



**CO-INFECTION OF TILAPIA LAKE VIRUS AND *Streptococcus agalactiae*
IN RED HYBRID TILAPIA [*Oreochromis niloticus* (Linnaeus, 1758) × *O.*
mossambicus (W. K. H. Peters, 1852)]**

By

LUKMAN BIN BASRI

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

November 2022

IB 2022 20

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



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

CO-INFECTION OF TILAPIA LAKE VIRUS AND *Streptococcus agalactiae* IN RED HYBRID TILAPIA [*Oreochromis niloticus* (Linnaeus, 1758) × *O. mossambicus* (W. K. H. Peters, 1852)]

By

LUKMAN BIN BASRI

November 2022

Chairman : Mohammad Noor Amal Azmai, PhD
Institute : Bioscience

Tilapia tilapinevirus or known as Tilapia Lake Virus (TiLV) is an emerging virus accountable for a viral disease in cultured and wild tilapia. TiLV has been responsible for a massive mortality of tilapia around the globe including Malaysia. Interestingly, most cases of TiLV-infected fish in Malaysia were co-present with bacterial pathogen including *Streptococcus agalactiae* that often resulted with a high death rate. The prominence of TiLV and *S. agalactiae* co-infection has not been explored concerning the interactions and mechanisms of these two infectious agents and their possible impact on the disease in tilapia. Thus, this study was conducted to determine the median lethal dose (LD₅₀), and pathogenicity of TiLV and *S. agalactiae* single and co-infection in red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) following intraperitoneal (IP) injection route. In this study, the red hybrid tilapias were challenged with different concentrations of TiLV (10⁴, 10⁵, and 10⁶ TCID₅₀/mL) and *S. agalactiae* (10³, 10⁴, 10⁵, 10⁶ and 10⁷ CFU/mL). Following the infections, the LD₅₀ of TiLV and *S. agalactiae* was determined at 10⁶ TCID₅₀/mL and 10⁴ CFU/mL, respectively. The clinical signs, and gross lesions of TiLV and *S. agalactiae* challenged fish were similar as observed in the naturally infected fish. Then, by referring to the obtained LD₅₀, the fish were challenged with 10⁶ TCID₅₀/mL of TiLV and 10³ CFU/mL of *S. agalactiae* following the single and co-infection studies. The co-infected fish showed a higher cumulative mortality (60.00% in TiLV-*S. agalactiae* co-infection, and 73.33% in *S. agalactiae*-TiLV co-infection) compared to the single infected fish (40.00% in TiLV infection, and 20.00% in *S. agalactiae* infection). Important histopathological findings such as intracytoplasmic inclusion bodies, and syncytial giant cells were also frequently observed in the co-infected fish with some of the lesions were significantly ($P < 0.05$) severe compared to the single infected fish. The viral and bacterial load recovered from the fish's brain and liver in qPCR analysis showed a significantly ($P < 0.05$) higher load pattern was observed in the co-infected fish following the introduction of the second pathogen compared to the single infected fish. The viral particles and

bacterial cells were also observed using the TEM analysis and important ultrastructural lesion was found in the infected organs. This study showed the red hybrid tilapia was susceptible to both pathogens following the IP challenge and the co-infection between TiLV and *S. agalactiae* synergistically worsened the disease severity in tilapia. The results could help in the future effective disease management strategies in cultured tilapia in Malaysia.

Keywords: Tilapia Lake Virus, *Streptococcus agalactiae*, clinical signs, histopathological changes, qPCR, TEM



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagaimemenuhi keperluan untuk Ijazah Sarjana Sains

**JANGKITAN BERSAMA ANTARA VIRUS TILAPIA TASIK DAN
Streptococcus agalactiae DALAM TILAPIA MERAH HIBRID [*Oreochromis
niloticus* (Linnaeus, 1758) × *O. mossambicus* (W. K. H. Peters, 1852)]**

Oleh

LUKMAN BIN BASRI

November 2022

Pengerusi : Mohammad Noor Amal Azmai, PhD
Institut : Biosains

Tilapia tilapinevirus atau lebih dikenali sebagai Virus Tilapia Tasik (TiLV) adalah virus baru dan bertanggungjawab terhadap jangkitan virus pada tilapia ternakan dan liar. TiLV telah menyebabkan kematian secara besar-besaran terhadap ikan tilapia di seluruh dunia termasuklah Malaysia. Menariknya, kebanyakan kes ikan yang dijangkiti TiLV di Malaysia telah hadir bersama patogen bakteria seperti *Streptococcus agalactiae* yang telah menyebabkan kadar kematian lebih tinggi. Kepentingan jangkitan bersama antara TiLV dan *S. agalactiae* belum pernah dikaji sebelum ini terutamanya mengenai interaksi dan mekanisme kedua-dua agen berjangkit ini dan kemungkinan kesannya terhadap tilapia. Justeru itu, kajian ini dijalankan bertujuan untuk mengenalpasti median dos membunuh (LD_{50}), dan patogenisiti jangkitan sendiri dan bersama antara TiLV dan *S. agalactiae* dalam ikan tilapia merah hibrid (*Oreochromis niloticus* × *O. mossambicus*) menerusi suntikan intraperitoneum (IP). Dalam kajian ini, ikan tilapia merah hibrid tersebut telah dicabar dengan kepekatan TiLV (10^4 , 10^5 , and 10^6 TCID₅₀/mL) dan *S. agalactiae* (10^3 , 10^4 , 10^5 , 10^6 and 10^7 CFU/mL). Menerusi cabaran tersebut, LD_{50} TiLV dan *S. agalactiae* telah ditentukan pada 10^6 TCID₅₀/mL dan 10^4 CFU/mL. Tanda klinikal, dan luka kasar ikan yang dicabar dengan TiLV dan *S. agalactiae* adalah sama seperti yang ditunjukkan oleh ikan yang dijangkiti secara semula jadi. Dengan merujuk kepada LD_{50} , ikan tersebut seterusnya telah dicabar dengan 10^6 TCID₅₀/mL TiLV dan 10^3 CFU/mL *S. agalactiae* untuk kajian jangkitan sendiri dan bersama. Ikan yang dijangkiti bersama telah menunjukkan kadar kematian yang tinggi (60.00% dalam jangkitan bersama TiLV-*S. agalactiae* dan 73.33% dalam jangkitan bersama *S. agalactiae*-TiLV) berbanding ikan yang dicabar secara sendiri (40.00% dalam jangkitan TiLV dan 20.00% dalam jangkitan *S. agalactiae*). Perubahan histopatologi yang penting seperti sel intrasitoplasmik dan sel gergasi telah dijumpai dalam ikan yang dijangkiti bersama dengan beberapa luka adalah lebih signifikan ($P < 0.05$) berbanding dengan ikan yang dicabar sendiri. Kadar virus dan bakteria yang diperolehi daripada organ otak dan hati ikan yang dicabar

melalui qPCR menunjukkan terdapat corak signifikan ($P < 0.05$) yang tinggi telah ditemui dalam ikan yang dijangkiti bersama selepas pengenalan patogen kedua berbanding dengan ikan yang dicabar secara sendiri. Partikel virus dan sel bakteria juga berjaya ditemui dengan menggunakan analisis TEM dan perubahan ultrastruktural yang penting telah ditemui dalam organ yang dijangkiti. Kajian ini menunjukkan bahawa ikan tilapia merah hibrid boleh dijangkiti dengan kedua-dua patogen tersebut menerusi suntikan IP dan jangkitan bersama antara TiLV dan *S. agalactiae* meneruskan lagi jangkitan dalam tilapia. Data yang diperolehi dalam kajian ini mampu membantu pengurusan penyakit ikan ternakan tilapia di Malaysia pada masa hadapan.

Keywords: Virus Tilapia Tasik, *Streptococcus agalactiae*, tanda klinikal, perubahan histopatologi, qPCR, TEM



ACKNOWLEDGEMENTS

With the name of almighty Allah S.W.T the most Merciful and Compassionate.

This study could not be completed without the helps, advice, and supports from my respectable supervisor, Assoc. Prof. Dr. Mohammad Noor Amal Azmai and other supervisory committee members, Assoc. Prof. Dr. Annas Salleh, and Assoc. Prof. Dr. Ina Salwany Md Yasin. Not forgotten, I would like to extend my gratitude to other members of the grant, Prof. Dr. Mohd Zamri Saad, and Dr. Nor Yasmin Abd Rahman.

