EVIDENCE OF VERTICAL AND HORIZONTAL TRANSMISSION OF *FRANCISELLA NOATUNENSIS* SUBSP. *ORIENTALIS* IN HYBRID RED TILAPIA (*OREROCHROMIS* SP.)

Mr. Vuong Viet Nguyen

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โรคฟรานซิสเซลโลซีสมีสาเหตุจากเชื้อฟรานซิสเซลล่า โอเรียนทอลลิส (*Franciscella orientalis*) เดิมชื่อ ฟรานซิสเซลล่า โนอาทูเนนซิส ซับสปีชีส์ โอเรียนทอลลิส (*Franciscella noatunensis* subsp. *orientalis*) ถูกรายงานว่าเป็นโรคอุบัติใหม่ที่สำคัญโรคหนึ่งของปลาที่เลี้ยงในเขตน้ำอุ่น อย่างไรก็ตามข้อมูลเกี่ยวกับการแพร่กระจายของเชื้อ ธรรมชาติที่ยุ่งยากของเชื้อ การมีชีวิตอยู่รอดและการคงอยู่ของเชื้อในสิ่งแวดล้อมและภูมิภาคต่างๆ ยังมีอยู่น้อย การศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบการแพร่กระจายของเชื้อจากพ่อแม่ไปสู่ลูกและการแพร่กระจายแบบแนวขวางโดยเวคเตอร์ และผลกระทบของเชื้อโรคอื่นๆ ต่อการระบาดของโรคฟรานซิสเซลโลซีสในปลานิลแดง เพื่อเป็นการพิสูจน์ว่าเชื้อ *F. orientalis* สามารถแพร่กระจายจากแม่ปลานิลที่ป่วยเป็นโรคฟรานซิสเซลโลซิสแบบไม่แสดงอาการในขณะที่อมไข่ที่ผสมแล้วไว้ในปากไปสู่รุ่นลูกที่ผ่านกระบวนการผลิตลูกปลานิลในรูปแบบปัจจุบัน จึงได้ทำการนำพ่อแม่ปลาที่ถูกทำให้ติดเชื้อ *F. orientalis* มาผสมพันธุ์กัน จากนั้นเมื่อได้ลูกปลาออกมาจึงทำการตรวจลูกปลาว่ามีเชื้อ *F. orientalis* อยู่หรือไม่ ผลการทดลองแสดงให้เห็นว่า รังไข่ และอัณฑะ ของพ่อแม่ปลาทั้ง 3 คู่ ตลอดจนทั้งไข่ที่ได้รับการผสมแล้ว ถุงไข่แดงของตัวอ่อน และลูกปลาในอายุ 5 วันและ 30 วัน ตรวจพบเชื้อ *F. orientalis* โดยวิธี PCR และ *in situ* DNA hybridization จากการทดลองในเรื่องการแพร่กระจายของเชื้อแบบแนวขวางโดยเวคเตอร์ พบว่าลูกน้ำยุงลายสามารถรับเชื้อ *F. orientalis* เมื่อนำไปแช่น้ำไว้รวมกับเชื้อ *F. orientalis* ที่ความเข้มข้น 0.895×10^{7} CFU mL^{-1} และเมื่อนำไปเลี้ยงไว้รวมกับปลานิลที่ติดเชื้อ *F. orientalis* ปลานิลแดงถูกนำมาทดลองโดยการให้กินลูกน้ำยุงลายที่มีเชื้อ *F. orientalis* ผลการทดลองพบว่าปลานิลแดงที่ให้กินลูกน้ำมีเชื้อ *F. orientalis* ที่เลี้ยงไว้ที่อุณหภูมิ 25 องศาเซลเซียส ตรวจพบเชื้อ *F. orientalis* ได้จาก PCR และมีรอยโรคทางจุลพยาธิวิทยาของก้อนแกรนูโลม่าซึ่งเป็นรอยโรคหลักของโรคฟรานซิสเซลโลซีสที่รุนแรงกว่าปลานิลที่เลี้ยงที่อุณหภูมิ 30 องศาเซลเซียส นอกจากนี้จากการศึกษาการติดเชื้อร่วมกันของเชื้อ *F. orientalis* และเชื้อโปรโตซัว *Ichthyophthirius multifiliis* (*Ich*) ในปลานิลแดงพบว่าปลานิลแสดงอาการของการติดเชื้อทั้ง 2 โรค การติดเชื้อร่วมกันนี้มีผลทำให้ปลานิลมีอัตราการตายที่สูงขึ้น วิทยานิพนธ์นี้ได้แสดงให้เห็นว่าเชื้อ *F. orientalis* สามารถแพร่กระจายจากพ่อแม่ไปสู่ลูกปลานิลแดงได้เมื่อมีการเพาะพันธุ์ปลานิลตามรูปแบบในปัจจุบันที่ปฏิบัติกันอยู่ สิ่งที่พบจากการทดลองนี้แสดงให้เห็นว่าลูกน้ำของยุงลายมีความสามารถในการแพร่กระจายเชื้อ *F. orientalis* ไปสู่ปลานิลแดงที่แข็งแรงปรกติ และลูกน้ำยุงลายอาจจะเป็นตัวการสำคัญที่เป็นตัวกักเชื้อ *F. orientalis* ในธรรมชาติ นอกจากนี้การติดเชื้อปรสิตภายนอกอย่างเช่น *Ich* สามารถเพิ่มความรุนแรงของโรคฟรานซิสเซลโลซีสที่เกิดจากเชื้อ *F. orientalis* ในปลานิลแดงได้

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Vuong Viet Nguyen : EVIDENCE OF VERTICAL AND HORIZONTAL TRANSMISSION OF *FRANCISELLA NOATUNENSIS* SUBSP. *ORIENTALIS* IN HYBRID RED TILAPIA (*OREROCHROMIS* SP.). Advisor: Asst. Prof. Dr. CHANNARONG RODKHUM Co-advisor: Assoc. Prof. Dr. SONTHAYA TIAWSIRISUP,Assoc. Prof. Dr. NAPADON PIRARAT

Francisellosis caused by *Franciscella orientalis* (formerly named: *Franciscella noatunensis* subspecies *orientalis*) has been reported as one of the most important emergent diseases of warm water fish. However, little is known about its transmission, fastidious nature, survival, and persistence in multiple environments and global presence. This study aims to investigate vertical transmission, horizontal transmission by vectors and effect of other organisms on the outbreak of francisellosis in hybrid red tilapia. To prove the transmission of *F. orientalis* from subclinically infected tilapia mouthbrooders to their offspring through the current practice of fry production in tilapia hatcheries, experimentally infected hybrid red tilapia broodstock were mated and their offspring were examined for the presence of *F. orientalis*. The results showed that the ovary and testis of all 3 pairs of the broodstock, as well as their fertilized eggs, yolk-sac larvae, 5, and 30-day old fry were *F. orientalis* positive by *F. orientalis*-specific PCR and *in situ* DNA hybridization. Upon the vector transmission experiment, mosquito larvae *Aedes aegypti* was able to acquire *F. orientalis* in immersion challenge test at a dose of 0.895 x 10^7 CFU mL^{-1} and cohabitated *with infected fish*. Hybrid red tilapia fed by infected pupae *at 25^oC* showed *more severe histopathological lesions* of typical granulomas resembling for francisellosis infection *and positive F. orientalis detection by specific PCR comparing with 30^oC. Additionally, co-infection challenge of F. orientalis* and *Ichthyophthirius multifiliis* in hybrid red tilapia successfully mimicked typical signs and histopathological manifestations of both diseases. Synergistic effect of the two pathogens infection in fish leading to the exacerbated mortality. In conclusion, the study provided evidence of vertical transmission of *F. orientalis* in hybrid red tilapia with the current practice in tilapia hatcheries. Our finding suggested mosquito larvae are available to acquire and transmit *F. orientalis* to healthy red tilapia and might be an important environment reservoir for the bacterium in nature. The ectoparasite *Ich* infection can enhance the severity of franciscellosis caused by *F. orientalis* infection in

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Figure 18 Mature trophont of Ichthyophthirius multifiliis and its horseshoe-shaped macronucleus (high magnification) observed in fresh-mounted smear from the gill filaments of hybrid red tilapia (Orochromis sp.). 60

Figure 19 Neighbor-joining tree (right) based on alignment of 488 bp-18S rDNA sequences derived from I. multifiliis in this study (marked by star) with that of other I. multifiliis obtained from GenBank. The partial 18S rDNA sequence of Cryptocaryon irritans was used as an outgroup. Bootstraps of 1000 replicates were performed, and percentage bootstrap values are shown at each branch point. The scale bar represents the nucleotide substitutions per site. 61

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Figure 20 Cumulative mortality of healthy Ich-free hybrid red tilapia (A) and (B) naturally Ich-infected hybrid red tilapia immersed with different doses of F. orientalis. Each treatment was performed in duplicates with 10 fish/group. Upper half of the SD bar is shown. 62

Figure 21 The naturally Ich-infected hybrid red tilapia challenged with F. orientalis showed white spots (arrows) on the skin epithelial layers, fins (A) and haemorrhage on skin (B), and white nodules on internal organs resembling francisellosis (C). By contrast, naturally Ich-infected without F. orientalis exposure revealed normal appearance externally and internally (D-F). 64

