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Comparison of antibiotic susceptibility and pathogenicity of wild and serially passaged *Streptococcus agalactiae* to Nile tilapia (*Oreochromis niloticus* L.)

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Abstract
The specific objectives of this study were to develop a spontaneously mutated *S. agalactiae* by serial passage in agar (T1 = control or wild, T2 = 10 days' serial passage, T3 = 20 days' serial passage, T4 = 30 days' serial passage, T5 = 40 days' serial passage and T6 = 50 days' serial passage) and to compare the antibiotic susceptibility and pathogenicity of wild and serially passaged *S. agalactiae* to Nile tilapia (*Oreochromis niloticus* L.).
S. agalactiae in T4 has resulted to increase zone of inhibition (ZOI) to penicillin and ampicillin when compared to T1. As the bacterial passage was increased to 50 days (T6), it resulted to significant decrease on the ZOIs to penicillin and ampicillin. For tetracycline, 40 days' serial passage (T5) was the most consistent in terms of decreased ZOIs as compared to T1; meanwhile, 30 days' serial passage (T4) had the most numbered increase in ZOIs. Therefore, T4 and T5 bacterial passages were needed in order to significantly change the ZOIs of *S. agalactiae* to the antibiotics used in this experiment.
In the pathogenicity experiment, except for T4 (20.00%), the wild *S. agalactiae* (T1 = 30.00%) had lower percent mortality as compared to serially passaged bacterium (T2 = 50.00%, T3 = 50.00%, T5 = 40.00%, T6 = 30.00%). Based on the onset of first mortality, it appeared that 40 (T5) and 50 days (T6) serial passages were less pathogenic as compared to T1. All of the infected fish share the same signs of bacterial infection.

Keywords: Mutation, spontaneous mutation, serial passage, *Streptococcus agalactiae*, zone of inhibition

1. 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 [1]. One of the most cultured fishes worldwide is tilapia (*Oreochromis* spp.) [2]. In fact, the production of tilapias made the fish as one of the most important species for the 21st century aquaculture [3] which also rose commercially in more than 100 countries [4]. Tilapia aquaculture offers economic and social benefits for rural communities. It also plays vital role in terms of worldwide employment. According to Food and Agriculture Organization [5], Nile tilapia (*O. niloticus* L.) is the most important farmed tilapia species, representing more than 73% of total tilapia production in 2010.
One of the main factors affecting fish production and efficiency is fish diseases and they represent a real danger for aquaculture [6]. Disease causing pathogens can be found on fish, water, surfaces of aquaculture equipment and facilities, slumps and filter beds. They can be transmitted by water, from fish to fish by vectors and by contaminated feed [7].
Streptococcosis has been recognized as one of the most serious bacterial diseases in tilapia culture and usually causes high mortality and lasts 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* [8-10]. 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 [11-12]. In recent years, however, the problems of *S. agalactiae* infection in tilapia culture have become gradually apparent. Severe streptococcal infection in tilapia caused by *S. agalactiae* has occurred in China, causing heavy losses [13].

S. agalactiae is a member of group B *Streptococcus* that can be either haemolytic [14] or non-haemolytic [15].Bacteria frequently encounter rapidly changing environments and their ability to adapt is the key for their survival. An understanding of the mechanisms and rates of genetic change is necessary if we want to gain insight into the future of these microscopic organisms. Microorganisms are masters of adaptation, and to co-exist, we must find ways to keep up, for example by limiting their rate of adaptation to antibiotics [16].

Serial passage refers to the process of growing bacteria in iterations. For instance, a particular bacterium will be grown in one culture medium, and then part of that bacterium will be removed and reintroduced into a new culture medium. The process of culturing is repeated with as many stages as desired, and then the final product is compared with the original bacterium. When serial passage is performed either *in vitro* or *in vivo*, bacterium that is being manipulated may evolve by mutating repeatedly [17]. In fact, exactly because serial passage allows for rapid evolution of a bacterium to its host, it can be used to study the evolution of antibiotic resistance; specifically, for determining what mutations could lead to the development of antibiotic resistance [17].

Mutations in an organism's genome can arise spontaneously, that is, in the absence of exogenous stress and prior to selection. Mutations are often neutral or deleterious to individual fitness but can also provide genetic diversity driving evolution. Mutagenesis in bacteria contributes to the already serious and growing problem of antibiotic resistance [18].

The general objective of the study was to compare the antibiotic susceptibility and pathogenicity of wild and serially passaged *S. agalactiae* isolated from diseased Nile tilapia. Specifically, the study aimed to (1) develop a spontaneously mutated *S. agalactiae* by serial passage in agar; (2) compare the antibiotic susceptibility of wild and serially passaged *S. agalactiae* by measuring the zone of inhibition; and (3) to compare the pathogenicity of wild and serially passaged *S. agalactiae* to Nile tilapia.