I would like to thank my lab mates, Jumria Sutra and Roslindawani Md Nor for their help on completing this study and to all my friends that helped me during my master's degree. Biggest thanks to my family for their unconditional loves and supports.

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

Mohamad Noor Amal Azmai, PhD

Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Ina Salwany Md Yasin, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Annas Salleh, PhD

Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 09 March 2023

Declaration by the 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 institutions;
- intellectual property from the thesis and the copyright of the thesis are fully-owned by Universiti Putra Malaysia, as stipulated in the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from the supervisor and the office of the Deputy Vice-Chancellor (Research and innovation) before the thesis is published in any written, printed or electronic form (including books, journals, modules, proceedings, popular writings, 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 in accordance with the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2015-2016) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software

Signature: _____ Date: _____

Name and Matric No.: Lukman bin Basri,

Declaration by Members of the Supervisory Committee

This is to confirm that:

- the research and the writing of this thesis were done under our supervision;
- supervisory responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2015-2016) are adhered to.

Signature: _____
Name of Chairman
of Supervisory
Committee: _____

Signature: _____
Name of Member of
Supervisory
Committee: _____

Signature: _____
Name of Member of
Supervisory
Committee: _____

TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vi
DECLARATION	viii
LIST OF TABLES	xiii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS AND SYMBOLS	xix
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	
2.1 Tilapia Lake Virus	3
2.1.1 Distribution	3
2.1.2 Virus taxonomy and classification	5
2.1.3 Host's agents	6
2.2.3.1 Susceptible species	6
2.2.3.2 Susceptible life stages	7
2.1.4 TiLV infection, characteristics, and diagnostics	7
2.1.4.1 Clinical signs, gross lesions, and histopathological changes	7
2.1.4.2 Cell culture, and electron microscopy	10
2.1.4.3 Molecular detections	11
2.2 <i>Streptococcus</i>	13
2.2.1 Streptococcosis	13
2.2.2 <i>Streptococcus agalactiae</i>	13
2.2.3 Host's agents	14
2.2.4 <i>S. agalactiae</i> infection, characteristics, and diagnostics	15
2.2.4.1 Clinical signs, gross lesions, and histopathological changes	15
2.2.4.2 Phenotypic and biochemical test	17
2.2.4.2 Molecular detections	18
2.3 The impacts of co-infection in tilapia culture	19
2.4 Co-infection of TiLV with bacterial pathogen	20
3 MATERIALS AND METHODS	
3.1 Viral and bacterial stock, isolation, and culture	21

3.2	Phenotypic and biochemical characterization of <i>S. agalactiae</i>	21
3.3	Viral genomic RNA extraction and cDNA synthesis	22
3.4	Bacterial DNA extraction	22
3.5	PCR detection	22
	3.5.1 Semi-nested PCR	22
	3.5.2 Conventional PCR	23
3.6	Determination of median lethal dose (LD ₅₀)	23
	3.6.1 Virus culture and preparation	23
	3.6.2 Serial dilution and virus titration	23
	3.6.3 Bacterial culture and preparation	24
	3.6.4 Serial dilution and colony forming unit determination	24
	3.6.5 Fish acclimatization	24
	3.6.6 Pathogen screening	25
	3.6.7 Fish challenge and monitoring	25
	3.6.8 LD ₅₀ determination	26
3.7	Single and co-infection	26
	3.7.1 Virus and bacteria concentration	27
	3.7.2 Fish acclimatization and pathogen screening	27
	3.7.3 Fish challenge and monitoring	27
	3.7.3.1 Single infection	27
	3.7.3.2 Co-infection	28
	3.7.3.3 Mortality rate, clinical signs, gross lesions, and sample collection	29
	3.7.4 Viral and bacterial load determination	30
	3.7.4.1 RNA and DNA extraction	30
	3.7.4.2 Real-time quantitative polymerase chain reaction (RT-qPCR)	30
	3.7.5 Histopathological preparation and assessment	31
	3.7.6 Electron microscopy analysis	31
3.8	Statistical analysis	32

4 RESULTS

4.1	<i>In-vitro</i> infection of TiLV	33
4.2	Phenotypic characterization of <i>S. agalactiae</i>	34
4.3	LD ₅₀ determination	37
	4.3.1 Viral and bacterial titre	37
	4.3.2 Mortality pattern of TiLV and <i>S. agalactiae</i> infection	38
	4.3.3 Clinical signs and gross lesions of challenged fish	40
	4.3.4 LD ₅₀ of TiLV and <i>S. agalactiae</i>	42
4.4	Pathogenicity of single and co-infection	44
	4.4.1 Mortality pattern	44

4.4.1.1	Single pathogen infection	44
4.4.1.2	Concurrent pathogen infection	45
4.4.2	Clinical signs and gross lesions of single and co-infected fish	46
4.4.2.1	Single TiLV infection	47
4.4.2.2	Single <i>S. agalactiae</i> infection	47
4.4.2.3	TiLV followed by <i>S. agalactiae</i> co-infection	48
4.4.2.4	<i>Streptococcus agalactiae</i> followed by TiLV co-infection	50
4.4.3	Viral and bacterial load	51
4.4.3.1	Comparison of TiLV load in the single and co-infected fish	53
4.4.3.2	Comparison of <i>S. agalactiae</i> load in the single and co-infected fish	55
4.4.4	Histopathological assessment	58
4.4.4.1	Single infection	59
4.4.4.2	Co-infection	60
4.4.4.3	Histopathological scoring	62
4.4.5	Electron microscopy analysis	64
4.4.5.1	Single infection	64
4.4.5.2	Co-infection	66
5	DISCUSSIONS	
5.1	LD ₅₀ , mortality, clinical signs, and gross lesions of TiLV and <i>S. agalactiae</i> infection in red hybrid tilapia (<i>Oreochromis niloticus</i> × <i>O. mossambicus</i>)	69
5.2	Comparison of mortality, clinical signs, and gross lesions between single and co-infection in red hybrid tilapia	70
5.3	Differences of histopathology and electron microscopy analysis of the single and concurrent infected red hybrid tilapia	72
5.4	Viral and bacterial load analysis	74
6	CONCLUSION AND RECOMMENDATION	76
	REFERENCES	78
	APPENDICES	89
	BIODATA OF STUDENT PUBLICATION	95
		96

LIST OF TABLES

Table		Page
2.1	Taxonomic classification of Tilapia Lake Virus, <i>Tilapia tilapinevirus</i> .	5
2.2	List of farmed and wild tilapia species affected by TiLV infection.	6
2.3	List of primers used in TiLV detection	12
2.4	Type of haemolysis activities and their appearances. (Buxton, 2005).	14
2.5	List of fish species affected by <i>S. agalactiae</i> infection.	15
2.6	List of primers used in <i>S. agalactiae</i> detection.	19
3.1	The scale of the gross lesions observed on the challenged fish.	26
4.1	Comparison of nucleotide and amino acid sequences of TiLV.	34
4.2	Phenotypic and biochemical test results of <i>S. agalactiae</i> .	35
4.3	Comparison of nucleotide and amino acid sequences of <i>S. agalactiae</i> .	37
4.4	Summary of viral inoculation following a serial dilution concentration.	37
4.5	Calculations of LD ₅₀ in red hybrid tilapia based on the Reed and Muench method.	42
4.6	Histopathological scoring of infected liver and brain following the single and co-infection, n=5.	63

