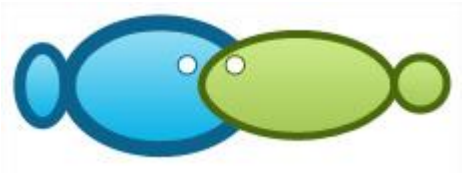

Antibiotic susceptibility and pathogenicity of *Streptococcus* spp. isolated from tilapia grow-out farms in Lubao, Pampanga, Philippines

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Abstract. This study was conducted in order to evaluate the antibiotic susceptibility and pathogenicity of *Streptococcus* spp. isolated on farmed Nile tilapia (*Oreochromis niloticus* L.), pond water and sediment in Lubao, Pampanga, Philippines. Sequencing of the 16S rRNA gene revealed that the *Streptococcus* spp. isolates belong to three species, namely *S. agalactiae*, *S. iniae* and *S. dysgalactiae*. *S. dysgalactiae*, as compared to *S. agalactiae* and *S. iniae*, showed the widest zone of inhibition in almost all of the antibiotics (gentamicin, penicillin, ampiclin, amoxicillin, oxolinic acid, vancomycin, tetracycline and chloramphenicol) used in this study, except for nalidixic acid. Based on the Clinical Laboratory Institute, *S. dysgalactiae* was classified as susceptible to all the antibiotics tested. *S. agalactiae* recorded the narrowest zone of inhibition in most of the antibiotics used and was classified as resistant to penicillin, ampicillin, amoxicillin and vancomycin, and susceptible to the remaining antibiotics (gentamicin, nalidixic acid, oxolinic acid, tetracycline and chloramphenicol). *S. iniae* was resistant to penicillin, ampicillin and amoxicillin, and was susceptible to the remaining antibiotics (gentamicin, nalidixic acid, oxolinic acid, vancomycin, tetracycline and chloramphenicol). *S. dysglactiae* and *S. agalactiae* showed an intermediate susceptibility to the tested antibiotics. The isolated *Streptococcus* spp. in this study could be best treated using gentamicin, tetracycline and chloramphenicol. Results of the pathogenicity experiment showed that *S. agalactiae* was the most pathogenic and virulent, with recorded 100% mortality 7 days post inoculation.
Key Words: antibiotics, bacteria, virulence, zone of inhibition.

Introduction. Fish is an important component of diets around the world. An estimated 1 billion people rely on fish as their main source of animal protein (Food and Agriculture Organization 2007). One of the most cultured fishes worldwide is tilapia (*Oreochromis* spp.) (Food and Agriculture Organization 2004), which becameone of the most important species for the 21st century's aquaculture (Fitzsimmons 2000) and had a commercial progression in more than 100 countries (Shelton & Popma 2006). Tilapia aquaculture offers economic and social benefits for rural communities. It also plays a vital role in terms of worldwide employment. According to the Food and Agriculture Organization, Nile tilapia (*Oreochromis niloticus* L.) is the most important farmed tilapia species, representing more than 73% of the total tilapia production in 2010 (Food and Agriculture Organization 2012). Streptococcosis has been recognized as one of the most serious bacterial diseases in tilapia culture and usually causes high mortality and epidemics lasting for a long period of time. Members of the genus *Streptococcus* are widely distributed in the world. The major species of *Streptococcus* which infect fish are *S. iniae*, *S. difficile*, *S. agalactiae*, *S. parauberis*, *S. dysagalactiae* and *S. shiloi* (Mata et al 2004; Eldar et al 1995). It has been reported that dozens of cultured and wild-ranging marine or freshwater fishes are susceptible to *Streptococcus* such as salmon, mullet, golden shiner, pinfish, eel, sea trout, tilapia, sturgeon, striped bass, rainbow sharks, red-tailed black sharks, danios, some cichlids and several species of tetras (Kitao 1993; Russo et al 2006).

Septicaemia caused by *Streptococcus* spp. is the most severe disease problem in intensively raised tilapia (Suresh 1999). Affected organs include brain, kidney and gut, among others. Most common disease signs include anorexia, exophthalmia, ascites and erratic swimming (Plumb 1999; Eldar et al 1995; Evans et al 2002; Salvador et al 2005). Recently, the tilapia-breeding industry has been hampered by outbreaks of *Streptococcus* spp. infection, which causes high mortality and major economic losses (Liu et al 2016). Antibiotics are used in aquaculture in attempts to control bacterial diseases. The wide range and frequent use of antimicrobial agents in aquaculture has resulted in the emergence of reservoirs of antimicrobial-resistant bacteria in fish and other aquatic animals, as well as in the aquatic environment (Akinbowale et al 2006). A high incidence of bacteria resistant to the antimicrobials used in aquaculture, including multi-drug resistant bacteria, has been found in fish farms and the surrounding aquatic environments (Inglis et al 1997).

The performance of antimicrobial susceptibility testing by the clinical microbiology laboratory is important to empirically select antimicrobial agents or to detect resistance in individual bacterial isolates (Reller et al 2009). For bacterial pathogens with unexplored resistance mechanisms, it is believed that empirical therapy or therapy based on experience is the most effective. Meanwhile, for bacterial isolates with acquired resistance mechanism, susceptibility testing is needed (Reller et al 2009). This study was conducted for the following reasons: (1) to isolate *Streptococcus* spp. from environmental samples and identify them using test kit and 16S rDNA sequencing; and (2) to evaluate the antimicrobial susceptibility profiles and pathogenicity of the identified isolates.

