MOLECULAR CHARACTERIZATION AND PATHOGENICITY OF Streptococcus agalactiae SEROTYPE IA ST7 AND III ST283 ISOLATED FROM CULTURED RED HYBRID TILAPIA IN PENINSULAR MALAYSIA

SYUHADA ROSLAN

FS 2019 88
MOLECULAR CHARACTERIZATION AND PATHOGENICITY OF
Streptococcus agalactiae SEROTYPE IA ST7 AND III ST283
ISOLATED FROM CULTURED RED HYBRID TILAPIA IN
PENINSULAR MALAYSIA

By

SYUHADA ROSLAN

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

August 2019
All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia
Aquaculture is one of the major contributors of economic resources and food production in Malaysia and tilapia (Oreochromis spp.) was one of the highest value in import and export market. But the production was reported to decrease from 51,554 MT to 35,996 MT from 2012 to 2016 respectively due to streptococcosis. Streptococcosis was caused by Streptococcus sp. which is the etiological agent of diseases in human and animals which could lead to different pathological effects. It was suggested that the different pathological effects post infection was caused by genetically distinction of the bacterial strain. Thus, this study was conducted to determine the molecular serotyping, virulence genes profiling and pathogenicity of Streptococcus agalactiae isolated from cultured red hybrid tilapia in Malaysia. A total of 256 isolates of S. agalactiae, isolated from fish in Peninsular Malaysia. Genotype of the bacterial collections was determined using PCR based on the capsular polysaccharide gene clusters and multilocus sequence typing. Subsequently, two serotypes were identified, which were 11% and 89% for serotype Ia ST7 and III ST283, respectively. The profiles of virulence genes were constructed using m-PCR with different types of the various published primers involving 14 standard virulence genes (fbsA, fbsB, pavA, scpB, lmb, cyle, cfb, spb1, hylB, rib, bca, bac, cspA and pbp1A/ponA). Serotype Ia showed different pattern of virulence genes profile compared to serotype III, where it lacked the lmb, scpB, pavA, fbsB, cyle, bca, cspA and bac genes, that differentiated the virulence level of the serotype. Median lethal dose 50 (LD₅₀) of S. agalactiae in red hybrid tilapia for serotypes Ia and III were calculated at 8.7 x 10³ CFU/mL and 6.3 x 10³ CFU/mL, respectively. There was no significant difference (p > 0.05) between the rate of mortality of red hybrid tilapia for both serotypes following intraperitoneal challenge. Histopathological
lesions included meningitis, necrosis of hepatocytes, coagulative tubular necrosis and hypocellular of spleen. Histopathological scoring and independent t-test showed that there was the significant difference (p < 0.05) between all lesions in brain, liver (except hepatonecrotic), and spleen but not in kidney. *Streptococcus agalactiae* serotype Ia ST7 showed severe lesions in all organs compared to *S. agalactiae* serotype III ST283. This study concluded that there were two serotypes (Ia and III) of *S. agalactiae* isolated from cultured red hybrid tilapia in Malaysia so far. Moreover, different serotypes of *S. agalactiae* consisted of different virulent characteristics. Red hybrid tilapia was susceptible to both serotypes following intraperitoneal challenge. Serotype Ia showed higher LD$_{50}$ compared to serotype III, but not significant (p > 0.05) in term of cumulative mortality.

Keywords: *Streptococcus agalactiae*, molecular serotyping, virulence gene, pathogenicity.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Master Sains

CIRI MOLEKUL DAN ANALISIS KEPATOGENAN OLEH *Streptococcus agalactiae* SEROTIPE IA ST7 DAN III ST283 DIPENCIL DARI IKAN TILAPIA MERAH HIBRID DI SEMENANJUNG MALAYSIA

