Evaluation of the Resistance Effect of Genetically Improved Red Tilapia Hybrid towards *Streptococcus agalactiae* Infection

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Abstract: This study was done in interest of getting disease-resistance red tilapia with high increase in body weight. The aim of this study was to evaluate the baseline generation from three populations of whether they have the resistance criteria towards *Streptococcus agalactiae*. In addition, the specific growth rate was calculated based on the fry nursing for 3 months to establish the correlation between growth and resistance towards *Streptococcus*. A total of 42 families were intraperitoneally injected with suspension containing 10^8 CFU/ml of *S. agalactiae*. 14 out of 42 families showed survival rate of 50% or more. There was a significant difference between family and survival of fish (Kruskall-Wallis $\chi 2 = 315.92$, $p = 0.000$). Thus, there is a potential to develop a resistance strain of tilapia towards *Streptococcus* disease through the selective breeding program.

Keywords: *Oreochromis* spp., *Streptococcus agalactiae*, *genetically improved*

Abstrak: Kajian ini dilakukan dengan tujuan untuk mendapatkan ikan tilapia merah yang rintang terhadap penyakit dengan ciri cepat membesar. Tujuan kajian ini adalah untuk menilai prestasi generasi asas daripada tiga populasi, sama ada mempunyai kriteria ketahanan terhadap *Streptococcus agalactiae*. Di samping itu, kadar pertumbuhan spesifik dikira berdasarkan tempoh asuhan selama 3 bulan untuk membuat korelasi di antara pertumbuhan dan rintangan terhadap *Streptococcus*. Sebanyak 42 famili telah disuntik di bahagian *intraperitoneal* dengan ampaian yang mengandungi 10^8 CFU/ml *S. agalactiae*. 14 daripada 42 famili menunjukkan kadar hidup sebanyak 50% atau lebih. Terdapat perbezaan yang ketara di antara famili dan kemandirian ikan (Kruskall-Wallis $\chi 2 = 315.92$, $p = 0.000$). Oleh itu, terdapat potensi untuk membangunkan strain tilapia yang rintang terhadap penyakit *Streptococcus* melalui program kacukan terpilih.

Introduction

Production of tilapia placed in high ranking in many countries such as Malaysia, China and Thailand (Amal & Zamri-Saad, 2011; Kayansamruaj *et al*., 2015; Ortega Asencios *et al*., 2016; Sun *et al*., 2016; Zhu *et al*., 2018). In Malaysia, tilapia is the second largest freshwater species in term of yearly production (DoF statistic, 2018). However, the production of tilapia is decreasing year by year starting from 2012 (38,842 mt) to 2018 (25,200 mt). Disease outbreak due to *Streptococcus* sp. infection has been found as a dominant factor that contribute to the reduction of tilapia production (Sun *et al*., 2016; Zhu e*t al*., 2015). In addition, this bacterium was also associated with disease

occurrences at cage culture in Kenyir and Banding Lake in Malaysia, which cause high mortalities and huge economic losses (Ismail *et al*., 2016; Rahmatullah *et al*., 2017). Previously, there were also reported outbreaks which happened in Brazil, Colombia and Guangdong, China caused by *Streptococcus* sp. (Costa *et al*., 2014; Figueiredo, *et al*., 2012; Hernández *et al*., 2009; Jimenez *et al*., 2011; Sun *et al*., 2016) with serious economic losses impact. Among the most isolated causative bacteria that caused streptococcosis disease in the tilapia industry worldwide were *S. agalactiae*, *S. iniae* and S. *dysgalactiae* (Abuseliana *et al*., 2010; Costa *et al*., 2014; Jimenez *et al*., 2011; Netto *et al*., 2011; Sun *et al*., 2016).

There are several methods engaged by farmers in order to decrease disease occurrences such as implementing biosecurity, therapeutants and vaccination strategies (Shoemaker *et al*., 2017; Liu *et al*., 2016). Vaccination method has been widely used and are effective to prevent the Streptococcal disease in tilapia (Liu *et al*., 2016). However, the use of vaccine will increase the operational cost of the farm and its effectiveness is not consistent especially when given by oral, immersion or spray method (Noraini *et al*., 2013). Alternative method of using antibiotic in feed is also not effective to kill the *Streptococcus*. Rahmatullah *et al*. (2017) found that *Streptococcus* was resistant towards antibiotics such as streptomycin, ampicillin, penicillin and erythromycin. The misuse of antibiotics could trigger the selection of antibiotic resistant bacteria and increase the risk to the environment and human health (Abutbul *et al*., 2004; Chu *et al*., 2016).