Material and Method

Collection of samples. Tilapia samples that were grown in 20 grow-out ponds in Lubao, Pampanga, Philippines from August 2017 to March 2018 were collected randomly by the use of a cast net. Composite water and sediment samples in every farm were collected using a sterile Kemmerer water sampler and grab sampler, respectively. The collected fish samples were kept in aerated plastic bags with pond water. Meanwhile, the water samples contained in sterilized polyethylene bottles and the sediment samples contained in plastic cups were temporarily stored in ice chests for the 4 to 5 hours of travel to the laboratory of the Freshwater Aquaculture Center-Central Luzon State University, Philippines for analysis.

Isolation of Streptococcus spp. Two series of 10-fold dilutions (10^{-1} and 10^{-2}) of tissue homogenates of tilapia organs (skin, kidney, liver, brain and foregut), pond water and pond sediment were separately made in Phosphate Buffered Saline. One hundred microliters (100 µL) of the diluted samples were plated onto a selective Edwards Medium. The plates were incubated for 18 to 24 h at 35 to 37°C. The colonies of *Streptococcus* appeared bluish to colourless in the selective medium. The isolated colonies were streaked to Trypticase Soy Agar (TSA) slants. The Edwards Medium is used for the rapid isolation of *S. agalactiae* and other streptococci in humans and animals. This medium contains crystal violet and thallium salts that are commonly used for selective isolation of streptococci. The medium also contains peptic digest of animal tissue and beef extract as sources of carbon, nitrogen and other essential nutrients. Sodium chloride helps to maintain the osmotic equilibrium of the medium. Esculin in the medium helps to differentiate esculin-positive (group D streptococci) organisms from esculin-negative (*S. agalactiae*) organisms. *Streptococcus* spp. colonies appear blue to colourless in the medium (Cruickshank et al 1975).

Biochemical characterization and identification of the bacteria isolates. The RapID STR System (Remel, USA) is comprised of RapID STR Panels and RapID STR Reagent. Each RapID STR Panel has several reaction cavities molded into the periphery of a plastic disposable tray. Reaction cavities contain dehydrated reactants and the tray allows simultaneous inoculation of each cavity with a predetermined amount of inoculum.

A suspension of the test organism in RapID Inoculation Fluid was used as the inoculum which rehydrates and initiates test reactions. After incubation of the panel, each test cavity was examined for reactivity by noting the development of a color. In some cases, reagents were added to the test cavities to provide a colour change. The resulting pattern of positive and negative test scores was used as the basis for identification of the test isolate by comparison of results to the reactivity patterns stored in the Electronic RapID Compendium (ERIC™) database or by use of the RapID STR Differential Chart.

16S rRNA gene amplification and clean-up. Prior to the PCR amplification, each gDNA extract was purified using 0.6x AMPure XP beads. The gene amplification includes the following components: genomic DNA, 27F and 1492R universal 16S primers, Taq buffer, DNA polymerase and DNTP mix. The cycling parameters on thermal cycler were set as follows: at 95°C for 5 minutes, 30 cycles of 95°C for 1 minute, at 60°C for 45 seconds, at 70°C for 1 minute at 72°C for 10 minutes and held at 4°C.

Capillary sequencing. The components in the incorporation of fluorescently labeled chain terminator ddNTPs included: amplicons, primers and AB Big Dye® Terminator v3.1 Cycle Sequencing Kit. The cycling parameters on Bio-Rad T100 Thermal Cycler were set as follows: a pre-held at 4°C, at 96°C for 1 minute, 25 cycles of 96°C for 10 seconds, at 50°C for 5 seconds, at 62°C for 4 minutes and held at 4°C. Ethanol precipitation was performed to remove the unincorporated ddNTPs, excess primers and primer dimers. A capillary electrophoresis on the ABI 3730xl DNA Analyzer was performed using a 50 cm 96-capillary array for the sample wells, POP7TM polymer and 3730xl Data Collection Software v3.1. Base calling was done on the Sequencing Analysis Software v5.4. The PCR products from the isolates were purified using the QIA quick PCR purification kit (Qiagen, Gemany), following the manufacturer's instructions. The purified PCR products were sent to a commercial company for sequencing.

Antimicrobial susceptibility testing. Antimicrobial susceptibility of the identified *Streptococcus* isolates was tested using Kirby Bauer disc diffusion technique (Bauer et al 1966). About 2 to 3 colonies were suspended in Trypticase Soy Broth (TSB) and were incubated at 37°C for 1 to 2 hours. The *Streptococcus* suspension was adjusted to the McFarland turbidity standards and was streaked in prepared TSA plates using L glass rods. After the inoculum has dried, the discs with antimicrobials commonly used in the treatment of ornamental fishes (Rodgers & Furones 2009) were placed on the surface of the inoculated plate using a sterile forceps (Bauer et al 1966). The antimicrobial discs (amoxicillin=20 µg; chloramphenicol=30 µg; penicillin=10 µg; tetracycline=30 µg; gentamicin=10 µg; vancomycin=30 µg; ampicilin=10 µg; nalidixic acid=20 µg and oxolinic acid=20 µg) (Rodgers & Furones 2009) were positioned in such a way that the minimum center distance was 24 mm and no closer than 10 to 15 mm from the edge of the Petri dish. In inverted position, the plates were incubated at 37°C and were observed after 24 hours of incubation. Using a ruler, the diameter of the zone of inhibition was measured in millimeters. The susceptible, intermediate and resistant categories were assigned on the basis of the critical points recommended by the Clinical and Laboratory Standards Institute (2012).