Oleh

SYUHADA ROSLAN

Ogos 2019

Pengerusi : Mohammad Noor Amal Azmai, PhD
Fakulti : Sains

Akuakultur ialah salah satu dari sumber ekonomi dan penghasilan makanan di Malaysia dan Tilapia (*Oreochromis* spp.) adalah salah satu penyumbang tertinggi dalam pasaran import dan ekspor. Walaupun itu, penghasilan tilapia dilaporkan telah menurun dari 51, 554 MT ke 35, 996 MT pada 2012 ke 2016, disebabkan oleh *Streptococcosis*. *Streptococcus* sp. iaitu ejen etiologi bagi penyakit manusia dan haiwan. Hal ini dicadangkan bahawa kesan patologi yang berbeza selepas jangkitan disebabkan oleh perbezaan genetik bagi jenis bakteria. Oleh itu, kajian ini dilakukan untuk menentukan penentuan serotipe molekular, profil gen virulen dan analisis patogenisiti *Streptococcus agalactiae* yang dipencil dari ikan tilapia merah di Malaysia. Sebanyak 256 sampel *S. agalactiae* dari Semenanjung Malaysia telah digunakan dalam kajian ini. Genotip bakteria telah ditentukan mengunakan kaedah tindak balas rantai polimer berdasarkan penentuan serotipe molekular dari kumpulan gen polisakarida kapsul dan jenis urutan multilokus di mana dua jenis serotipe telah ditemukan iaitu 11% dan 89% untuk serotipe ST7 dan ST283. Profil gen virulen telah dibina menggunakan kaedah m-PCR berdasarkan primer yang berbeza melibatkan 14 gen virulen standard (*fbsA, fbsB, pavA, scpB, lmb, clyE, cfb, spb1, hylB, rib, bca, bac, cspA dan pbp1A/ponA*). Serotip ia menunjukkan corak yang berbeza berbanding serotipe III dimana ianya tiada gen *lmb, scpB, pavA, fbsB, cly, bca, cspA* dan *bac* gen, yang membezakan tahap kevirulenan kedua-dua serotipe. Dos kematian 50 (LD₅₀) bagi serotipe Ia ialah $8.7 \times 10^{3}$ CFU/ml manakala $6.3 \times 10^{3}$ CFU/ml bagi serotipe III. Tiada perbezaan bermakna antara kematian disebabkan oleh serotipe la dan III ($p > 0.05$). Perubahan histopatologi termasuk meningitis, nekrosis sel hepatosit, nekrosis koagulan dengan sitoplastma merah dan hiposel dalam limpa. Penilaian keterukan lesi histopatologi dan analisis t-
test telah merekod perbezaan yang bermakna (p < 0.05) pada lesi otak, hati (kecuali hepatonekrotik), dan limpa tetapi tidak bermakna pada ginjal. Kajian ini merumuskan yang terdapat dua serotipe (Ia dan III) S. agalactiae yang dipencil dari ikan tilapia hibrid merah di Malaysia setakat ini. Tambahan pula, serotipe yang berbeza mempunyai karakter virulensi yang berbeza. Ikan tilapia hibrid merah juga mudah dijangkiti oleh kedua-dua serotipe menggunakan kaedah intraperitoneal. Serotipe Ia menunjukkan kadar LD₅₀ yang tinggi berbanding serotipe III, tetapi tidak bermakna dari segi kumulatif kadar kematian.

Kata kunci: Streptococcus agalactiae, penentuan serotipe molekular, gen virulen, kepatogenan.
ACKNOWLEDGEMENTS

All praises to ALLAH S.W.T. for bounties of mercy and guidance which enabled me to complete this thesis. Indeed, without His Help and Will, nothing is achievable. I had completed this report as fulfillment of the requirement for Master of Science (Bacteriology) by research.

First of all, I would like to thank Universiti Putra Malaysia for financially supported my study under the Graduate Research Fellowship (GRF) scholarship and chances to study and gain experience here. I would like to thank the Department of Biology, Faculty of Science, UPM, for giving me opportunities to learn many things and write this thesis. I also would like to express my profound gratitude to my supervisor Assoc. Prof. Dr. Mohammad Noor Amal Azmai who encouraged, guided and advised me along the way. I am extremely grateful and gratitude with much appreciation for his guidance by sharing his knowledge and his supportive character to enhance quality of this thesis.

I also would like to take this opportunity to express my deep sense of gratitude to all my supervisory committee members, Prof. Dr. Mohd Zamri Saad, Assoc. Prof. Dr. Ina Salwany Md Yasin, and Assoc. Prof. Dr. Muskazli Mustapha for the professional advice. On the other hand, appreciation is credited to Dr. Nurrul Shaqinah Nasruddin from Universiti Kebangsaan Malaysia, who patiently guided me through the honors project and helped me with the ideas. Also, Mrs. Latifah Hanan from Histopathology Laboratory, Faculty of Veterinary Medicine, UPM, special thank for her guidance, monitoring and suggestions all the while through conducting my experiment.

