UNIVERSITI PUTRA MALAYSIA

LETHAL DOSE, CLINICAL SIGNS, PATHOLOGICAL CHANGES AND DISEASE DEVELOPMENT OF *Streptococcus agalactiae* FOLLOWING INTRAPERITONEAL EXPOSURE TO JAVANESE MEDAKA (*Oryzias javanicus, Bleeker* 1854)

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By

SITI SUHAIBA BINTI MASTOR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

LETHAL DOSE, CLINICAL SIGNS, PATHOLOGICAL CHANGES AND DISEASE DEVELOPMENT OF *Streptococcus agalactiae* FOLLOWING INTRAPERITONEAL EXPOSURE TO JAVANESE MEDAKA (*Oryzias javanicus*, Bleeker 1854)

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March 2018

Chairman: Mohammad Noor Amal Azmai, PhD

Faculty: Science

This study was conducted to determine the median lethal dose, clinical signs, pathological changes and disease development of *Streptococcus agalactiae* in Javanese medaka (*Oryzias javanicus*, Bleeker 1854) model, following intraperitoneal exposure. Javanese medaka was collected from estuary area of Sungai Pelek, Sepang, Selangor, and brought to the laboratory for quarantine and acclimatization. The fish were then challenged from 10^2 - 10^8 CFU/mlof virulent *S. agalactiae* via intraperitoneal injection. Mortalities and clinical signs were observed until 240 h post infection (hpi), while the dead fish were collected for bacterial isolation and histological analyses. Median lethal dose 50% (LD_{50}) of *S. agalactiae* in Javanese medaka was determined at 5.3 × 10^2CFU/ml. Most of the infected fish showing lethargy, erratic swimming pattern, exophthalmia and necrosis at the injection site. The histopathological changes were mainly generalised congestion of the internal organs. *Streptococcus agalactiae* were successfully isolated from the dead fish. In the disease development studies, the number of Javanese medaka mortalities following infection by 10^3 CFU/ml of *S. agalactiae* was directly proportional with concentration of *S. agalactiae* in fish and severity of histopathological findings through 96 hpi. Clinical signs and histopathological assessment also showed that infected fish displayed similar findings compared to several the natural host of *S. agalactiae*. This study concluded that Javanese medaka was

susceptible towards *S. agalactiae* infection and could be a potential alternative test organism for study of streptococcocis in fish.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains

DOS KEMATIAN, TANDA KLINIKAL, PERUBAHAN PATOLOGI DAN PERKEMBANGAN PENYAKIT OLEH *STREPTOCOCCUS AGALACTIAE* KEATAS MODEL MEDAKA JAWA (*ORYZIAS JAVANICUS*, BLEEKER 1854), MELALUI PENDEDAHAN SECARA SUNTIKAN ANTARA PERITONEAL

Oleh

SITI SUHAIBA BINTI MASTOR

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Pengerusi: Mohammad Noor Amal bin Azmai, PhD
Fakulti: Sains

Kajian ini telah dilakukan untuk mengkaji dos median kematian, tanda-tanda klinikal, perubahan patologi serta perkembangan penyakit oleh *Streptocococus agalactiae* ke atas medaka Jawa (*Oryzias javanicus*, Bleeker 1854). Medaka Jawa diambil dari Sungai Pelek, Sepang dan dibawa pulang ke makmal untuk penyesuaian kepada suasana makmal. Ikan-ikan tersebut didedahkan kepada beberapa siri kepekatan bakteria *S. agalactiae* daripada 10^2 hingga 10^8 CFU/ml melalui suntikan di ruangan antara peritoneal medaka Jawa. Kematian serta tanda-tanda klinikal direkod sehingga 240 jam selepas suntikan, manakala ikan yang mati diambil untuk pemencilan bakteria dan analisis histologi. Dos kematian yang dikenalpasti ialah 5.3×10^2 CFU/ml. Kebanyakan ikan yang dijangkiti menunjukkan tanda-tanda klinikal seperti keletihan, berenang dengan tidak menentu, eksoftalmia dan nekrosis di kawasan suntikan. Perubahan histopatologi pula menunjukkan lebih kepada kesesakan sel darah merah dalam organ dalaman ikan. *Streptococcus agalactiae* berjaya dibuktikan kehadirannya daripada ikan yang telah mati. Kajian perkembangan penyakit menunjukkan bahawa jumlah kematian selari dengan kepekatan bakteria *S. agalactiae* CFU/g sepanjang 96 hpi. Keputusan kajian menunjukkan bahawa medaka Jawamudah dijangkiti oleh*S. agalactiae* dan berpotensi untuk menjadi organisma alternatif didalam kajian streptococcosis bagi ikan.

