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Oreochromis niloticus shows a higher prevalence of Beta-haemolytic streptococci when are maintained in cages than in ponds

Oreochromis niloticus exhibe una prevalencia mayor de Streptococcus beta hemolítico cuando se mantienen en jaulas en comparación con estanques

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ABSTRACT

Positive cases of *Streptococcus* in tilapia cause serious economic losses, however, there are no reports on the prevalence of Streptococcus in tilapia farmed within ponds and cages in the tropics, this is the first report about it. The goal was to compare the prevalence of beta-haemolytic streptococci in tilapia (*Oreochromis niloticus*) from pond and cage culture farming, in Chiapas, Mexico, also, evaluate the susceptibility to antibiotics. 155 fish between 250-300 gr were collected in 31 farms (12 ponds and 19 cages). The highest prevalence (45.16%, $P<0.05$) of beta-haemolytic streptococci was in fish with clinical manifestations from cages, compared to fish from ponds. *S. agalactiae* and *S. alactolyticus* were identified. Signs in fish infected with *Streptococcus agalactiae* included exophthalmia, ascites, cerebral congestion, hemorrhages, and melanization. 100% of the *S. agalactiae* strains were susceptible to beta-lactams, clindamycins, among others. There is a higher prevalence of beta-haemolytic streptococci in fish farming in cages compared to pond culture. The information generated in this study favors the implementation of sanitary strategies to optimize tilapia farming in tropical regions.
Keywords: pond culture, cage culture, prevalence, beta-haemolytic streptococci, *Oreochromis niloticus*.

RESUMEN

Los casos positivos a *Streptococcus* en tilapia ocasionan graves pérdidas económicas, sin embargo, no hay informes sobre la prevalencia de *Streptococcus* en tilapia cultivada en estanque y jaula en el trópico, este es el primer informe al respecto. El objetivo fue comparar la prevalencia de *Streptococcus* beta hemolíticos en tilapia (*Oreochromis niloticus*) procedentes de cultivos en estanque y jaula, en Chiapas, México, además, evaluar la susceptibilidad hacia los antibióticos. Se recolectaron 155 peces entre 250-300 gr en 31 granjas (12 estanques y 19 jaulas). La mayor prevalencia (45.16 %, $P<0.05$) de *Streptococcus* beta hemoliticos., fue en peces con manifestaciones clínicas en jaulas, comparado con peces de

estanques. Se identifico principalmente a *S. agalactiae* y *S. alactolyticus*. Los signos en peces infectados por *Streptococcus agalactiae* fueron exoftalmia, ascitis, congestión cerebral, hemorragias y melanización. El 100% de las cepas de *S. agalactiae* fueron susceptibles a betalactámicos, clindamicinas, entre otros. Existe mayor prevalencia de *Streptococcus* spp., en los peces mantenidos en jaula comparado con el cultivo de estanque. La información generada en este estudio favorece a la implementación de estrategias sanitarias para optimizar la cría de tilapia en las regiones del trópico.
Palabras claves: cultivo tipo estanque, cultivo tipo jaula, prevalencia, *Streptococcus* beta hemolítico, *Oreochromis niloticus*.

INTRODUCTION

Globally, tilapia (*Oreochromis niloticus*) is the second most farmed fish after carp (Ng & Romano, 2013) and it is a food with great acceptance in the local and international market. In Mexico, tilapia plays a fundamental role as food and economic income for the families that produce it, this has intensified tilapia production, increasing aquaculture systems from 4% in 1950 to 52 % in 2018 (Berger, 2020). Chiapas is one of the main tilapia producing regions in Mexico and has four of the main hydroelectric dams in the nation, which houses the largest tilapia producers; in 2020, it reached almost 31 thousand tons of product, followed by other producing regions with less than 9 thousand tons (SADER, 2021). However, during tilapia farming, different bacteria causing infections with fatal consequences have been identified, among these are *Aeromonas hydrophila*, *Francisella noatunensis* subsp. *orientalis*, *Flavobacterium columnare*, *Vibrio vulnificus*, *Streptococcus iniae* and *Streptococcus agalactiae* (group B Streptococcus, GBS) (Dangwetngam *et al*., 2016).