After all, I was fortunate to have supportive siblings Nur Fasihah, Nur Hana, Suhaili Auni, Nurlina Wahida, Amir Muslim and especially my beloved parent Mr. Roslan Ibrahim and Mrs. Zamihah Ismail. Special thanks to them for giving positive moral and financial support while I am conducting my experiment. And not to forget my helpful friend, Nurul Najwa Farihah and Jumria Sutra for always be there whenever I need a helping hand in finishing my study.
I certify that a Thesis Examination Committee has met on 8 August 2019 to conduct the final examination of Syuhada binti Roslan on her thesis entitled "Molecular Characterization and Pathogenicity of Streptococcus agalactiae Serotype Ia ST7 and Serotype III ST283 Isolated from Cultured Red Hybrid Tilapia in Peninsular Malaysia" 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.

Members of the Thesis Examination Committee were as follows:

Shamarina bt Shohaimi, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Hishamuddin b Omar, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Lee Seong Wei, PhD
Associate Professor
Faculty of Agro Based Industry
Universiti Malaysia Kelantan
Malaysia
(External Examiner)

NOR AZOWA IBRAHIM, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 October 2019
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 the Supervisory Committee were as follows:

Mohammad Noor Amal Azmai, PhD  
Associate Professor  
Faculty of Science  
Universiti Putra Malaysia  
(Chairman)

Mohd Zamri Saad, PhD  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

Ina Salwany Md Yasin, PhD  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

Muskhazli Mustafa, PhD  
Associate Professor  
Faculty of Science  
Universiti Putra Malaysia  
(Member)

ZALILAH MOHD SHARIFF, PhD  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of the thesis are fully owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012,
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writing, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: ________________________   Date: __________________

Name and Matric No.: _______________________________________

© COPYRIGHT UPM
Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to:

Signature: ____________________________________________________________
Name of Chairman of Supervisory Committee: ____________________________

Signature: ____________________________________________________________
Name of Member of Supervisory Committee: ____________________________

Signature: ____________________________________________________________
Name of Member of Supervisory Committee: ____________________________

Signature: ____________________________________________________________
Name of Member of Supervisory Committee: ____________________________
TABLE OF CONTENTS

ABSTRACT                              i
ABSTRAK                                iii
ACKNOWLEDGEMENTS                       v
APPROVAL                               vi
DECLARATION                            vii
LIST OF TABLES                         xiii
LIST OF FIGURES                        xvi
LIST OF ABBREVIATIONS AND SYMBOLS      xvii

CHAPTER

1 INTRODUCTION
1.1 Background of study  1
1.2 Problem statements  3
1.3 Objectives  4

2 LITERATURE REVIEW
2.1 Streptococcosis  5
2.2 Streptococcus agalactiae  6
2.3 Streptococcus agalactiae infection in animal and human  7
2.4 Identification and detection of Streptococcus agalactiae  10
  2.4.1 Phenotypic and biochemical test of Streptococcus agalactiae  10
  2.4.2 PCR-based methods and 16S rRNA gene sequencing  11
2.5 Molecular typing of Streptococcus agalactiae  11
  2.5.1 Molecular serotyping based on cellular component
    2.5.1.1 Cellular components and pathogenic characteristics  12
    2.5.1.2 Capsular polysaccharide (cps) genes  13
  2.5.2 Multilocus sequence typing (MLST)
    2.5.2.1 Variation in sequence type (ST) of Streptococcus agalactiae  16
2.6 Virulence characteristics of Streptococcus agalactiae  17

3 MATERIALS AND METHODS
3.1 Bacterial stock, isolation and culture  20
3.2 Phenotypic and biochemical characterizations  20
3.3 Bacterial genomic DNA extraction  21
3.4 PCR and 16S rRNA sequencing analysis 21
3.5 Singleplex PCR serotyping based on cps genes cluster 21
3.6 Multilocus sequence typing 23
3.7 Determination of virulence genes profiles 24
3.8 Pathogenicity trials preparation 26
3.8.1 Bacterial culture and preparation 26
3.8.2 Serial dilution and colony forming unit counting 26
3.8.3 Fish acclimatization 27
3.8.4 Bacterial passage 27
3.8.5 Fish challenge and monitoring 27
3.8.6 Median lethal dose determination 28
3.8.7 Histopathological preparation and scoring 28
3.8.8 Statistical analysis 29