Pathogenicity test. Pathogenicity test of the representative isolates of identified *Streptococcus* spp. was conducted using healthy tilapia (100 to 120 g; free from physical and behavioral signs of diseases). Groups of fish (n=5 $tank^{-1}$) were acclimatized in aquaria (120 × 30 × 45 cm^3), supplied with 120 L freshwater and maintained at 35°C, with aeration for about two weeks. The fish were fed with a commercial diet twice daily at a rate of 2% of its bodyweight. Half of the water was replaced and the uneaten feeds were siphoned out daily. The bacteria were grown in TSB overnight and the density of the bacterial suspension was enumerated using a spread plate method on TSA. Each fish from the groups was intraperitoneally injected with 0.1 mL of the bacterial suspension with a density of 10^8 CFU mL^{-1} of the representative isolates. A control group was included, where fish were injected with the same volume of sterile Phosphate Buffered

Saline (PBS). Clinical signs and morbidity were recorded daily for one week and the experiment was terminated when 100% morbidity or mortality has occurred among the challenged groups. Newly dead or moribund fish were examined and tissues as described above were inoculated onto Edwards Medium to confirm the cause of morbidity or mortality.

Statistical analysis. Analysis of variance (ANOVA) was used in determining the significant differences in the zones of inhibition of the various antibiotics against the isolated *Streptococcus* spp. The survival in the pathogenicity experiment was also statistically compared using ANOVA. A Tukey's test was used for the comparison of means.

Results

Purification, gram-staining, microscopy and catalase testing of presumptively identified Streptococcus spp. Around 114 isolates from fish, pond water and sediment, that grew in the Edwards Medium, were chosen and each of the colony was purified in the same medium by applying the quadrant streak method. The target bacterium is characterized as Gram-positive, occurs in chain and is catalase negative. The colony that did not satisfy the characteristics of the target bacterium was removed from the list. From the original 114 colonies, the list was narrowed down to 85 and eventually to 15 isolates (6 from fish, 5 from sediment and 4 from water). The final 15 isolates were subjected to RapID STR System for identification.

Identification of the bacterial isolates using RapID STR system. The summarized result of the 15 isolates that were subjected to the RapID STR system is shown in Table 1. Of the 15 bacterial isolates, three were identified as *S. agalactiae* (isolates 9, 69 and 114), seven as *Streptococcus* spp. (isolates 11, 12, 20, 44, 45, 55 and 74) and three as *Enterococcus* spp. (isolates 10, 13 and 17). Meanwhile, two of the isolates (6 and 56) were not identified using the test kit.

Identification of the bacterial isolates using 16S ribosomal RNA sequencing. Ten out of the 15 isolates that were identified as *S. agalactiae* (isolates 9, 69 and 114) and *Streptococcus* spp. (isolates 11, 12, 20, 44, 45, 55 and 74) were subjected to 16 S rRNA sequencing. The DNA from the isolates was used as template for PCR amplification using 16S rRNA specific primers (27F and 1492R primers). Seven out of 10 successful amplifications (isolates 9, 12, 20, 45, 55, 69 and 114) were evidenced by the expected 1.5 kb sized amplicon, in the agarose gel (Figure 1).

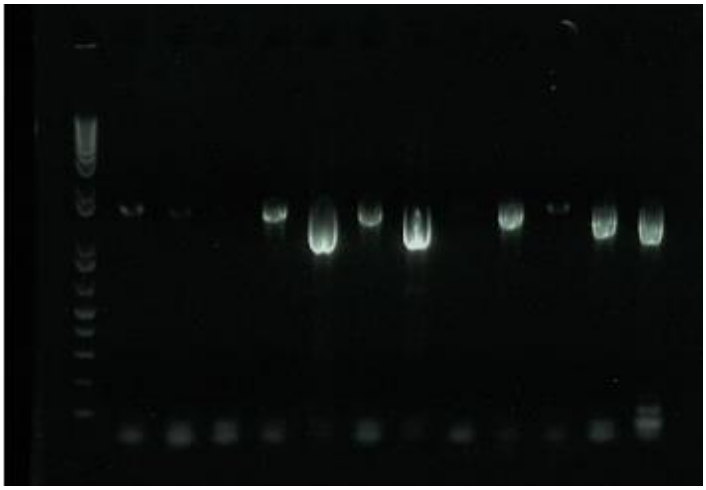

Figure 1. Agarose gel electrophoresis of amplified 16S rRNA gene region with ~1,500 bp size (Left to right: Invitrogen 1 Kb plus DNA ladder, positive control, negative control, 44, 20, 12, 55, 9, 74, 45, 11, 114 and 69).

Table 1

Summarized results for the identification of the bacterial isolates using the RapID STR System

Code	*Source*	*Biochemical test*													*Remarks*
		ARG	*ESC*	*MNL*	*SBL*	*RAF*	*INU*	*GAL*	*GLU*	*NAG*	*PO_4*	*TYR*	*HPR*	*LYS*	*PYR*	
6	Sediment	+	+	-	+	+	-	-	+	-	+	+	-	+	+	Unidentified
9	Fish	+	-	-	-	-	-	-	+	-	+	+	-	+	+	*S. agalactiae*
10	Fish	+	+	+	+	+	+	+	-	+	-	-	-	+	+	*Enterococcus* spp.
11	Sediment	+	-	-	+	-	-	-	+	-	+	+	-	+	-	*Streptococcus* spp.
12	Fish	+	-	-	-	-	-	-	+	-	+	+	-	+	+	*Streptococcus* spp.
13	Fish	-	+	+	+	+	-	+	-	-	-	-	-	-	+	*Enterococcus* spp.
17	Water	-	+	+	+	+	-	+	-	-	-	-	-	-	+	*Enterococcus* spp.
20	Water	+	-	-	-	-	-	-	+	-	+	+	-	+	+	*Streptococcus* spp.
44	Sediment	+	-	+	+	-	-	-	+	-	+	+	-	+	-	*Streptococcus* spp.
45	Fish	-	+	+	-	-	-	-	-	+	+	-	-	+	-	*Streptococcus* spp.
55	Water	-	+	+	-	-	-	-	-	+	+	-	-	+	-	*Streptococcus* spp.
56	Water	+	+	-	-	+	-	-	-	+	+	+	-	-	+	Unidentified
69	Fish	+	-	-	-	-	-	-	+	-	+	+	-	+	+	*S. agalactiae*
74	Water	+	-	-	-	-	-	-	+	-	+	+	-	-	-	*Streptococcus* spp.
114	Sediment	+	-	-	-	-	-	-	+	-	+	+	-	+	+	*S. agalactiae*

