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ANTIBIOTICS SUSCEPTIBILITY OF *Streptococcus agalactiae* ISOLATED FROM TILAPIA POND WATER IN LUBAO, PAMPANGA, PHILIPPINES

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ABSTRACT

The main purpose of this study was to isolate, quantify and assess the antibiotics susceptibility of *Streptococcus agalactiae* from tilapia pond water in Lubao, Pampanga, Philippines.

Highest CFU/mL of *S. agalactiae* was recorded in Farm 3 (270 CFU/mL). There was no big difference in CFU/mL among farms because they share the same management practices.

For antibiotics with dose of 10 µg, gentamicin was the most effective against *S. agalactiae* isolates with zone of inhibition from 17.00 to 22.80 mm. Nalidixic acid (19.80 to 22.00 mm) at 20 µg had wider zone of inhibition as compared to amoxicillin (7.20 to 15.00 mm) of the same dose. At 30 µg dose, tetracycline (21.60 to 25.60 mm) was more effective than chloramphenicol (18.40 to 26.20 mm) and vancomycin (7.00 to 19.00 mm).

All isolates were resistant to penicillin and ampicillin at 10 µg dose. Four of the isolates were resistant to amoxicillin at 20 µg dose and vancomycin at 30 µg dose. Meanwhile, *S. agalactiae* isolates were 100% susceptible to tetracyline at 30 µg dose and 80% susceptible to

chloramphenicol at 30 µg dose. Lastly, *S. agalactiae* was intermediate to susceptible to gentamicin at 10 µg dose and nalidixic acid at 20 µg dose.

The dosages of penicillin, ampicillin, amoxicillin and vancomycin should be increased when it will be used for treating tilapia infected by *S. agalactiae*. Tetracycline and chloramphenicol at 30 µg dose could be recommended as best treatment for tilapia infected with the bacterium.

Keywords: *Streptococcus agalactiae*, Nile tilapia, antibiotics, zone of inhibition

INTRODUCTION

Streptococcosis is a disease caused by two diverse groups of bacteria which are *Streptococcus* spp. and *Lactococcus* spp. that infect a wide range of hosts, including humans [1, 2, 3]. Streptococcosis outbreaks in tilapia have been reported from many parts of the world [4, 5, 6, 7] with total global losses in production of around US$250 M in 2008 [8, 9]. According to several studies, high mortality rates (50 to 70%) in *Streptococcus*-infected fishes are frequently associated with intensive aquaculture systems [9, 10, 11]. The most significant causative bacteria of streptococcosis in fish worldwide include *S. iniae*, *S. agalactiae*, *S. parauberis*, *S. difficile*, *S. shiloi* and *S. dysagalactiae* [5, 6, 12, 13, 14, 15, 16, 17, 18].

Under natural conditions, one of the main pathways of disease transmission appears to be through indirect contact via the water in culture systems [2, 9, 20, 21, 22, 23, 24, 25]. Bacteria are usually excreted in the feces of these fish and survive in the water, and become infective to other fishes [24]. *Streptococcus* spp. can survive for long periods in water, mud and ponds, and even in the equipment used in normal operations [26].

Tilapia ponds in Pampanga are highly dependent in river and irrigation canal as major water sources. *Streptococcus* spp. along with other pathogenic bacteria such as *Staphylococcus aureus*, *S. epidermidis*, *Aeromonas hydrophila*, *Vibrio vulnificus*, *V. parahaemolyticus* and *Pseudomonas aeruginosa* was isolated in tilapia ponds in Pampanga and water sources such as Pampanga River and irrigation canals [27].

Streptococcal infections respond to antibiotic therapy, but the disease cannot be legally controlled with antibiotics all the way to the market because the withdrawal period for all effective antibiotics is longer than it takes for the streptococcal infection to return. Furthermore, it is only a matter of time

before *Streptococcus* develops resistance to the antibiotics. In fact, streptococcal strains at several facilities have already developed resistance to some antibiotics [28].