2. Materials and Methods

2.1. Isolation and Purification of *S. agalactiae*

Two series of 10-fold dilutions (10^{-2} and 10^{-3}) of kidney of moribund tilapia was made in sterile distilled water. One hundred microliters of the diluted sample were streaked into Edwards Modified Medium (EMM) plates. The plates were incubated at 37 ˚C for 18 to 24 hours. *S. agalactiae* colonies appear blue to colorless in EMM.

Three colonies from the EMM plate were purified in Trypticase Soy Agar (TSA) plates using streak plate method. The purified colonies were separately inoculated in TSA tubes.

2.2. Spontaneous mutation of *S. agalactiae*

Purified *S. agalactiae* colony was serially passaged in TSA slants for 10, 20, 30, 40 and 50 times. The slants were incubated at 37 ˚C for 18 to 24 hours. *S. agalactiae* at different passages was evaluated for antibiotic susceptibility and pathogenicity to Nile tilapia.

2.3. Preparation of filter paper discs

Holes of approximately 7 mm was made in Whatman Filter Paper No. 3 using a puncher. The discs were autoclaved at 15 lbs pressure for 30 minutes.

2.4. Preparation of antibiotic stock solution

Standard antibiotic powders (Penicillin, Ampicillin and Tetracycline) were purchased in drugstores. In order to obtain a stock solution of 20 µg/µL, a known weight of the above mentioned antibiotics were dissolved in sterile distilled water. The stock solution was diluted at the time of disc preparation to obtain the working solution of 10 mL. The concentrations of antibiotic solutions that were evaluated are presented in Table 1. Using a micropipette, a fixed volume of 20 µl was loaded on each disc one by one.

Table 1: Computed volume of stock solution in each concentration of working solution.

Concentration of stock solution (µg/µl)	Volume of stock solution (mL)	Concentration of working solution (µg/µl)	Volume of working solution (mL)
20	0.00	0	10
20	0.13	5	10
20	0.25	10	10
20	0.50	20	10
20	1.00	40	10
20	1.50	60	10
20	2.00	80	10
20	2.50	100	10
20	5.00	200	10
20	10.00	400	10

2.5. Drying and impregnation of discs

The tube containing the serially passaged bacterium was incubated at 37 °C for 1 to 2 hours. The bacterial suspension was adjusted to 0.5 McFarland turbidity standards and evenly spread in TSA plates using a sterile cotton swab. After the inoculum was dried, the prepared antibiotic discs were placed on the surface of the inoculated plate using sterile forceps. The plates with discs were incubated at 37 °C and observed after 24 hours of incubation. The diameter of the zone of inhibition was measured in millimeters using ruler. The susceptible, intermediate and resistant categories were assigned on the basis of the critical points recommended by the Clinical and Laboratory Standards Institute [19].

2.6. Preparation of *S. agalactiae*

About 2 to 3 colonies *of* the serially passaged *S. agalactiae* were suspended in 5 mL TSB. The bacterial suspension was adjusted to 10^8 cells/mL using McFarland turbidity standards.

2.7. Acclimatization of the experimental fish

Nile tilapia weighing 25 to 30 g and free from diseases were acclimatized in aquaria for two weeks. Feeding was

provided at 5% rate. The aquaria were provided with aerators.

2.8. Challenge test

Glass aquaria with water capacity of 50 L and dimension of 60 cm × 30 cm × 42 cm were used in pathogenicity test. Each aquarium was stocked with 10 pieces of acclimatized tilapia.

Briefly, 0.1 mL of the serially passaged *S. agalactiae* with approximate concentration of 10^8 cells/mL was injected intraperitoneally to the experimental fish. Tilapia in control group were injected with the same volume of sterile distilled water. The fish were fed twice a day at 5% body weight. Clinical signs and morbidity were recorded daily for a period of four weeks. The set-up was terminated when 100% morbidity or mortality has occurred among the challenged group. Dead or moribund fish were necropsied, and smears from kidney was serially diluted (10^{-2} dilution) and spread aseptically on TSA plates. The plates were incubated at 37 °C for 18 to 24 hours. Mortalities were valid if the recovered colonies in TSA plates are comparable to known colony of *S. agalactiae.*

2.9. Data analysis

Analysis of variance (ANOVA) was used in determining significant differences in the zone of inhibition and survival of unchallenged and challenged fish. Tukey's test was used for the comparison of means.