4 RESULTS
4.1 Phenotypic characterization of *Streptococcus agalactiae* 30
4.2 Genotypic characterization of *Streptococcus agalactiae* 31
4.2.1 Bacterial identification using PCR method 31
4.2.2 Singleplex PCR for serotyping of *Streptococcus agalactiae* 32
4.2.3 Distribution of *Streptococcus agalactiae* serotype Ia ST7 and III ST283 based on sampling sites 33
4.2.4 MLST of *Streptococcus agalactiae* 34
4.2.5 Virulence genes profiling of *Streptococcus agalactiae* 35
4.3 Pathogenicity of *Streptococcus agalactiae* infection in red hybrid tilapia (*Oreochromis niloticus* x *O. mossambicus*) 36
4.3.1 Colony forming unit per milliliter (CFU/ml) 36
4.3.2 Reisolation of *Streptococcus agalactiae* from challenged fish 37
4.3.3 Mortality pattern of *Streptococcus agalactiae* following infection by serotype Ia ST7 and III ST283 38
4.3.4 Median lethal dose of *Streptococcus agalactiae* for serotype Ia ST7 and III ST283 40
4.3.5 Clinical signs and gross lesions of infected fish 40
4.3.5.1 External and internal clinical signs and gross lesions of serotype Ia ST7 42
4.3.5.2 External and internal clinical signs and gross lesions of serotype III ST283 43
4.3.6 Comparative rate of mortality 45
4.4 Histopathological assessment red hybrid tilapia (Oreochromis niloticus x O. mossambicus) following infection by Streptococcus agalactiae

4.4.1 Brain section
4.4.2 Liver section
4.4.3 Kidney section
4.4.4 Spleen section

5 DISCUSSION
5.1 Phenotypic characterization of Streptococcus agalactiae
5.2 Genotypic characterization of Streptococcus agalactiae
5.2.1 Phenotypic-genotypic PCR identification of Streptococcus agalactiae
5.2.2 Serotyping of Streptococcus agalactiae based on cps gene cluster
5.2.3 Virulence gene profiling of Streptococcus agalactiae
5.2.4 MLST of Streptococcus agalactiae for isolates used in pathogenicity challenge
5.3 Clinical signs, gross lesions, mortality and lethal dose (LD_{50}) of Streptococcus agalactiae infection in red hybrid tilapia (Oreochromis niloticus x O. mossambicus)
5.4 Histopathological assessment of Streptococcus agalactiae infection in red hybrid tilapia