Streptococcosis is one of the major infectious diseases threatening tilapia farming globally and is caused by various Streptococcus species such as *S. iniae*, *S. agalactiae*, *S. parauberis*, *S. difficile*, *S. shiloiy* and *S. dysgalactiae*. *S. agalactiae* and *S. iniae* species stand out for their high incidence (Jantrakajorn *et al*., 2014; Pradeep *et al*., 2016; Mishra *et al*., 2018). Many *Streptococcus* species are pathogenic to other hosts and has been considered as an important causative agent of zoonoses (Sun *et al*., 2016). In humans, *S. iniae* can cause cellulitis, bacteremia, septic arthritis, meningitis and endocarditis, while *S. agalactiae* can cause meningitis and pneumonia (Mishra *et al*., 2018). Streptococcosis caused by the beta hemolytic group, is a multifactorial disease with high mortality of fish close to commercialization, i.e. fish weighing more than 200 g and close to a commercial size (Chu *et al*., 2016). In aquaculture systems the negative economic effect due to acute infections is quite high, for example, worldwide commercial losses of 250 million dollars were reported, as well as mortality above 50 % during 2008 (Osman *et al*., 2017). It is known that the development of the disease and the appearance of symptoms depend largely on the type of strain, serotype, host diversity, age, immune status of the fish and environmental conditions (Chu *et al*., 2016). The incidence of *Streptococcus* spp. cases is subject to certain times of the year influenced by stress generated by inadequate management and natural interactions that could generate an inappropriate environment, these variants have the potential to compromise the physiology in fish and develop severe infection (UICN, 2007; Huicab *et al*., 2016). Counteracting this imbalance by antibiotics is the main option; however, this causes environmental effects by residues in the water, resistance of pathogenic microorganisms that affect fish and human health (Dangwetngam *et al*., 2016).

Recurrent cases of mortality in tilapia prompted the interest in identifying the prevalence and presentation of bacteria, mainly *Streptococcus* spp. being one of the bacterial genera with species reported in tropical areas (Ortega *et al*., 2019). The objective of this study was to compare the prevalence of beta-hemolytic *Streptococcus* in tilapia (*Oreochromis niloticus*) cultured in cages and ponds, from farms located in Chiapas, Mexico. Additionally, the aim was to find out if the Streptococcus strains found are related to antibiotic resistance.

MATERIAL AND METHODS

Study population

The work was carried out from winter 2018 to autumn 2020. Tilapia were acquired from 31 farms in the main bodies of water located in Chiapas, Mexico. Samples were taken from 19 floating cages maintained in the main dams known as Malpaso, La Angostura, Peñitas and Lagunas de Catazajá, located in Mezcalapa, Ostuacán, Ocozocoautla de Espinoza and Catazajá municipalities respectively. Fish were also sampled from 12 farms with ponds, located in Comitán, Palenque, Tzimol, Tecpatán, Reforma, Trinitaria and Chiapa de Corzo municipalities From these fish, 95 were from cage systems (34 apparently healthy fish and 61 with clinical signs), while 60 fish were from pond systems (46 apparently healthy and 14 with clinical signs). The fish were kept in concrete or geomembrane ponds and the cages were made of galvanized steel. Clinical signs of the selected fish were erratic swimming, exophthalmia, ascites, and superficial wounds under the fins, small areas of scaling to extensive inflammatory and ulcerative areas.