Figure 22 Detection of F. orientalis (A) and Ich (B) from I. multifiliis+F. orientalis coinfected fish and Ich-infected fish. In (A), F. orientalis PCR detection was performed using F. orientalis-specific primers and DNA extracted from spleen tissues as template; 1-9, the naturally Ich-infected fish immersed with F. orientalis; 10-13, the naturally Ich-infected fish control. –ve, no template control; +ve, positive control using DNA extracted from VMCU-FNO131 isolate. In (B), I. multifilis detection was conducted using newly designed primers based on 18S rDNA sequences. DNA extracted from gill and mucus were used as PCR template. 1-6, the moribund coinfection fish; 7-12, the naturally Ich-infected fish control at end of experiment (19 dpc). –ve, no template control; +ve, DNA of I. multifiliis previously confirmed by 18S rDNA sequence analysis. Note that different individual fish were used in (A) and (B). 65

Figure 23 Naturally Ich-infected hybrid red tilapia immersion challenged with F. orientalis showed presence of trophonts (arrow) of I. multifiliis, severe consolidation, hyperplasia of secondary lamellae (A) and multiple granulomas in spleen (C). Fish in control group of Ich alone revealed normal gill lamellae (B) and hyperplasia of splenic cells (D). 67

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CHAPTER 1

INTRODUCTION

Significant and rationale of research

Franciscella orientalis (formerly named: *Franciscella noatunensis* subspecies *orientalis*) is a causative agent of piscine francisellosis in various warm water fish species (Colquhoun and Duodu, 2011). Presently, francisellosis was described as worldwide distribution and responsible for major economic losses in aquaculture industries globally. Particularly, tilapia has been considered as the most susceptible host resulting in high mortality up to 95% and 23 CFU can be lethal dose in tilapia fingerlings (Colquhoun and Duodu, 2011; Soto et al., 2009b). In Thailand, tilapia consisting of hybrid red tilapia (*Oreochromis* sp.) and nile tilapia (*Oreochromis niloticus*) has been leading in top production of cultured freshwater fish (FAO, 2014). And, francisellosis has been reported outbreak in aquaculture system in various regions in Thailand (Jantrakajorn and Wongtavatchai, 2016; Nguyen et al., 2016).

Horizontal transmission of francisellosis in fish has been proved by experimental challenge including injection, immersion, cohabitation, direct exposure to contaminated water (Ellingsen et al., 2011; Soto et al., 2009a; Soto and Revan, 2012). Recent studies found that reproductive organs (ovary and testis) of infected tilapia revealed multiple white nodules resulted in several granulomas in histological analysis (Mauel et al., 2007; Ortega et al., 2016). However, vertical transmission of the disease to offspring has not been confirmed yet. Knowledge about the routes of infection is of vital importance for disease prevention and control. In order to develop proper management strategies and effective biosecurity programmes for advancing sustainable tilapia aquaculture, the vertical transmission route of *Fno* in tilapia should be the first priority to investigate.

Mosquitoes are considered to be the major vectors of the bacterium *Francisella tularensis*, a member of genus Francisella, which causes tularaemia in United States,

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several European countries (Eliasson et al., 2002; Read et al., 2008). Many research studies have demonstrated the presence of *Francisella* genes in all stages of mosquitoes (*Aedes aegypti*) infected with *Francisella* species (Backman et al., 2015; Lundstrom et al., 2011; Thelaus et al., 2014). It is noted that *F. tularensis* subsp. *holarctica* was transstadially maintained from orally infected larvae to adult mosquitoes and that 25 % of the adults exposed as larvae were positive for the presence of *F. tularensis*-specific sequences for at least 2 weeks (Thelaus et al., 2014). In aspect of vector transmission of piscine francisellosis, and how the bacterium persists between outbreaks is still less information. Laboratory studies have proved that several groups of parasites (ciliate protozoa; *Argulus* spp; *Caligus* spp.; salmon louse) are ability act as vectors of both bacterial and viral infection in aquatic animal (AHNE, 1985; Novak et al., 2016; Xu et al., 2012b). On another hand, the mosquito larvae are naturally distributed in tilapia culture area that francisellosis outbreak. Although several studies reported that many crustacean parasites were concerned as vector transmission of pathogenic agents in fish. There lacks information of mosquito larvae in transmission of aquatic pathogens.

It is commonly accepted that multiple pathogens are responsible for disease outbreaks in cultured farm. In previous studies, the ectoparasite *Ichthyophthirius multifillis* (*Ich*) concurrently occurred with various pathogens such as *Streptococcus agalactiae* (Xu et al., 2009), *Edwardsiella ictaluri* (Xu et al., 2012c), *Aeromonas hydrophila* (Xu et al., 2012a), *F. orientalis* (Mauel et al., 2003; Mauel et al., 2007; Soto et al., 2009b). However, no literature available account for the role of *I. multifillis* in outbreak of francisellosis. Ichthyophthiriasis caused by protozoan *Ich* probably the most prevalent parasite disease of freshwater fish caused by protozoa. The disease is recognized as a serious agent leads to mass kill among different fish species in wild and culture fish worldwide (Matthews, 2005; Ventura and Paperna, 1985). It is noted that *F. orientalis* and *Ich* are two common pathogens of tilapia that share similar optimum

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temperature (cool water temperature) for disease manifestation (Birkbeck et al., 2011; Matthews, 2005). According to our observation, the both pathogens are usally concurrent infection in the disease cultured tilapia. The purpose of this study was to investigate whether *Ich*-infected tilapia be more susceptible with *F. orientalis*.

Research Questions

1. Does *F. orientalis* transmits from hybrid red tilapia broodstock to their offsprings?
2. Are mosquito larvae a transmission vector of francisellosis?
3. Does *I. multifillis* enhance horizontal transmission of *F. orientalis*?

Objectives of study

1. To investigate whether *F. orientalis* transmitted vertically.
2. To evaluate whether mosquito larvae can serve as a reservoir/vector for francisellosis transmission to healthy tilapia.
3. To investigate whether parasitism of *I. multifillis* enhances susceptibility of tilapia to *F. orientalis.*

Keywords (Thai): โรคติดเชื้อฟรานซิสเซลล่า การแพร่กระจายเชื้อแบบแนวขวาง

โรคติดเชื้ออิคไทรอฟทิริเอซิส ลูกน้ำยุง การแพร่กระจายเชื้อจากพ่อแม่ไปสู่ลูก

Keywords (English): francisellosis, horizontal transmission, Ichthyophthiriasis, mosquito larvae, vertical transmission

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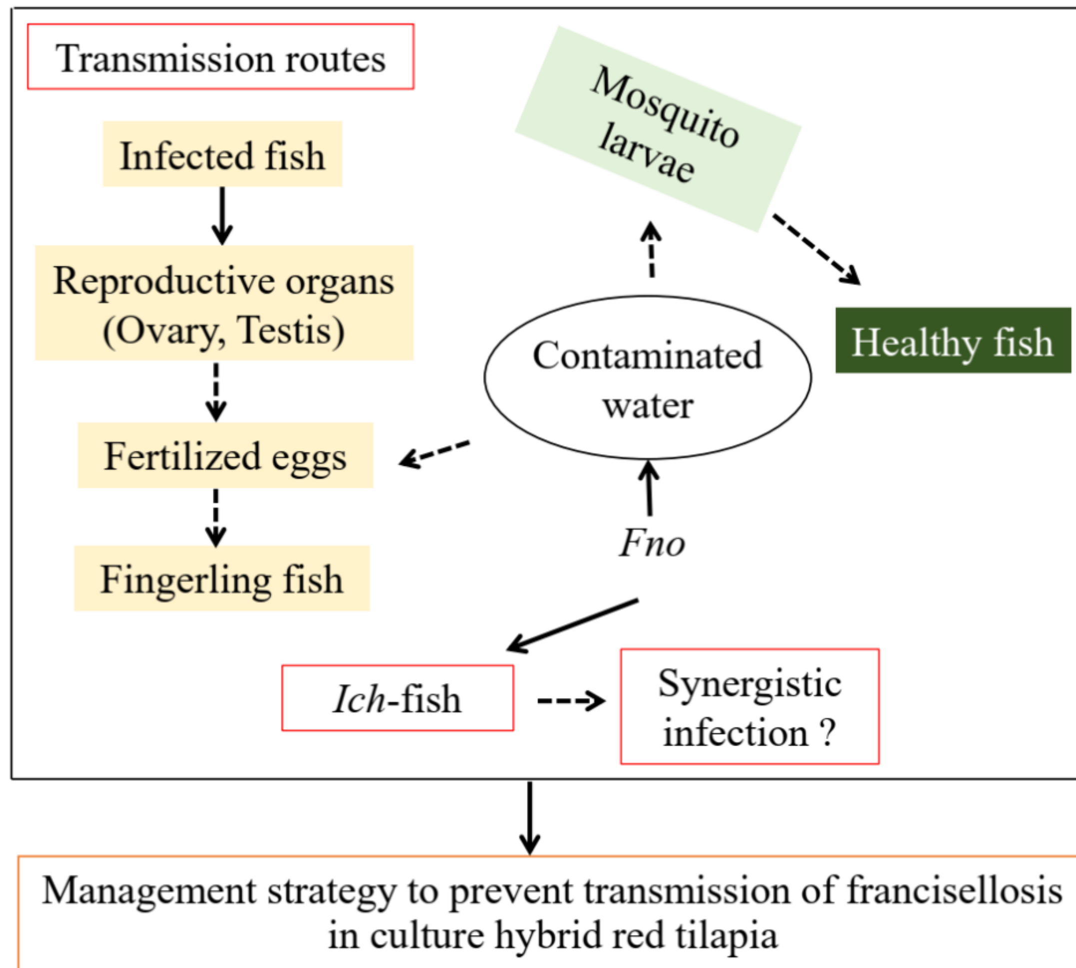
Hypotheses

1. *F. orientalis* is able to transmit vertically.
2. Mosquito larvae could be a reservoir/vector of francisellosis.
3. *I. multifillis* could encourage horizontal transmission of *F. orientalis*.