ARG = L-arginine, ESC = esculin, MNL = mannitol, SBL = sorbitol, RAF = raffinose, INU = inulin, GAL = p-Nitrophenyl-α-D galactoside, GLU = p-Nitrophenyl-α-D-glucoside, NAG = p Nitrophenyl-n-acetyl-β,D-glucosaminide, PO4 = p-Nitrophenyl phosphate, TYR = tyrosine β-napthylamide, HPR = hydroxyproline β-naphthylamide, LYS = lysine β-napthylamide, PYR = pyrrolidine β-naphthylamide.

PCR products were subjected to sequencing using the same set of primers. BLAST analysis of sequences derived from both primers revealed that isolates 9 and 114 were *S. agalactiae*, isolates 12 and 20 were *S. iniae* and isolate 55 was *S. dysgalactiae*. Using the RapID STR System, isolates 11 and 45 were identified as *Streptococcus* spp.; however, the BLAST analysis showed that the two isolates belong to the genus *Enterococcus faecalis* sp.

Antibiotics profiles of Streptococcus spp. The zones of inhibition (ZOI) exhibited by *S. agalactiae*, *S. iniae* and *S. dysgalactiae* to a set of antibiotics that are commonly used in treating bacterial infection in ornamental fishes are shown in Table 2 and Figures 2 to 4. *S. dysgalactiae* showed the widest ZOI to almost all of the antibiotics (gentamicin, penicillin, ampicilin, amoxicillin, oxolinic acid, vancomycin, tetracycline and chloramphenicol) used in this study, except for nalidixic acid. Based on the Clinical and Laboratory Standard Institute (CLSI), *S. dysgalactiae* was listed as susceptible to all the antibiotics in Table 2. *S. agalactiae* recorded the narrowest ZOI to most of the antibiotics tested (gentamicin, penicillin, ampicillin, amoxicillin, oxolinic acid, vancomycin and chloramphenicol) and was noted to be resistant to penicillin, ampicillin, amoxicillin and vancomycin, while being susceptible to the remaining antibiotics (gentamicin, nalidixic acid, oxolinic acid, tetracycline and chloramphenicol). *S. dysgalactiae* and *S. agalactiae* were intermediate and *S. iniae* was resistant to penicillin, ampicillin and amoxicillin, and susceptible to the remaining antibiotics (gentamicin, nalidixic acid, oxolinic acid, vancomycin, tetracycline and chloramphenicol).

The Nile tilapia infected with *S. dysgalactiae* could be treated using any of the antibiotics listed in Table 2 but the highest ZOI was recorded using 10 µg of gentamicin (30.90±5.69 mm); the ZOI of gentamicin was statistically comparable ($p>0.05$) to most of the antibiotics used, except for the nalidixic acid (19.90±3.78 mm) ($p\leq0.05$). The antibiotic tetracycline at 30 µg dose had the widest ZOI (24.40±1.58 mm) against *S. agalactiae* and the recorded ZOI of this antibiotic was significantly higher compared to the ZOI of gentamicin (18.30±1.64 mm), penicillin (8.00±0.00 mm), ampicillin (8.40±1.26 mm), amoxicillin (7.70±6.74 mm), oxolinic acid (18.80±1.03 mm), vancomycin (7.50±0.53 mm) and chloramphenicol (19.50±1.58 mm) ($p\leq0.05$). In treating fish infected with *S. agalactiae*, gentamicin is more recommended because the bacterium was already susceptible even at lower dose of 10 µg. The widest ZOI for *S. iniae* was recorded using chloramphenicol doses of 30 µg (28.20±1.48 mm), with statistical significance compared to gentamicin (26.50±2.72 mm), penicillin (8.50±0.97 mm), ampicillin (9.10±1.29 mm), amoxicillin (8.20±0.63 mm), vancomycin (19.80±1.40 mm) and tetracycline (18.80±1.81 mm) ($p\leq0.05$); however, the bacterium was already susceptible to gentamicin at a low dose, of 10 µg (26.50±2.72 mm) (Table 2).