6 CONCLUSION AND RECOMMENDATION
6.1 Conclusions
6.2 Recommendations

REFERENCES
APPENDICES
BIODATA OF STUDENT
PUBLICATION
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>List of fish species infected by streptococcal-related bacterial.</td>
</tr>
<tr>
<td>2.2</td>
<td>Type of hemolysis activities and properties (Buxton et al., 2005)</td>
</tr>
<tr>
<td>2.3</td>
<td>Common nucleic acid amplification methods used in clinical laboratories (Gullet and Molte, 2015)</td>
</tr>
<tr>
<td>2.4</td>
<td>The list of <em>S. agalactiae</em> serotype (Ia to IX) and gene contents.</td>
</tr>
<tr>
<td>2.5</td>
<td>The presence of virulence genes in <em>S. agalactiae</em> serotype Ia and III isolated from Nile tilapia (Kannika et al., 2017).</td>
</tr>
<tr>
<td>2.6</td>
<td>The 14 GBS virulence genes and its function (Lin et al., 2011).</td>
</tr>
<tr>
<td>3.1</td>
<td>Specific primers and sequence used in this study (Imperi et al., 2010)</td>
</tr>
<tr>
<td>3.2</td>
<td>Oligonucleotide primer used for GBS MLST (Jones et al., 2003)</td>
</tr>
<tr>
<td>3.3</td>
<td>Multiplex PCR based on 14 human virulence gene (Kannika et al., 2017).</td>
</tr>
<tr>
<td>4.1</td>
<td>Biochemical test results of <em>Streptococcus agalactiae</em>.</td>
</tr>
<tr>
<td>4.2</td>
<td>Summarization of <em>S. agalactiae</em> serotype Ia ST7 and III ST283 based on year of sampling and localities.</td>
</tr>
<tr>
<td>4.3</td>
<td>The virulence gene profiles of <em>S. agalactiae</em> serotype Ia and III isolated from red hybrid tilapia grouped based on adhesion, invasion and immune evasin function.</td>
</tr>
<tr>
<td>4.4</td>
<td>Summarization of serial dilution result</td>
</tr>
<tr>
<td>4.5</td>
<td>Mortality pattern of red hybrid tilapia following IP challenge with different serotype of <em>Streptococcus agalactiae</em></td>
</tr>
<tr>
<td>4.6</td>
<td>Histological lesions scoring of selected organs following infection with <em>Streptococcus agalactiae</em> serotype Ia ST7 and III ST283, n=5.</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Clinical signs showed by fish infected by <em>Streptococcus agalactiae</em>. (a) Exophthalmia of fish eye; (b) Swelling and hemorrhage at the gall bladder and the liver of fish; (c) Congestion of the viscera; (d) Yellow and dark red nodules in the muscle near the vertebra (Anshary et al., 2014; Liu et al., 2014; Musa et al., 2014; Zamri-Saad et al., 2014).</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>Histopathological observation of <em>Streptococcus agalactiae</em> infection in fish. (a) Congested blood vessels with swollen endothelium and thrombosis in liver (b) Meninges (arrow) of a red tilapia naturally infected by S. agalactiae showing thickening due to infiltration of heterophils (c-d) Arrow showed accumulation of bacteria while asterisk marks the necrosis center (Liu et al., 2014; Zamri-Saad et al., 2014; Pradeep et al., 2016).</td>
<td>9</td>
</tr>
<tr>
<td>2.3</td>
<td>Conserved and variable region of 16S rRNA gene (with length) (Sharma, 2016).</td>
<td>12</td>
</tr>
<tr>
<td>2.4</td>
<td>Illustration of adhesion, invasion and immune evasion of pathogenic bacteria (Foster et al., 2004).</td>
<td>18</td>
</tr>
<tr>
<td>4.1</td>
<td>Amplification of <em>S. agalactiae</em> using specific primer from isolates of nine location sampling site. Lane 1 to 9, PCR product obtained showing expected band size of 192 bp. PCR products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining.</td>
<td>31</td>
</tr>
<tr>
<td>4.2</td>
<td>Amplification <em>cps</em> genes of <em>S. agalactiae</em> using two primer pairs. Lane M1 and M2 are the DNA ladder 1kb and 100 bp; Lane 1 and 2 are the band size for serotype Ia (272 bp and 688 bp); Lane 3 and 4 are the band size for serotype III (352 bp and 688 bp). PCR products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining.</td>
<td>32</td>
</tr>
<tr>
<td>4.3</td>
<td>Map of distribution of <em>S. agalactiae</em> serotype Ia ST7 and III (ST283) based on nine sampling sites.</td>
<td>33</td>
</tr>
<tr>
<td>4.4</td>
<td>Amplification MLST housekeeping genes for <em>S. agalactiae</em> using seven primer pairs. Lane M is the DNA ladder 1kb; Lane 1 to 7 are the band size for <em>adhP</em>, <em>pheS</em>, <em>atr</em>, <em>glnA</em>, <em>sdhA</em>, <em>glck</em>, and <em>tkt</em> gene. PCR products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining.</td>
<td>34</td>
</tr>
<tr>
<td>4.5</td>
<td>(a) Amplification of 14 standard virulence genes of <em>S. agalactiae</em> for serotype Ia and (b) serotype III. Set 1 to set 3 referred to different set of multiplex PCR and set 4 was singleplex PCR analysis independently. The</td>
<td>36</td>
</tr>
</tbody>
</table>
band obtained showing expected band size as stated in the diagram. M lane is molecular marker 1kb DNA ladder (Promega). PCR products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining.

4.6 *Streptococcus agalactiae* reisolated from infected fish post revirulent on BA media plate showing β-hemolytic properties from various organ; (E) = Eye, (B) = Brain, (K) = Kidney and (L) = Liver.