Conceptual framework



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CHAPTER 2

LITERATURE REVIEW

2.1 Tilapia culture and disease

Tilapia is a common term used to designate a group of commercially important food fish belonging to the family Cichlidae. In this group, hybrid red tilapia (*Oreochromis* sp.) and nile tilapia (*Oreochromis niloticus*) are well known as major freshwater food-fish in world aquaculture. Its production had increased annually from 3,165,000 tons in 2010 to 5,377,000 tons in 2016 accounted for 10% of fresh water fish world aquaculture production (FAO, 2018). Thailand has for decade placed on top five of countries tilapia production (Ragna et al., 2016). In Thailand, tilapia took a lead of freshwater fish production for more than a decade (FAO, 2010). However, the increasing area and production of tilapia culture resulted in challenge in in an increase in incidence and severity of disease agents in tilapia. Amongst the most common and virulent pathogens found causing mortalities in cultured tilapia are the bacterial pathogens: *Flavobacterium columnare*, *Edwardsiella tarda*, *Aeromonas* sp., *Vibrio* sp., *Francisella* sp., *Streptococcus iniae*, and *Streptococcus agalactiae* (Agnew and Barnes, 2007; Colquhoun and Duodu, 2011; Declercq et al., 2013; Griffin et al., 2016; Liu et al., 2016; Peepim et al., 2016). Recently, Francisellosis in wild and culture fish emerged as an agent causing significant mortalities and economic losses.

2.2 Francisellosis in tilapia

Francisellosis in fish is caused by Gram-negative bacteria, belongs to genus *Francisella* family *Francisellaceae*. In this genus, only two species including *Francisella noatunensis* subsp. *orientalis* (*Fno*) and *Francisella noatunensis* subsp. *noatunensis* (*Fnn*) were described as aetiological agents of Franciscellosis in warm and cool water fish, respectively (Birkbeck et al., 2011; Colquhoun and Duodu, 2011). Recently, Ramirez-Paredes (*et al*., 2020) indicated *F. noatunensis* subsp. *noatunensis* and *F.*

noatunensis subsp. *orientalis* clearly distinguish base on whole genome derived parameters, metabolic fingerprinting and chemotaxonomic analyses. *F. noatunensis* subsp. *orientalis* was reclassified as *F. orientalis* (*Fo*). Initial description of *F. noatunensis* subsp. *noatunensis* was proposed as a novel *F. noatunensis* subsp. *chilensis* subsp. nov. with type strain PQ1106^{T} isolated from farmed Atlantic salmon in Chile and *F. noatunensis* (*Fn*) included isolates from wild and farmed Atlantic cod in Northern Europe (Ramirez-Paredes et al., 2020). Francisellosis was first mentioned by (Chern and Chao, 1994) in cultured tilapia in Taiwan and referred to *Rickettsia*-like organism (RLO). The disease was then described under named *Piscirickettsia*-like syndrome (PRS) in both freshwater and marine fish (Mauel and Miller, 2002). In 2007, Mauel *et al* were the first to isolate the bacterium from a natural disease outbreak in culture nile tilapia (*Oreochromis niloticus*). Based on molecular identification, organism was classified in genus *Francisella* (Mauel et al., 2007). The later successes in isolation and molecular methods proved that most of PRS cases caused by *Francisella* spp (Birkbeck et al., 2011; Colquhoun and Duodu, 2011). The bacteria is a Gram-negative, facultative intracellular, non-motile, pleomorphic coccoid, catalase positive and cytochrome oxidase negative (Colquhoun and Duodu, 2011). According to phylogenetic analysis of bacteria belonging to the genus *Francisella*, the two fish pathogens (*Fno* and *Fnn*) and (*F. phiomoragia*), an opportunistic human pathogen, were placed in one clade and another clade contains mammal pathogen (*F. tularensis, F. novicida, F. hispaniensis and Wolbachia persica*) (Sjödin et al., 2012).

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Tilapia suffering with infection by francisellosis can show a variety of clinical *signs and lesions such as* cachexia, anorexia, anemia, and slow swimming. The gross pathology observed in chronic infection was an enlarged spleen and kidney with appearance of numerous white nodules (granulomas) (Fig.1). In some cases, white nodules also were seen in gill, intestine and liver. While in acute cases death occurred before gross internal manifestations of the disease appeared (Birkbeck et al., 2011;

Colquhoun and Duodu, 2011). Smears from infected tissues stained with Wright-Giemsa showed macrophages containing coccoid, Gram-negative intracellular organisms (Fig.1). Histopathologically, the pathological changes were observed in almost every organs, with the most dominant in the gills, spleen, and kidneys and less frequent in liver, heart, intestine. A typical granuloma was characterized by infiltration of macrophages, lymphocytes and neutrophils in a necrotic core which contained small pleomorphic cocco bacilli and surrounded by fibrous capsule (Hsieh et al., 2007; Mauel et al., 2003).

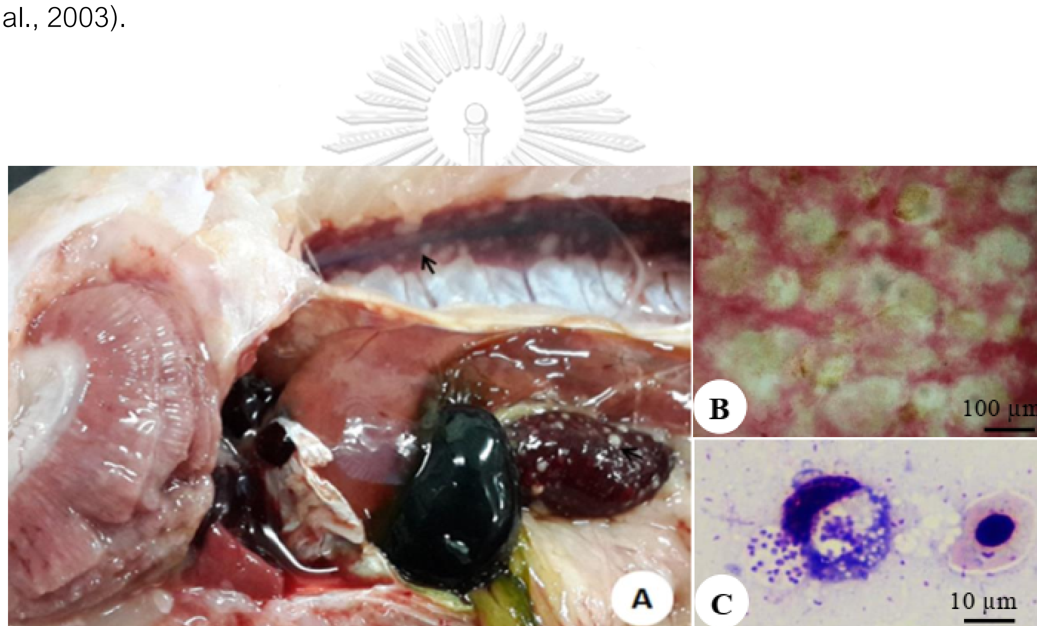

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Figure 1 Tilapia manifestation of francisellosis showed typical presentation of white nodules in spleen, kidneys and gills (arrow) (A), that exposed severe granulomatous inflammatory response under microscopy (B), and composed of small pleomorphic rods intracellular bacteria (C) (Nguyen et al., 2016)

Up to date, infection of *F. orientalis* was reported in various fish species with worldwide distribution (summarized in table 1). Almost these observations suggested that tilapia is the main host for *F. orientalis* and usually resulted in moderate mortalities around 40% (Colquhoun and Duodu, 2011; Ortega et al., 2016). In common with other *Francisella* bacteria, *F. orientalis* poses high virulent infection in susceptible host. As in

the previous report, only injection doses of 23 bacteria were able to cause mortalities and serious pathological lesions in head kidney and spleen tissues of nile tilpia (Soto et al., 2009a).

Isolation of *F. orientalis* from infected tilapia has been challenging by its fastidious nature, contamination by other opportunistic bacteria and antibiotic treatment. It is well known that Francisella bacteria grow slowly on selective cysteine heart agar as early visible colonies were observed at day three. Therefore, inoculated plates maybe overgrown with contaminated bacteria which can grow faster resulted in a false negative (Mauel et al., 2007; Soto et al., 2009b). From the infected tilapia, *F. orientalis* is able to be isolated on selective media cystine heart agar plus bovine haemoglobin and ampicillin (50 µg mL^{-1}) and polymixin B (100 units mL^{-1}) (Soto et al., 2009b). The bacteria can be successfully sub-cultured on chocolate II agar, and Mueller-Hinton base with the addition of 3% fetal bovine serum, 1% glucose and 0.1% cysteine as recommended by Soto *et al.* (2009b), or cystine heart agar supplemented with 5% sheep blood, as recommended by Mikalsen *et al.* (2009). On the selective media, colonies of *F. orientalis* appears as convex, smooth, mucoid and grey at 48 h incubation at 20 – 30 ^{o}C. But the optimal temperature for cultured bacteria was 28 to 30 ^{o}C (Soto et al., 2009b).