Several drugs have been tested for the treatment of streptococcosis. Oxytetracycline incorporation in feeds (75 and 100 mg/kg body weight) was effective in controlling *S. iniae* in blue tilapias (*O. aureus*) [29]. Some reports concluded that erythromycin was effective against streptococcal infections in cultured yellowtails [30] and rainbow trout [31] at doses of 25 to 50 mg/kg/day for 4 to 7 days. Doxycycline, oxytetracycline, kitasamycin, oleandomycin, josamycin and lincomycin have also been used to control streptococcosis in the cultured yellowtail in Japan [31].

This study was conducted in order to: (1) isolate and quantify *S. agalactiae* from tilapia pond water in Lubao, Pampanga, Philippines and; (2) to evaluate the antibiotics susceptibility of the *S. agalactiae* isolates.

MATERIALS AN D METHODS

Collection of Pond Water Samples

The 22 municipalities of Pampanga cover an estimated 34,055 ha of fishponds allotted for rearing both freshwater and brackishwater fishes, and other aquatic organisms [32]. The study site, which is the municipality of Lubao is located at the southwestern part of Pampanga and is composed of 44 barangays. It is bounded by the municipalities of Guagua on the north, Sasmuan on the east, Floridablanca on the west and Orani, Bataan on the south.

Five tilapia grow-out farms near the Pampanga River have served as the collection sites. Composite pond water samples in each tilapia farm were collected using sterile Kemmerer water sampler. Water samples stored in polyethylene bottles were kept in ice chest and were immediately transported to the laboratory for analysis.

Surveying and GPS Reading

The tilapia grow-out farms were surveyed using a pre-tested questionnaire designed by the Freshwater Aquaculture Center-Central Luzon State University. The exact location of the farm was known using handheld GPS equipment.

Isolation and Quantification of *S. agalactie*

Two series of 10-fold dilutions (10^0 and 10^{-1}) of pond water samples were made in sterile distilled water. One hundred microliters of the diluted samples were plated onto Edward Medium Modified (EMM). EMM is a selective medium for the rapid isolation of *S. agalactiae* and other streptococci. *S. agalactiae* colonies appear colorless to blue in color [33]. The plates

were incubated for 18 to 24 h at 35 to 37 °C. Plates with 25 to 250 colonies were only considered as valid count. The counts were expressed as colony forming unit/mL (CFU/mL) using the formula:

$$\text{CFU/mL} = \frac{\text{average no. of colonies x dilution factor}}{\text{volume plated (mL)}}$$

In every collection site, one colony of *S. agalactiae* was selected and inoculated in test tube containing Trypticase Soy Agar (TSA). A total of five colonies were used in antimicrobial susceptibility testing.

Antimicrobial Susceptibility Testing

About 2 to 3 colonies of *S. agalactiae* were suspended in Trypticase Soy Broth (TSB) and incubated further for 1 to 2 hours at 37 °C. The bacterial suspension was adjusted to 0.5 McFarland turbidity standards and was streaked in prepared TSA plate using a sterilized cotton swab. After the inoculum has dried, the discs with antimicrobials commonly used in aquaculture and ornamental fishes [34] were placed on the surface of the inoculated plate using sterile forceps [35]. The antimicrobial discs (amoxicillin = 20 µg; chloramphenicol = 30 ug; penicillin = 10 µg; tetracycline = 30 µg; gentamicin = 10 µg; vancomycin = 30 µg; ampicilin = 10 µg; nalidixic acid = 20 µg) [34] were positioned such that the minimum center distance is 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) [36].

Statistical Analysis

Statistical difference in zone of inhibitions among antibiotic discs was compared using One Way Analysis of Variance. Comparison of means was done using Tukey's Test.