Table 2

Antibiotics profile of Streptococcus spp. isolated from tilapia grow-out ponds in Lubao, Pampanga, Philippines

Antibiotics	*ZOI (mm) S. agalactiae*	*CLSI*	*ZOI (mm) S. iniae*	*CLSI*	*ZOI (mm) S. dysgalactiae*	*CLSI*
Gentamicin (10 µg)	18.30±1.64^{c}	S	26.50±2.72^{b}	S	30.90±5.69^{a}	S
Penicillin (10 µg)	8.00±0.00^{b}	R	8.50±0.97^{b}	R	16.60±2.27^{a}	S
Ampicillin (10 µg)	8.40±1.26^{b}	R	9.10±1.29^{b}	R	20.10±0.88^{a}	S
Nalidixic (20 µg)	20.80±1.55^{ab}	S	23.60±1.90^{a}	S	19.90±3.78^{b}	S
Amoxicillin (20 µg)	7.70±6.74^{b}	R	8.20±0.63^{b}	R	16.70±1.70^{a}	S
Oxolinic acid (20 µg)	18.80±1.03^{b}	S	25.10±0.88^{a}	S	24.80±0.92^{a}	S
Vancomycin (30 µg)	7.50±0.53^{c}	R	19.80±1.40^{b}	S	22.50±3.03^{a}	S
Tetracycline (30 µg)	24.40±1.58^{a}	S	18.80±1.81^{b}	S	26.30±3.64^{a}	S
Chloramphenicol (30 µg)	19.50±1.58^{b}	S	28.20±1.48^{a}	S	29.40±4.20^{a}	S

Resistant (R) = $\leq$ 14 mm; Intermediate (I) = 15 to 19 mm; Susceptible (S) = $\geq$ 20 mm. Means (±SD) not sharing a common superscript are significantly different ($p\leq0.05$).

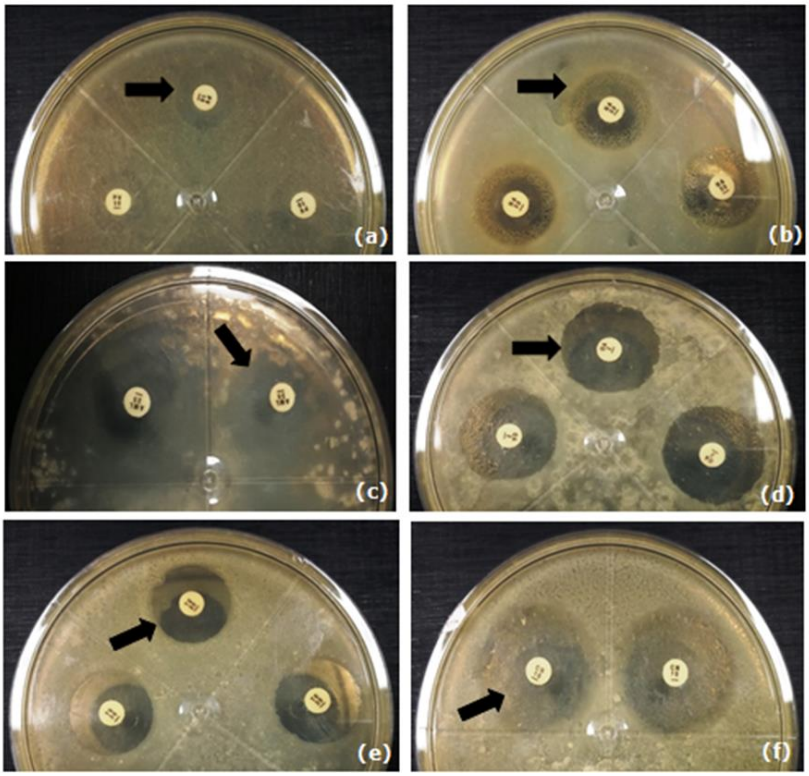

Figure 2. Zone of inhibition of *Streptococcus dysgalactiae* isolated from tilapia obtained from grow-out ponds in Lubao, Pampanga, Philippines (a. penicillin, b. nalidixic acid, c. amoxicillin, d. oxolinic acid, f. tetracycline and g. chloramphenicol).

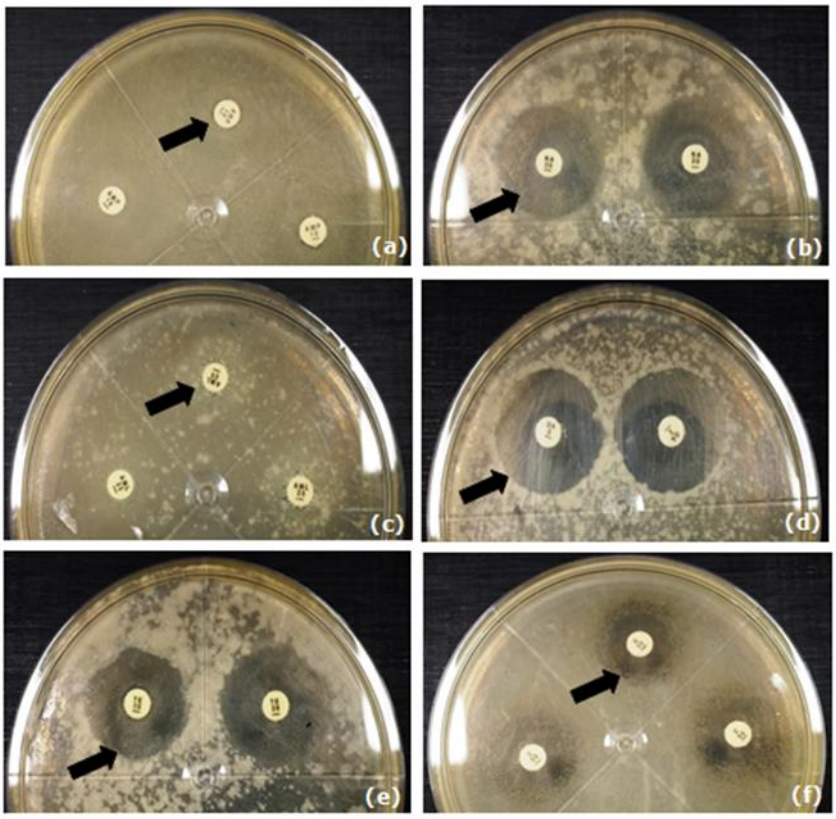

Figure 3. S Zone of inhibition of *Streptococcus agalactiae* isolated from tilapia obtained from grow-out ponds in Lubao, Pampanga, Philippines (left to right: a. penicillin, b. nalidixic acid, c. amoxicillin, d. oxolinic acid, e. tetracycline and f. chloramphenicol).