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Table 1 Updated susceptible hosts of fastidious bacteria; *F. orientalis*

Hosts	Location	Life stage	References
Tilapia, *Oreochromis* spp	Taiwan	Fry to adult	(Chern and Chao, 1994)
Tilapia	United States	Fry to adult	(Soto et al., 2011)
Tilapia	Costa Rica	Fry to adult	(Soto et al., 2009b)
Tilapia	Indonesia		(Ottem et al., 2009)
Tilapia	United Kingdom	Fingerling	(Jeffery et al., 2010)
Tilapia	Brazil	Fry to adult	(Lewisch et al., 2014)
Tilapia	Mexico	Fry to adult	(Ortega et al., 2016)
Ornamental Malawi cichlids (various species)	Australia		(Lewisch et al., 2014)
Tilapia	China	Fry to adult	(Lin et al., 2016)
Tilapia	Thailand	Juvenile to adult	(Nguyen et al., 2016)
Threeline grunt (*Parapristipoma trilineatum*)	Japan		(Kamaishi et al., 2005)
Ornamental cichlid (11 different species)	Taiwan	Juvenile to adult	(Hsieh et al., 2007)
Hybrid striped bass, *Morone chrysops* x *M. saxtilis*	United States	Juvenile to adult	(Ostland et al., 2006)
Indo-Pacific reef fish (6	United States		(Camus et al., 2013)

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different species)			

2.3 Environments factor affecting francisellosis outbreak

Fish infectious disease is the result of a complex reaction between pathogens in the water, susceptible hosts, and an unfavorable environment conditions (Plumb and Hanson, 2011). In this sense, the environment factors are essential to understand the pathogenesis of pathogens and to develop practical prevention method and successful aquaculture. In culture tilapia, outbreak of francisellosis disease was closely related to water temperature, in which the disease often occurred at temperature of lower than 26 °C (Birkbeck et al., 2011; Colquhoun and Duodu, 2011). Mauel *et al.* (2003) reported that wild and cultured tilapia on the island of Oahu, Hawaii were more susceptible to francisellosis in the water temperature ranging from 21.5 to 26.3 °C, causing 96% mortality within 24 days post challenge. But dead fish were not found in the water temperature maintained between 26.5 and 29.2 °C. Similar findings were observed by Soto *et al.* (2012), mass mortality of experiment tilapia cultured at 25 °C, while few dead fish were recorded at 30 °C. In addition, the concentration of bacteria loaded in spleen tissue and histopathological changes in target organs of treatment 25 °C were higher than those at 30 °C.

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Moreover, temperature condition and sea water also have an effect on persistence and culturability of *F. orientalis* in water microcosms. Soto and Revan (2012) reported that the greatest number of bacterial colonies was counted at 20 °C followed by 25°C and 30°C in both filtered sea and freshwater microcosms. And, suspended bacterium in seawater microcosms persist for longer periods of time than those in freshwater. However, *F. orientalis* is not able to replicate in non-nutritious environment. In this regard, *F. orientalis* rapidly decrease in colony counts when inoculated in water microcosms. Moreover, the pathogenic properties of the bacteria suspended in water microcosms appear to decrease after only 24 h and was not pathogenic to nile tilapia

(*Oreochromis niloticus*) following an immersion challenge after 96 h (Soto and Revan, 2012).

2.4 Transmission and reservoir of *F. orientalis*

Moreover, the water temperate, age of fish also seems to be an important impact on outbreak of francisellosis in culture tilapia. Although the disease affects all ages of fish, disease occurred in young fish is more acute and producing higher mortality comparing with those in adult. (Hsieh et al., 2006; Soto et al., 2013). Horizontal transmission of francisellosis has been proved in the previous cohabitation experiment. The disease can occur via direct contact with infected fish or waterborne. Progression of transmission would be faster under injury from capture activities and optimal environmental conditions such as low water quality and temperature. The disease most likely happens during the transportation of the fish especially when turbidity and water temperature were fluctuated (Mauel et al., 2003; Mauel et al., 2007).

2.5 The impact of ectoparasites on freshwater fish

Fish ectoparasites attach themselves to the skin and gills of the host. Various ectoparasites including protozoans; monogeneans; leeches; crustaceans cause significant infestation in many kinds of fish (Matthews, 2005). In many cases, low infections generally cause little damage to the skin tissues so that do not lead to fish mortality. However, higher infection level of ectoparasites can cause severe damage on fish population especially, under the high densities stock in farm. Some of the most pathogenic ectoparasites are ciliate protozoans *Ichthyophthirius multifiliis* (Hanson et al., 2008); *Trichodina* spp (Wellborn and Thomass, 1967); *Argulus* spp (Taylor et al., 2006) which has been reported as main problem in nature and culture freshwater fish. In this part, the brief information of dominant parasite *I. multifillis* will be discussed.

Ichthyophthiriasis (also known as white spot disease) is probably the most prevalent parasite disease of freshwater fish caused by protozoan *Ichthyophthirius*

multifiliis (*Ich*). The disease is recognized as a serious agent leads to mass kill among different fish species in wild and culture fish worldwide (Matthews, 2005; Ventura and Paperna, 1985). Naturally outbreak of *I. multifiliis* in pre-spawning and spawning sockeye salmon was blamed for the 153.6 million fewer fry produced in the Skeena River watershed, Canada (Traxler et al., 1998). Infection by this parasite causes particularly losses of 42% of channel catfish producers in U.S in 2002 (Hanson et al., 2008). *Ich* infection causes lesion on skin and gills of fish, leading to loss of the respiratory, depletion of energy reserves, impaired haemopoiesis. The typical signs of ichthyophthiriasis is multiple white spots on the skin, fin and gills of fish (Matthews, 2005). Key factors in it dominate prevalence are wide temperature tolerance, wide range of susceptible host and direct life cycle. The long list susceptible host of this disease includes rainbow trout (Heinecke and Buchmann, 2013), common carp (Gonzalez et al., 2007), grass carp (Yulin, 1996), nile tilapia (Xu et al., 2009).

I. multifillis is an obligate ectoparasite and was first named by Fouquet (1876) (Fouquet, 1876) base on its large number of offspring produced at encysment. Its life cycle has three developmental stages including a reproductive stage (tomocyst), an infective stage (theront) and a parasitic stage (trophont) (McCartney et al., 1985; Wei et al., 2013). Infective theront swim in water to find hosts (Murphy and Lewbart, 1995). One it successfully burrows into the host epithelium, theront transforms to trophont and parasites there until reach to tomont stage (Matthews, 2005). The mature tomont leaves the host, attaches to substrates and encysting. The encysted tomont divides rapidly to produce its offspring, called tomites. Following several divisions, tomites come out tomocyst and turn to infective theront (Murphy and Lewbart, 1995). The disease is able to diagnosis base on clinical signs and wet mount method to identify *I. multifillis*. In previous works, identification of *Ich* based on the unique characteristics of trophont such as a horsehaped macronucleus, spherical cell which surrounded with a thick layer of cilia (Matthews, 2005;

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McCartney et al., 1985). Currently, 18S rDNA sequence was a useful tool to distinguish *I. multifillis* and other Oligohymenophorea species (Wright and Lynn, 1995).

2.6 Role of ectoparasite on disease transmission in cultured fish

In the aquaculture system, fish are naturally exposed to multiple pathogens. Therefore, it is not surprising that co-infection frequently occurs in cultured fish. Previous studies have shown that primary infected with *I. multifillis* resulted in reducing resistant of fish to secondary bacterial infection. For example, the significant higher mortality and more persistent was recorded in group of *Ich*-infected gold fish to *Aeromonas hydrophila* when compared to single infected groups (Liu and Lu, 2004). Similarly, primary infestation of *I. multifillis* enhanced susceptibility of channel catfish to *A. hydrophila*, *Edwardsiella ictalurid* (Xu et al., 2012a; Xu et al., 2012c). In nile tilpia, damage on body surface caused by *I. multifillis* facilitates invasion of *Streptococcus iniae* lead to higher mortality than the fish were exposed to only single pathogen (Xu et al., 2009).

Many ectoparasites are known to be vectors and/or reservoirs of the pathogens, which the parasites have the potential to contribute to pathogens transmission. It was supposed that dissemination of pathogens may occur while the parasites moving between hosts (AHNE, 1985; Nylund et al., 1994). In previous studies, various fish viral pathogens were isolated from ectoparasite such as infectious salmon aneamia virus (ISAV, Nylund et al., 1993); salmonid alphavirus (SAV, (Petterson et al., 2009)) from salmon lice *Lepeophtheirus salmonis*, infectious haematopoietic necrosis (IHN) virus from *Piscicola salmositica* and a copepod *Salmincola* sp. (Mulcahy et al., 1990) viral hemorrhagic septicemia virus (VHSV) from leech *Myzobdella lugubris* and amphipod zooplankton *Diporeia* spp. (Faisal and Winters, 2011). In addition, several bacterial pathogens were also detected in the parasite including *Aeromonas salmonicida* (Novak et al., 2016), *Tenacibaculum maritimum*; *Pseudomonas fluorescens*; *Vibrio* spp. (Barker

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et al., 2009) in salmon lice, *Edwardsiella ictalurid* in *I. multifillis* (Xu et al., 2012c). Under laboratory conditions, previous studies have indicated the vector potential of *L. salmonis* to IHNV (Jakob et al., 2011) and *A. salmonicida* (Novak et al., 2016). Furthermore, bacteria (Liu and Lu, 2004; Xu et al., 2012b) and virus (LOBO-DA-CUNHA and AZEVEDO, 1992) has been detected in the trophonts of *I. multifillis*. Interestingly, bacteria *E. ictalurid* was able to persist and replicate during tomont division, resulting spread of bacteria in their offspring, theronts. And, 60% fish exposed to these theronts was positive with *E. ictaluri* by specific PCR detection (Xu et al., 2012b). This study proposed *I. multifillis* was a potential vector for *E. ictaluri*.