Euthanasia of tilapia was in accordance with the principles and procedures for the humane slaughter of domestic and wild animals in Mexico (NOM-033-SAG/ZOO-2014). The fish study was evaluated and was authorized by the bioethics commission of the University of Sciences and Arts of Chiapas (Oficio No. LMEB/010718).The fish were extracted from the culture sites and kept alive until the moment of sacrifice, following the technique of Rey *et al*, (2002) the fish were desensitized by means of a cut in the spinal cord. For bacterial isolation, a fragment of liver, spleen, brain, heart and kidney tissue was collected. The samples were collected in a 1000 µl Eppendorf tube with sterile brain heart infusion broth (BHI) for transport. Once in the laboratory, the suspension was left to incubate in constant agitation at 30 °C for 24 hours.

Isolation and identification

Samples were inoculated on 5 % ram blood agar (BD-BBL) and were incubated at 30 °C for 24 hours. Grayish-white, circular, whole-bordered colonies surrounded by a transparent halo from total erythrocyte hemolysis were selected. Additionally, colonies with distinct phenotypes suggestive of other Gram-positive cocci and Gram-negative bacteria were identified. Secondary identification tests such as Gram staining technique, CAMP test and catalase were used. The Vitek 2 automated microbial identification system (bioMérieux) was used for final identification.

Antimicrobial susceptibility analysis

The minimum inhibitory concentration (MIC) technique was used through the Vitek 2 automated microbial identification system (bioMérieux) for 14 antibiotics: Benzylpenicillin (PCG), Ampicillin (AMP), Cefotaxime (CTX), Ceftriaxone (CRO), Levofloxacin (LVX), Moxifloxacin (MXF), Erythromycin (ERY), Clindamycin (CLI), Linezolid (LZD), Vancomycin (VAN), Tetracycline (TET), Tigecycline (TGC), Chloramphenicol (CHL) and Trimethoprim/sulfamethoxazole (SXT). The MIC values obtained were compared with the standards established by the Clinical Laboratory Standards Institute (CLSI).

Statistical analysis

Significant associations between the prevalence of bacterial-positive fish and risk factors were determined by univariate analysis using the Chi-square test (Fisher's exact test when the expected frequencies were below 5). A value of $P< 0.05$ was considered statistically significant. The analysis was performed using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, USA).

RESULTS

Streptococcus beta hemolyticus was identified in almost half of the farms with cages (45.16 %, $P<0.05$), this universe represented the highest number of farms with dead fish (32.3 %) and clinical signs (35.48 %), compared to farms growing fish in ponds (Table 1).

Table 1. Proportion of beta-hemolytic *Streptococcus* beta hemolyticus in tilapia culture farms

Infrastructure	Water source	Farms N total	Farms Positive	Farms Mortality	Farms signs	ID
Floating cage	surface	19	14(45.16 %) *	10 (32.3 %) *	11(35.48 %) *	*S. agalactiae* *S. alactolyticus*
Pond	Surface/ subterranean	12	2 (6.45 %)	1 (3.23 %)	1(3.23 %)	*S. agalactiae*
Total	2	31	16 (51.61 %)	11 (35.48 %)	12 (38.71 %)	2

* Statistically significant difference (P<0.05)

From 155 tilapia collected, 28.3 % (44) showed bacterial growth corresponding to beta-hemolytic *Streptococcus*, mainly *Streptococcus agalactiae* and *Streptococcus alactolyticus*, the latter in only one case.

In caged fish, *Streptococcus agalactiae* positive cases represented 40 % (38/95), specifically 47.5 % (29/61) in fish with clinical signs and 26.4 % (9/34) in asymptomatic fish.

In pond, 10 % (6/60) of the fish were positive for *Streptococcus agalactie*, of which the bacterium was isolated in 28.5 % (4/14) with clinical signs and in 4.3 % (2/46) of the asymptomatic fish, (specimens did not show signs and lesions suggestive of streptococcosis).

Signs in fish infected in situ were erratic swimming, distension, exophthalmia, while pathological examination showed ascites, cerebral congestion, hemorrhages, melanization, ulcers and necrosis at the base of the caudal peduncle (Figure 1). Conjunctively in organs, there was enlargement and microscopic changes of fibrinous-granulomatous type (Figure 2).