LIST OF FIGURES

Figure		Page
2.1	Global distribution of TiLV.	4
2.2	Worldwide map showing regions with potential and confirmed of TiLV infection.	4
2.3	(A) Affected tilapia showing gross pathology of skin erosions and skin ulcers (arrowhead). (B) The affected tilapias were showing typical exophthalmos, and abdominal distended. (C) Experimentally infected tilapia showing haemorrhagic skin (arrow), mild exophthalmos, and swelling of the abdomen. (D) Photograph of experimentally infected tilapia exhibited bilateral exophthalmia, and mild scale protrusion. (E&F) Co-infected red hybrid tilapia showing haemorrhagic skin particularly on the operculum, caudal, dorsal, and caudal fin area.	8
2.4	(A) The infected liver tissue showing foamy cytoplasm and formation of syncytial hepatocytes. (B) The presence of intracytoplasmic inclusion bodies on the affected liver tissue. (C) Kidney tissue showing severe infiltration of inflammatory cells and formation of the syncytial cells. (D) Liver tissue showing pancreatic necrosis and infiltration of lymphocytes. (E) Increased melanomacrophage centre (MMC) found on the spleen tissue. (F) The presence of syncytial cells and infiltration of lymphocytes on the brain.	9
2.5	(A) CPE (arrow) observed on the E-11 cells within 5 days of TiLV post-inoculation. (B) CPE with cell shrinkage and syncytial formation (arrow) within 4 days of TiLV post-inoculation.	10
2.6	(A) The presence of virion with diameter of 55 to 60 nm on the infected E-11 cell culture. (B) Typical appearance of virion within the cytoplasm of hepatocytes (arrow).	11
2.7	Global distribution of streptococcosis causative species.	13
2.8	Gross lesions observed on the affected tilapia. (A) Bilateral exophthalmia. (B) Corneal opacity. (C) Congestion of the viscera. (D) Softening of the brain.	16
2.9	Histopathological changes of <i>S. agalactiae</i> infected fish. (A) Infected liver exhibited blood congestion with swollen endothelium (arrows) and thrombosis. (B) Extensive infarction of tissue adjacent observed on the liver section.	17

	(C) Thickening of meningeal layer on the brain of infected fish. (D) Severe blood congestion observed on the brain section. (E) Kidney section exhibited tubular and coagulative necrosis with eosinophilic cytoplasm. (F) Hyperactive production of MMC (arrows) on the spleen of infected fish.	
3.1	The experimental set up for the single TiLV and <i>S. agalactiae</i> infection in the red hybrid tilapia.	28
3.2	The experimental set up for the co-infection of TiLV and <i>S. agalactiae</i> in the red hybrid tilapia.	29
4.0	(A) Mock infected E-11 cells. (B) TiLV-infected E-11 cells showing CPE at 4-7 dpi; cells shrinkage (arrows), and cells vacuolation (stars).	33
4.1	Agarose gel electrophoresis showing the 415 and 250 bp bands of the TiLV genes from the inoculated E-11 cells. Lane M is the 100 bp DNA marker; Lane N is the mock infected E-11 cells; Lane P is the positive control; Lane 1 to 2 is the TiLV-positive E-11 cells. #Marks band are from cross-hybridizations between amplified products.	34
4.2	(A) Pure culture of <i>S. agalactiae</i> on the BA plates after 24 h at 30°C. (B) Microscopic view of <i>S. agalactiae</i> under 100× magnification.	36
4.3	Agarose gel electrophoresis showing the 1500 bp bands of the <i>S. agalactiae</i> genes from the cultured pure colonies. Lane M is the 1000 bp DNA marker; Lane N is the negative control; Lane 1 to 4 is the <i>S. agalactiae</i> isolates.	36
4.4	Cumulative mortality of red hybrid tilapia following IP-injection with 10^6 , 10^5 , and 10^4 TCID ₅₀ /mL of TiLV.	39
4.5	Cumulative mortality of red hybrid tilapia following IP-injection with 10^7 , 10^6 , 10^5 , 10^4 and 10^3 CFU/mL of <i>S. agalactiae</i> .	40
4.6	External and internal gross lesions observed on the challenged red hybrid tilapia with different TiLV concentration. (A) Mild skin ulcer (star) and severe haemorrhages on the dorsal fin (arrow) of the challenged fish. (B) Spleen and gall bladder enlargement (white arrows).	41
4.7	External and internal gross lesions observed on the challenged red hybrid tilapia with different <i>S. agalactiae</i> concentration. (A) Control red hybrid tilapia. (B) Moderate skin ulcer (red arrows) observed on the challenged fish	41

- body together with mild corneal opacity (arrow). (C) Mild skin haemorrhages and severe pectoral fin rot (arrow) on the challenged fish. (D) Swollen liver in the challenged fish (arrow).
- 4.8 The plotted LD₅₀ graph for (A) TiLV infection and (B) *S. agalactiae* infection. 43
- 4.9 Cumulative mortality of challenged red hybrid tilapia following the 10⁶ TCID₅₀/mL of TiLV and 10³ CFU/mL of *S. agalactiae* single infection until 20 dpi. 45
- 5.0 Cumulative mortality of challenged red hybrid tilapia following the co-infection. The *S. agalactiae* was co-injected at 13 dpi in TiLV infection following *S. agalactiae* co-infection group, and TiLV was co-injected at 2 dpi in *S. agalactiae* infection following TiLV co-infection group. 46
- 5.1 External and internal gross lesions observed on the challenged red hybrid tilapia following single TiLV (10⁶ TCID₅₀/mL) infection. (A & B) Severe haemorrhages on the dorsal fin and operculum area (arrows) at 16 dpi. (C) Moderate skin ulcer on the caudle peduncle (arrow) at 17 dpi. (D) Pale liver (arrow) together with watery internal organs observed at 17 dpi. 47
- 5.2 External and internal gross lesions observed on the challenged red hybrid tilapia following single *S. agalactiae* (10³ CFU/mL) infection. (A) Skin ulcer, and erosion observed on the caudal peduncle (star) together with the haemorrhages on the operculum area (arrow) at 2 dpi. (B) Exophthalmia (arrow) on the right eye of the challenged fish at 3 dpi. (C) Severe skin ulcer observed at 6 dpi on the caudle peduncle (arrow). (F) Gall bladder enlargement (arrow) observed at 6 dpi. 48
- 5.3 External and internal gross lesions observed on the challenged red hybrid tilapia following TiLV (10⁶ TCID₅₀/mL) and *S. agalactiae* (10³ CFU/mL) co-infection. (A) Skin ulcer, and erosion observed on the caudal peduncle (arrow) together with the rotten caudal fin (star) observed at 16 dpi. (B) The challenged fish exhibited haemorrhages on the caudal peduncle area (arrow) at 16 dpi. (C) Mild haemorrhagic spot on the dorsal fin and operculum (arrow) at 17 dpi. (D) Severe corneal opacity (arrow) on the challenged fish at 17 dpi. (E) Enlargement of the internal organs (circle) at 18 dpi. (F) Green patches located at the liver organs (arrow) observed at 19 dpi. 49
- 5.4 External and internal gross lesions observed on the challenged red hybrid tilapia following *S. agalactiae* (10³ 51

CFU/mL) and TiLV (10^6 TCID₅₀/mL) co-infection. (A & B) The challenged fish exhibited haemorrhages on the dorsal fin, anal fin, and operculum area (arrows) at 4 dpi. (E) Skin ulcer, and erosion observed on the caudal peduncle area (arrow) at 6 dpi. (D) Severe corneal opacity (arrow) observed on challenged fish at 8 dpi. (E) Enlargement of the internal organs (circle) observed at 8 dpi. (F) Green patches located at the liver organs (arrow) at 8 dpi.

- 5.5 (A) Standard curve of TiLV. (B) Standard curve of *S. agalactiae*. 52
- 5.6 The relationship between TiLV load (log TCID₅₀/mL) in the brain of red hybrid tilapia following single and co-infection. *Streptococcus agalactiae* was concurrently injected at 13 dpi. Asterisk mark (*) represents significant difference at $P < 0.05$ between each TiLV load. 53
- 5.7 The relationship between TiLV load (log TCID₅₀/mL) in the liver of red hybrid tilapia following single and concurrent infection. *Streptococcus agalactiae* was concurrently injected at 13 dpi. Asterisk mark (*) represents significant difference, $P < 0.05$ between each TiLV load. 54
- 5.8 The relationship between *S. agalactiae* load (log CFU/mL) in the brain of red hybrid tilapia following the single and co-infection. TiLV was concurrently injected at 2 dpi. Asterisk mark (*) represents significant difference at $P < 0.05$ between each *S. agalactiae* load. 56
- 5.9 The relationship between *S. agalactiae* load (log CFU/mL) in the liver of red hybrid tilapia following the single and co-infection. TiLV was concurrently injected at 2 dpi. Asterisk mark (*) represents significant difference at $P < 0.05$ between each *S. agalactiae* load. 57
- 6.0 The photomicrograph of (A) normal liver and (B) normal brain. 58
- 6.1 The photomicrograph of infected liver and brain following single TiLV infection. (A) Severe hepatic congestion (arrows) in the hepatic sinusoids and veins. (B) Presence of numerous eosinophilic intracytoplasmic inclusion bodies (arrows) in the hepatocytes. Syncytial cells were occasionally observed (inset). (C) Mild cerebral and meningeal congestion (arrows) involving capillaries. 59
- 6.2 The photomicrograph of infected liver and brain following single *S. agalactiae* infection. (A) Moderate hepatic congestion observed in hepatocytes. (B) Severe cerebral 60

