en estanques de cultivo de tilapia, debido a que este factor podría causar estrés y afectar la respuesta inmune, además puede afectar el perfil metabólico del pez, aumentando la susceptibilidad a patógenos oportunistas,

También se registró la presencia de genes de resistencia a tetraciclina de tejidos de tilapia identificados con ADN de *S. agalactiae*, en estanques tratados con oxitetraciclina y florfenicol. Esto demuestra que el uso continuo de antibióticos promueve la selección de ARG, lo cual probablemente reduzca su eficacia. Además pueden existir otras comunidades bacterianas que pueden contribuir a la dispersión de ARGs en las granjas acuícolas.

En conclusión, este trabajo reportó la detección molecular de dos especies de *Streptococcus* y ARGs, brindando información que permita un control rápido y efectivo de estos patógenos en el cultivo de tilapia a nivel mundial. Sin embargo, como perspectivas de futuro, los estudios complementarios sobre a distribución de serotipos de *Streptococcus* sp. y los genes de resistencia a los antibióticos de la tilapia cultivada en Costa Rica podrían contribuir al conocimiento de las infecciones por *S. agalactiae* y *S. iniae* en el cultivo de tilapia alrededor del mundo.

3. Recomendaciones

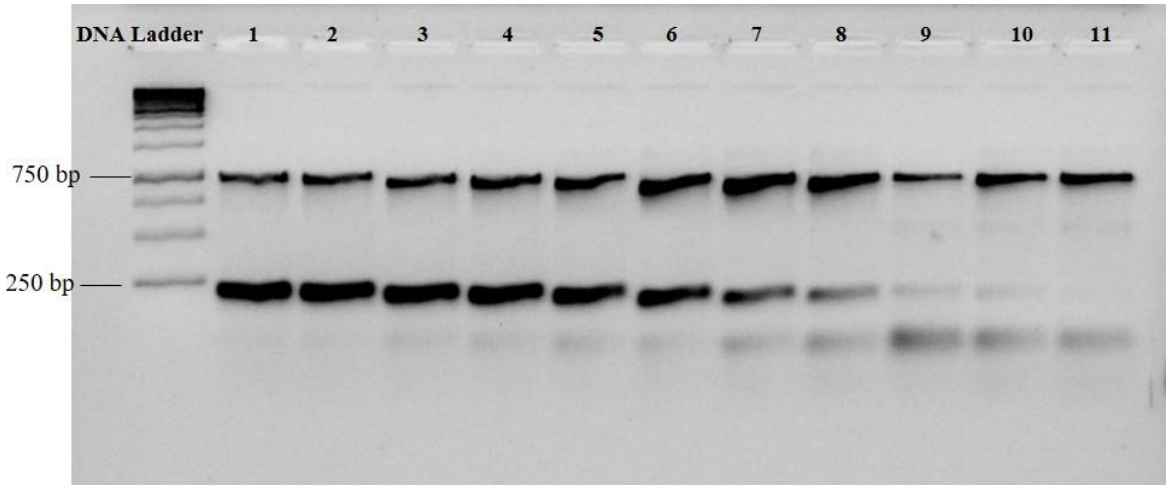
Se recomienda realizar el aislamiento de *S. agalactiae* y *S. iniae* a partir de los tejidos de tilapias, con el fin de contar con aislados para pruebas de susceptibilidad microbiana hacia los antibióticos que se utilizan más frecuentemente en acuicultura en el país. Para complementar y comparar estos resultados con los datos de la detección molecular de genes de resistencia. También se podría realizar análisis de expresión de estos genes de resistencia, para comprobar que realmente se están expresando estos marcadores.

Además, es recomendable, realizar estudios de serotipificación de *S. ag*alactiae, de la cual se conoce que existen dos serotipos que causan el brote de la enfermedad en la tilapia. Con esta información se podría aportar datos relevantes para que los productores puedan diseñar un programa de vacunación de protección cruzada a los peces.