Bacterial cultures revealed *Streptococcus* beta hemolyticus in the organs, mainly *Streptococcus agalactie*. In the cage-type system a higher frequency of bacteria was isolated from organs, brain was the most recurrent region with 11 % (17), followed by kidney 10 % (16), liver 9.6 % (15), spleen 6 % (10) and heart 6 % (10).

In the organ samples obtained from fish in a pond system, *Streptococcus beta-hemolyticus* isolates were in lower proportion, 2 % in the spleen and 1 % for the other organs.

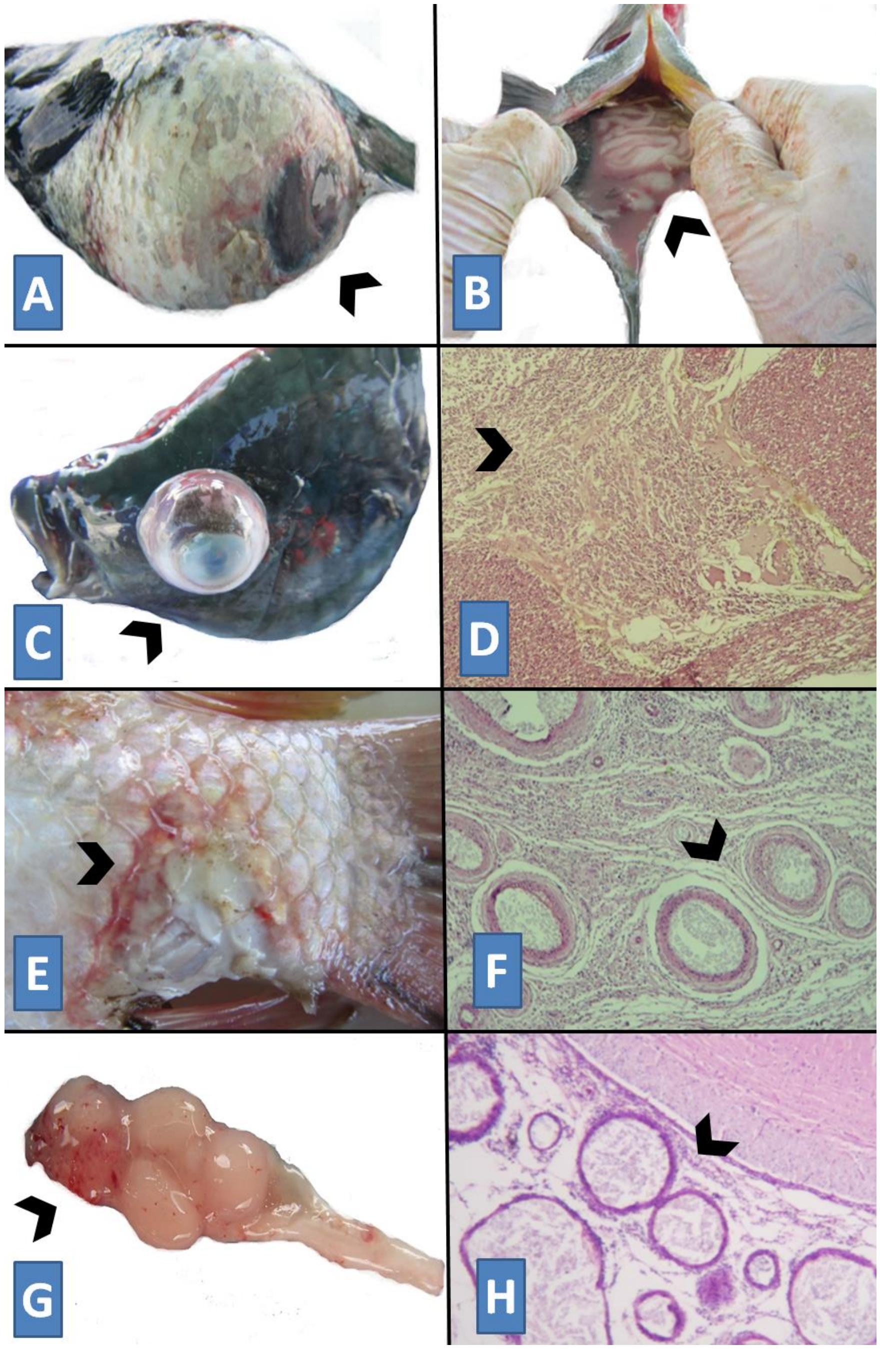

Figure 1. Tilapia. Arrowheads indicate macroscopic and microscopic findings. A. Distention of the coelomic cavity due to ascites B. Peritoneal fibrinous cellomitis C. Eye with exophthalmia, hemorrhage and corneal opacity D. Severe diffuse lymphoplasmacytic panophthalmitis E. Ulceration, hemorrhage and caseous necrosis at the base of the caudal peduncle F. Severe multifocal fibrinous-granulomatous dermatitis and myositis G. Brain with hemorrhage and diffuse fibrinous exudate H. Severe diffuse diffuse fibrino-granulomatous meningoencephalitis. Hematoxylin-eosin stain, 10X-40X. *Streptococcus agalactie* positive culture