RESULTS AND DISCUSSION

Surveying and GPS Reading

The five tilapia farms that served as collection sites for water samples were all located in Barangay Bancal Pugad, Lubao, Pampanga, Philippines. This barangay was near in one of the tributaries of Pampanga River (Table 1).The five farms were engaged on medium (3 to 6 ha) to large-scale (>7 ha) tilapia grow-out operation. The farm owners believed that the combination of drying and liming was already enough to prepare their tilapia ponds. All of the farms relied on water source coming from Pampanga River. Water

exchange was frequently done, at least once a week. Level of management was intensive with stocking density of >9 tilapia/m^2. The cultured tilapia was dependent on commercial feeds. All of them already experienced fish diseases and fish kill, and abrupt/extreme environmental conditions. In case of disease problem, the normal remedies being done were application of salt and lime as treatments, water exchange and early harvest of tilapia (Table 1).

Quantification of *S. agalactiae*

Edward Medium Modified contains crystal violet and thallium salts which are responsible for the selective isolation of streptococci and inhibition of other types of bacteria. The medium also contains esculin that helps to differentiate esculin-positive (group D streptococci) organisms from esculin-negative (*S. agalactiae*) organisms [37].

Highest CFU/mL was recorded in Farm 3 (270 CFU/mL) followed by Farm 2 and Farm 4 (260 CFU/mL), Farm 1 (255 CFU/mL) and Farm 5 (250 CFU/mL) (Table 2). There was no big difference in CFU/mL among farms because they share the same source of water and management practices.

Based upon interview results, the possible risk factors for the occurrence of *S. agalactiae* in pond water were high stocking density, full feeding, unsafe source of water and incomplete pond preparation practices.

The single presence of the pathogen in the aquatic environment is not enough to induce the disease. Risk factors such as stress could affect the physiology of tilapia and increase their susceptibility to the disease agent [10]. Some of the most common stressors related to outbreaks of streptococcosis include high temperatures (above 31 °C) or strong temperature fluctuations (>1 °C), high salinity, pH above 8, low concentration of dissolved oxygen (< 1 mg/L), poor water quality (high concentrations of ammonium or nitrite), high stock density of individuals per-unit-area of culture (>25 kg/m^2), and the concomitant effects of routine handling and harvesting of animals [10, 26, 38, 39].

Antimicrobial Susceptibility Testing

For antibiotics with dose of 10 µg, gentamicin was the most effective against *S. agalactiae* isolates with zone of inhibition from 17.00 to 22.80 mm. Zone of inhibition of penicillin in Farm 2 isolate (10.60±1.67 mm) and of ampicillin in Farm 1 isolate (14.60±1.52 mm) was significantly higher as compared to the rest of isolates. Lowest zone of inhibition of gentamicin was recorded in Farm 5 isolate (17.00±0.71 mm) and it was

significantly lower as compared to the rest (Table 3).

At 20 µg dose, nalidixic acid (19.80 to 22.00 mm) had wider zone of inhibition as compared to amoxicillin (7.20 to 15.00 mm). Highest zone of inhibition of amoxicillin was observed in Farm 1 isolate (14.60±1.52 mm) and it was significantly higher from the rest. Meanwhile, the zone of inhibition of nalidixic acid in Farm 3 isolate (22.00±1.00 mm) was significantly higher to Farm 2 isolate only (19.20±0.45 mm) (Table 3).

Tetracycline (21.60 to 25.60 mm) at 30 µg dose was more effective than chloramphenicol (18.40 to 26.20 mm) and vancomycin (7.00 to 19.00 mm) of the same dosages based upon the range of zone of inhibition. Farm 3 isolate recorded the highest zone of inhibition for chloramphenicol (26.20±0.84 mm) and vancomycin (19.00±1.23 mm) when compared to other isolates. Meanwhile, tetraycline was most effective to Farm 4 isolate (25.60±1.14 mm) (Table 3).