- and meningeal congestion (arrows) and thickness of meningeal layer due to oedema (double headed arrow).
- 6.3 The photomicrograph of infected liver and brain following TiLV and *S. agalactiae* co-infection. (A) Severe and generalized hepatic lipidosis with common observations of syncytial cells (insets). (B) Severe infiltration of lymphocyte (arrow) accompanied by meningeal redness (star). 61
- 6.4 The photomicrograph of infected liver and brain following TiLV and *S. agalactiae* co-infection. (A) Severe and generalized hepatic lipidosis with common observations of syncytial cells (insets). (B) Cerebral congestion (arrows) and thickness of meningeal layer due to oedema (double headed arrow). 61
- 6.5 The ultrastructural micrograph of infected liver in TiLV infection group. (A) The presence of viral particles (arrows) with diameter of 55 to 100 nm in the cytoplasm of hepatocytes (bar = 1 μ m). (B) Swollen hepatocytic mitochondria (arrows) (bar = 1 μ m). 65
- 6.6 The ultrastructural micrograph of infected liver in *S. agalactiae* infection group. The presence of bacterial cells (arrows) with diameter of 1 to 2 μ m in a vascular lumen of the liver (bar = 2 μ m). 66
- 6.7 The electron micrograph of infected liver in TiLV followed by *S. agalactiae* co-infection group revealed (A) the presence of intracytoplasmic vesicles containing immature viral particles (arrows) between hepatocyte tubular organelles (bar = 1 μ m), (B) presence of bacterial cells (arrow) in a lumen of capillary in the liver (bar = 1 μ m), and (C) numerous, severely swollen mitochondria (arrows) in the cytoplasm of infected hepatocytes (bar = 500 μ m). 67
- 6.8 The electron micrograph of infected liver in *S. agalactiae* followed by TiLV co-infection group revealed (A) the presence multiple bacterial cells (arrows) in the capillary lumen in the liver (bar = 2 μ m), (B) viral particles resembling the TiLV in the cytoplasm of hepatocytes (bar = 1 μ m), and (C) multiple swollen mitochondria (arrows) in the cytoplasm of infected cells (bar = 2 μ m). 68

LIST OF ABBREVIATIONS AND SYMBOLS

MT	Metric tonnes
PCR	Polymerase chain reaction
BA	Blood agar
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
H ₂ O ₂	Hydrogen peroxide
bp	Base pair
TEM	Transmission electron microscopy
TSA	Trypticase soy agar
TSB	Trypticase soy broth
ng	Nanogram
mM	Micromolar
mL	Milliliter
μL	Microliter
MgCl ₂	Magnesium chloride
dnTPs	Deoxyribonucleotide triphosphate
min	Minutes
dpi	Days post infection
CFU	Colony forming unit
TCID ₅₀	Tissue culture infectious dose
IP	Intraperitoneal injection
LD ₅₀	Median lethal dose
API	Analytical profile index

GBS	Group B <i>Streptococcus</i>
β	Betta
γ	Gamma
α	Alpha
%	Percentage



CHAPTER 1

INTRODUCTION

Background of study

Tilapia (*Oreochromis* spp.) is a common cultured freshwater fish which serve as one of the main protein sources globally (FAO, 2016). In aquaculture industry, tilapia was produced extensively from all over the world for two main purposes: commercialization and economical purpose. In Malaysia, 44.7% of the total freshwater aquaculture production is dominated by tilapia (FAO, 2016). The first and the third highest Malaysian freshwater aquaculture production were recorded for catfish (*Clarias* spp.) and striped catfish (*Pangasianodon* spp.). The annual Malaysian tilapia production between 2007 to 2018 was recorded at 351, 545 MT with estimated wholesale value of USD 61.5 million (Saba et al., 2020). Meanwhile, at the end of 2020, the production of tilapia globally was estimated at 7 million tons making them as a second most important freshwater fish in the world (FAO, 2021). These values significantly indicated that tilapia farming industry plays an important role in the aquaculture industry for this country and the world (DOF, 2018).

On the other hand, Tilapia Lake Virus (TiLV) disease (TiLVD) is an emerging novel viral disease which has been responsible for high mass mortalities of farmed and wild tilapia (Waiyamitra et al., 2018). The disease also known as syncytial hepatitis of tilapia (SHT) and 'tilapia one month mortality syndrome' as it caused syncytial hepatitis and mortalities of tilapia's fry or juveniles within one month of transferred from hatcheries to grow out cages (Del-Pozo et al., 2017; Surachetpong et al., 2017). The TiLVD was primarily caused by *Orthomyxo*-like virus called Tilapia Lake Virus (TiLV) (*Tilapia tilapiinevirus*). The TiLV has been reported recently to cause a viral disease in tilapia in several Asian countries including Thailand (Surachetpong et al., 2017; Dong et al., 2017b), Malaysia (Abdullah et al., 2018; Amal et al., 2018), Indonesia (Koesharyani et al., 2018), and Philippines (OIE, 2017a). The first case caused by TiLV was reported to occur in Israel (Eyngor et al., 2014) followed by Ecuador (Ferguson et al., 2014). Other than tilapia, TiLV also found susceptible to infect other fish species including wild river carp (Abdullah et al., 2018), zebrafish (Rakus et al., 2020), and giant gourami (Jaemwimol et al., 2018).

Meanwhile, the streptococcosis remains as one of the main diseases that caused problems in tilapia farming industry in Malaysia (Syuhada et al., 2020). One of the main pathogens that caused streptococcosis in Malaysia is *Streptococcus agalactiae*. *Streptococcus agalactiae* is a Gram-positive bacterium that could infect wide range of organisms including humans and animals (Pradeep et al., 2016). Unlike other *Streptococcus* spp., *S. agalactiae* belongs to the Lancefield group B *Streptococcus* (GBS) that usually caused neonatal sepsis and meningitis in human and fish (Wang et al., 2017). Besides, it was also reported

that the *S. agalactiae* usually appeared with chained or non-chained group and could be haemolytic or non-haemolytic (Wang et al., 2017).

Co-infection occurs when the host organisms was infected simultaneously or non-simultaneously (secondary pathogen) by two or more different pathogens and the two or more infectious pathogens were actively together infecting the host organisms (Kotob et al., 2017). Most of the time, the co-infection increased and worsened the diseased severity in fish compared to the single infection (Nicholson et al., 2020). In previous study in Malaysia, *Aeromonas veronii* was concurrently isolated from TiLV-diseased red hybrid tilapia (Amal et al., 2018) and similarly, multiple bacteria including *S. agalactiae* and *A. hydrophila* were also concurrently isolated from the TiLV-infected red hybrid tilapia (Basri et al., 2020). Besides, in other countries, several bacterial species including *Flavobacterium*, *Streptococcus*, and *Aeromonas* spp. were also found in the TiLV-infected tilapia (Nicholson et al., 2017; Surachetpong et al., 2017). Therefore, these reports indicated the potential threat of co-infection to occur in tilapia farming industry, especially in Malaysia.

Problem statement

Freshwater aquaculture industry, especially tilapia farming has become one of the major contributors of income and food sources in Malaysia. However, the presence of infectious agents including TiLV and *S. agalactiae* have caused problems towards tilapia farming and consequently resulted with enormous economic losses (Amal and Zamri-Saad, 2011). To make it worse, most of the TiLV-diseased fish in Malaysia were co-infected with bacterial pathogen including *S. agalactiae* which has worsened and increased the disease severity in tilapia. Since the study on virulency and pathogenicity of the co-infection between TiLV and *S. agalactiae* is still lacking, therefore, a complete *in-vivo* study should be conducted to gain a better understanding on the pathogenicity of the co-infection between TiLV and *S. agalactiae* in tilapia. Nevertheless, by studying on the pathogenicity of single and co-infection between these two infectious agents, the data could provide an important information for the effective future tilapia farming management to counter the tilapia diseases.