3. Results and Discussions

3.1. Antibiotic susceptibility to penicillin

In general, majority of serially passaged *S. agalactiae* (T2 to T6) have resulted to increase zone of inhibition (ZOI) of the bacterium to penicillin. These were evident on the following dosages of penicillin: ZOI of T1 or the wild *S. agalactiae* was significantly higher as compared to T2, T3 and T4 at 10 µg/20 µl, in T2, T3 and T4 at 20 µg/20 µl, in T4 at 40 µg/20 µl, 80 µg/20 µl, 100 µg/20 µl and 400 µg/20 µl, and in T3 and T4 at 200 µg/20 µl. It appeared that serially passaged *S. agalactiae* for 30 days (T4) was the most consistent in terms of increased ZOIs as compared to control (T1). As the bacterial passage was increased to 50 days (T6), it resulted to significant decrease on the ZOIs. Therefore, 30 days (T4) or 50 days (T6) bacterial passage was needed in order to change the ZOIs of *S. agalactiae* to penicillin (Table 2).

According to CLSI, the classification of wild and serially passaged *S. agalactiae* was different only at 20 µg/20 µl where wild (T1) was intermediate and serially passaged (T2 to T6) was susceptible to penicillin. The serially passaged *S. agalactiae* could be prevented at lower penicillin dose (20 µg/20 µl) as compared to its wild counterpart (>20 µg/20 µl). As the dosage of penicillin increase beyond 20 µg/20 µl, both of the wild and serially passaged *S. agalactiae* were classified as susceptible (Table 3).

Penicillin is one of the major β-lactam antibiotics. The basic structure of this antibiotics is a thiazolidine ring connected to a β-lactam ring, to which is attached to an acyl side chain [20]. The penicillin as well as other β-lactam antibiotics are bactericidal drugs. They kill susceptible bacterial by inhibiting the synthesis of the bacterial peptidoglycan cell wall [21-22]. The penicillin and cephalosporin interfere with this last step in peptidoglycan synthesis by acting as an analog of the D-alanine-D-alanine portion of the N-acetyl muramic moiety. The conformation of penicillin is very similar to that of D-alanine-D-alanine, so the enzymes involved in the transpeptidation reaction react with the β-lactam nucleus, inactivating the transpeptidase reaction and results in the formation of new peptidoglycan chains that are not cross-linked and lack tensile strength. Weak points develop in the growing cell wall, which result in cell rupture due to osmotic lysis [21]. Resistance to penicillin may occur due to any of the three mechanisms: (a) preventing the drug from reaching its target: diminished permeability of the bacterial cell to the antibiotic; (b) altering the target: alteration(s) of the penicillin-binding proteins; and (c) inactivating the antibiotic: bacterial production of inactivating enzymes, referred to as β-lactamases [23].

The *S. agalactiae* isolated from tilapia pond soil in Lubao, Pampanga, Philippines was found to be intermediate to susceptible to penicillin at dose of 10 µg with ZOI range of 14.60 mm to 33.20 mm [24]. Meanwhile, *S. agalactiae* from tilapia pond water was found to be resistant to the same dose of penicillin [25].

Table 2: Comparison on the zone of inhibition of wild and serially passaged *S. agalactiae* to penicillin of various dosages.

Treatment	Zone of Inhibition (mm)									
	0 µg/ 20 µl	**5 µg/ 20 µl**	**10 µg/ 20 µl**	**20 µg/ 20 µl**	**40 µg/ 20 µl**	**60 µg/ 20 µl**	**80 µg/ 20 µl**	**100 µg/ 20 µl**	**200 µg/ 20 µl**	**400 µg/ 20 µl**
T1 (Control)	0.00± 0.00^{a}	18.25± 1.16^{ab}	18.50± 0.93^{b}	19.75± 0.89^{d}	24.13± 1.36^{bc}	25.50± 1.31^{ab}	25.63± 0.74^{bc}	26.38± 0.74^{bc}	28.25± 1.04^{b}	30.25± 0.46^{bc}
T2	0.00± 0.00^{a}	18.88± 1.13^{a}	20.75± 1.75^{a}	21.63± 1.60^{b}	24.75± 0.71^{ab}	23.50± 1.20^{cd}	25.75± 0.16^{b}	26.00± 1.07^{c}	27.75± 0.89^{b}	29.38± 0.52^{c}
T3	0.00± 0.00^{a}	19.00± 0.53^{a}	20.13± 0.35^{a}	21.25± 0.46^{bc}	23.13± 0.64^{c}	24.25± 0.47^{bc}	25.25± 0.46^{bc}	27.38± 0.52^{ab}	29.63± 0.52^{a}	31.25± 0.89^{ab}
T4	0.00± 0.00^{a}	18.75± 0.71^{ab}	20.50± 1.20^{a}	23.50± 0.76^{a}	25.38± 0.52^{a}	26.13± 0.64^{a}	27.50± 0.53^{a}	28.38± 0.92^{a}	29.88± 1.13^{a}	32.13± 0.99^{a}
T5	0.00± 0.00^{a}	17.50± 0.53^{bc}	18.00± 0.76^{bc}	20.50± 0.53^{bcd}	23.63± 0.52^{bc}	24.63± 0.52^{bc}	24.63± 0.52^{c}	25.88± 0.64^{c}	27.75± 0.46^{b}	27.88± 0.64^{d}
T6	0.00± 0.00^{a}	16.63± 0.92^{c}	16.88± 0.64^{d}	20.00± 0.93^{cd}	21.38± 0.74^{d}	22.25± 0.46^{d}	23.50± 0.53^{d}	25.25± 0.46^{c}	26.50± 0.53^{c}	27.50± 0.76^{d}