Salah *et al*. (2017) found that beta-glucans (immunostimulants) at certain dosages can modulate immune-related genes in Nile tilapia. Zhu *et al*. (2017) also found that a total of 2822 genes has been notably expressed in tilapia spleen when challenged with *Streptococcus agalactiae*. Research on getting increase in genetic gain of performance traits in Nile tilapia (*Oreochromis niloticus*) has been ongoing (Eknath *et al*., 2007; Ponzoni *et al*., 2005) while the interest to get disease resistance strain has recently received attention after the disease outbreak occurrence (Shoemaker *et al*., 2017). It is possible to search for a disease resistance strain according to the fact that immune response is all regulated by immune genes (Zhu *et al*., 2018). Thus, the objective of this study was to identify candidates using challenge test that have the potential to be developed as disease-resistant strain in the selective breeding programs for breeding purposes.

Materials and Methods

Fish preparation

A total of 780 red tilapia hybrid (*Oreochromis* spp.) with body weight ranging between 20.2 g to 238.4 g were used, which comprised of 42 families. Each fish was recognized by its PIT tag number. PIT tags were implanted into juvenile tilapia after 3 months of nursing. Fishes were then grown in the communal ponds for a maximum period of 5 months before harvested and selected for the challenge test. Prior to the experiment, kidney, liver, eye and brain from five fishes from each batch were subjected to bacteriological examination. Only *S. agalactiae*-free fishes were used in the challenge test.

Fish culture

The fish were divided into 4 groups or batches based on the harvesting period. Four batches of fish consisted of 8 to 12 families each, were challenged in 4 different time period. Fish from each family were randomly chosen and separated between male and female. The quantity of fish depends on the total number of fish harvested and were varied between 10 to 30 fish per family. Each batch run has a control group (30 fishes) consisted of the respective families challenged at that time. Stocking of fish was set at 28 to 32 fish per tank according to the total number of male and female at each batch. The fish were kept in 2000 l fiberglass tanks with 1000 l water and fed twice daily with

commercial pellet (32% crude protein, Dindings) in the ratio of 3% wet weight per day. All fishes were acclimatized in the laboratory for at least 7 days prior to the experiment. Fish were anesthetized with clove oil before the injection and off-feed for 2 days after the injection procedure.

Preparation of inoculum

S. agalactiae used as inoculum in the experiment was provided by National Fish Health Research Center (NaFisH), Penang, Malaysia. The isolated strain was stored in TSA (Tryptic Soy Agar - Merck) slant agar until its use. Ten identical colonies were picked and inoculated into BHI (Brain-heart infusion) broth and incubated in an orbital shaker at 150 rpm, 30°C for 18 h. For each batch, *S. agalactiae* culture was measured at 540 nm using spectrophotometer to achieve OD 0.6 with a concentration of 1.5×10^9 CFU/ml when counting on TSA (Merck) plates. Dilutions in TSB (Merck) were performed from the original concentration to obtain 1.0×10^8 CFU/ml inoculum and confirmed by counting on TSA plates. In order to verify the purity of the inoculum, one aliquot from the culture was inoculated in TSA (Merck) containing 5% human blood and incubated at 30°C incubator for 24 h.

Challenge test

Challenge fish was intraperitoneally injected with 0.1 ml of bacterial suspension containing 1×10^8 CFU/ml for each of 50 g body weight fish while the control fish was injected with brain heart infusion broth without the bacteria. Water temperature was continuously monitored at interval of 1 hour using LogTag temperature recorder. Clinical signs and mortality were recorded at 24 h post-injection intervals for 14 days. Fin clips were taken from live and dead fishes after end of experiment. The surviving fish was humanely disposed after the experiment.

Swabs were aseptically collected from the brain, liver, eye and kidney of a freshly dead fish and streaked onto tryptic soy agar supplemented with 5% human blood and incubated at 30°C for 24 h (Rahmatullah *et al*., 2017). A single colony from each initial plate was transferred to a new blood agar plate to obtain pure cultures. Then, the gram-positive, oxidase-negative, and catalase-negative isolates were identified using the API® 20 Strep Kit (BioMérieux, France) according to the manufacturer's instructions.