Objectives

The objectives of this present study are:

1. To determine the median lethal dose (LD₅₀) of Tilapia Lake Virus and *Streptococcus agalactiae* infection in red hybrid tilapia.
2. To analyze the pathogenicity of single infection of Tilapia Lake Virus and *Streptococcus agalactiae* in red hybrid tilapia.
3. To identify and compare the pathogenicity of co-infection between Tilapia Lake Virus and *Streptococcus agalactiae* in red hybrid tilapia.

REFERENCES

- Abdel-Latif, H. M., Dawood, M. A., Menanteau-Ledouble, S., & El-Matbouli, M. (2020). The nature and consequences of co-infections in tilapia: A review. *Journal of Fish Diseases*, 43(6), 651-664.
- Abdullah, A., Ramly, R., Ridzwan, M. M., Sudirwan, F., Abas, A., Ahmad, K., & Kua, B. C. (2018). First detection of tilapia lake virus (TiLV) in wild river carp (*Barbonymus schwanenfeldii*) at Timah Tasoh Lake, Malaysia. *J. Fish Dis*, 41, 1459-1462.
- Abdullah, S., Omar, N., Yusoff, S. M., Obukwho, E. B., Nwunuji, T. P., Hanan, L., & Samad, J. (2013). Clinicopathological features and immunohistochemical detection of antigens in acute experimental *Streptococcus agalactiae* infection in red tilapia (*Oreochromis* spp.). *SpringerPlus*, 2(1), 1-7.
- Ahasan, M. S., Keleher, W., Giray, C., Perry, B., Surachetpong, W., Nicholson, P., & Waltzek, T. B. (2020). Genomic characterization of tilapia lake virus isolates recovered from moribund Nile tilapia (*Oreochromis niloticus*) on a farm in the United States. *Microbiology resource announcements*, 9(4), e01368-19.
- Aich, N., Paul, A., Choudhury, T. G., & Saha, H. (2021). Tilapia Lake Virus (TiLV) disease: current status of understanding. *Aquaculture and Fisheries*.
- Aisyhah, M. A. S., Amal, M. N. A., Zamri-Saad, M., Siti-Zahrah, A., & Shaqinah, N. N. (2015). *Streptococcus agalactiae* isolates from cultured fishes in Malaysia manifesting low resistance pattern towards selected antibiotics. *Journal of fish diseases*, 38(12), 1093-1098.
- Ali, A., Hassan, D., Saleha, A. A., Siti, K. B., & Milud, A. (2010). *Streptococcus agalactiae* the etiological agent of mass mortality in farmed red tilapia (*Oreochromis* sp.). *Journal of Animal and Veterinary Advances*, 9(20), 2640-2646.
- Ali, F. A., Hassan, H., Saleha, A. A., Siti, K. B., & Milud, A. (2011). Pathogenicity of *Streptococcus agalactiae* isolated from a fish farm in Selangor to juvenile red tilapia (*Oreochromis* sp.). *Journal of Animal and Veterinary Advances*, 10(7), 914-919.
- Amal, A. M. N., Nur-Nazifah, M., Siti-Zahrah, A., Sabri, M. Y., & Zamri-Saad, M. (2008). Determination of LD₅₀ for *Streptococcus agalactiae* infections in red tilapia and GIFT.
- Amal, M. N. A., & Zamri-Saad, M. (2011). Streptococcosis in tilapia (*Oreochromis niloticus*): a review. *Pertanika Journal of Tropical Agricultural Science*, 34(2), 195-206.

- Amal, M. N. A., Ismail, A., Saad, M. Z., Yasin, I. S. M., Nasruddin, N. S., Mastor, S. S., & Mohamad, N. (2019). Study on *Streptococcus agalactiae* infection in Javanese medaka (*Oryzias javanicus* Bleeker, 1854) model. *Microbial pathogenesis*, 131, 47-52.
- Amal, M. N. A., Koh, C. B., Nurliyana, M., Suhaiba, M., Nor-Amalina, Z., Santha, S., & Zamri-Saad, M. (2018). A case of natural co-infection of Tilapia Lake Virus and *Aeromonas veronii* in a Malaysian red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) farm experiencing high mortality. *Aquaculture*, 485, 12-16.
- Amal, M. N. A., Zamri-Saad, M., Siti-Zahrah, A., & Zulkafli, A. R. (2013). Transmission of *Streptococcus agalactiae* from a hatchery into a newly established red hybrid tilapia, *Oreochromis niloticus* (L.) × *Oreochromis mossambicus* (Peters), farm. *Journal of Fish Diseases*, 36(8), 735-739.
- Anshary, H., Kurniawan, R. A., Sriwulan, S., RamLi, R., & Baxa, D. V. (2014). Isolation and molecular identification of the etiological agents of streptococcosis in Nile tilapia (*Oreochromis niloticus*) cultured in net cages in Lake Sentani, Papua, Indonesia. *SpringerPlus*, 3(1), 1-11.
- Ansorge, R. (2021, April 17). Bacterial and viral infections. Retrieved on 1 January 2023 from <https://www.webmd.com/a-to-z-guides/bacterial-and-viral-infections>.
- Assis, G. B. N., Tavares, G. C., Pereira, F. L., Figueiredo, H. C. P., & Leal, C. A. G. (2017). Natural coinfection by *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *Orientalis* in farmed Nile tilapia (*Oreochromis niloticus* L.). *Journal of fish diseases*, 40(1), 51-63.
- Bacharach, E., Mishra, N., Briese, T., Eldar, A., Lipkin, W. I., & Kuhn, J. H. (2017). ICTV taxonomic proposal 2016.016 a-Dm. A. v2. *Tilapinevirus*. Create the unassigned genus *Tilapinevirus*.
- Bacharach, E., Mishra, N., Briese, T., Zody, M. C., Kembou Tsofack, J. E., Zamostiano, R., & Lipkin, W. I. (2016). Characterization of a novel orthomyxo-like virus causing mass die-offs of tilapia. *Mbio*, 7(2), e00431-16.
- Baltimore, D. (1971). Expression of animal virus genomes. *Bacteriological reviews*, 35(3), 235.
- Barato, P., Martins, E. R., Melo-Cristino, J., Iregui, C. A., & Ramirez, M. (2015). Persistence of a single clone of *Streptococcus agalactiae* causing disease in tilapia (*Oreochromis* sp.) cultured in Colombia over 8 years. *Journal of fish diseases*, 38(12), 1083-1087.
- Bartholomew, J. W., & Mittwer, T. (1952). The gram stain. *Bacteriological reviews*, 16(1), 1-29.

- Basri, L., Nor, R. M., Salleh, A., Md. Yasin, I. S., Saad, M. Z., Abd. Rahaman, N. Y., Barkham, T., & Amal, M. N. A. (2020). Co-Infections of Tilapia Lake Virus, *Aeromonas hydrophila* and *Streptococcus agalactiae* in farmed red hybrid tilapia. *Animals*, 10(11), 2141.
- Behera, B. K., Pradhan, P. K., Swaminathan, T. R., Sood, N., Paria, P., Das, A., & Jena, J. K. (2018). Emergence of tilapia lake virus associated with mortalities of farmed Nile tilapia *Oreochromis niloticus* (Linnaeus 1758) in India. *Aquaculture*, 484, 168-174.
- Berridge, B. R., Bercovier, H., & Frelief, P. F. (2001). *Streptococcus agalactiae* and *Streptococcus difficile* 16S–23S intergenic Rdna: genetic homogeneity and species-specific PCR. *Veterinary Microbiology*, 78(2), 165-173.
- Biller, J. D., & Takahashi, L. S. (2018). Oxidative stress and fish immune system: phagocytosis and leukocyte respiratory burst activity. *Anais da Academia Brasileira de Ciências*, 90, 3403-3414.
- Bradley, J. E., & Jackson, J. A. (2008). Measuring immune system variation to help understand host-pathogen community dynamics. *Parasitology*, 135(7), 807-823.
- Braunstein, H., Tucker, E. B., & Gibson, B. C. (1969). Identification and significance of *Streptococcus agalactiae* (Lancefield group B). *American journal of clinical pathology*, 51(2), 207-13.
- Buxton, R. (2005). Blood agar plates and hemolysis protocols. *American Society for Microbiology*, 1-9.
- Cao, J., Liu, Z., Zhang, D., Guo, F., Gao, F., Wang, M., & Lu, M. (2022). Distribution and localization of *Streptococcus agalactiae* in different tissues of artificially infected tilapia (*Oreochromis niloticus*). *Aquaculture*, 546, 737370.
- Chaput, D. L., Bass, D., Alam, M., Hasan, N. A., Stentiford, G. D., Aerle, R. V., & Tyler, C. R. (2020). The segment matters: Probable reassortment of tilapia lake virus (TiLV) complicates phylogenetic analysis and inference of geographical origin of new isolate from Bangladesh. *Viruses*, 12(3), 258.
- Chauhan, R. (2014). Fungal attack on *Tilapia mossambicus* in culture pond, leading to mass mortality of fishes. *Int J Phram Sci Rev Res*, 7.
- Contreras, H., Vallejo, A., Mattar, S., Ruiz, L., Guzmán, C., & Calderón, A. (2021). First report of tilapia lake virus emergence in fish farms in the department of Córdoba, Colombia. *Veterinary World*, 14(4), 865.
- Debnath, P. P., Delamare-Deboutteville, J., Jansen, M. D., Phiwsaiya, K., Dalia, A., Hasan, M. A., & Rodkhum, C. (2020). Two-year surveillance of tilapia

