UNIVERSITI PUTRA MALAYSIA

ROLE OF BIOFILM FORMATION ON THE PATHOGENESIS OF Streptococcus agalactiae INFECTION IN RED HYBRID TILAPIA Oreochromis niloticus x Oreochromis mossambicus

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By

ISIAKU ABDULSALAM IBRAHIM

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

December 2016

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DEDICATION

This work is dedicated to my lovely parents

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

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December 2016

Chairman : Assoc. Prof. Sabri Md. Yusoff, PhD
Faculty : Veterinary Medicine

Streptococcus agalactiae (group B *Streptococcus*, GBS) is an important pathogen of aquatic animals that has led to significant economic loss due to frequent disease outbreak and mortalities in fish worldwide. Tilapia has shown an unusual susceptibility to GBS infection, which is often characterised by acute septicaemia or chronic meningoencephalitis. While acute septicaemia is a sequel to an invasive infection, the mechanism of chronic meningoencephalitis in fish is not fully understood. However, most pathogens that induce acute invasive diseases are capable of developing biofilm associated chronic lesions. Biofilm is an assemblage of irreversibly attached microbial organisms within generated matrix of extracellular polymeric substances. The aim of this research was to investigate the role of biofilm in piscine GBS infection in red hybrid tilapia. A putative piscine GBS biofilm strain was selected and analysed for biofilm formation *in vitro*. The piscine GBS strain exhibited a weak attachment to polystyrene plates by standard crystal violet assay. Furthermore, fluorescent *in situ* hybridization and confocal laser scanning microscopy revealed discrete aggregates of attached piscine GBS around the brain meningeal surface of the orally exposed experimental tilapia. Importantly, these organised aggregates were first detected at a time point corresponding to the transition from an acute to chronic infection. The aggregates where embedded in a polysaccharide containing matrix and became intractable to antibiotic treatment despite earlier *in vitro* susceptibility on sensitivity test. The eye and stomach had no aggregates suggestive of the sessile life style. Intracellular bacterial aggregates, such as within erythrocytes and ventricular ependyma of the brain were also observed. Leukocytic infiltrates predominantly macrophages were readily seen around biofilms, while

erythrocytes appeared often coagulated and severely injured as shown by increased expression of heat shock protein 70. Moreover, an effective adaptive immune response was not detected during the period of study. The erythrocytes may have facilitated invasion of GBS into the brain of tilapia. The present research demonstrates for the first time, that biofilm is associated with persistence of piscine GBS and development of chronic meningoencephalitis in the experimental tilapia**.** It provides a foundation for further investigation and the development of a holistic framework to prevent GBS infection in fish. Current approaches including vaccine strategies need to be reviewed to account for the biofilm phenotype.

Keywords: Tilapia, *Streptococcus agalactiae,* meningoencephalitis, biofilm, *in situ* hybridization

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Veterinar

PERANAN PEMBENTUKAN BIOFILEM KE ATAS PATOGENESIS *Streptococcus agalactiae* PADA TILAPIA HYBRID MERAH *Oreochromis niloticus* x *Oreochromis mossambicus*

Oleh

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Streptococcus agalactiae (Streptokokus kumpulan B, GBS) merupakan patogen penting dalam haiwan akuatik yang membawa kepada kerugian ekonomi yang ketara disebabkan oleh kekerapan wabak penyakit dan kematian ikan di seluruh dunia. Ikan tilapia telah menunjukkan kecenderungan jangkitan GBS yang selalunya dikategorikan sebagai septisemia akut atau meningoensefalitis kronik. Oleh kerana septisemia akut merupakan ekoran dari jangkitan invasif, mekanisme bagi penyakit meningoensefalitis kronik pada ikan masih belum difahami sepenuhnya. Walau bagaimanapun, kebanyakan patogen yang menggalakkan penyakit invasif akut ini mampu membentuk biofilem yang berkaitan lesi kronik. Biofilem adalah gabungan organisma mikrob yang berkeadaan kekal di antara matrik janaan dari bahan-bahan polimerik luar sel. Kajian ini bertujuan untuk mengkaji peranan biofilem terhadap jangkitan GBS dalam tilapia hibrid merah. Biofilem jenis *piscine* putatif telah dipilih dan dianalisa untuk formasi biofilem secara *in vitro*. *Piscine* GBS telah menunjukkan pelekatan yang lemah kepada plat polisterin oleh asai standard kristal violet. Tambahan, hibridiasi pendarflour *in situ* dan mikroskopi imbasan laser sefokus menunjukkan agregat diskret oleh GBS di sekitar permukaan meningeal otak yang terdedah secara oral dalam tilapia. Pentingnya, agregat yang teratur telah dikesan awal pada titik masa yang sepadan dengan transisi daripada jangkitan akut kepada jangkitan kronik. Agregat tersebut yang terbenam dalam polisakarida yang mengandungi matrik dan menjadi sukar untuk dirawat dengan rawatan walaupun pada awalnya lebih cenderung dengan ujian sensitiviti. Mata dan perut yang tiada agregat menandakan gaya hidup yang sesil. Bakteria intrasel beragregat seperti di antara sel eritrosit dan ventrikel ependima otak juga turut diperhatikan. Penyusupan leukosit kebanyakannya