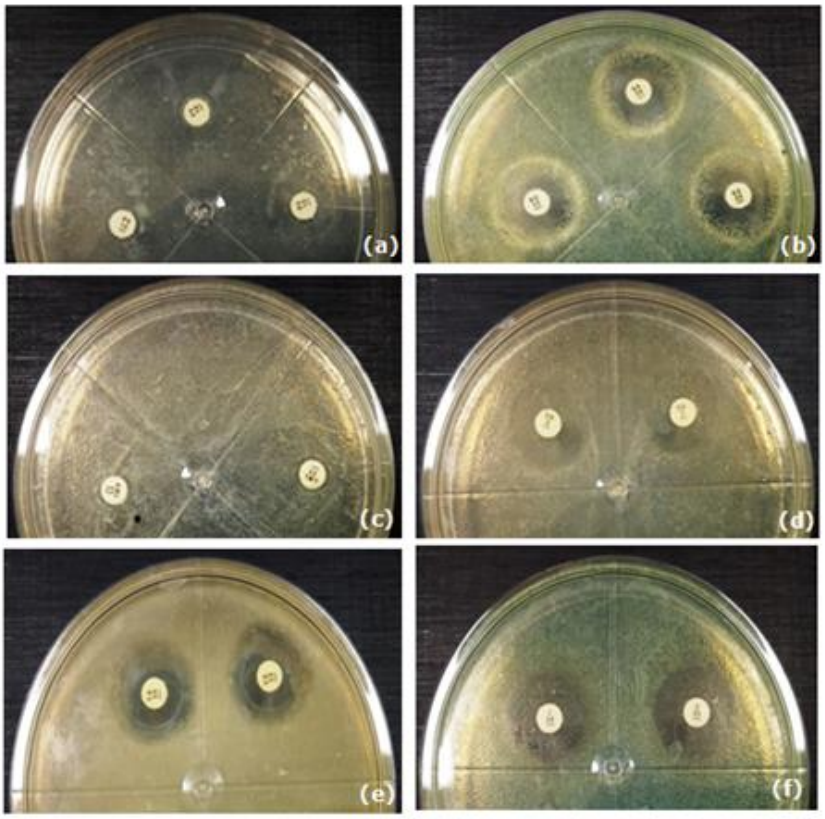

Figure 4. Zone of inhibition of *Streptococcus iniae* isolated from tilapia obtained from grow-out ponds in Lubao, Pampanga, Philippines (left to right: a. penicillin, b. nalidixic acid, c. amoxicillin, d. oxolinic acid, e. vancomycin and f. chloramphenicol).

Pathogenicity of the different Streptococcus spp. The weights of the Nile tilapia samples that were used in the pathogenicity experiment ranged from 103.21 to 105.12 g. The fish in the control group was intraperitoneally injected with sterile PBS, while fish in T1, T2 and T3 were injected with 108 CFU mL^{-1} of *S. agalactiae*, *S. iniae* and *S. dysgalactiae*, respectively.

In the control group, no signs of bacterial infection were observed until the pathogenicity set-up was terminated. The fish were responsive to feeding until day 7 post inoculation (PI), with no recorded mortality. The highest mortality was recorded in fish infected with *S. agalactiae* (100.00±0.00%) and this was statistically different to the mortality recorded in fish infected with *S. iniae* (40.00±0.00%) and *S. dysgalactiae* (0.00±0.00%) ($p<0.05$) (Table 3).

Table 3

Mortality (%) and recovered bacterium of Nile tilapia experimentally challenged with *Streptococcus* spp.

	Mortality (%)	*Recovered bacterium*
Control (injected with PBS)	No mortality	No recovery of bacterium
Treatment 1 (injected with *S. agalactiae*)	100.00±0.00^{a}	Recovered in all parts
Treatment 2 (injected with *S. iniae*)	40.00±0.00^{b}	Recovered in all parts except in brain
Treatment 3 (injected with *S. dysgalactiae*)	0.00±0.00^{c}	Recovered in all parts except in brain

Means (±SD) not sharing a common superscript are significantly different ($p \leq 0.05$).

The organs (skin, kidney, liver, brain and foregut) of the fish infected with *Streptococcus* spp. were individually harvested and streaked in Edwards Medium for the recovery of the bacterium. All of the organs of the Nile tilapia infected with *S. agalactiae* were found positive to the bacterium because of the recovery of bluish to colorless colonies in the selective medium. In the case of the fish infected with *S. iniae* and *S. dysgalactiae*, the bluish to colorless colonies were recovered in the skin, kidney, liver and foregut but not in the brain of the infected tilapia (Table 3). This study hypothesized that mortality would be even greater if the inoculated bacterium reached the brain of the infected fish.