The isolates were classified whether resistant, intermediate or susceptible to the various antibiotics on the basis of critical points recommended by the Clinical and Laboratory Standards Institute (2012) [36]. All five isolates were resistant to penicillin and ampicillin at 10 µg dose. Four of the isolates were resistant to amoxicillin at 20 µg dose and vancomycin at 30 µg dose (Table 4). Resistance to these antibiotics is mediated by the permeability barrier and/or efflux pumps, naturally insensitive target enzymes, regulational, mutational or recombinational changes in the target enzymes genes and acquired drug-resistance genes [40]. Specifically, for β-lactam antibiotics such as penicillin, the bacterium resistance could be due to production of β-lactamase, decreased penetration through the outer cell-membrane to access the cell wall enzymes and the resistance of the target penicillin binding protein (PBP) to binding by β-lactam agents [41]. To increase the spectrum of action of amoxicillin against microorganisms, it should be combined with clavulanic acid, a β-lactamase inhibitor. Clavulanic acid is a suicide inhibitor of bacterial β-lactamase enzyme from *Streptomyces clavuligerus* [42]. Ampicillin when administered alone has weak antibacterial activity against most organisms, but when given in combination with β-lactam antibiotics prevents antimicrobial inactivation by microbial lactamase [43]. In the study, the dosages of penicillin, ampicillin, amoxicillin and vancomycin should be increased when it will be used for treating tilapia infected by *S. agalactiae*.

Meanwhile, *S. agalactiae* isolates were 100% susceptible to tetracyline at 30 µg dose and 80% susceptible to chloramphenicol at 30 µg dose (Table 4). Chloramphenicol has been shown to exert a strong inhibitory action on microbial protein synthesis [44]. In the present study, these two antibiotics could be recommended as best treatment for tilapia infected with the bacterium. On the other hand, tetracyclines possess antibacterial activity by binding to the 30S ribosomal subunit of a susceptible organism. Following ribosomal binding the tetracycline interferes with the binding of aminoacyl-tRNA to the messenger RNA molecule/ribosome complex; this disrupts the bacterial protein synthesis [45]. Tetracycline binds with the 70S ribosomes found in mitochondria and can also inhibit protein synthesis in mitochondria [46].

Lastly, *S. agalactiae* was intermediate to susceptible to gentamicin at 10 µg dose and nalidixic acid at 20 µg dose (Table 4). Aminoglycoside such as gentamicin is able to inhibit the protein synthesis in bacteria by binding to one of the ribosomal subunits [47], and are effective against aerobic Gram-negative rods and certain Gram-positive bacteria. Gentamicin is less toxic and is widely used for infections caused by Gram-negative rods (*Escherichia, Pseudomonas, Shigella* and *Salmonella*). Meanwhile, nalidixic acids are able to interfere with DNA replication and transcription in bacteria [48].

Table 1: Summary of management practices of farm collection sites

Farm	Location/Address	Scale of Operation	Pond Preparation	Water Source	Water Exchange	Level of Management	Feeding	Problems Incurred	Preventive/Control Measures
1	Bancal Pugad N 14˚54.757' E 120˚ 34.560'	Large-scale	Drying and liming	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Salt treatment, liming, early harvest
2	Bancal Pugad N 14˚54.583' E 120˚ 34.945'	Medium-scale	Drying	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Salt treatment, early harvest
3	Bancal Pugad N 14˚57.247' E 120˚ 36.597'	Large-scale	Drying	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Water exchange, liming
4	Bancal Pugad N 14˚58.145' E 120˚ 35.695'	Medium-scale	Drying	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Salt treatment, liming, early harvest
5	Bancal Pugad N 14˚54.935' E 120˚ 35.047'	Medium-scale	Drying and liming	River	Frequent	Intensive	Full feeding	Fish diseases, fish kill, abrupt/extreme environmental conditions	Salt treatment, early harvest

Table 2: Colony forming unit (CFU) of *S. agalactiae* per mL of pond water in five collection sites

Farm	Number of *S. agalactiae* Colonies		CFU/mL	Log10 CFU/mL
	10^0 Dilution	10^{-1} Dilution		
1	26	8	255	2.407
	25	2		
	9	0		
2	1	0	260	2.415
	0	0		
	26	4		
3	8	0	270	2.431
	27	5		
	8	0		
4	3	1	260	2.415
	26	23		
	8	1		
5	25	3	250	2.400
	1	0		
	0	0		