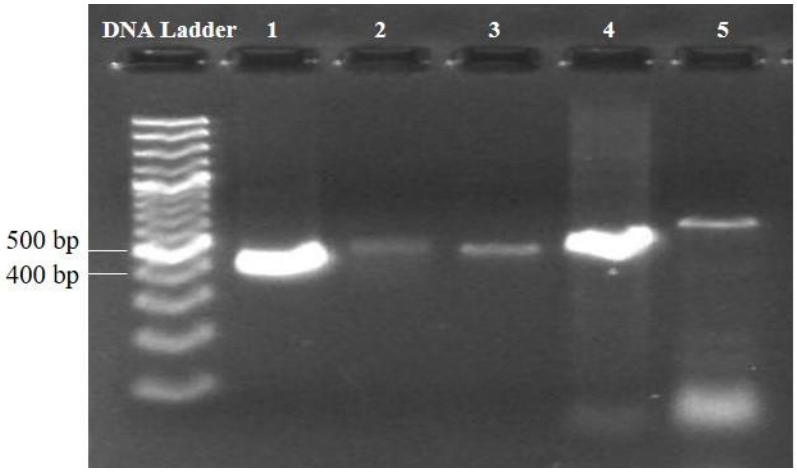
Como perspectivas a futuro, se considera importante utilizar la histopatología, ya que es una herramienta de diagnóstico útil para estudios clínicos asociados a enfermedades en peces.

Este estudio fue del tipo exploratorio, por lo tanto, es relevante dar continuidad mediante la ampliación del muestreo a otras regiones de producción de tilapia del país. Con el propósito de obtener datos de la distribución de estos patógenos y los genes de resistencia.

4. Anexos



Anexo 1. Ensayo de optimización por PCR dúplex para la detección de *S. agalactiae* y *S. iniae*, con controles positivos. Los carriles muestran diferentes temperaturas de hibridación probadas: 50, 50,8, 51,8, 53,2, 54,9, 56,8, 58,6, 60,0, 61,1, 61,7 y 62 ° C. Escalera de ADN GeneRuler de 1 kb (Thermo Scientific).



Anexo 2. Ensayo de PCR de punto final para la detección de genes de resistencia a los antimicrobianos: *tet*(M) (muestras 1, 2, 3 y 4) y *tet*(O) (muestras 4 y 5). Los carriles muestran algunas muestras representativas: 1 = estanque 133 cerebro, 2 = estanque 51 ojos, 3 = estanque 133 ojos, 4 = estanque 136A bazo, 5 = estanque 49 ojos. Escalera de ADN GeneRuler 100 bp Plus (Thermo Scientific).

Anexo 3. Evaluación de la presencia del gen *tet*(O) por PCR punto final mediante la amplificación de una región parcial en muestras de diferentes órganos de tilapia (*O. niloticus*)

Pond number	Organ			
	Eyes	**Spleen**	**Brain**	**Liver**
49	+	-	-	-
51	-	-	-	-
53	-	-	-	-
56	-	-	-	-
57	-	-	-	-
129	+	+	+	-
133	+	-	+	+
134	-	-	-	-
135A	-	-	-	+
135B	-	-	-	-
136A	-	-	-	-
136B	-	-	-	-
137	-	-	-	-
138A	+	+	-	-
138B	-	-	-	-
140	-	-	-	-
141A	-	-	-	-
141B	-	-	-	-
142	-	-	-	-
143A	-	-	-	+
143B	-	-	-	-
144	-	-	-	-
145A	-	-	-	-
145B	-	-	-	-
146A	-	+	+	+
146B	-	-	-	-
147A	-	-	+	+
147B	-	-	-	-
148A	-	-	-	-
148B	-	-	-	-

Anexo 4. Evaluación de la presencia del gen *tet*(M) por PCR punto final mediante la amplificación de una región parcial en muestras de diferentes órganos de tilapia (*O. niloticus*)

Pond number	Organ			
	Eyes	Spleen	Brain	Liver
49	+	-	-	-
51	+	-	-	+
53	-	-	-	-
56	-	-	-	-
57	-	-	-	-
129	-	-	-	+
133	+	-	+	-
134	-	-	-	-
135A	-	-	-	-
135B	-	-	-	-
136A	-	-	-	-
136B	-	-	-	-
137	-	-	-	-
138A	-	-	-	-
138B	-	-	-	-
140	-	-	-	-
141A	-	-	-	-
141B	-	-	-	-
142	-	-	-	-
143A	-	-	-	-
143B	-	-	-	-
144	-	-	-	-
145A	-	-	-	-
145B	-	-	-	-
146A	-	-	-	-
146B	-	+	-	-
147A	-	-	-	-
147B	-	-	-	-
148A	-	-	-	-
148B	-	-	-	-