Note: T1 = control or wild, T2 = serially passaged for 10 days, T3 = serially passaged for 20 days, T4 = serially passaged for 30 days, T5 = serially passaged for 40 days, T6 = serially passaged for 50 days.

Table 3: CLSI classification of the zone of inhibition of wild and serially passaged *S. agalactiae* to penicillin of various dosages.

Treatment	CLSI Classification									
	0 µg/ 20 µl	5 µg/ 20 µl	10 µg/ 20µl	20 µg/ 20µl	40 µg/ 20µl	60 µg/ 20µl	80 µg/ 20µl	100 µg/ 20µl	200 µg/ 20µl	400 µg/ 20 µl
T1 (Control)	R	I	I	I	S	S	S	S	S	S
T2	R	I	S	S	S	S	S	S	S	S
T3	R	I	S	S	S	S	S	S	S	S
T4	R	I	S	S	S	S	S	S	S	S
T5	R	I	I	S	S	S	S	S	S	S
T6	R	I	I	S	S	S	S	S	S	S

Note: Resistant (R) = ≤ 14 mm; Intermediate (I) = 15 to 19 mm; Susceptible (S) = ≥ 20 mm

3.2 Antibiotic susceptibility to ampicillin

The ampicillin susceptibility of *S. agalactiae* as affected by serial passaged (T2 to T6) was inconsistent as shown by increase or decrease on the measured ZOIs as compared to control (T1). Significant increase in ZOIs as compared to control (T1) were recorded in T2 and T4 at 10 µg/20 µl, in T4 at 20 µg/20 µl, 40 µg/20 µl, 60 µg/20 µl, 80 µg/20 µl, 100 µg/20 µl and 400 µg/20 µl, and in T3 and T4 at 200 µg/20 µl. Meanwhile, significant decrease in ZOIs as compared to control (T1) were recorded in T2, T3, T5 and T6 at 5 µg/20 µl, in T6 at 10 µg/20 µl, 20 µg/20 µl, 40 µg/20 µl, 60 µg/20 µl, 80 µg/20 µl, 100 µg/20 µl and 400 µg/20 µl, and in T4 and T5 at 200 µg/20 µl. Consistent with results on ampicillin, serially passaged *S. agalactiae* for 30 days (T4 from 10 µg/20 µl to 400 µg/20 µl) have resulted to increase ZOIs to ampicillin. Therefore, 30 days (T4) was the bacterial passage needed in order to significantly change the ZOIs of *S. agalactiae* to ampicillin (Table 4).

CLSI classification based on ZOIs revealed that wild (T1) and serially passaged (T2 to T6) *S. agalactiae* were intermediate to susceptible to ampicillin at 5 µg/20 µl to 10 µg/20 µl dosages. Meanwhile starting at 20 µg/20 µl, the bacteria were already susceptible to the antibiotics. Therefore, the dose as low as 20 µg/20 µl was considered effective in treating fishes infected with wild or serially passaged *S. agalactiae* (Table 5).

Ampicillin is used to treat infections by many Gram-positive and Gram-negative bacteria. It was the first "broad spectrum" penicillin with activity against Gram-positive bacteria, including *S. pneumoniae*, *S. pyogenes*, some isolates of *Staphylococcus aureus*, *Trueperella* and some *Enterococcus*. It is one of the few antibiotics that works against multi-drug resistant *E. faecalis* and *E. faecium* [26]. Activity against Gram-negative bacteria includes *Neisseria meningitidis*, some *Haemophilus influenzae*, and some of the Enterobacteriaceae [26-27]. Ampicillin acts as an irreversible inhibitor of the enzyme transpeptidase, which is needed by bacteria to make the cell wall. It inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to cell lysis; therefore, ampicillin is usually bacteriolytic [28].