Table 3: Zone of inhibition of the antibiotics used against the five *S. agalactiae* farm isolates

Antibiotics/Dosages	Zone of Inhibition (mm)				
	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Penicillin (10 µg)	7.40 ± 0.55^{b}	10.60 ± 1.67^{a}	8.00 ± 0.00^{b}	8.00 ± 0.00^{b}	8.00 ± 0.00^{b}
Gentamicin (10 µg)	21.00 ± 1.23^{ab}	18.20 ± 0.84^{cd}	22.80 ± 0.84^{a}	19.60 ± 1.14^{bc}	17.00 ± 0.71^{d}
Ampicillin (10 µg)	14.60 ± 1.52^{a}	10.40 ± 1.14^{b}	10.20 ± 0.84^{b}	7.40 ± 0.55^{c}	9.40 ± 0.89^{b}
Amoxicillin (20 µg)	15.00 ± 0.71^{a}	10.40 ± 0.55^{b}	8.40 ± 0.89^{c}	7.20 ± 0.45^{d}	9.84 ± 2.90^{cd}
Nalidixic acid (20 µg)	19.80 ± 1.64^{ab}	19.20 ± 0.45^{b}	22.00 ± 1.00^{ab}	21.20 ± 0.84^{ab}	20.40 ± 2.07^{ab}
Chloramphenicol (30 µg)	22.20 ± 0.84^{b}	20.00 ± 0.71^{cd}	26.20 ± 0.84^{a}	18.40 ± 1.34^{d}	20.60 ± 0.89^{c}
Tetracycline (30 µg)	22.60 ± 2.41^{bc}	24.80 ± 1.92^{ab}	21.60 ± 0.89^{c}	25.60 ± 1.14^{a}	23.20 ± 0.84^{abc}
Vancomycin (30 µg)	10.20 ± 3.11^{b}	10.00 ± 1.58^{bc}	19.00 ± 1.23^{a}	7.00 ± 0.00^{c}	8.00 ± 0.00^{bc}

Note: Different superscript was significant at $p<0.05$

Table 4: Classification of isolates on the basis of critical points recommended by the Clinical andLaboratory Standards Institute (2012)

Antibiotics/Dosages	Categories				
	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Penicillin (10 µg)	Resistant	Resistant	Resistant	Resistant	Resistant
Gentamicin (10 µg)	Susceptible	Intermediate	Susceptible	Intermediate	Intermediate
Ampicillin (10 µg)	Resistant	Resistant	Resistant	Resistant	Resistant
Amoxicillin (20 µg)	Intermediate	Resistant	Resistant	Resistant	Resistant
Nalidixic acid (20 µg)	Intermediate	Intermediate	Susceptible	Susceptible	Susceptible
Chloramphenicol (30 µg)	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible
Tetracycline (30 µg)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Vancomycin (30 µg)	Resistant	Resistant	Intermediate	Resistant	Resistant

Note: Resistant = $\leq$ 14 mm; Intermediate = 15 to 19 mm; Susceptible = $\geq$ 20 mm

CONCLUSION

S. agalactiae isolates from tilapia pond water were found to be 100% and 80% susceptible to tetracycline and chloramphenicol, respectively at 30 µg dose. These two antibiotics could be recommended as best treatment for tilapia infected with *S. agalactiae*. Meanwhile, *S. agalactiae* isolates were 100% resistant to penicillin and ampicillin at 10 µg dose, and 80% resistant to amoxicillin at 20 µg dose and vancomycin at 30 µg dose. The dosages of penicillin, ampicillin, amoxicillin and vancomycin should be increased when it will be used for treating tilapia infected by *S. agalactiae*. Lastly, *S. agalactiae* isolates were intermediate to susceptible to gentamicin at 10 µg dose and nalidixic acid at 20 µg dose.

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