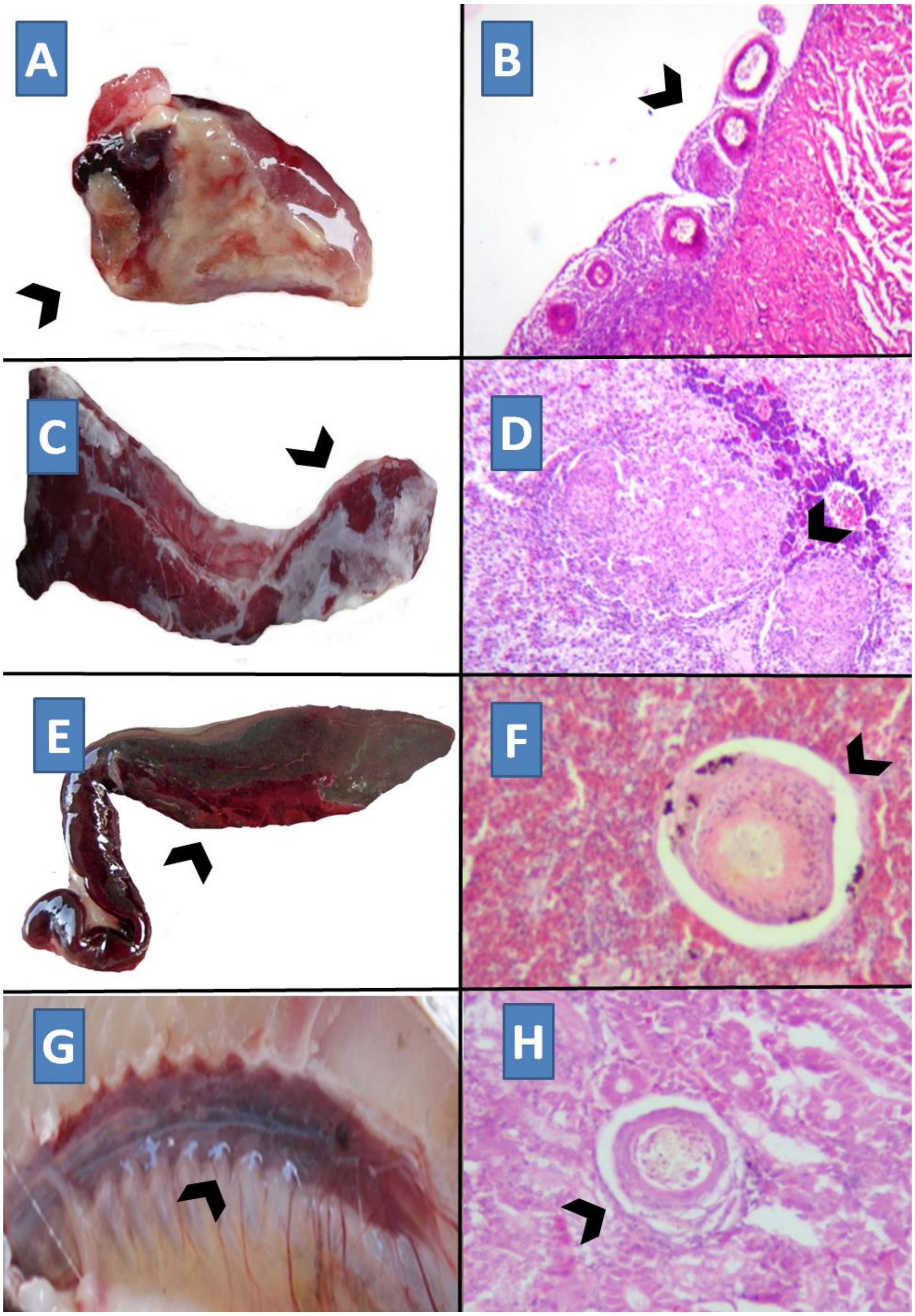

Figure 2. Tilapia. Arrowheads indicate macroscopic and microscopic findings. A. Cardiomegaly and pericardium with severe diffuse fibrinous exudate B. Severe diffuse diffuse fibrinous-granulomatous pericarditis C. Severe diffuse fibrinous-hemorrhagic hepatomegaly D. Severe multifocal granulomatous granulomatous hepatitis E. Severe diffuse fibrinous splenomegaly F. Severe multifocal granulomatous splenitis G. Severe multifocal nodular nephromegaly H. Severe multifocal granulomatous posterior nephritis. Hematoxylin-eosin stain, 10X-40X. Culture positive for *Streptococcus agalactie*

In cages, the course of the disease was acute, while in ponds the lesions were less evident, with a chronic and even asymptomatic course.
The sites where tilapia farms are located in Chiapas represent one of the main water resources of the Mexican tropics, located in the Grijalva river basin. Considering this criterion, the presence of *Streptococcus* beta hemolyticus was observed more frequently in farms with cages that are recurrently located inside dams (45.16 %, P<0.05), compared to the frequency of *Streptoccoccus* beta hemolyticus in fish kept in ponds (6.45 %), which capture water from rivers and wells for filling (Table 2).

Table 2. Prevalence of *Streptoccoccus* beta hemolyticus as a function of water bodies

Water source	Infrastructure	Water body	Farms total	Farms Positives
Surface	Cage	Hydroelectric dam	19	14 (45.16 %)*
		River	4	0
Subterranean	Pond	Well	8	2 (6.45 %)

* Statistically significant difference (P<0.05)

Antibiotic susceptibility testing

In response to antibiotics, *S. agalactiae* was sensitive to most of the antibiotics tested: benzylpenicillin, ampicillin, cefotaxime, ceftriaxone, levofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, vancomycin, tetracycline, tigecycline, and chloramphenicol. However, for all *S. agalactiae* strains, inhibition concentrations of up to 10 µg/ml of trimethoprim/sulfamethoxazole (SXT), followed by 2 µg/ml with linezolid (LZD) and chloramphenicol (CHL) were required. In the case of *S. alactolyticus* they presented sensitivity values for benzylpenicillin, ampicillin, cefatoxime, levofloxacin, moxifloxacin, clindamycin, vancomycin and tigecycline. However, it demanded concentrations of 16 µg/ml with tetracycline (TET) and 8 µg/ml with erythromycin (ERY).