The *S. agalactiae* isolated from tilapia pond soil in Lubao, Pampanga, Philippines was found to be intermediate to susceptible to ampicillin at dose of 10 µg with ZOI range of 15.20 mm to 25.60 mm [24]. Meanwhile, *S. agalactiae* from tilapia pond water was found to be resistant to the same dose of ampicillin [25].

Table 4: Comparison on the zone of inhibition of wild and serially passaged *S. agalactiae* to ampicillin of various dosages.

Treatment	Zone of Inhibition (mm)									
	0 µg/20µl	5 µg/20 µl	10 µg/20 µl	20 µg/20 µl	40 µg/20 µl	60 µg/20 µl	80 µg/20 µl	100 µg/20 µl	200 µg/20 µl	400 µg/20 µl
T1 (Control)	0.00± 0.00^{a}	20.00± 0.77^{a}	19.13± 0.38^{bc}	21.88± 0.83^{bc}	24.25± 1.16^{b}	25.75± 0.71^{b}	25.00± 0.53^{bc}	26.38± 0.52^{bc}	27.38± 1.30^{c}	29.88± 0.35^{b}
T2	0.00± 0.00^{a}	18.50± 0.76^{bc}	21.00± 0.53^{a}	22.25± 0.46^{bc}	24.25± 0.71^{b}	25.25± 0.46^{b}	24.75± 1.16^{bc}	22.88± 1.46^{d}	24.00± 1.07^{d}	26.75± 1.49^{c}
T3	0.00± 0.00^{a}	17.63± 0.52^{cd}	20.00± 0.76^{ab}	22.50± 0.93^{ab}	24.00± 0.53^{b}	25.63± 0.52^{b}	25.63± 1.19^{b}	26.88± 1.64^{b}	29.50± 0.83^{b}	31.63± 2.07^{b}
T4	0.00± 0.00^{a}	19.38± 0.74^{ab}	21.00± 0.93^{a}	23.63± 0.92^{a}	27.00± 0.76^{a}	28.38 ± 1.19^{a}	29.63± 0.74^{a}	30.13± 0.64^{a}	33.00± 0.76^{a}	35.75± 0.89^{a}
T5	0.00± 0.00^{a}	17.00± 0.53^{d}	18.50± 0.93^{c}	21.75± 0.89^{bc}	23.13± 0.83^{b}	25.13 ± 0.35^{b}	24.13 ± 0.35^{c}	24.88± 0.35^{c}	27.00± 0.53^{c}	27.75± 0.71^{c}
T6	0.00± 0.00^{a}	16.75± 0.46^{d}	17.13± 0.64^{d}	20.00± 1.07^{c}	21.63± 0.52^{d}	23.00 ± 0.00^{d}	24.25± 0.71^{c}	24.88± 0.85^{c}	26.13± 0.64^{c}	27.75± 0.89^{c}

Note: T1 = control or wild, T2 = serially passaged for 10 days, T3 = serially passaged for 20 days, T4 = serially passaged for 30 days, T5 = serially passaged for 40 days, T6 = serially passaged for 50 days.

Table 5: CLSI classification of the zone of inhibition of wild and serially passaged *S. agalactiae* to penicillin of various dosages.

Treatment	CLSI Classification									
	0 µg/20 µl	5 µg/20 µl	10 µg/20 µl	20 µg/20 µl	40 µg/20 µl	60 µg/20 µl	80 µg/20 µl	100 µg/20 µl	200 µg/20 µl	400 µg/20 µl
T1 (Control)	R	S	I	S	S	S	S	S	S	S
T2	R	I	S	S	S	S	S	S	S	S
T3	R	I	S	S	S	S	S	S	S	S
T4	R	I	S	S	S	S	S	S	S	S
T5	R	I	S	S	S	S	S	S	S	S
T6	R	I	S	S	S	S	S	S	S	S

Note: Resistant (R) = ≤ 14 mm; Intermediate (I) = 15 to 19 mm; Susceptible (S) = ≥ 20 mm.