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I certify that a Thesis Examination Committee has met on 12 March 2018 to conduct the final examination of Siti Suhaiba Mastor on her thesis entitled "'Lethal dose, clinical signs, pathological changes and disease development of *Streptococcus agalactiae* following intraperitoneal exposure to Javanese medaka (*Oryzias javanicus*, Bleeker 1854)' " in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of Supervisory Committee were as follows:

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I hereby confirm that:

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This is to confirm that:

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LIST OF ABBREVIATIONS

˚C	Degree Celcius
%	Percentage
LD_{50}	Median lethal dose
mm	Millimeter
mgL^{-1}	Miligram per liter
ppt	Parts per thousand
°	Degree
N	North
E	East
µm	Micrometer
m	Meter
cm	Centimeter
USD	US Dollar
PCR	Polymerase chain reaction
IHC	Immunohistochemistry
β	Beta
γ	Gamma
GBS	Group B *Streptococcus*
H&E	Hematoxylin and eosin
hr	Hour
BHIA	brain heart infusion agar
TSA	tryptone soy agar
THBA	Todd-Hewitt broth agar
mg/kg/day	Milligram per kilogram per day
µS/cm	Micro-Siemens per centimeter
NH_4^+	Ammonia
L	Liter
NO_2^-	Nitrite
NO_3^-	Nitrite
$CaCO_3$	Calcium carbonate
g	Gram
cc	Cubic centimeter
G	Gauge
CFU/ml	Colony forming unit per mililiter
rpm	Revolutions per minute
MS222	Triacane-metasulphonate
µL	microliter
EtOH	Ethanol
IP	Intraperitoneal
CFU/g	Colony forming unit per gram

CHAPTER 1

INTRODUCTION

1.1 Introduction

Many studies have been conducted in establishing fish as test organism in various research disciplines. Zebrafish, *Danio rerio* (Hamilton, 1822), for example, is excellently playing its role as the model organism in various studies relating to disease, ecotoxicology, neurology, genetic and etc., since it display many interesting criteria as a test organism (Ribas & Piferrer, 2014). Around 320 million of years ago, this particular species was evolving in South Asia with its geographical distribution predominantly in Bangladesh, Nepal and India (Spence et al., 2008). Recently, zebrafish has been introduced around the globe for the purpose of research (Bier & McGinnis, 2008).

However, the introduction of a non-native species in an ecosystem is always likely to cause an ecological problems if the species is able to integrate itself successfully into the ecosystem, resulting in possible detrimental interactions with native species or even on ecosystem functioning (Gozlan et al., 2010). The non-native species which introduced to the environment are also able to give impacts in terms of competition (Gurevitch et al., 1992; Fausch, 1998; Potapov & Lewis, 2004; Simon et al., 2004; Caiola & Sostoa, 2005; McDowall, 2006;), predation (McDowall, 2006; Bampfylde & Lewis, 2007;Yonekura et al., 2007), transmission of a novel disease (Gozlan et al., 2006), hybridization and habitat modification (McDowall, 2006).

Recently, researchers were looking for new native fish species to be established as test organism, in order to reduce the ecological and environmental impacts. Javanese medaka, *Oryzias javanicus* (Bleeker, 1854), is a small fish which massively studied and amenable in many areas of research. It holds many other model organism's characteristics such as short life cycles, and can be found in a diverse groups distributed around Asia (Magtoon & Termvidchakorn, 2009). They live in brackish water, freshwater and also the saltwater. In the same genus with Javanese medaka, Japanese medaka, *Oryzias latipes* (Temminck & Schlegel, 1846) is one of the most established fish model species, where they are widely used in experimental of vertebral biology for many years (Ismail & Yusof, 2011). Based on Koyama et al., (2006), Javanese medaka is commonly found in estuarine waters of southern to eastern Asia.