4.7 The similar clinical signs and gross lesions showed in both serotype Ia ST7 and III ST283. (a) The rotten anal fin (arrow); (b) The abdominal distention (star); (c) The corneal opacity (arrow) of the infected fish; (d) Normal healthy control red hybrid tilapia post-challenged with 500µl of sterile TSB.

4.8 The internal lesions of fish infected by *Streptococcus agalactiae* serotype Ia ST7. (a) Swollen gall bladder (star) and the white patch of the liver (arrow); (b) green patches (star) observed on the liver; (c) pale liver (star) for the fish challenged with *S. agalactiae* with concentration of $8.7 \times 10^5$ CFU/ml.

4.9 The external (a-c) and internal (d-h) lesions of red hybrid tilapia infected with *S. agalactiae* serotype III ST283. (a) Internal hemorrhage operculum; (b) Hemorrhagic tissue at the anal part (star); (c) Abdominal distention (star) and hemorrhage at the anus (arrow); (d) focal pale liver tissue and green patches (arrow); (e) hemorrhagic of liver tissue (arrow) (f) generalized pale liver; (g) centered paleness at spleen (star); (h) swollen intestine causing the abdominal distention.

4.10 Brain of fish infected by *Streptococcus agalactiae* serotype Ia ST7 (a-d) and serotype III ST283 (e-f). (a) Congestion of blood vessel in the stratum periventriculare region (arrow); (b) thickening of meningeal layer due to infiltration of heterophil (arrow); (c) congestion of blood vessel in meningeal layer (arrow); (d) presence of red neuron at the stratum marginale line (arrow); (e) presence of red neuron in the inner region (arrow); (f) mild thickening of meningeal layer due to infiltration of heterophil (arrow).

4.11 Liver of fish infected by *S. agalactiae* serotype Ia ST7. (a) The straight line showed the division of normal and infarcted area; (b) Infarction at surrounding area of central vein (star); (c) Presence of thrombus and inflammatory cell at the portal triad of the liver; (d) Presence of inflammatory cells at the lumen of the vein (arrow).

4.12 Liver of fish infected by *Streptococcus agalactiae* serotype III ST283. (a) Generalized hepatonecrotic cells; (b) the occurrence of swollen hepatocyte cells
(SH) compared to the normal cell; (c) slightly hemorrhages shown in sinusoid (arrow); (d) inflammation at the central vein (arrow); (e) necrosis at the perivascular area (arrow).

4.13 Kidney of fish infected by *Streptococcus agalactiae* serotype Ia ST7 (a-d) and III ST283 (e-f). (a) Tubular and coagulative necrosis with eosinophilic cytoplasm (star); (b) generalized necrosis; (c) generalized moderate coagulative necrosis with eosinophilic cytoplasm; (d) bacterial load with glomerulus and tubular necrosis (star); (e) generalized necrosis; (f) coagulative tubular necrosis with eosinophilic cytoplasm.

4.14 Spleen of fish infected by *Streptococcus agalactiae* serotype Ia ST7 (a-b) and III ST283 (c-d). (a) The straight line divides the condition of normal and infarcted area of the spleen; (b) presence of hypocellular area due to the lack of oxygen supply (arrow); (c) presence of hypocellular area (arrow); (b) hyperactive production of MMC (arrow).
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT</td>
<td>Metric ton</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>cps</td>
<td>Capsular polysaccharide</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
</tr>
<tr>
<td>MLST</td>
<td>Multilocus sequence typing</td>
</tr>
<tr>
<td>BA</td>
<td>Blood agar</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Oxygen</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>GBS</td>
<td>Group B <em>Streptococcus</em></td>
</tr>
<tr>
<td>MLEE</td>
<td>Multilevel event extraction</td>
</tr>
<tr>
<td>CG</td>
<td>Clonal group</td>
</tr>
<tr>
<td>CC</td>
<td>Clonal complex</td>
</tr>
<tr>
<td>TSA</td>
<td>Trypticase soy agar</td>
</tr>
<tr>
<td>TSB</td>
<td>Trypticase soy broth</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>mM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>secs</td>
<td>Seconds</td>
</tr>
<tr>
<td>°C</td>
<td>Degree celcius</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>hpi</td>
<td>Hour post-infection</td>
</tr>
<tr>
<td>LD$_{50}$</td>
<td>Lethal dose 50</td>
</tr>
<tr>
<td>API</td>
<td>Analytical profile index</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Background of study

Aquaculture is one of the biggest industry in Malaysia and ranked top 15 world producer in 2014 with estimated production is 521,000 tones (FAO, 2016). It was reported that aquaculture 8.9% of total national agriculture domestic product (GDP) which created an estimated 1,753,900 million jobs for Malaysian (DOMS, 2016).