3.3. Antibiotic susceptibility to tetracycline

In general, majority of serially passaged *S. agalactiae (*T2 to T6) have resulted to decrease ZOIs of the bacterium to tetracycline. Significant reductions in ZOIs as compared to control (T1) were recorded in T2, T5 and T6 at 5 µg/20 µl, in T2, T3 and T5 at 10 µg/20 µl, in T2 at 20 µg/20 µl, in T2, T3 and T5 at 60 µg/20 µl, in T2 at 100 µg/20 µl, in T2 and T5 at 200 µg/20 µl, and in T5 at 200 µg/20 µl. It appeared that serially passaged *S. agalactiae* for 40 days (T5) was the most consistent in terms of decreased ZOIs as compared to control (T1). Meanwhile, serially passaged *S. agalactiae* for 30 days (T4) had the most significantly increased value in ZOIs. Therefore, 30 days (T4) bacterial passage was needed in order to significantly change the ZOIs of *S. agalactiae* to tetracycline (Table 6).

At 5 µg/20 µl to 20 µg/20 µl, the CLSI classification of wild *S. agalactiae* (T1) was intermediate to tetracycline. It was found out that T4, T5 and T6 at dosage of 20 µg/20 µl were susceptible to tetracycline. Their CLSI classification was changed to susceptible starting at 40 µg/20 µl. The CLSI classification of serially passaged *S. agalactiae* was inconsistent from 5 µg/20 µl to 20 µg/20 µl doses of tetracycline. Starting in T4, T5 and T6 at dosage of 20 µg/20 µl, the CLSI classification of the two groups of *S. agalactiae* were consistently susceptible (Table 7).

Tetracycline has a broad spectrum of antibiotic action. Originally, it possessed some level of bacteriostatic activity against almost all medically relevant aerobic and anaerobic bacterial genera, both Gram-positive and Gram-negative, with a few exceptions, such as *Pseudomonas aeruginosa* and *Proteus* spp., which display intrinsic resistance [28]. Tetracycline binds to the 30S ribosomal subunit of the bacterium and it interferes with the binding of aminoacyl tRNA to the messenger RNA molecule/ribosome complex, thus, disrupting the bacterial protein synthesis [29]. However, acquired resistance has proliferated in many pathogenic organisms and greatly eroded the formerly vast versatility of this group of antibiotics. Resistance amongst *Staphylococcus* spp., *Streptococcus* spp., *Neisseria gonorrhoeae*, anaerobes, members of the Enterobacteriaceae, and several other previously sensitive organisms is now quite common. Bacteria usually acquire resistance to tetracycline from horizontal transfer of a gene that either encodes an efflux pump or a ribosomal protection protein. Efflux pumps actively eject tetracycline from the cell, preventing the build-up of an inhibitory concentration of tetracycline in the cytoplasm [28]. Ribosomal protection proteins interact with the ribosome and dislodge tetracycline from the ribosome, allowing for translation to continue [30].

The *S. agalactiae* isolated from tilapia pond soil in Lubao, Pampanga, Philippines was found to be susceptible to tetracycline at dose of 30 µg with ZOI range of 22.20 mm to 258.00 mm [24]. Meanwhile, *S. agalactiae* from tilapia pond water was found to be susceptible at 40 µg dose of ampicillin [25].

Table 6: Comparison on the zone of inhibition of wild and serially passaged *S. agalactiae* to tetracycline of various dosages.

Treatment	Zone of Inhibition									
	0 µg/ 20 µl	**5 µg/ 20 µl**	**10 µg/ 20 µl**	**20 µg/ 20 µl**	**40 µg/ 20 µl**	**60 µg/ 20 µl**	**80 µg/ 20 µl**	**100 µg/ 20 µl**	**200 µg/ 20 µl**	**400 µg/ 20 µl**
T1 (Control)	00.00± 00.00^{a}	18.38± 0.74^{ab}	19.75± 0.52^{ab}	19.38± 0.52^{d}	20.13± 0.64^{d}	23.63± 0.52^{a}	24.13± 0.74^{bc}	24.38± 0.52^{c}	26.50± 0.76^{b}	27.13± 0.64^{bc}
T2	00.00± 00.00^{a}	15.38± 0.52^{d}	15.88± 0.64^{e}	15.50± 0.53^{e}	20.00± 0.76^{d}	21.63± 0.74^{bc}	23.38± 1.06^{c}	23.13± 1.06^{d}	25.25± 0.46^{cd}	26.63± 0.74^{cd}
T3	00.00± 00.00^{a}	17.63± 0.52^{bc}	18.25± 0.46^{d}	19.88± 0.35^{cd}	20.38± 0.52^{d}	20.75± 0.71^{c}	21.00± 0.93^{c}	24.38± 0.74^{c}	26.63± 0.52^{b}	28.13± 0.83^{b}
T4	00.00± 00.00^{a}	18.63± 0.52^{a}	20.38± 0.92^{a}	23.63± 0.74^{a}	24.63± 0.74^{a}	24.38± 0.74^{a}	26.50± 0.53^{a}	27.13± 0.64^{a}	27.50± 0.53^{ab}	29.25± 0.89^{a}
T5	00.00± 00.00^{a}	17.25± 0.89^{c}	18.50± 0.53^{cd}	21.00± 0.5^{b}	21.63± 0.52^{c}	22.50± 1.07^{b}	23.38± 0.52^{c}	24.00± 0.53^{cd}	24.50± 0.53^{d}	25.88± 0.64^{d}
T6	00.00± 00.00^{a}	14.75± 0.46^{d}	19.38± 0.5^{bc}	20.25± 0.46^{bc}	23.13± 0.64^{b}	23.77± 0.46^{a}	24.75± 0.46^{b}	25.50± 0.53^{b}	25.88± 0.30^{bc}	27.38± 0.71^{bc}