ialah makrofaj dapat dilihat di sekitar biofilem sementara kemunculan eritrosit kerap dalam keadaan membeku dan luka teruk seperti yang ditunjukkan dengan peningkatan ekspresi kejutan haba protein 70. Disamping itu, tindak balas imun mudah suai efektif tidak dapat dikesan sepanjang tempoh kajian. Eritrosit mungkin memudahkan GBS untuk menyerang bahagian otak tilapia. Kajian terkini menunjukkan buat pertama kalinya, biofilem yang berkaitan dengan GBS berterusan dan pembentukan kronik meningoensefalitis dalam eksperimen tilapia. Ia turut menyediakan asas kepada penyelidikan lanjutan dan pembangunan satu rangka kerja yang menyeluruh untuk mencegah jangkitan GBS dalam ikan. Pendekatan semasa termasuk strategi vaksin perlu dikaji semula untuk diskripsi fenotip biofilem.

Kata kunci: Tilapia, *Streptococcus agalactiae*, biofilem, meningoensefelitis, hibridasi *in situ*

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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 Veterinary Science in Pathology. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AB-PAS	Alcian blue and periodic acid Schiff
ANOVA	Analysis of variance
AOPI	Acridine orange and propidium iodide
AP	Ancillary protein
BBB	Blood brain barrier
BHIB	Brain heart infusion broth
BKD	Branched chained alpha-keto acid dehydrogenase
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CC	Clonal complex
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
COX	Cyclooxygenase
Cy3	Cyanine 3
DAB	Diaminobenzidine
DAPI	Diamidino-phenylindone
DB	Denaturation Buffer
ddH_2O	Deionised distilled water
DNA	Deoxyribonucleic acid
DVM	Doctor of veterinary medicine
eDNA	Extracellular DNA
ELISA	Enzyme-linked immunosorbent assay
EPS	Exopolymeric substances
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate

GALT	Gut associated lymphoid tissues
GAPDH	Glyceraldehyde-phosphate dehydrogenase
GAS	General adaptive syndrome
GBS	Group b streptococcus
H&E	Haematoxylin and eosin
HB	Hybridization Buffer
HRP	Horseradish peroxidase
HSP70	Heat shock protein 70
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL	Interleukin
iTRAQ	Isobaric tagging for relative and absolute quantification
MS 222	Tricaine methanesulfonate
NaCl	Sodium chloride
NYSC	National youth service corp
OD	Optical density
PAMPs	Pathogen associated membrane patterns
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween 20
PCR	Polymerase chain reaction
PDE	Professional diploma in education
PFA	Paraformaldehyde
PI	Pilus island
PK	Pyruvate kinase
PRR	Pathogen recognition receptors
PVC	Polyvinyl chloride
QS	Quorum sensing

RAS	Recirculating aquaculture system
SD	Standard deviation
SEM	Scanning electron microscopy
Srt	Sortase
SSC	Saline sodium citrate
ST	Sequence type
TCR	T-cell receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Tris-HCl	Tris hydrochloric acid

CHAPTER 1

INTRODUCTION

1.1 Background of study

The existence of bacterial organisms in nature as biofilm is recently well acknowledged, and when present on tissues it is often associated with disease chronicity (Hall-Stoodley & Stoodley 2009). Biofilm is an assemblage of surface associated, irreversibly attached microbes within generated matrix of extracellular polymeric substance (Donlan 2002). Unlike freely suspended or planktonic cells, they are characterised by a reduce growth rate and the regulation of specific genes (Donlan 2002, Cheng et al., 2010).