Statistical analysis

In order to compare the mortality rates in each family, the Kruskall-Wallis test (non-parametric ANOVA tests) was used to compare the independent variable according to mortality of each family. Spearman's correlation coefficients were computed to test the associations of body weight and specific growth rate with the mortality of fish.

Results and Discussion

The highest mortality rate was recorded during the first week of post-infection. After 24 h post-infection, the clinical signs observed were erratic swimming, lethargy, and loss of appetite, however not all the infected fish showed the symptom. Swab samples taken from the organs of the dead fish in the first 3 days confirmed the presence of *S. agalactiae*. Other clinical signs such as corneal opacity, bleeding on the base of pectoral, dorsal and caudal fins, and dark color at the abdominal parts were appeared mostly after 3 days of injection. 14 out of 42 families showed survival rate of 50% or more i.e. UC3A (90%), CU1A (80%), CU3A (80%), CU3B (78.3%), JJ1A (76.2%), UU2B (75.0%), JJ2A (72.2%), CC5A (70.0%), JU3B (69.2%), JC4A (66.7%), CC4A (58.8%), UU3A (57.1%), JU2A (50.0%) and UC1A (50.0%) (Figure 1).

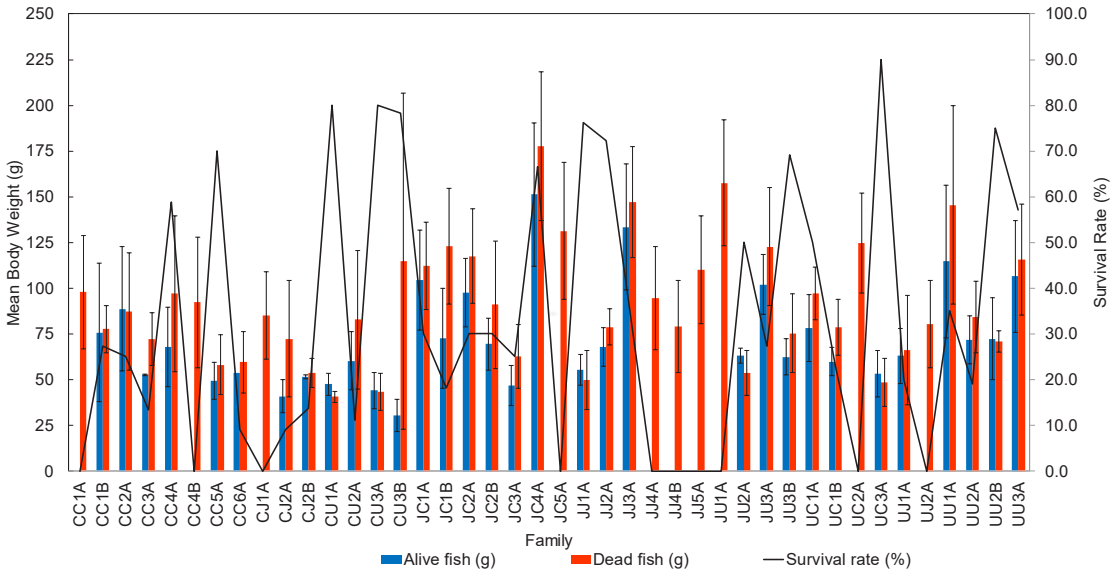

Figure 1: Cumulative percentage of the survival rate and mean body weight of the fish challenged with *S. agalactiae*.

The mean, minimum and maximum temperature value recorded during the challenge test were 26.5°C ± 0.1 (SE), 24.8°C and 27.9°C; respectively. There was a significant difference between family and survival of fish (Kruskall-Wallis $\chi 2 = 315.92$, $p = 0.000$) with larger units of the mean rank, having a high survival rate (Table 1). A negative correlation was found between survival of fish and body weight (Spearman's rho = -0.315, $p < 0.001$ (0.000)).