DISCUSSION

In aquaculture, *S. agalactiae* is one of the most reported pathogens in tilapia, its isolation in open environments is linked to regions with thermal fluctuations, causing up to 60 % mortality due to acute infections (Yuasa *et al*., 2008), in tropical regions the average temperatures in summer range from 12 to 27 °C while in winter from 6 to 22 °C. The severity of the infection is determined by the strain and its virulence; up to 80 % mortality has been reported for aggressive strains (Sudpraseart *et al*., 2021). In this study, Streptococcus beta hemolyticus was evidenced as the bacterium associated with visible infection pictures, with a farm mortality rate of 35. However, in cages and ponds, *Streptococcus* beta hemolyticus positive fish were observed. There were no cases of mortality, even in farms without sanitary problems, however, these were less frequent, Therefore, the possibility of infections caused by *Streptococcus* beta as secondary agents to stress caused by other pathogens such as parasites or viruses, poor nutrition, mechanical damage, among many others, cannot be ruled out. In tilapia is influenced by multiple factors and is unrelated to the presence of the bacterium alone (Amal & Zamri, 2011; Mishra *et al*., 2018). The virulence of *S. agalactiae* has been related to environmental conditions, the disease in fish from regions with temperatures below 30 °C has presented the asymptomatic form as opposed to temperatures above 30 °C where fish evidenced a clinical course of disease, mortality and a severe inflammatory response (Chu *et al*., 2016). Virulence gene profiling can also be a cause of asymptomatic cases, differences between gene clusters that are linked to in apparent *S. agalactiae* infection in adult tilapia have been reported (Sun *et al*., 2016), so more precise morphological identification of the genetic qualities of these pathogens is suggested. The present study was carried out in a tropical region in southern Mexico, with a warm humid climate in more than half of its territory and an average annual temperature of 30 °C, reaching maximum water temperatures above 30 °C, which enhances periodic cases of streptococcosis, accompanied by lesions and mortality in adult animals close to harvest. The high presence of *Streptococcus* spp. in cages located in the dams could be attributed to the speed of the water current. It changes significantly throughout the year; in addition, keeping these volumes of liquid in storage causes the aquatic habitat to be influenced by the speed of water dragging from low to no speed (Ramos & Montenegro, 2012; Mohammad *et al*., 2015). These changes can exacerbate eutrophication processes, causing oxygen depletion during the first hours of the day (FAO, 2012), a situation that predisposes to a vulnerable and critical scenario for fish, favoring mortality. Due to this, monitoring of the area is suggested to identify the optimal and critical periods for fish farming. On the contrary, those pond culture sites are less vulnerable and this is in agreement with our results. This study showed that tilapia cultured in floating cages are usually more vulnerable than those in ponds to *Streptococcus* spp. infections, which causes uncertainty due to environmental changes that may occur and increase fish health problems, so constant sanitary monitoring is necessary. Infectious cases in caged tilapia are often linked to more than one pathogen, among the most frequent are *S. agalactiae*, *S. iniae* (Ortega *et al*., 2017), *Enterococcus* sp, (Sandoval *et al*., 2013) and *L. garvieae* (Chu *et al*., 2016; Anshary *et al*., 2014). The latter induces clinical signs similar to *Streptococcus* spp. and has been recovered from suspected streptococcosis outbreaks (Bwalya *et al*., 2020).

S. alactolyticus is an atypical species of *Streptococcus* in tilapia. This species is described within the *S. bovis*/*S. equinus* complex (SBSEC) that is almost exclusively associated with domestic animals, as reported in the microbiota of pigs, chickens, pigeons and canines, in humans it has been isolated between 29-55 % of individuals with inflammatory bowel disease, colon cancer and endocarditis (Almeida *et al*., 2016). *S. alactolyticus* strain reported in this work was obtained from a lesion with purulent content in the direction of the caudal peduncle, which can be attributed to a possible transmission of the pathogen by contact of fish feces floating in the water. This suggests an important route of transmission to susceptible fish together with fluctuating water and environmental parameters, in addition to a saturated population load that induce a vulnerable state of the fish and the beginning of an infectious outbreak, without underestimating its potential response to antibiotics, with a predisposition to resistance that limits treatment options.