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CHAPTER 3

EVIDENCE OF VERTICAL TRANSMISSION OF *FRANCISELLA ORIENTALIS* IN HYBRID RED TILAPIA (*OREOCHROMIS* SP.)

Abstract

F. orientalis has been reported as an important bacterial pathogen causing significant mortality (30-95%) in farmed tilapia in broad geographic areas. However, we found that there was a proportion of broodfish in our laboratory that appeared healthily but which tested positive for *F. orientalis*. We therefore hypothesized that *F. orientalis* might be able to be transmitted from subclinically infected tilapia mouthbrooders to their offspring through the current practice of fry production in tilapia hatcheries. To prove this, experimentally infected hybrid red tilapia broodstock were mated and their offspring were examined for the presence of *F. orientalis*. In this study, three pairs of infected broodfish were mated for natural spawning and fertilized eggs from each couple were then collected from the female mouths for artificial incubation. The newly hatched larvae were cultured for 30 days and sample collection was performed at different developmental stages i.e. yolk-sac larvae, 5 and 30-day old fry. The results showed that the ovary and testis of all 3 pairs of the broodstock, as well as their fertilized eggs and offspring were *F. orientalis* positive by *F. orientalis*-specific PCR and *in situ* DNA hybridization. In summary, this study revealed that with the current practice in tilapia hatcheries, *F. orientalis* might be able to transmit from subclinically infected tilapia mouthbrooders to their offspring. Therefore, using *F. orientalis*-free broodfish in tilapia hatcheries should be considered in order to produce *F. orientalis*-free tilapia fry.

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3.1 Introduction

Francisellosis is a systemic disease that is caused by Gram-negative, small coccobacillus bacterium, *F. orientalis* (Ramirez-Paredes et al., 2020). It has been reported worldwide and is responsible for considerable economic losses in various warm water fish species (Colquhoun and Duodu, 2011), and tilapia in particular, has been considered as the most susceptible host resulting in mortality levels of up to 95% and as little as 23 colony forming units (CFU) can be lethal for tilapia fingerlings (Colquhoun and Duodu, 2011; Soto et al., 2009a). Since the first case of *F. orientalis* was reported in Thailand in 2013, the disease has been reported in farmed tilapia in several provinces (Jantrakajorn and Wongtavatchai, 2016; Nguyen et al., 2016). According to the private sector, francisellosis is presently considered as one of the top three most important infectious diseases of farmed tilapia in Thailand.

Currently, the horizontal transmission of *F. orientalis* has been proven by experimental challenge using different infection routes e.g. injection, immersion, cohabitation between infected and healthy fish or direct exposure to contaminated water (Soto et al., 2014; Soto et al., 2009a; Soto et al., 2013). Previous studies found that reproductive organs (ovary and testis) of infected tilapia showed multiple white nodules that formed granulomatous inflammation in histopathology analysis. Thus, vertical transmission of *F. orientalis* is potentially suspected (Mauel et al., 2007; Ortega et al., 2016). So far only one published work by Pradeep *et al.* (2017) supported the potential of *F. orientalis* vertical transmission by performing artificial fertilization using naturally infected broodfish and examining the presence of *F. orientalis* from their reproductive organs and offspring. However, *F. orientalis* detection was carried out by a single technique called loop mediated isothermal amplification (LAMP). In the current study, eggs were collected from natural mouthbrooding fish in an experiment set up similar to current practices in tilapia hatchery. The hybrid red tilapia (*Oreochromis* sp.) broodfish were subclinically infected by pre-exposure to *F. orientalis*. Confirmation of *F. orientalis*

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in the fish reproductive organs and their progeny was performed by a combination of PCR, histology and *in situ* hybridization (ISH) assays.

3.2 Material and methods

3.2.1 Experimental fish

This project has been reviewed and approved by the Biosafety Committee (approval no. IBC 1831055) and Animal Ethics Committee (approval no. CU-ACUP 1931007) from Chulalongkorn University. Clinically healthy four-month-old hybrid red tilapia (initial body weight 30 ± 6 g) were kindly provided by Kamphaengsaen Fisheries Research Station, Faculty of Fisheries, Kasetsart University, Thailand. The fish were acclimatized in two 1-m^3 fiber glass tanks containing chlorine-free water at a temperature of 26.5 ± 0.5^{o}C for two weeks. The fish were fed with commercial tilapia pellet feed (CP) containing ~30% crude protein at the rate of 5% biomass twice per day. The tank contained air stones and cotton filters. The water and cotton filters were replaced two times per week and water parameters (pH, nitrite, total ammonia) were checked daily during the experimental period. Ten fish were randomly selected for bacterial and parasitic examination to verify that the fish were healthy prior to the challenge experiment. It should be noted that for *F. orientalis* examination, species-specific PCR (Dong et al., 2016a) and bacterial culture using cysteine heart agar (CHA) (Soto et al., 2009b) were performed with the spleen and reproductive organs to ensure that the fish were not infected with *F. orientalis*.

3.2.2 Bacterial preparation

F. orientalis strain VMCU-FNO131 originally isolated from farmed hybrid red tilapia suffering the piscine francisellosis in Thailand (Nguyen et al., 2016) was used in this study. The bacterium was recovered from glycerol stock and prepared as described previously (Nguyen et al., 2016; Soto et al., 2009b). The actual number of *F.*

orientalis used in challenge tests was evaluated through tenfold serial dilution using a standard plate count method.

3.2.3 Experimental design

An experimental design for investigating the *F. orientalis* transmission in the present study is illustrated in Figure 1. In order to obtain subclinically infected broodstock, a sub-lethal dose of the *F. orientalis* isolate VMCU-FNO131 (2.88×10^5 CFU mL^{-1}) previously identified from a median lethal dose (LD_{50}) was used (Nguyen, 2015). Using this dose, 18 male and 18 female fish were immersed for 30 min in two 20-L tanks containing the bacterium before being transferred to two 1-m^3 tanks. At 10-day post challenge (dpc), 4 males and 4 females were randomly collected for confirmation of presence of the *F. orientalis* infection. The remaining broodstock were observed and maintained for use in the mating experiment.

To investigate fish maturity, the broodstock were starved for one day before being checked individually. The males that showed reddish color of protruded papilla and the females that released eggs after wiping their abdomen were selected for breeding (Rothbard and Pruginin, 1975). Each pair of a total of three pairs of the broodstock were then transferred to a 50-L glass aquarium tank in a flow water system with a water temperature of 26.5 ± 0.5^{o}C. To encourage breeding, 50% of the water in the tanks was changed daily. Behavior of the fish was monitored continuously until eggs were spawned, fertilized, and scooped into female mouths naturally in the tank. These events occurred approximately 5 to 6-week post *F. orientalis* challenge. The fertilized eggs were then collected from the female's mouth and washed with water that was treated by ultraviolet light (UV) one week before using. Subsequently, the eggs were artificially incubated in round-bottomed hatching chamber as previously described. (P.J et al., 2011). After hatching period, the larvae of each family were cultured in a 50-L aquarium tank with filtered chlorine treated water for 30 days. Water parameters were

checked daily during the experiment period. The larvae were fed with powdered feed (28% protein, CP) twice a day.

After mating and fertilized eggs were collected from the mouth of female broodfish, the parental fish were humanly terminated for *F. orientalis* diagnosis. The collected samples in this task included spleen tissues (50 mg) and reproductive organs (50 mg) of individual broodfish, pool of 10 fertilized eggs, 10 yolk-sac larvae, 10 five-day old fry, and 10 thirty-day old fry from each family. Three sets of the samples were prepared and used for i) bacterial isolation, ii) preservation in 95% ethanol for PCR detection and iii) preservation in 10% buffered formalin for histology and *in situ* hybridization (ISH) assay (see below).

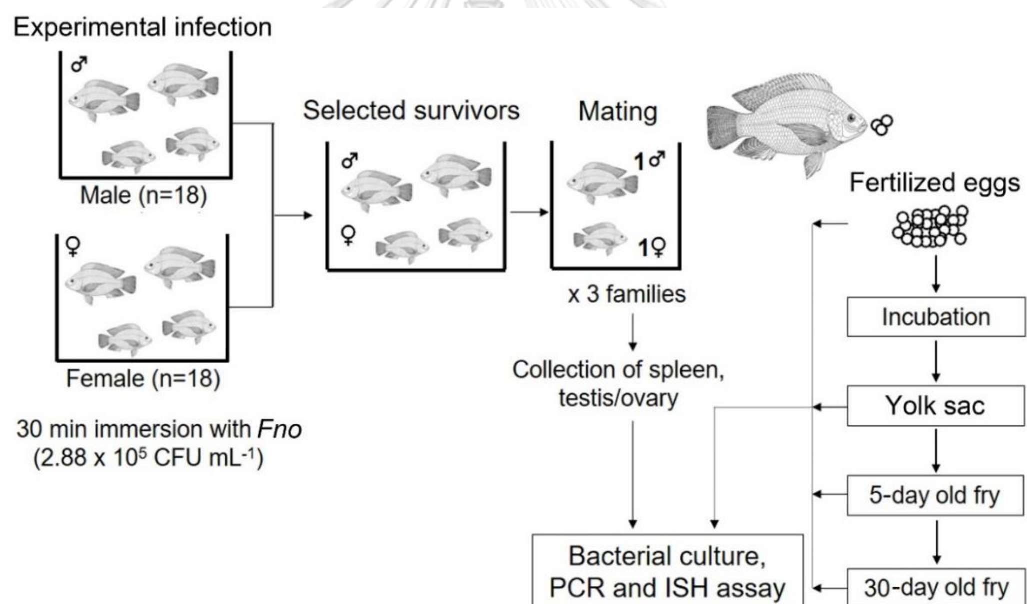

Figure 2 Experimental design for investigating the transmission of F. noatunensis subsp. orientalis (Fno) from hybrid red tilapia broodstock (Oreochromis sp.) to their offspring. The broodstock were immersed with an under-lethal dose of Fno before being selected to mate and produce fry. The fertilized eggs were collected from females' mouth for artificial incubation until the late fry stage. The samples of each family including spleen, ovary, testis of the broodfish, fertilized eggs, yolk sac, 5-day old fry and 30-day old fry were analyzed for the presence of Fno using bacterial culture, PCR and ISH assay.