All of the Nile tilapia that were experimentally injected with *S. agalactiae* (T1) showed clinical signs of infection (*e.g.* sloughing of scale, bulging of eyes, reddening of the body) 1-day post inoculation (PI) (Figure 5), which corroborated the study of Evans et al (2002), stating that the onset of clinical sign happens within 24 hours PI. The infected dead fish was dissected and it manifested watery internal organs, enlargement of spleen and liver and petechial haemorrhage in liver and brain tissues (Figure 6). These internal manifestations of disease in the *S. agalactiae*-challenged tilapia were also observed in the study of Rodkhum et al (2011). For the *S. iniae*-infected Nile tilapia (T2), the first clinical sign of infection appeared 1-day PI in one fish. At day 2 PI, some of the injected fish became lethargic, had reddened body and was non-responsive to feeding. The occurrence of fin rot in one of the infected fishes happened in day 3 PI; the first lethal case was also recorded during this day. 4 days PI, none of the remaining fish were eating well. Although no mortality was recorded in fish infected with *S. dysgalactiae* (T3), the first clinical signs of infection were observed 1-day PI, such as sloughing of scales and lethargy. At day 2 PI, additional signs of bacterial infection (reddening of the body) were noticed in the same fish. All of the fish became non-responsive to feeding after 6 days.

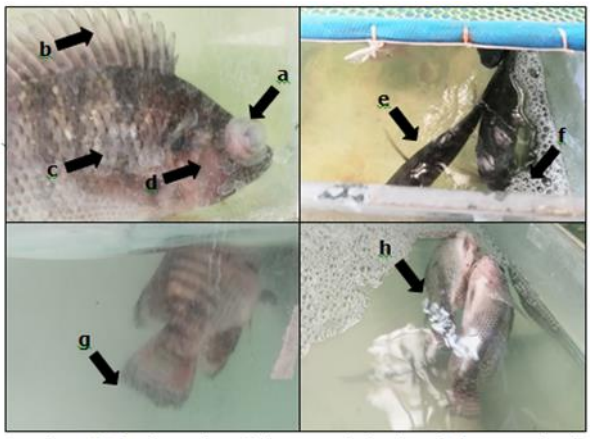

Figure 5. Observations during the pathogenicity set-up: bulged eye (a), overproduction of mucus (b), sloughing of scales (c), reddening (d), blackening of the skin (e), fish gulped for air (f), rotted caudal fin (g) and dead fish (h) (original photos).

Figure 6. Watery stomach and spleen of *Streptococcus* infected tilapia (original photos).

Discussion. Remel RapIDTM STR System is a qualitative micromethod employing conventional and chromogenic substrates for the identification of medically important streptococci and related organisms which have been isolated from human clinical specimens. The tests used in RapID STR System are based on microbial degradation of specific substrates detected by various indicator systems. The RapID STR System is intended to facilitate the identification of Lancefield groups A, B, C, D and G of streptococci, viridans streptococci and *Streptococcus pneumoniae*, *Enterococcus* spp., Aerococcus spp., *Gemella* spp., *Leuconostoc* spp., *Pediococcus* spp., *Weisella confusa*, and *Listeria monocytogenes* (Lennette et al 1980; Balows et al 1991). This identification system was also used in the study of Abuseliana et al (2001), reporting a 99.81% identification rate for all the Streptococcus spp. isolates.

Other studies have shown that *S. iniae*, *S. agalactiae* and other species of Streptococcus isolated from different hosts have a considerable diversity of biochemical characteristics, including acid production from carbohydrates (Buller 2004; Cagirgan 2004; Garcia et al 2008; Sharifiyazdi et al 2010). Most *S. agalactiae* isolated from fish exhibited a negative reaction to the mannitol test (Buller 2004; Garcia et al 2008), but some strains from cattle showed a positive reaction (Garcia et al 2008). Differences in the lactose fermentation had been observed among *Lactococcus garvieae* strains. For example, *L. garvieae* from rainbow trout in Iran and *L. garvieae* ATCC 43921, as well as isolates from rainbow trout in Turkey were positive to the lactose fermentation (Cagirgan 2004; Sharifiyazdi et al 2010). As these studies demonstrate, differences in biochemical properties may provide important information on the physiological profile of the organism. However, phenotypic characteristics alone cannot provide a definitive identification at the species level. Molecular methods have the ability to identify pathogenic species and to discriminate at the species and strains level (Cunningham 2002) and therefore these were used in the current study.

In a study conducted by Reyes et al (2018), *S. agalactiae* tilapia isolates from Pampanga, Philippines were found resistant to penicillin and ampicillin at 10 µg doses, amoxicillin at 20 µg doses and vancomycin at 30 µg doses. Meanwhile, the isolates were susceptible to tetracyline at 30 µg doses and chloramphenicol at 30 µg doses. Lastly, the isolates were intermediate to susceptible to gentamicin at 10 µg doses and nalidixic acid at 20 µg doses. The results of Reyes et al (2018) were comparable in this present study, except for gentamicin and nalidixic acid, for which *S*. agalactiae was categorized as susceptible. In a separate study conducted by Reyes et al (2019), *S. agalactiae* isolates from tilapia pond sediment in Pampanga, Philippines were susceptible to gentamicin, nalidixic acid, chloramphenicol and tetracycline, and intermediate to susceptible to penicillin, ampicillin, amoxicillin and vancomycin. Compared to this present study, the antibiotics penicillin, ampicillin, amoxicillin and vancomycin fall under the resistant category. In the study of Abuseliana et al (2001), the susceptibility of *S. agalactiae* isolates to amoxicillin, ampicillin and erythromycin was high, while it was intermediate or less sensitive to chloramphenicol, linomycin, rifampicin, vancomycin, gentamicin, sulfamethoxazole + trimethoprime and tetracycline. Meanwhile, *S. agalactiae* isolated from Thailand (Suanyuk et al 2005) and Kuwait (Duremdez et al 2004) were resistant to sulfamethoxazole + trimethoprime in contrast to the result of Abuseliana et al (2001). Some antibiotics have been used such as the oxytetracycline, which has been effective in controlling *S. iniae* in blue tilapia. Other reports have documented the effectiveness of antibiotics such as the erythromycin, doxycycline, kitasamycin and lincomycin in *S. iniae*-infected tilapia (Darwish & Griffin 2002). According to Suanyuk et al (2005), *S. iniae* was sensitive to 10 antibiotics, including ampicillin, chloramphenicol, erythromycin, gentamicin, nitrofurantoin, norfloxacine, oxytetracycline, penicillin G, sulphamethoxazol/trimethoprim and trimethoprim; the bacterium was resistant to nalidixic acid and oxolinic acid. In this present study, except penicillin, *S. iniae* was also susceptible to ampicillin, chloramphenicol and gentamicin. In contrary to Suanyuk et al (2005), the present study found that *S. iniae* was susceptible to nalidixic acid and oxolinic acid. *S. dysgalactiae* was completely susceptible to β-lactam antibiotics, and the result of their study was comparable the the present study, in the case of penicillin and ampicillin. The non-susceptible rates for ciprofloxacin, clarithromycin, clindamycin, and