Note: T1 = control or wild, T2 = serially passaged for 10 days, T3 = serially passaged for 20 days, T4 = serially passaged for 30 days, T5 = serially passaged for 40 days, T6 = serially passaged for 50 days.

Table 7: CLSI classification of the zone of inhibition of wild and serially passaged *S. agalactiae* to tetracycline of various dosages.

Treatment	CLSI Classification									
	0 µg/20 µl	**5 µg/20 µl**	**10 µg/20 µl**	**20 µg/20 µl**	**40 µg/20 µl**	**60 µg/20 µl**	**80 µg/20 µl**	**100 µg/20 µl**	**200 µg/20 µl**	**400 µg/20 µl**
T1 (Control)	R	I	I	I	S	S	S	S	S	S
T2	R	I	I	I	S	S	S	S	S	S
T3	R	I	I	I	S	S	S	S	S	S
T4	R	I	I	S	S	S	S	S	S	S
T5	R	I	I	S	S	S	S	S	S	S
T6	R	R	I	S	S	S	S	S	S	S

Note: Resistant (R) = ≤ 14 mm; Intermediate (I) = 15 to 19 mm; Susceptible (S) = ≥ 20 mm

3.4. Pathogenicity of wild and serially passaged *S. agalactiae*

The observation on the pathogenicity of wild (T1) and serially passaged (T2 to T6) *S. agalactiae* to Nile tilapia lasted for two weeks. Except for T4 (20.00%), the wild *S. agalactiae* (T1 = 30.00%) had lower percent mortality as compared to serially passaged bacterium (T2 = 50.00%, T3 = 50.00%, T5 = 40.00%, T6 = 30.00%). First record of mortality in T1 was observed within 24 hours of injection of the bacterium. For T2, T3 and T4, mortality was first recorded after 3 to 4 days of injection. More than 7 days have passed before mortality was recorded in T5 and T6.

Based upon the onset of first mortality, it appeared that serially passaged *S. agalactiae* for 40 (T5) and 50 days (T6) were less pathogenic as compared to the wild (T1). Before the death of fish in various treatments, clinical signs of bacterial infection were already observed. All of the fish in the treatments had the same signs of bacterial infection such as bulging of eyes, erratic swimming, increase aggressiveness such as biting of caudal fin and cessation of feeding. In a study conducted by Giordano et al. [31], the Nile tilapia challenged with *S. agalactiae* showed alterations in behavior and similar clinical signs such as anorexia, lethargy, erratic swimming, exophthalmia and ascites. Macroscopically, skin hemorrhage, splenomegaly, hepatomegaly with organ paleness and visceral adherences were observed. The highest mortality coefficients were observed in days 1 to 2 after inoculation of the bacterium with accumulated mortality of 44.4%. Except for T1 (66.67%), 100% recovery of *S. agalactiae* was observed in the kidney of dead fish. The colonial morphology of the recovered bacterium in the kidney of dead tilapia in treatments was comparable to the injected bacterium during the pathogenicity test. The injected and recovered *S. agalactiae* colonies were characterized as yellow, small in size, round in shape, entire margin, convex elevation and smooth in texture. The recovery of the injected *S. agalactiae* served as a proof that the same bacterium was the one responsible for the recorded mortality and observed clinical signs of infection (Table 8).

Table 8: Results on mortality based on observed clinical signs of infection and recovered bacterium in the pathogenicity experiment.