In this experiment, a non-infected control family of hybrid red tilapia was treated in the same manner and respective samples of reproductive organs, fertilized eggs, yolk-sac larvae and fry were preserved for PCR analysis.

3.2.4 Bacterial isolation

Spleen tissues, eggs, testis of broodstock, fertilized eggs, larvae, fingerling fish originated from 3 pair families were aseptically collected and washed carefully in distilled water three times. The samples were grilled in 100 µl normal saline. The collected suspension was streaked on selective cystine heart agar plate added 10% sheep blood, Polymixin B 100 units mL^{-1}, Ampicillin 50 µg mL^{-1}. Plates will be incubated at 28°C for 5 days (Soto et al., 2009b). Individual colony was sub-cultured under the same condition to obtain pure isolates. Suspected isolates (Gram negative, non-motile, pleomorphic coccoid, catalase positive, cytochrome oxidase negative and required cysteine for growth) were selected to identify by specific PCR for *F. orientalis*.

3.2.5 DNA Extraction and PCR condition

The sample set preserved in alcohol described above was individually ground in 60 µL of Tris-EDTA (TE) buffer and heated at 65°C for 10 min. After a brief centrifugation, the upper layer was subjected to DNA extraction using the Wizard® Genomic DNA Purification kit (Promega, USA) according to the manufacturer's instructions. The DNA was then eluted with nuclease-free water, quantified using the NanoDrop spectrophotometer (Thermo Scientific, US), and tested for the presence of *F. orientalis* using an improved PCR detection protocol.

One-tube semi-nested PCR assay was developed in this study to increase the *F. orientalis* detection sensitivity. The target sequence was a unique hypothetical protein gene sequence (GenBank accession no. JQ780323) described to be specific for *F. orientalis* strains (Duodu et al., 2012). A published primer pairs FnoF1/FnoR1 (FnoF1, 5'-

GGC GTA ACT CCT TTT AGC TTC C-3'and FnoR1, 5'- TTA GAG GAG CTT GGA AAA GCA-3') (Dong et al., 2016a) in combination with a newly designed primer FnoRev2 (5'-AGG TAT GCA GTC TAC TTC TAA TG-3') designed based on this region using Primer2 software (v.0.4.0) (Untergasser et al., 2012) were used. A 25 μL of PCR reaction was composed of 12.5 μL of Master Mix (Go-Taq®Green, Promega, US); 4 μL of DNA template (150–200 ng); and 0.6, 0.4 and 0.4 μL of primer FnoF1 (5'- GGC GTA ACT CCT TTT AGC TTC C-3'), FnoR1 (5'- TTA GAG GAG CTT GGA AAA GCA-3') and FnoRev2 (5'-AGG TAT GCA GTC TAC TTC TAA TG-3'), respectively. PCR conditions consisted of initial denaturation at 94°C for 3 min; 40 cycles of amplification at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; final extension at 72°C for 5 min. Expected PCR products of 375 and 203 bp were generated by FnoF1/FnoRev2 and FnoF1/FnoR1 primers, respectively. Amplified products were electrophoresed with 1% agarose gel and visualized under UV light.

The newly established semi-nested PCR was performed sensitivity test with 10-fold serial dilutions (200 ng to 0.2 fg) of genomic DNA from the bacterial isolates VMCU-FNO131. The specificity of PCR assay was tested with DNA extracted from a healthy hybrid red tilapia and 9 common fish bacterial pathogens recovered from diseased fish (Table 2). DNA concentration of each bacterial isolate was quantified using the NanoDrop spectrophotometer (Thermo Scientific) and adjusted to 200 ng μL^{-1}. In addition, semi-nested PCR product from 4 different fish samples were purified using wizard® SV Gel and PCR Clean-up system (Promega, USA) and sequenced by 1st BASE Pte Ltd. (Malaysia) using appropriate primers (FnoF1/FnoR1 and FnoF1/FnoRev2). A BLAST search of NCBI were performed with those sequences to confirm accuracy of amplified products.

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Table 2: Genomic DNA of bacterial isolates used to verify specificity of PCR assay

Number	Species	Host	References
1	*Edwardsiella ictaluri*	Striped catfish	(Dong et al., 2015b)
2	*E. tarda*	Tilapia, Thailand	Laboratory strain
3	*Streptococcus niae*	Tilapia, Thailand	Laboratory strain
4	*S. agalactiae*	Tilapia, Thailand	(Dong et al., 2015a)
5	*Hahella chejuensis*	Tilapia, Thailand	(Senapin et al., 2016)
6	*Flavobacterium columnare*	Striped catfish	(Dong et al., 2015b)
7	*Aeromonas hydrophila*	Tilapia, Thailand	(Dong et al., 2015a)
8	*A. veronii*	Tilapia, Thailand	(Dong et al., 2015a)
9	*A. schubertii*	Tilapia, Thailand	Laboratory strain

3.2.6 Histopathological analysis

Samples, including spleen tissue of broodstock, fertilized eggs, testis, five-day old fry, thirty-day old fingerlings of each family that collected as mention above, were used for histopathological assessment and DNA *in situ* hybridization analysis. In brief, the samples were fixed in 10% neutral buffered formalin overnight and then placed in 70% ethanol. Afterwards the tissues were dehydrated by incubating in increasing concentrations of ethanol (70–100%) and then transferred to xylene. The tissues were infiltrated and embedded in paraffin. The paraffin-embedded tissues will be cut at 5 μm thickness and the sections will be picked up onto HistoGrip (Zymed, San Francisco, CA, USA) coated glass slides. The paraffin sections were deparaffinized with xylene and then rehydrated in alcohol series and distilled water. The sections were stained with hematoxylin and eosin (H&E) for histopathological examination.

3.2.7 *In situ* hybridization method

The samples that were positive with *F. orientalis* by PCR test, were further used for *in situ* hybridization assay. The method for *in situ* hybridization were then conducted as previous described (Dong et al., 2016a; Senapin et al., 2016). Specific primers for *F. orientalis* targeting the unique hypothetical gene fragment were used to amplified template for digoxygenin (DIG)-labeled probe preparation using a commercial PCR DIG-labeling mix (Roche Molecular Biochemicals, Germany) (Dong et al., 2016a). A control probe was be produced from none *F. orientalis* template. The probe was purified using a Favorgen Gel/PCR Purification Kit (Taiwan) and used in standard *in situ* hybridization assays with fish tissue sections as previously described (Dong et al., 2016a; Senapin et al., 2016).

The paraffin-embedded tissues on HistoGrip coated slide were deparaffinized 3 times in xylene, and then re-hydrated through ethanol series (100%–50%), distilled water, and finally in TNE buffer (100 mM Tris–HCl, 10 mM EDTA, pH 8.1). The tissue sections were treated with 10 μg mL^{-1} proteinase K for 30 min at 37 °C. After that, the tissue sections were treated with 0.5 M EDTA for 1 h and then post-fixed with 4% paraformaldehyde at 4°C for 5 min. After washing in water, the sections were placed in pre-hybridization buffer (4 × SSC, 0.6 M NaCl, and 0.06 M $Na_3C_6H_5O_7$) and equilibrated at 37°C for 10 min with 50 (*v/v*) de-ionized formamide) before adding the prepared probes. Each probe was added to hybridization buffer (containing 50% deionized formamide, 50% dextran sulfate, 50 × Denhardt's solution (Sigma), 20 × SSC, 10 mg mL^{-1} salmon sperm DNA (Invitrogen, Breda, the Netherlands). Both tissue sections and probes were heated at 95 °C for 15 min and then placed on ice for 10 min. Each specific probe was applied to the tissue sections and covered with cover slips and

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incubated overnight at 42°C in a moist-chamber. After incubation, the tissue sections were washed in graded sodium citrate solution series, for 30 min in 2 × SSC (37°C), 30 min in 1 × SSC (42°C), 30 min in 0.5 × SSC (42°C), and then equilibrated for 5 min in buffer I (1 M Tris–HCl, 1.5 M NaCl, pH 7.5). The sections were incubated for 30 min at room temperature in Buffer II (Buffer I containing 0.1% Triton X-100 and 2% normal sheep serum) before an addition of alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500 in buffer II) for 1 h. Unbound antibody were washed off twice for 10 min each with Buffer I, and the sections will be equilibrated for 10 min in buffer III (100 mM Tris–HCl, 1.5 M NaCl, 50 mM $MgCl_2$, pH 9.5). The signal was developed by addition of NBT–BCIP substrate. The tissues were counter stained with methyl green which specific for cell nuclei. The tissue slides were observed and photographed under a microscope equipped with a digital camera.