Many species of *Streptococcus* naturally exist within these organised surface associated microbial communities (Nobbs et al., 2009). Importantly, *Streptococcus agalactiae* also known as group B *Streptococcus* (GBS) was shown to exhibit the biofilm phenotype *in vitro* (Rinaudo et al., 2010, Borges et al., 2012). This pathogen affects humans, ruminants and fish. In fish, it has led to a significant economic loss due to disease outbreaks and high mortalities in global aquaculture (Delamare-Debouteville et al., 2014, Guo et al., 2014).

Piscine GBS infection in Malaysia was first reported in the late 1990s and have since been a serious burden in tilapia culture. Outbreak was earlier confined to Pahang (1997), Terengganu and Kelantan (2002) at Pahang River, Kenyir Lake and Pergau lake respectively, but it is currently widespread in Peninsular Malaysia (Zamri-Saad et al., 2014). The annual revenue from tilapia production has therefore reduced concomitantly. In part, this is because, current treatments and vaccines developed for use against disease by piscine GBS have been ineffective (Sudheesh et al., 2012, Zamri-Saad et al., 2014).

While it has been suggested that juvenile tilapia is an important source of infection to fish farms (Anshary et al., 2014), persistence of GBS in brood stock and its possible acquisition by either egg, larvae, fry or fingerlings require investigation. Indeed, the closely related *Streptococcus pyogenes* and *Streptococcus suis* have been involved in biofilm associated persistence (Grenier et al., 2009, Oliver-Kozup et al., 2011). Biofilm is also very common in aquatic environments, providing a niche for potentially pathogenic bacteria (Parsek & Singh 2003) such as piscine GBS.

The pathogenicity of GBS in tilapia has been ascertained, however, knowledge of its interaction with the respective host is still not fully understood (Abuseliana et al., 2011, Guo et al., 2014, Su et al., 2015). Indeed, it is unknown if piscine GBS has the ability to form biofilm *in vitro* such as the aquatic environment or within the fish host. Therefore, the main aim of this study was to determine biofilm formation by piscine GBS and its role in the pathogenesis of infection in red hybrid tilapia.

1.2 Objectives of the study

The specific objectives set for the study were:

1. to develop and quantify piscine GBS biofilm using a microtitre plate assay.
2. to investigate *in vivo* GBS biofilm formation in the brain, eye and stomach of red hybrid tilapia.
3. to determine the microscopic changes associated with GBS biofilm formation in the brain, eye and stomach of red hybrid tilapia.

1.3 Hypothesis

1. **Null:** piscine GBS does not exhibit biofilm phenotypic characteristics.
2. **Alternate:** piscine GBS exhibit biofilm phenotypic characteristics.

1.4 Statement of problems

1. Piscine GBS is presently a cause of persistent infection, disease outbreak, mortality and high economic loss for tilapia culture in Malaysia and other parts of the world.
2. The pathogenesis of GBS in tilapia is still not fully understood, and the role of biofilm is currently unknown.
3. Biofilm formation is a potential challenge for current attempts to develop effective vaccines and other control strategies against GBS in aquaculture.

1.5 Justification of the study

Piscine GBS is the most important bacterial pathogen in tilapia culture. However, the interaction of GBS with the tilapia host is still not fully understood. In humans, GBS is thought to evade tissue responses and persist through the mechanism of biofilm formation. This underscores the need for a comparative investigation of the biofilm mechanism in the lower vertebrate whose defence system has also completely evolved – with components of both

innate and adaptive immunity. In addition, persistence of GBS in tilapia has been observed in aquaculture and biofilm formation may be involved.

1.6 Significance of the study

The findings of this study further explains the pathogenesis of GBS in tilapia. Accordingly, it provides an additional basis for testing the most effective strategy for prevention, and adoption of better measures in the control of disease by GBS in aquaculture.

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