Cases positive for *Streptococcus* spp in fish describe a variety of signs that demonstrate the disease lethality in its systemic extent. Erratic swimming, exophthalmia, corneal opacity, hemorrhage, hemorrhagic cerebral congestion and ascites are evidence of the pathogen presence, followed by petechiae, lethargy, inappetence, anal prolapse, cachexia, melanization, skin ulceration; internally, organ enlargement and congestion, meningitis, adhesions, whitish material in the heart and other histopathological findings are described (Hernandez, 2009; Laith, 2017; Legario *et al*., 2020; García, 2021; Sudpraseart *et al*., 2021). The appearance of signs and severity of cases can vary according to the type of disease presentation either acute or chronic (He *et al*., 2021). The increase in environmental temperature plays a fundamental role in the speed of appearance of lesions in fish, this was demonstrated in Nile tilapia inoculated with *Streptococcus agalactiae* and subjected to temperatures higher than 30 °C, since the time of appearance of lesions was reduced and contributed to high mortality rates (Marcusso *et al*., 2015).

In the bacteriological analysis of fish organs cultured in cages, an infection that reaches the brain was evidenced, a situation that limits the motor actions of the fish, and predisposes that the drugs administered by the feed are not effective, since due to the course of the disease the fish stop feeding. On the other hand, in fish cultured in ponds the disease prevailed in spleen, in this sense the most vulnerable fish are separated, monitored and a great part of them manage to recover. Prevention has always been the best option to reduce the incidence of pathogens; however, in cases of high fish mortality, the use of antibiotics has become the treatment option. In aquaculture, the use of antibiotics is reduced mainly to the pharmacological families of amphenicols, fluoroquinolones and tetracyclines. In reference to these families, MIC analysis revealed the effectiveness to inhibit bacteria with most of the antibiotics tested, mainly chloramphenicol (amphenicol), levofloxacin, moxifloxacin (fluoroquinolones), in contrast, a resistance of S. alactolyticus to tetracycline was observed. A worrying variation of inhibitory concentrations was evidenced that may suggest long-term resistance, so further work is suggested to understand more about this phenomenon. In this sense, *S. alactolyticus* is referenced as part of the microbiota; drugs that enter through the feed exert greater interaction with the microorganisms that inhabit the intestine. It could reduce the effectiveness of the antibiotic at recommended doses and increase the risk of dissemination of resistant bacteria when organic matter is released into the water, however, further studies are suggested in this regard.

Although cages allow higher production, they also lack stable parameters due to their dependence on the environment, so having a system for recording, monitoring and proper diagnosis will help to take the necessary management and sanitary control measures to mitigate disease risks. Similarly, having monitoring data allows contemplating environmental risks, which although they cannot be predicted, they must be adjusted to changing conditions and be the starting point to establish the best time of the year for aquaculture (Pulido, 2012). In the epidemiological field, the identification of risk factors is one of the first strategies to limit the access of pathogens, monitoring aspects of origin and introduction of live fish, parameters, water quality, fomites or live vectors. The host and the environment, as well as the management plan should be made according to the site, conditions and environmental constraints, especially water temperature, which is a very important factor in the epidemiological triad, without omitting those sudden changes, can act as a stress inducer (Oidtmann *et al*., 2013).

CONCLUSIONS

A higher prevalence of beta-hemolytic Streptococcus was evident in fish cultured in cages compared to fish cultured in ponds. Bacteriological analysis revealed that *S. agalactiae* was the predominant species in diseased fish, followed by *S. alactolyticus*. In addition, *Streptococcus* spp. strains isolated from tilapia were susceptible to all antibiotics tested, however, a demand for high concentrations of erythromycin, tetracycline, chloramphenicol and trimethoprim/sulfamethoxazole was evident. The information generated in this study suggests a better control of the risk factors that can act as stress inducers in fish cultured in cages, in order to mitigate infectious outbreaks in tilapia and optimize the potential of aquaculture in the tropics.

Declaration of conflict of interest

Authors declare that the study was conducted in the absence of commercial or financial relationships that could be interpreted as a potential conflict of interest and all subjects gave informed consent prior to inclusion in the study.

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Errata Erratum

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