Table 1: Family ranking based on Kruskal-Wallis Test

No	Family	Mean Rank	No	Family	Mean Rank	No	Family	Mean Rank
1	CC1A	259.50	15	JC1B	332.05	29	JU2A	459.00
2	CC4B	259.50	16	UU2A	335.50	30	UC1A	459.00
3	CJ1A	259.50	17	CC3A	339.30	31	UU3A	487.50
4	JC5A	259.50	18	UJ1A	339.30	32	CC4A	494.21
5	JJ4A	259.50	19	UC1B	351.58	33	JC4A	525.50
6	JJ4B	259.50	20	CC2A	359.25	34	JU3B	535.73
7	JJ5A	259.50	21	JC3A	359.25	35	CC5A	538.80
8	JU1A	259.50	22	CC1B	368.32	36	JJ2A	547.67
9	UC2A	259.50	23	JU3A	368.32	37	UU2B	558.75
10	UJ2A	259.50	24	JC1A	379.20	38	JJ1A	563.50
11	CC6A	295.77	25	JC2A	379.20	39	CU3B	571.76
12	CJ2A	295.77	26	JC2B	379.20	40	CU1A	578.70
13	CU2A	303.83	27	UU1A	399.15	41	CU3A	578.70
14	CJ2B	313.91	28	JJ3A	404.59	42	UC3A	618.60

*larger units of mean rank having a high survival rate

Several researchers have found that certain genes will be expressed when exposed to pathogenic bacteria. Zhu *et al*. (2018) found 5756 unique genes differentially expressed greater than

2-fold when challenged with pathogenic bacteria and resistant fish showed strong abilities in pathogen recognition, antigen presentation and immune activation than the susceptible fish. Shoemaker *et al*. (2017) confirmed the presence of high additive genetic component in the survival fish injected with *S. iniae* (estimated heritability 0.52 ± 0.12) and with *S. agalactiae* (estimated heritability 0.38 ± 0.11). Meanwhile ß-glucan can modulate the immune-related genes in Nile tilapia, which showed better protective effect when challenged with *S. iniae* (Salah *et al*., 2017). Pradeep *et al*. (2016) has confirmed the vertical transmission of streptococcosis since *S. agalactiae* and *S. iniae* were found present in milt, unfertilized eggs.

The main clinical signs observed in this experiment were the same as found by several researchers (Yanong & Francis-Floyd, 2002; Rahmatullah *et al*., 2017). Darkening of skin was also observed at upper parts of abdominal area and might be the result of the accumulation of hemorrhagic ascites along with reddish-brown mucous content in the intestine, deposition of a fibrinoid material on the epicardium and hemorrhagic brownish appearance of the retro-bulbar tissue and meninges (Iregui *et al*., 2014; Zamri-Saad *et al*., 2010). There was no clinical signs observed on the external body of the dead fish after 24 h post-infection. The signs only appeared on the dead fish by the naked eyes after 3 days of post-infection and the same observation was also found by Filho *et al*. (2009). It was also observed that there was no mass mortality occurred after 10 days of post-infection. Filho *et al*. (2009) assumed the establishment of cellular effective immune response towards the bacteria infection occurred after 21 and 28 days. According to Zhu *et al.* (2015), many gene was expressed differentially as early as 5 h and reduced to baseline at 50 h after the infection of *S. iniae*. Thus, this might contribute to fish death during the early hour of post-injection.

The highest temperature recorded during the challenge test in this experiment was 27.9°C while the lowest was at 24°C. This might decrease the pathogenicity effect of *Streptococcus* to cause death to the tilapia tested as found by Kayansamruaj *et al*. (2014). They found that high temperature at 35°C gave higher accumulated mortalities (85%) compared with low temperature 28°C (45% mortalities) when S. *agalactiae* was injected to Nile tilapia. High water temperature in the range of 27.5–31.0°C might also play a major role in the outbreak of streptococcosis in the lake (Rahmatullah *et al*., 2017). However, the mortality pattern of the tested fish was high since some of the families showed 100% mortality rate regardless of the low room temperature. It might be due to the immune response and tolerance to various pathogens can also be lowered by temperatures (Ndong *et al*., 2007).

Small fish was proned to the *Streptococcus* disease compared to bigger fish. Studies on streptococcosis in Malaysia have found that the weight of tilapia susceptible to *S. agalactiae* is between 100 - 300 g (Amal & Zamri-Saad, 2011; Amal *et al*., 2015). It is possible that the family that have high survival rate and challenged with *S. agalactiae* at smaller size doesn't possessed the resistance gene or express the immunity gene. In the future, it is most crucial that only big-sized fishes will be challenged to get a better result. However, Sun *et al*. (2016) couldn't confirm the existence of infection in the adult tilapia as they isolated the bacteria mostly from juvenile stage.

In conclusion, 14 families were found to have prospect to become broodstock for disease resistance strain. The search for a disease resistance tilapia strain will provide an alternative method besides vaccines and antibiotics usage to combat the infection of *Streptococcus* sp., which eventually will help to boost up again the aquaculture production.

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