minocycline were approximately 55, 37, 23 and 25%, respectively. Differences in resistance and sensitivity to antibiotics among the same species could be due to the environmental variability, serotype variety and frequently non-guided use of chemotherapy in the aquaculture field (Abuseliana et al 2001).

In the present investigation, a 50% mortality was already recorded in T1 as early as 1-day PI while a 100% mortality was recorded 3 days PI, at a dose of 108 CFU mL^{-1}, indicating the virulence of the local *S. agalactiae* isolate. In the work of Rodkhum et al (2011), the *S. agalactiae*-infected tilapia had a 10% mortality 2 days PI, at a dose of 108 CFU mL^{-1}, which increased to 40% at the 7^{th} day PI. Meanwhile, the research of Evans et al (2002) revealed that a mortality rate of 30% was reported in the first 24 hours PI with *S. agalactiae* at a concentration of 1.1×105 CFU $fish^{-1}$. The other studies reported 48% and 90% mortalities at a dose of 9 x 108 CFU mL^{-1} and 1.5 x 108 CFU $fish^{-1}$, respectively, 7 days PI (Pretto-Giordano et al 2010). The virulence (100% mortality 7 days PI) factors of *S. agalactiae* according to Evans et al (2002) include proteins and enzymes linked to cell surface metabolism such as capsule genes, haemolysin and adhesion genes. For the *S. iniae*-infected Nile tilapia (T2), the first clinical sign of infection appeared 1-day PI in one fish. At day 2 PI, some of the injected fish became lethargic, had reddened body and was non-responsive to feeding. The occurrence of fin rot in one of the infected fishes happened in day 3 PI; the first mortality was also recorded during this day. 4 days PI, the remaining fish were not eating well. The recorded mortality 7 days PI was 40% in fish injected with *S. iniae*. According to Ye et al (2011), this bacterium reduces feeding efficiency and weight gain, thereby affecting the productive performance of infected organisms. *S. iniae* adhered to, then invaded the epithelial cells, but its persistence and replication inside the cells were short-lived (Eyngor et al 2007), and this might be the reason for the lower mortality recorded in fish infected with *S. iniae*. The study of Anshary et al (2014) showed that the mean survival time of tilapia injected with *S. iniae* (0.1 mL of 1.0 × 107 CFU mL^{-1}) was 2.5 days; all of the fish injected with *S. iniae* suspension showed clinical signs and died within 6 days PI. Although no mortalities were recorded in fish infected with *S. dysgalactiae* (T3), the first clinical signs of infection were observed 1-day PI. The virulence of *S. dysgalactiae* is mainly based on its cell surface properties such as high hemagglutination and hydrophobic properties which determine the main adhesion and invasive pathogenic mechanism of the pathogen (Abdelsalam et al 2009). This has been confirmed by Abdelsalam et al (2009), who have indicated that *S. dysgalactiae* isolates were able to adhere to and invade fish epithelial cell line.

Conclusions. BLAST analysis of sequences derived from the DNA sequence analysis revealed that the isolates belong to *S. agalactiae*, *S. iniae* and *S. dysgalactiae*. *S. dysgalactiae*, as compared to *S. agalactiae* and *S. iniae*, showed the widest ZOI in almost all of the antibiotics used in this study and was generally classified as susceptible, based upon CLSI. *S. agalactiae* recorded the narrowest ZOI in most of the antibiotics used and was classified as resistant to susceptible. *S. iniae* showed intermediate reactions to the antibiotics used. Results of the pathogenicity experiment revealed that *S. agalactiae* was the most pathogenic and virulent, with a recorded 100% mortality 7 days PI. The infection caused by the isolated Streptococcus spp. in this study could be best treated using gentamicin, tetracycline and chloramphenicol.

Acknowledgements. This work was financially supported by the Department of Science and Technology.

Conflict of interest. The authors declare no conflict of interest.

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Received: 13 January 2022. Accepted: 01 June 2022. Published online: 17 June 2022.
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How to cite this article:
Reyes A. T., Raymundo A. K., Baldrias L. R., Paller V. G. V., Dalmacio I. F., 2022 Antibiotic susceptibility and pathogenicity of *Streptococcus* spp. isolated from tilapia grow-out farms in Lubao, Pampanga, Philippines. AACL Bioflux 15(3):1389-1402.