lake virus (TiLV) reveals its wide circulation in tilapia farms and hatcheries from multiple districts of Bangladesh. *Journal of fish diseases*, 43(11), 1381-1389.

Del-Pozo, J., Mishra, N., Kabuusu, R., Cheetham, S., Eldar, A., Bacharach, E., & Ferguson, H. W. (2017). Syncytial hepatitis of tilapia (*Oreochromis niloticus* L.) is associated with orthomyxovirus-like virions in hepatocytes. *Veterinary pathology*, 54(1), 164-170.

Department of Fisheries. (2013). Annual Fisheries Statistics, Department of Fisheries, Ministry of Agriculture & Agro-based Industry. Putrajaya, Malaysia, 2018.

Devi, P., Khan, A., Chattopadhyay, P., Mehta, P., Sahni, S., Sharma, S., & Pandey, R. (2021). Co-infections as modulators of disease outcome: minor players or major players?. *Frontiers in Microbiology*, 1894.

Dong, H. T., Rattanarojpong, T., & Senapin, S. (2017a). Urgent update on possible worldwide spread of tilapia lake virus (TiLV).

Dong, H. T., Siriroob, S., Meemetta, W., Santimanawong, W., Gangnonngiw, W., Pirarat, N., & Senapin, S. (2017b). Emergence of tilapia lake virus in Thailand and an alternative semi-nested RT-PCR for detection. *Aquaculture*, 476, 111-118.

Dong, H. T., Ataguba, G. A., Khunrae, P., Rattanarojpong, T., & Senapin, S. (2017c). Evidence of TiLV infection in tilapia hatcheries from 2012 to 2017 reveals probable global spread of the disease. *Aquaculture*, 479, 579-583.

Dong, H. T., Nguyen, V. V., Le, H. D., Sangsuriya, P., Jitrakorn, S., Saksmerprom, V., & Rodkhum, C. (2015). Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms. *Aquaculture*, 448, 427-435.

Duremdez, R., Al-Marzouk, A., Qasem, J. A., Al-Harbi, A., & Gharabally, H. (2004). Isolation of *Streptococcus agalactiae* from cultured silver pomfret, *Pampus argenteus* (Euphrasen), in Kuwait. *Journal of fish diseases*, 27(5), 307-310.

Eickhoff, T. C., Klein, J. O., Daly, A. K., Ingall, D., & Finland, M. (1964). Neonatal sepsis and other infections due to group B beta-hemolytic streptococci. *New England Journal of Medicine*, 271(24), 1221-1228.

El-Sayed, A. F. M. (2006). Tilapia culture in salt water: environmental requirements, nutritional implications and economic potentials. *Avances en Nutricion Acuicola*.

- Evans, J. J., Klesius, P. H., Gilbert, P. M., Shoemaker, C. A., Al Sarawi, M. A., Landsberg, J., & Al Zenki, S. (2002). Characterization of β -haemolytic Group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus* L., and wild mullet, *Liza klunzingeri* (Day), in Kuwait. *Journal of fish diseases*, 25(9), 505-513.
- Eyngor, M., Zamostiano, R., Kembou Tsofack, J. E., Berkowitz, A., Bercovier, H., Tinman, S., & Eldar, A. (2014). Identification of a novel RNA virus lethal to tilapia. *Journal of clinical microbiology*, 52(12), 4137-4146.
- FAO. (1996). *Fisheries and aquaculture in sub-saharan Africa: Situation and outlook in 1996*. Retrieved on 13 September 2022.
- FAO. (2016). *Contributing to food security and nutrition for all*. Retrieved on 13 September 2022.
- FAO, (2021). *Tilapia sector growth to resume after shaking off pandemic effect*. Retrieved on 30 December 2022.
- Fathi, M., Dickson, C., Dickson, M., Leschen, W., Baily, J., Muir, F., & Weidmann, M. (2017). Identification of Tilapia Lake Virus in Egypt in Nile tilapia affected by 'summer mortality' syndrome. *Aquaculture*, 473, 430-432.
- Fathi, S., Harun, A. N., Rambat, S., & Tukiran, N. A. (2018). Current issues in aquaculture: lessons from Malaysia. *Advanced science letters*, 24(1), 503-505.
- Feldman, A. T., & Wolfe, D. (2014). Tissue processing and hematoxylin and eosin staining. In *Histopathology* (pp. 31-43). Humana Press, New York, NY.
- Ferguson, H. W., Kabuusu, R., Beltran, S., Reyes, E., Lince, J. A., & del Pozo, J. (2014). Syncytial hepatitis of farmed tilapia, *Oreochromis niloticus* (L.): a case report. *J Fish Dis*, 37(6), 583-589.
- Gao, F. Y., Pang, J. C., Lu, M. X., Zhu, H. P., Ke, X. L., Liu, Z. G., & Wang, M. (2018). Molecular characterization, expression and functional analysis of NOD1, NOD2 and NLRC3 in Nile tilapia (*Oreochromis niloticus*). *Fish & shellfish immunology*, 73, 207-219.
- Geng, Y., Wang, K. Y., Huang, X. L., Chen, D. F., Li, C. W., Ren, S. Y., & Lai, W. M. (2012). *Streptococcus agalactiae*, an emerging pathogen for cultured ya-fish, *Schizothorax prenanti*, in China. *Transboundary and emerging diseases*, 59(4), 369-375.
- Hansen, P. A. (1953). A note on the name *Streptococcus agalactiae* Lehmann and Neumann. *International Journal of Systematic and Evolutionary Microbiology*, 3(1), 21-23.
- ICTV. (2020). *Virus taxonomy: 2020 release*. Retrieved on 24 June 2021.