However, the progressive effort in development of this sector lead to several effects such as fish stock depletion, national environmental issues, media influences on spreading false information, non-compliance towards halal aquaculture, and diseases that affected cultured fish (Fathi et al., 2018). One of the highest contributor in local import and export market is tilapia (*Oreochromis* spp.). Tilapia (*Oreochromis* spp.) was the second highest harvested freshwater fish in Malaysia with a total production of 35,996 metric tons (MT) in 2016 (DOF, 2018). The first and third highest were recorded for catfish (*Clarias* spp.) and striped catfish (*Pangasianodon* spp.) with a total production of 50,534 MT and 13,914 MT, respectively.

The wholesale value of tilapia was highest at RM 259 million, followed by catfish and striped catfish, at RM 208 million and RM 138 million, respectively. These values significantly indicated that tilapia farming is among the important aquaculture industry in this country (DOF, 2018). It was reported by DOF (2018) that the total production of tilapia was decreased due to several factors especially streptococcus. In Malaysia, streptococcus was the caused by *Streptococcus agalactiae* and *S. iniae* (Zamri-Saad et al., 2014).

*Streptococcus agalactiae* is a Gram-positive bacterium that associated with severe invasive diseases to human and animals. It is a type of bacteria that can cause diseases in aquatic animals, especially fish as well as mammals and reptiles (Amal and Zamri-Saad, 2011). It was reported that *S. agalactiae* strains can be haemolytic or non-haemolytic (Wang et al., 2013).

The common clinical signs and gross lesions showed by fish when infected by this pathogen including exophthalmia, lethargy, intraocular hemorrhage, opaqueness in cornea and classic manifestation of brain infection (Wang et al., 2013). The clinical signs showed by tilapia infected by *S. agalactiae* can cause
lesion which could be characterized by curvature of the spinal cord, stiffness and bleeding at the base of the fins (Asencios et al., 2016). *Streptococcus agalactiae* infection can cause mortality due to chronic infection and some cases can be categorized as severe acute infection (Pretto-Giordano et al., 2010; Kayansamruaj et al., 2014).

Several potential control and prevention measures of streptococcosis has been suggested by Zamri-Saad et al. (2014) such as selection of hatcheries, treatment of water supply and fish fry, antibiotics and vaccinations. Apart from all suggested prevention which is focusing on the fish management only, the study of the pathogen itself are also important to determine the reason of infection. Yet, there were lack of genetic information of *Streptococcus* sp. reported in Malaysia.

The genomic study is a must for the development of effective vaccine for streptococcosis prevention. In studying epidemiology of *S. agalactiae*, knowing the genetic variation is important to verify the distinct strain, serotype and sequence type of the species. Molecular serotyping through polymerase chain reaction (PCR) and sequencing of serotype-specific gene fragment within capsular polysaccharide (*cps*) genes are among the excellent methods in studying epidemiology of this bacteria (Manning et al., 2005). Generally, serotypes are groups within a single species of microorganisms, such as bacteria or virus, which share distinctive surface structures while *cps* gene is the gene that is related to virulence characteristics of the bacteria (Berridge et al., 2001).

Molecular serotyping of *S. agalactiae* can be discriminated based on two methods, which are based on *cps* serotyping and multilocus sequence typing (MLST) methods (Versalovic et al., 2002; Imperi et al., 2010; Yildirim et al., 2011; Kannika et al., 2017). The molecular typing for *S. agalactiae* is determined by the detection of antigenic structure, *cps* gene clusters using 19 specific primers (Imperi et al., 2010). *Streptococcus agalactiae* has 10 different capsular serotypes which are Ia, Ib, and II-IX (Yao et al., 2013; Li et al., 2014; Kayansamruaj et al., 2014; Kannika et al., 2017). Group B *Streptococcus* (GBS) generally produced one of the 10 antigenically distinct *cps*, which are thought to play a key role in its virulence. In addition, the *cps* gene is a major virulence factor in invasive disease caused by *S. agalactiae* (Chideroli et al., 2017).