3.3 Results

3.3.1 Specificity and sensitivity of semi nested PCR assay

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In order to increase limited detection, the one-tube semi-nested PCR assay was developed base on a published primer pair FnoF1/FnoR1 (203 bp) (Dong et al., 2016a) in combination with newly designed primer FnoRev2 targeting larger fragment (375 bp) in a unique sequence of a hypothetical protein gene of *F. orientalis* species. The nested PCR was proved specific for *F. orientalis* when testing with different common bacterial pathogens. Figure 3 showed 2 target amplicons were obtained from isolates of *F. orientalis* strain VMCU-FNO131 whereas no cross-amplification to DNA extracted from a healthy hybrid red tilapia and 9 common fish bacterial pathogens recovered from diseased fish. In addition, DNA sequence of semi nested PCR products generated

using new primer pair FnoF1/FnoRev2 from ovary, testis, fertilized eggs, larvae, 5-day old, and 30-day old fish matched 100% identical to hypothetical protein gene sequence of *F. orientalis* in database (GenBank accession no. JQ780323) (Figure 4). The newly established one-tube semi-nested PCR has the limit detection of 20 fg genomic DNA that is 100-fold more sensitive than a 203 bp-single PCR (Figure 5).

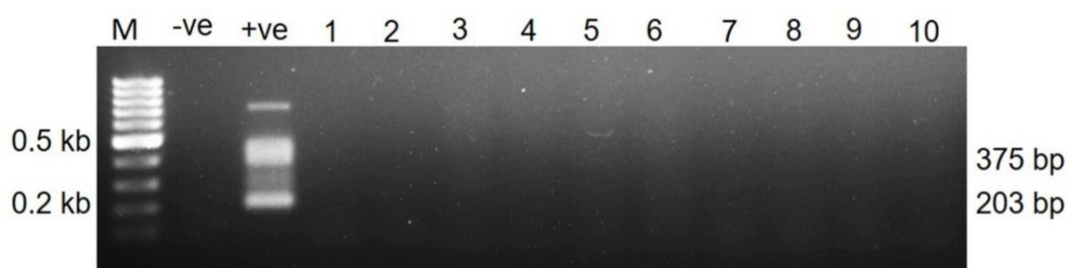

Figure 3: Specificity test of the one tube semi-nested PCR assay. M, DNA marker; lanes 1-10 were *Edwardsiella ictaluri, E. tarda, Streptococcus iniae, S. agalactiae, Hahella chejuensis, Flavobacterium columnare, Aeromonas hydrophila, A. veronii, A. schubertii* and DNA extracted from a healthy red tilapia, respectively; +ve, positive control using *F. orientalis* extracted DNA as template; -ve, no template control.

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Fno          CGTAACTCCTTTTAGCTTCCTAGAACATCAATCTCAGTTAGAACGAAAAGATTATACAAT 60
Ovary        CGTAACTCCTTTTAGCTTCCTAGAACATCAATCTCAGTTAGAACGAAAAGATTATACAAT 60
Testis       CGTAACTCCTTTTAGCTTCCTAGAACATCAATCTCAGTTAGAACGAAAAGATTATACAAT 60
Fertilized   CGTAACTCCTTTTAGCTTCCTAGAACATCAATCTCAGTTAGAACGAAAAGATTATACAAT 60
Larvae       CGTAACTCCTTTTAGCTTCCTAGAACATCAATCTCAGTTAGAACGAAAAGATTATACAAT 60
5-day old    CGTAACTCCTTTTAGCTTCCTAGAACATCAATCTCAGTTAGAACGAAAAGATTATACAAT 60
30-day old   CGTAACTCCTTTTAGCTTCCTAGAACATCAATCTCAGTTAGAACGAAAAGATTATACAAT 60
             ************************************************************
Fno          AACAATAACATTTCTTGGAGAAAATTATTCGCAAATAGCTCGTACAGGAACAGCCAAATT 120
Ovary        AACAATAACATTTCTTGGAGAAAATTATTCGCAAATAGCTCGTACAGGAACAGCCAAATT 120
Testis       AACAATAACATTTCTTGGAGAAAATTATTCGCAAATAGCTCGTACAGGAACAGCCAAATT 120
Fertilized   AACAATAACATTTCTTGGAGAAAATTATTCGCAAATAGCTCGTACAGGAACAGCCAAATT 120
Larvae       AACAATAACATTTCTTGGAGAAAATTATTCGCAAATAGCTCGTACAGGAACAGCCAAATT 120
5-day old    AACAATAACATTTCTTGGAGAAAATTATTCGCAAATAGCTCGTACAGGAACAGCCAAATT 120
30-day old   AACAATAACATTTCTTGGAGAAAATTATTCGCAAATAGCTCGTACAGGAACAGCCAAATT 120
             ************************************************************
Fno          AACACATATCTCATGGGAAACAAATTCAAAAGGAATTATATTTAAATGCCAAAATGAATA 180
Ovary        AACACATATCTCATGGGAAACAAATTCAAAAGGAATTATATTTAAATGCCAAAATGAATA 180
Testis       AACACATATCTCATGGGAAACAAATTCAAAAGGAATTATATTTAAATGCCAAAATGAATA 180
Fertilized   AACACATATCTCATGGGAAACAAATTCAAAAGGAATTATATTTAAATGCCAAAATGAATA 180
Larvae       AACACATATCTCATGGGAAACAAATTCAAAAGGAATTATATTTAAATGCCAAAATGAATA 180
5-day old    AACACATATCTCATGGGAAACAAATTCAAAAGGAATTATATTTAAATGCCAAAATGAATA 180
30-day old   AACACATATCTCATGGGAAACAAATTCAAAAGGAATTATATTTAAATGCCAAAATGAATA 180
             ************************************************************
Fno          TGCTTTTCCAAGCTCCTCTAA 201
Ovary        TGCTTTTCCAAGCTCCTCTAA 201
Testis       TGCTTTTCCAAGCTCCTCTAA 201
Fertilized   TGCTTTTCCAAGCTCCTCTAA 201
Larvae       TGCTTTTCCAAGCTCCTCTAA 201
5-day old    TGCTTTTCCAAGCTCCTCTAA 201
30-day old   TGCTTTTCCAAGCTCCTCTAA 201
             *********************
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Figure 4 Multiple alignment of 201 bp sequences obtained from direct sequencing of the semi-nested PCR products from all samples in one representative family and the sequence of the hypothetical protein gene of *Francisella orientalis* strain FSC771 (GenBank accession no. JQ780323). All samples sequence showed 100% identity to the published sequence of *F. orientalis.*

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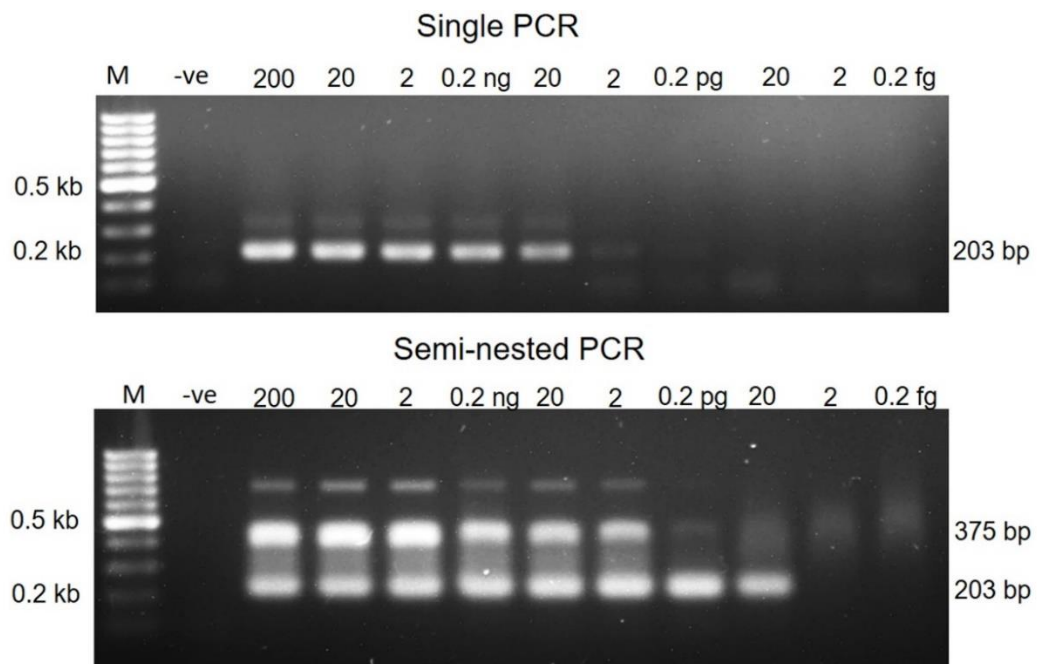

Figure 5 Sensitivity test of single PCR and one-tube semi-nested PCR using 10-fold serial dilutions of *F. orientalis* genomic DNA (200 ng to 0.2 fg genomic DNA per PCR reaction). M, DNA marker; -ve, negative control.

3.3.2 Establishment of subclinically *F. orientalis*-infected broodstock in the laboratory

Using a sub-lethal dose of *F. orientalis* for immersion challenge, only two fish died at 10 dpc during 6 weeks-period while the majority of the experimental broodfish appeared to be unaffected externally. Eight out of the 34 remaining fish were then randomly selected for histopathological and PCR examination. The internal organs of these fish were abnormally enlarged with the presence of white nodules in the spleen and head kidney, a typical sign of francisellosis. Additionally, the spleens of all examined fish were positive for *F. orientalis* by specific PCR test (Figure 6). The results indicated that a population of subclinically *F. orientalis*-infected broodfish was successfully established in the laboratory.