- Imperi, M., Pataracchia, M., Alfarone, G., Baldassarri, L., Orefici, G., & Creti, R. (2010). A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of *Streptococcus agalactiae*. *Journal of microbiological methods*, 80(2), 212-214.
- Jaemwimol, P., Rawiwan, P., Tattiyapong, P., Saengnual, P., KamLangdee, A., & Surachetpong, W. (2018). Susceptibility of important warm water fish species to tilapia lake virus (TiLV) infection. *Aquaculture*, 497, 462-468.
- Jansen, M. D., Dong, H. T., & Mohan, C. V. (2019). Tilapia lake virus: a threat to the global tilapia industry?. *Reviews in Aquaculture*, 11(3), 725-739.
- Johnson-Summer, M. (2003). Principles of Disease and Epidemiology Chapter 14 Introduction Pathology, Infection and Disease.
- Kayansamruaj, P., Pirarat, N., Kondo, H., Hirono, I., & Rodkhum, C. (2015). Genomic comparison between pathogenic *Streptococcus agalactiae* isolated from Nile tilapia in Thailand and fish-derived ST7 strains. *Infection, Genetics and Evolution*, 36, 307-314.
- Kayansamruaj, P., Pirarat, N., Kondo, H., Hirono, I., & Rodkhum, C. (2014). Draft genome sequences of *Streptococcus agalactiae* strains isolated from Nile tilapia (*Oreochromis niloticus*) farms in Thailand. *Genome announcements*, 2(6), e01300-14.
- Kembou Tsofack, J. E., Zamostiano, R., Watted, S., Berkowitz, A., Rosenbluth, E., Mishra, N., & Bacharach, E. (2017). Detection of tilapia lake virus in clinical samples by culturing and nested reverse transcription-PCR. *Journal of Clinical Microbiology*, 55(3), 759-767.
- Klesius, P. H., Shoemaker, C. A., & Evans, J. J. (2008). *Streptococcus*: a worldwide fish health problem. In *Proceedings of the 8th International Symposium on Tilapia in Aquaculture* (pp. 83-107). Cairo, Egypt.
- Koesharyani, I., Gardenia, L., Widowati, Z., Khumaira, K., & Rustianti, D. (2018). Studi kasus infeksi tilapia lake virus (TiLV) pada ikan nila (*Oreochromis niloticus*). *Jurnal Riset Akuakultur*, 13(1), 85-92.
- Kotob, M. H., Menanteau-Ledouble, S., Kumar, G., Abdelzaher, M., & El-Matbouli, M. (2017). The impact of co-infections on fish: a review. *Veterinary research*, 47(1), 1-12.
- Laith, A. A., Ambak, M. A., Hassan, M., Sherif, S. M., Nadirah, M., Draman, A. S., & Najiah, M. (2017). Molecular identification and histopathological study of natural *Streptococcus agalactiae* infection in hybrid tilapia (*Oreochromis niloticus*). *Veterinary world*, 10(1), 101.
- Liamnimitr, P., Thammatorn, W., Sonicha, U., Tattiyapong, P., & Surachetpong, W. (2018). Non-lethal sampling for Tilapia Lake Virus detection by RT-QPCR and cell culture. *Aquaculture*, 486, 75-80.

- Lim, S. Y., Ooi, A. L., & Wong, W. L. (2016). Gill monogeneans of Nile tilapia (*Oreochromis niloticus*) and red hybrid tilapia (*Oreochromis* spp.) from the wild and fish farms in Perak, Malaysia: infection dynamics and spatial distribution. *SpringerPlus*, 5(1), 1-10.
- Machimbirike, V. I., Jansen, M. D., Senapin, S., Khunrae, P., Rattanaojpong, T., & Dong, H. T. (2019). Viral infections in tilapines: More than just tilapia lake virus. *Aquaculture*, 503, 508-518.
- Mian, G. F., Godoy, D. T., Leal, C. A. G., Yuhara, T. Y., Costa, G. M., & Figueiredo, H. C. P. (2009). Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. *Veterinary microbiology*, 136(1-2), 180-183.
- Mishra, A., Nam, G. H., Gim, J. A., Lee, H. E., Jo, A., & Kim, H. S. (2018). Current challenges of *Streptococcus* infection and effective molecular, cellular, and environmental control methods in aquaculture. *Molecules and cells*, 41(6), 495.
- Mohamad, N., Mohd Roseli, F. A., Azmai, M. N. A., Saad, M. Z., Md Yasin, I. S., Zulkiply, N. A., & Nasruddin, N. S. (2019). Natural concurrent infection of *Vibrio harveyi* and *V. alginolyticus* in cultured groupers in Malaysia. *Journal of aquatic animal health*, 31(1), 88-96.
- Montoya-López, A., Moreno-Arias, C., Tarazona-Morales, A., Olivera-Angel, M., & Betancur, J. (2019). Body shape variation between farms of tilapia (*Oreochromis* sp.) in Colombian Andes using landmark-based geometric morphometrics. *Latin American Journal of Aquatic Research*, 47(1), 194-200.
- Mugimba, K. K., Chengula, A. A., Wamala, S., Mwega, E. D., Kasanga, C. J., Byarugaba, D. K., & Munang'andu, H. M. (2018). Detection of tilapia lake virus (Ti LV) infection by PCR in farmed and wild Nile tilapia (*Oreochromis niloticus*) from Lake Victoria. *Journal of fish diseases*, 41(8), 1181-1189.
- Mugimba, K. K., Lamkhannat, M., Dubey, S., Mutoloki, S., Munang'andu, H. M., & Evensen, Ø. (2020). Tilapia lake virus downplays innate immune responses during early stage of infection in Nile tilapia (*Oreochromis niloticus*). *Scientific reports*, 10(1), 1-12.
- Mugimba, K. K., Tal, S., Dubey, S., Mutoloki, S., Dishon, A., Evensen, Ø., & Munang'andu, H. M. (2019). Gray (*Oreochromis niloticus* x *O. aureus*) and red (*Oreochromis* spp.) Tilapia show equal susceptibility and proinflammatory cytokine responses to experimental Tilapia Lake Virus infection. *Viruses*, 11(10), 893.
- Nagl, S., Tichy, H., Mayer, W. E., Samonte, I. E., McAndrew, B. J., & Klein, J. (2001). Classification and phylogenetic relationships of African tilapia fishes inferred from mitochondrial DNA sequences. *Molecular phylogenetics and evolution*, 20(3), 361-374.

- Najiah, M., Aqilah, N. I., Lee, K. L., Khairulbariyah, Z., Mithun, S., Jalal, K. C. A., & Nadirah, M. (2012). Massive mortality associated with *Streptococcus agalactiae* infection in cage-cultured red hybrid tilapia *Oreochromis niloticus* in Como River, Kenyir Lake, Malaysia. *Journal of Biological Sciences*, 12(8), 438-442.
- Nicholson, P., Fathi, M. A., Fischer, A., Mohan, C., Schieck, E., Mishra, N., & Jores, J. (2017). Detection of Tilapia Lake Virus in Egyptian fish farms experiencing high mortalities in 2015. *Journal of Fish Diseases*, 40(12), 1925-1928.
- Nicholson, P., Mon-on, N., Jaemwimol, P., Tattiyapong, P., & Surachetpong, W. (2020). Coinfection of tilapia lake virus and *Aeromonas hydrophila* synergistically increased mortality and worsened the disease severity in tilapia (*Oreochromis* spp.). *Aquaculture*, 520, 734-746.
- Noor-Shahirah, Z., Saydatunzawiyah, Y., & Norshida, I. (2018). Health examination of cultured red hybrid tilapia from Setiu Marine Pond Farm Terengganu. *Journal Of Agrobiotechnology*, 9(1S), 232-238.
- Ohta, A., & Nishiyama, Y. (2011). Mitochondria and viruses. *Mitochondrion*, 11(1), 1-12.
- OIE. (2017a). *Immediate notifications and follow-ups. Tilapia lake virus, the Philippines*. Retrieved on 20 June 2021.
- OIE. (2017b). *Immediate notifications and follow-ups. Tilapia lake virus, Chinese Taipei*. Retrieved on 20 June 2021.
- OIE. (2018a). *Immediate notification. Tilapia lake virus, Peru*. Retrieved on 21 June 2021.
- OIE. (2018b). *Immediate notification. Tilapia lake virus, Mexico*. Retrieved on 21 June 2021.
- Pasnik, D. J., Evans, J. J., & Klesius, P. H. (2008). Influence of tricaine methanesulfonate on *Streptococcus agalactiae* vaccination of Nile tilapia (*Oreochromis niloticus*). *Veterinary research*, 28-33.
- Patterson, M. J. (1996). *Streptococcus. Medical Microbiology. 4th edition*.
- Petti, C. A., Polage, C. R., & Schreckenberger, P. (2005). The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. *Journal of clinical microbiology*, 43(12), 6123-6125.
- Phares, C. R., Lynfield, R., Farley, M. M., Mohle-Boetani, J., Harrison, L. H., Petit, S., & Schrag, S. J. (2008). Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. *Jama*, 299(17), 2056-2065.