Many advantageous featured in medaka as an experimental animal. Throughout the embryonic development, the embryos were totally transparent yet particularly pigment-less mutant, ever since it is an oviparous fish, where the embryonic stage occurs externally (Wakamatsu et al., 2001). Quintet and STIII are the examples of strains which transparent even in the adult stage. Moreover, medaka is able to adapt with low temperature which is its natural tolerance. It is a temperate-zone fish where it is able to survive without thermostatic regulator. The environmental temperature able to control the embryonic development rate, for instance, arresting the embryonic development at 10˚C and recommences at 25˚C (Kinoshita et al., 2009).

Nevertheless, *Streptococcus agalactiae* is a kind of wide range infectious bacteria infecting humans, terrestrial and aquatic animals (Wongsathein, 2012). Various of disease outbreaks had been reported from this bacteria species, thus, has been responsible as causative agent for neonatal meningitis, pneumonia, sepsis, soft tissue and osteomyelitis infections in humans (Brochet et al., 2006; Johri et al., 2006). Bolaños et al., (2005) mentioned that this particular bacteria species latent to infect pregnant women and elderly people as well as immune-compromised adults which lead to mortality, particularly those with malignancies, diabetes mellitus, liver cirrhosis and a history of previous surgery.

On the other hand, ruminants are also susceptible to the infection by *S. agalactiae*. This pathogen prone to inhabit the mammary glands occasioning in clinical and sub-clinical mastitis in cattle which can utterly affect milk quality and production (Phuektes et al., 2001). Besides, it has also been isolated from several other animals presenting with a disease including frogs, mice, dogs, cats, hamsters, chickens, guinea pigs, horses, emerald monitors, monkeys, camels, bottlenose dolphins and captive saltwater crocodiles (Zappuli et al., 2005; Evans et al., 2006; Bishop et al., 2007).

Fish farmers also faced a huge economic loss due to streptococcocis. In acute *Streptococcus* infection, the fish mortality could be observed between 1 to 7 days, and was able to reach up to 50% of mortality. Recently, *Streptococcus inae* had just identified in farmed tilapia (*Oreochromis aureus*) in Mexico (Ortega et al., 2017) meanwhile, *S. difficile* (Berridge et al., 2001) were reported to cause diseases in rainbow trout. In Malaysia, Amal et al.,(2013) stated that red tilapia (*Oreochromis* sp.) were mainly suffered by *S. agalactiae* infection as first reported in late 1990's. Lately, reported outbreaks and infections of *S. agalactiae* in tilapia in Malaysia have been widespread (Amal et al., 2013).

1.2 Problem statements

Unintentionally introduction of alien fish species for utilization as test organisms could potentially annihilate local species, thus, threatening the stability of the existing water ecosystem. Moreover, Javanese medaka has never been evaluated as a model organism in bacterial fish diseases study especially for streptococcocis.

Thus, this study was assessed the availability of Javanese medaka as the native model organism, as another alternative for fish model instead of using non-native species for research purposes in this country.

1.3 Objectives

This study was conducted in order to evaluate the capability of Javanese medaka as an alternative test organism for streptococcocis study in fish.

Thus, the objectives of this study are:

i. to determine the median lethal dose (LD_{50}) of *Streptococcus agalactiae* following intraperitoneal exposure to Javanese medaka *Oryzias javanicus*;
ii. to determine the clinical signs and pathological changes in Javanese medaka following intraperitoneal exposure to *S. agalactiae*;
iii. to assess the disease development of *S. agalactiae* following intraperitoneal exposure to Javanese medaka.

1.4 Hypothesis

It is hypothesized that Javanese medaka is suitable as native test organism for study of streptococcosis in fish.

Thus, the null hypotheses (H_0) for this study are:

i. median lethal dose (LD_{50}) of *S. agalactiae* following intraperitoneal exposure to Javanese medaka cannot be determined;
ii. clinical signs and pathological changes do not shown by Javanese medaka following intraperitoneal exposure by *S.*

agalactiae and are not comparable to the previous studies using different fish species;

iii. there is no development of streptococcosis observed in Javanese medaka following intraperitoneal exposure by *S. agalactiae*

Thus, the alternative hypotheses (H_a) for this study are:

i. median lethal dose (LD_{50}) of *S. agalactiae* following intraperitoneal exposure to Javanese medaka can be determined;

ii. clinical signs and pathological changes shown by Javanese medaka following intraperitoneal exposure by *S. agalactiae* are comparable to the previous studies using different fish species;

iii. there is development of streptococcosis observed in Javanese medaka following intraperitoneal exposure by *S. agalactiae*

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