Treatment	Mortality (%)	Clinical Signs of Infection	Recovery of Bacterium (%)
T1 (Control)	30.00	Bulging of eyes, lethargy in swimming	66.67
T2	50.00	Erratic swimming, increase aggressiveness, cessation in feeding	100.00
T3	50.00	Bulging of eyes, erratic swimming, cessation in feeding, increase aggressiveness	100.00
T4	20.00	Bulging of eyes, erratic swimming, cessation in feeding, increase aggressiveness	100.00
T5	40.00	Bulging of eyes, erratic swimming, cessation in feeding, increase aggressiveness	100.00
T6	30.00	Bulging of eyes, erratic swimming, cessation in feeding, increase aggressiveness	100.00

3.5. Bacterial serial passage and mutation

Serial passage can either be performed *in vitro* or *in vivo*. In the *in vitro* method, a strain of bacteria will be isolated and allowed to grow for a period of time. After the sample has grown for some time, part of it will be transferred to a new environment and allowed to grow for the same period of time. This process will be repeated as many times as desired [32-33]. When serial passage is performed either *in vitro* or *in vivo*, the bacterium that is being manipulated may evolve by mutating repeatedly. Identifying and studying mutations that occur through serial passage often reveals information about the bacterium being studied. Accordingly, after serial passage has been performed, it can be valuable to compare the resulting sample of bacteria to the original, noting any mutations that have occurred and what the collective effect of these mutations is. A variety of significant outcomes are possible. The virulence or pathogenicity of the bacterium may be changed [17], for example, or a bacterium could evolve to become adapted to a different host environment than that in which it is typically found [17]. Notice that relatively few passages are necessary to produce a noticeable change in a bacterium; for instance, a bacterium can typically adapt to a new host within ten or so passages [17]. In fact, exactly because serial passage allows for rapid evolution of a bacterium to its host, it can be used to study the evolution of antibiotic resistance; specifically, for determining what mutations could lead to the development of antibiotic resistance.

Whereas serial passages experiments are insufficient to predict antibiotic susceptibility/resistance, they are still useful for detecting those genes in which mutation can confer susceptibility/resistance. To identify chromosomally encoded determinants that can alter antibiotic susceptibility, comprehensive transposon-tagged libraries have been used [34-36]. By this approach, those mutants that increase resistance but also those genes whose inactivation increase susceptibility can be identified. Serial passage experiments are also useful for analyzing compensatory mutations that can ameliorate fitness costs in the host cell or affecting the stability of the resistance mechanism, while retaining the original resistance. Compensation has been shown to occur *in vitro*, in animal models [37-41], and in humans [38, 40] or a number of different pathogens [42-43]. Identification of mutations can be done by various methods depending on the organism and our knowledge of the system. Sequencing of PCR-amplified suspected target genes to identify the most probable mutations is a useful first step. In organisms like *Escherichia coli* and *Salmonella typhimurium*, and when the mutant has an easily detectable phenotype, use of standard genetic tools often allow rapid genetic mapping of the mutation. In organisms where genetic manipulation is difficult, the only option might be DNA sequencing [44].

4. Conclusion

In general, the 30 days (T4) serially passaged *S. agalactiae* have resulted to increase ZOIs to penicillin and ampicillin when compared to its wild counterpart (T1). As the bacterial passaged was increased to 50 days (T6), it resulted to significant decrease on the ZOIs to penicillin and ampicillin. In the antibiotic susceptibility, as the dosage of penicillin increased beyond 20 µg/20 µl, both the wild and serially passaged bacteria were classified as susceptible. In ampicillin, starting at 20 µg/20 µl, the bacteria were already susceptible to antibiotics. For tetracycline, the serially passaged *S. agalactiae* for 40 days (T5) was the most consistent in terms of decrease ZOIs as compared to control (T1); meanwhile, serially passaged *S. agalactiae* for 30 days (T4) had the most numbered increase in ZOIs. In general, 30 days (T4) bacterial passaged was needed in order to significantly change the ZOIs of *S. agalactiae* to the three used antibiotics in this experiment. The CLSI classification of serially passaged *S. agalactiae* was inconsistent from 5 µg/20 µl to 20 µg/20 µl doses of tetracycline. Starting in T4, T5 and T6 at dosage of 20 µg/20 µl, the CLSI classification of the two groups of *S. agalactiae* were consistently susceptible.

In the pathogenicity experiment, except for T4 (20.00%), the wild *S. agalactiae* (T1 = 30.00%) had lower percent mortality as compared to the serially passaged bacterium (T2 = 50.00%, T3 = 50.00%, T5 = 40.00%, T6 = 30.00%).

Based on the occurrence of first mortality, it appeared that serially passaged *S. agalactiae* for 40 (T5) and 50 days (T6) were less pathogenic as compared to the wild (T1). All of the fish in the treatments obtained the same signs of bacterial infection. The colonial morphology of the recovered bacterium in the kidney of dead tilapia in treatments was comparable to the injected bacterium during the pathogenicity test.

5. References

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