- Pradeep, P. J., Suebsing, R., Sirthammajak, S., Kampeera, J., Jitrakorn, S., Saksmerprome, V., & Withyachumanarnkul, B. (2016). Evidence of vertical transmission and tissue tropism of Streptococcosis from naturally infected red tilapia (*Oreochromis* spp.). *Aquaculture Reports*, 3, 58-66.
- Qasem, J. A., Al-Zenki, S., & Al-Marzouk, A. (2010). Identification and characterization of *Streptococcus agalactiae* isolates using 16S rRNA sequencing and cellular fatty acid composition analysis. *Pakistan journal of biological sciences: PJBS*, 13(1), 9-15.
- Raabe, V. N., & Shane, A. L. (2019). Group B streptococcus (*Streptococcus agalactiae*). *Microbiology spectrum*, 7(2), 7-2.
- Rahman, M. M., Rahman, M. A., Monir, M. S., Haque, M. E., Siddique, M. P., Khasruzzaman, A. K. M., & Islam, M. A. (2021). Isolation and molecular detection of *Streptococcus agalactiae* from popped eye disease of cultured Tilapia and Vietnamese koi fishes in Bangladesh. *Journal of Advanced Veterinary and Animal Research*, 8(1), 14.
- Rakus, K., Mojzesz, M., Widziolek, M., Pooranachandran, N., Teitge, F., Surachetpong, W., & Adamek, M. (2020). Antiviral response of adult zebrafish (*Danio rerio*) during tilapia lake virus (TiLV) infection. *Fish & shellfish immunology*, 101, 1-8.
- Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *American journal of epidemiology*, 27(3), 493-497.
- Regan, C. T. (1920). The classification of the fishes of the family *Cichlidae* I. The Tanganyika genera. *Annals and Magazine of Natural History*, 5(25), 33-53.
- Reiner, K. (2010). Catalase test protocol. *American society for microbiology*, 1-6.
- Reynolds, J. (2005). Serial dilution protocols. *American Society for Microbiology*, 2005, 1-7.
- Robinson, J. A., & Meyer, F. P. (1966). Streptococcal fish pathogen. *Journal of Bacteriology*, 92(2), 512-512.
- Saba, A. O., Ismail, A., Zulkifli, S. Z., Shohaimi, S., Jamil, N. R., Nawi, N. M., & Amal, M. N. A. (2020). Checklists, production trends, and potential ecological and socioeconomic impacts of non-native freshwater fishes in Malaysia: a review. *Aquatic Invasions*, 15(4), 646-670.
- Salvador, R., Muller, E. E., Freitas, J. C. D., Leonhardt, J. H., Pretto-Giordano, L. G., & Dias, J. A. (2005). Isolation and characterization of *Streptococcus* spp. Group B in Nile tilapias (*Oreochromis niloticus*) reared in hapas nets and earth nurseries in the northern region of Parana State, Brazil. *Ciência Rural*, 35, 1374-1378.

- Sathish, S., Chidambaram, P., Uma, A., & Yuvarajan, P. (2021). Prevalence of parasites in tilapia farms and their management practices in Tamil Nadu, India. *Journal of Entomology and Zoology Studies*, 9(2), 678-689.
- Sebastião, F. D. A., Lemos, E. G., & Pilarski, F. (2015). Validation of absolute quantitative real-time PCR for the diagnosis of *Streptococcus agalactiae* in fish. *Journal of microbiological methods*, 119, 168-175.
- Senapin, S., Shyam, K. U., Meemetta, W., Rattanarojpong, T., & Dong, H. T. (2018). Inapparent infection cases of tilapia lake virus (TiLV) in farmed tilapia. *Aquaculture*, 487, 51-55.
- Shabayek, S., & Spellerberg, B. (2017). Acid stress response mechanisms of group B streptococci. *Frontiers in Cellular and Infection Microbiology*, 7, 395.
- Shields, P., & Cathcart, L. (2010). Oxidase test protocol. *American society for microbiology*, 1-9.
- Slotved, H. C., Kong, F., Lambertsen, L., Sauer, S., & Gilbert, G. L. (2007). Serotype IX, a proposed new *Streptococcus agalactiae* serotype. *Journal of clinical microbiology*, 45(9), 2929-2936.
- Surachetpong, W., Janetanakit, T., Nonthabenjawan, N., Tattiyapong, P., Sirikanchana, K., & Amonsin, A. (2017). Outbreaks of tilapia lake virus infection, Thailand, 2015–2016. *Emerging infectious diseases*, 23(6), 1031.
- Surachetpong, W., Roy, S. R. K., & Nicholson, P. (2020). Tilapia lake virus: The story so far. *Journal of Fish Diseases*, 43(10), 1115-1132.
- Swaminathan, T. R., Nithyanantham, S. R., Narendrakumar, L., Dharmaratnam, A., Sood, N., Pradhan, P. K., & Lal, K. K. (2021). Co-infection of *Lactococcus garvieae* and Tilapia Lake virus (TiLV) in Nile tilapia *Oreochromis niloticus* cultured in India. *Diseases of Aquatic Organisms*, 147, 127-140.
- Syuhada, R., Zamri-Saad, M., Ina-Salwany, M. Y., Mustafa, M., Nasruddin, N. N., Desa, M. N. M., & Amal, M. N. A. (2020). Molecular characterization and pathogenicity of *Streptococcus agalactiae* serotypes Ia ST7 and III ST283 isolated from cultured red hybrid tilapia in Malaysia. *Aquaculture*, 515, 734543.
- Tattiyapong, P., Dachavichitlead, W., & Surachetpong, W. (2017). Experimental infection of Tilapia Lake Virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.). *Veterinary microbiology*, 207, 170-177.
- Tattiyapong, P., Sirikanchana, K., & Surachetpong, W. (2018). Development and validation of a reverse transcription quantitative polymerase chain reaction for tilapia lake virus detection in clinical samples and

- experimentally challenged fish. *Journal of Fish Diseases*, 41(2), 255-261.
- Thairu, Y., Nasir, I. A., & Usman, Y. (2014). Laboratory perspective of gram staining and its significance in investigations of infectious diseases. *Sub-Saharan African Journal of Medicine*, 1(4), 168.
- Thangaraj, R. S., Ravi, C., Kumar, R., Dharmaratnam, A., Saidmuhammed, B. V., Pradhan, P. K., & Sood, N. (2018). Derivation of two tilapia (*Oreochromis niloticus*) cell lines for efficient propagation of Tilapia Lake Virus (TiLV). *Aquaculture*, 492, 206-214.
- Waiyamitra, P., Piewbang, C., Techangamsuwan, S., Liew, W. C., & Surachetpong, W. (2021). Infection of Tilapia tilapinevirus in Mozambique Tilapia (*Oreochromis mossambicus*), a Globally Vulnerable Fish Species. *Viruses*, 13(6), 1104.
- Waiyamitra, P., Tattiyapong, P., Sirikanchana, K., Mongkolsuk, S., Nicholson, P., & Surachetpong, W. (2018). A TaqMan RT-QPCR assay for tilapia lake virus (TiLV) detection in tilapia. *Aquaculture*, 497, 184-188.
- Wang, R., Li, L., Huang, Y., Huang, T., Tang, J., Xie, T., & Huang, W. (2017). Pathogenicity of human ST23 *Streptococcus agalactiae* to fish and genomic comparison of pathogenic and non-pathogenic isolates. *Frontiers in microbiology*, 8, 1933.
- Wei, S., Zhang, Z., Li, Y., Hu, M., Yu, A., Zhang, H., ... & Lin, L. (2016). Epidemic and antibiotic resistance of *Streptococcus agalactiae* isolated from tilapia (GIFT *Oreochromis niloticus*) in Guangdong Province. *Journal of Fisheries of China*, 40(3), 503-511.
- Ye, X., Li, J., Lu, M., Deng, G., Jiang, X., Tian, Y., & Jian, Q. (2011). Identification and molecular typing of *Streptococcus agalactiae* isolated from pond-cultured tilapia in China. *Fisheries Science*, 77(4), 623-632.
- Yi, M., Wang, M., Li, Z., Liu, Z., Song, C., Zhang, D., & Lu, M. (2019). An investigation into the effects of *Streptococcus agalactiae* on the 5-HT system and the behavior of GIFT tilapia (*Oreochromis niloticus*). *Aquaculture Reports*, 15, 100232.
- Zamri-Saad, M., Amal, M. N. A., & Siti-Zahrah, A. (2010). Pathological changes in red tilapias (*Oreochromis* spp.) naturally infected by *Streptococcus agalactiae*. *Journal of Comparative Pathology*, 143(2-3), 227-229.
- Zhang, Z. (2021). Research Advances on Tilapia Streptococcosis. *Pathogens*, 10(5), 558.