Besides, MLST is another reliable approach in studying the epidemiology and molecular typing of bacterial species. MLST produced accurate and portable data in the study of evolutionary and population biology (Urwin and Maiden, 2003). Apart of studying the extracellular antigen, MLST is the study of unambiguous sequence-based typing method which aiming at the variation targeted housekeeping genes that usually of about 500 bp in size (Tien et al., 2011). *Streptococcus agalactiae* consists of 1349 sequence type (ST) around the world (PubMLST, 2019) while the common ST found in Asia such as China and Singapore were the ST7, ST10, ST19, ST283 and ST485 (Kalimuddin et al., 2017; Wang et al., 2018).
The genotypic characterization for constructing virulence genes profile of *S. agalactiae* is based on 14 virulence genes found in human isolates, which includes *fbsA* (the fibrinogen-binding protein FbsA), *fbsB* (the fibrinogen-binding protein FbsB), *pavA* (a fibrinogen-binding protein), *scpB* (C5a peptidase), *lmb* (laminin-binding protein), *cylE* (β-haemolysin/cytolysin), *cfb* (CAMP factor), *spb1* (haemolysin III), *hylB* (hyaluronate lyase), *rib* (the surface protein rib), *bca* (C-α protein), *bac* (C-β protein), *cspA* (the serine protease cspA), and *pbp1A/ponA* (penicillin-binding protein A) (Lin et al., 2011). By conducting multiplex PCR analysis, the virulence genes profile can be categorized under three virulence gene categories, which are adhesins, invasins and immune evasins (Willis and Whitfield, 2013; Laczeski et al., 2014).

Each of the virulence gene carries different roles in pathogenicity in the host after infection of the organism. GBS infection can be determined by the virulence factor encoded by these virulence genes including *cps* gene clusters (Hannoun et al., 2009). For example, the *bca* gene coding for Alpha-C protein, a surface protein that helps the bacteria to enter the host cells (Sadowi et al., 2010). Each group of virulence gene plays different role where (1) adhesin promotes the adhesion of bacteria to the host cell surface, (2) invasins allow the adhesive bacteria to cross the mucosal and the blood-brain barrier and (3) immune evasins facilitate escape from host immunity. Usually, pathogenicity analysis was conducted to prove the correlation between serotype and virulence of bacteria by conducting the challenge test of *S. agalactiae* infection on the susceptible fish (Kannika et al., 2017).

### 1.2 Problem statements

Freshwater aquaculture, especially tilapia farming is one of the major contributor of food source in Malaysia. However, streptococcosis has been emerged as an important disease in this industry and resulted to enormous economic losses (Yanong et al., 2010; Amal and Zamri-Saad, 2011). Nevertheless, streptococcosis also escalate the food insecurity and might possibly harm the health of the consumers. As the genetic information of *S. agalactiae* isolates is still lacking in Malaysia, the molecular research can give a beneficial information to solve this problem. Moreover, it was realized that *S. agalactiae* have several strains with different ST and antigenic properties. Besides, by studying the genetic materials of different strain of the isolates, the comparison of virulence level and infection causes by particular strain can be compare by conducting the bacterial challenge to the host animal. Hence, the molecular epidemiology studies of *S. agalactiae* is needed to provide bacterial information towards the future vaccine strategy for tilapia farming.
1.3 Objectives

The objectives of this present study are:

1. To identify the molecular serotyping of *Streptococcus agalactiae* isolates from cultured hybrid tilapia in Malaysia based on capsular polysaccharide (cps) genes and multilocus sequence typing (MLST).

2. To determine the virulence genes profiling of *Streptococcus agalactiae* based on the standard virulence genes found in human.

3. To investigate the pathogenicity of *Streptococcus agalactiae* serotype Ia ST7 and III ST283 in red hybrid tilapia (*Oreochromis niloticus* x *O. mossambicus*).
REFERENCES


DOF (Department of Fisheries of Malaysia). (2018). Fisheries statistic. DOF. Malaysia.


**Streptococcus agalactiae** from Nile Tilapia (Oreochromis niloticus) based on UPLC-MS/MS. Veterinary Microbiology, 210: 174-182.


agalactiae isolated from tilapia farms in Thailand by multiplex PCR. Journal of Applied Microbiology, 122: 1497-1507.