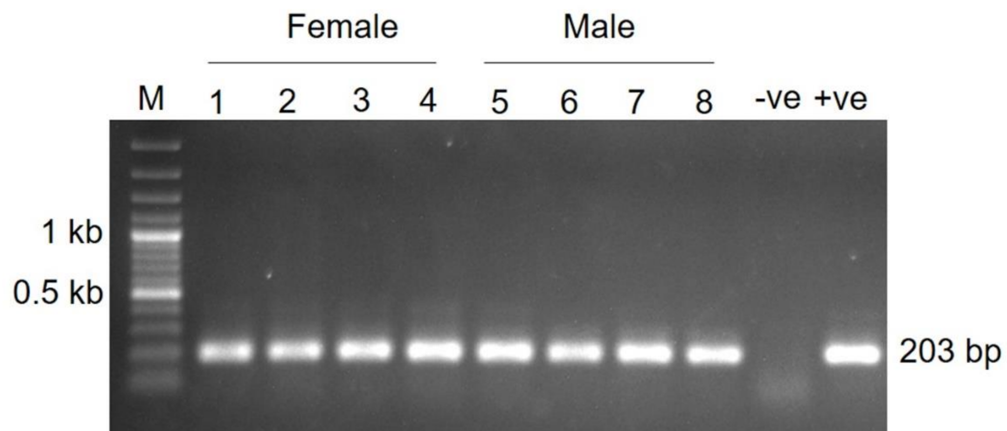

Figure 6 Detection of *F. orientalis* in experimentally infected broodfish using Fno-specific PCR assay by Dong et al. (2016a). M, DNA Marker; 1-4, spleen of female broodfish; 5-8, spleen of male broodfish; +ve, positive control using *F. orientalis* extracted DNA as template; -ve, no template control.

3.3.3 Evidence of *F. orientalis* in the gonad tissues of broodfish

Externally, all 3 pairs of the infected broodfish still showed normal appearance post challenge. Internally, the spleen, liver, and head kidney were enlarged. Presence of white nodules-like granulomas was noticed on the ovaries of all 3 female broodfish but were not seen on the testes (Figure 7). Using a newly established one-tube semi-nested PCR assay, it was shown that the spleen and gonad tissues of 6 broodfish from 3 infected families tested positive for *F. orientalis* (Figure 8, lanes 1-4). DNA sequences of representative 203 bands were sequenced and exhibited 100% identity to *F. orientalis* sequences in the GenBank database. Respective samples from non-infected control broodfish tested negative for *F. orientalis* (Figure 8, lane 1-4).

Histopathologically, presence of granulomas forming feature, the typical feature of francisellosis was not observed in the testis and ovary but clearly presented in the spleen of all broodfish (Table 3, Figure 9). ISH results shown in Figure 10 using an *F. orientalis*-specific probe confirmed the results obtained with the PCR assay. Reactive

signals were detected in oocyte cytoplasm and their membranes as well as various locations in the testis of broodfish (Figure 10). With respect to bacterial isolation, *F. orientalis* was not successfully cultured from the spleen, ovary or testis of the broodfish using CHA medium, a selective medium for *F. orientalis*.

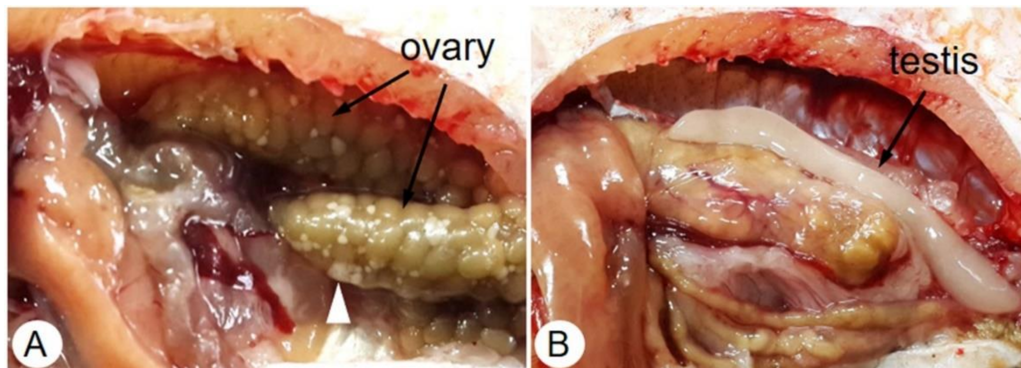

Figure 7 Female (A) and Male (B) of hybrid red tilapia broodfish subclinically infected with *F. orientalis*. White nodules-like granulomas were observed in the ovary (triangle) but not in the testis. No lesions were detected in the non-infected broodstock.

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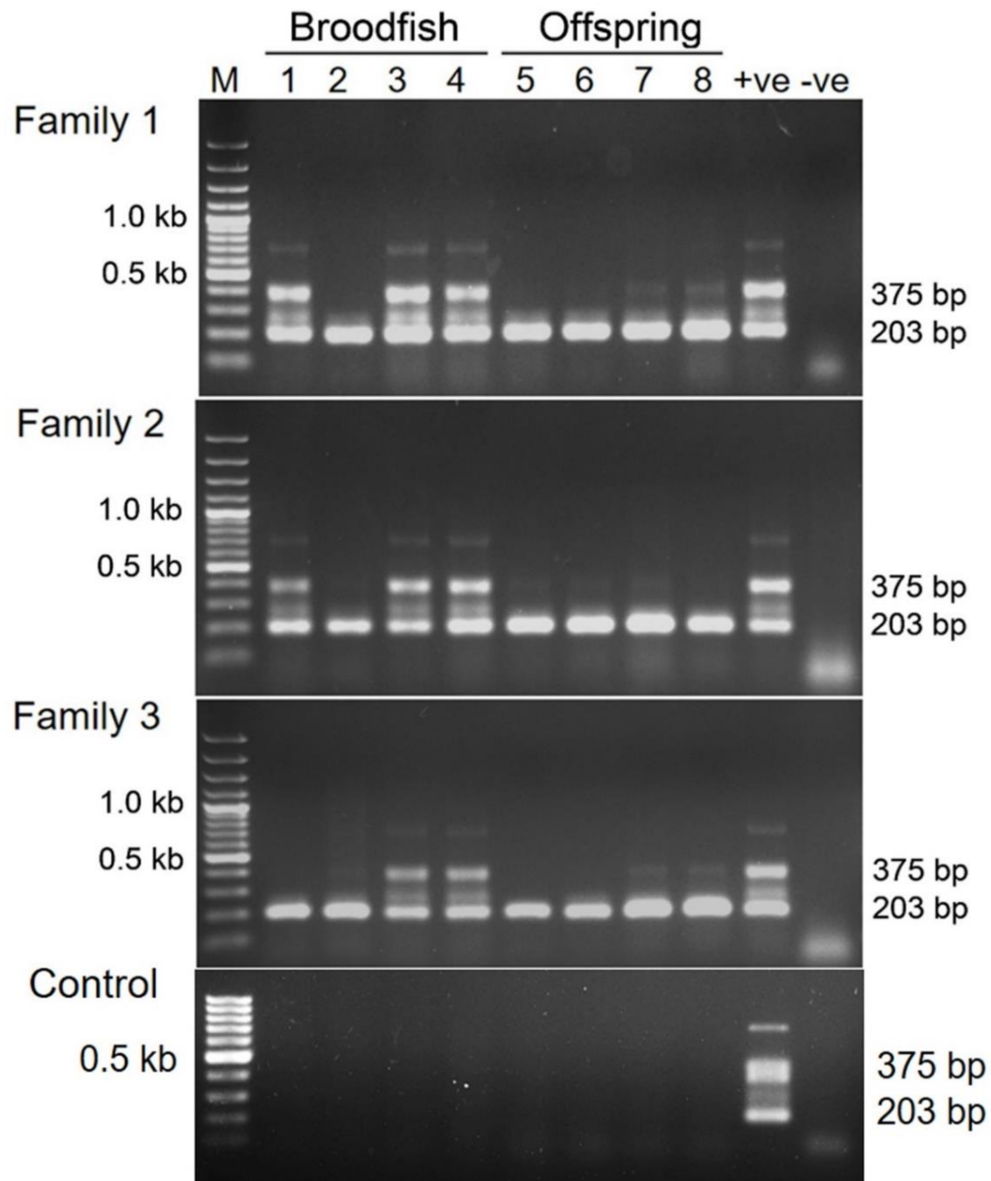

Figure 8 Detection *of F. orientalis* in different life stages of three infected families and one non-infected control Figure 9 Detection of family of hybrid red tilapia using specific PCR. M, DNA Marker; 1, ovary; 2, testis; 3, spleen of female; 4, spleen of male; 5, fertilized eggs; 6, yolk-sac larvae; 7, 5-day old fry; 8, 30-day old fry; +ve, positive control using *F. orientalis* extracted DNA as template; -ve, no template control. Note that ~700 bp band derived from cross hybridization of amplified products.

3.3.4 Detection of *F. orientalis* in different developmental stages of the infected broodfish's offspring

The samples derived from *F. orientalis*-infected broodfish's offspring including fertilized egg, yolk-sac larvae, 5-day old fry, and 30-day old fry were also tested for the presence of *F. orientalis* by bacterial isolation, PCR and ISH. Similar to broodfish samples, *F. orientalis* was unable to be cultured from the offspring samples using CHA medium. Despite no visually abnormal signs being noticed in all development stages of the offspring, all of them tested positive for *F. orientalis* using specific PCR (Table 3, Figure 8). All tested samples yielded 203 bp-nested products (Figure 8, lanes 5-8), indicating low bacterial loads in the tissues. Sequencing of representative PCR products revealed 100% identity to the target sequence of PCR assay (Figure 4). Consistent with the PCR results, the ISH using *F. orientalis*-specific probe revealed weak reactive signals in the larvae and fry samples compared to no signals in sections from the control. Representative ISH staining of the offspring samples are shown in Figure 10.

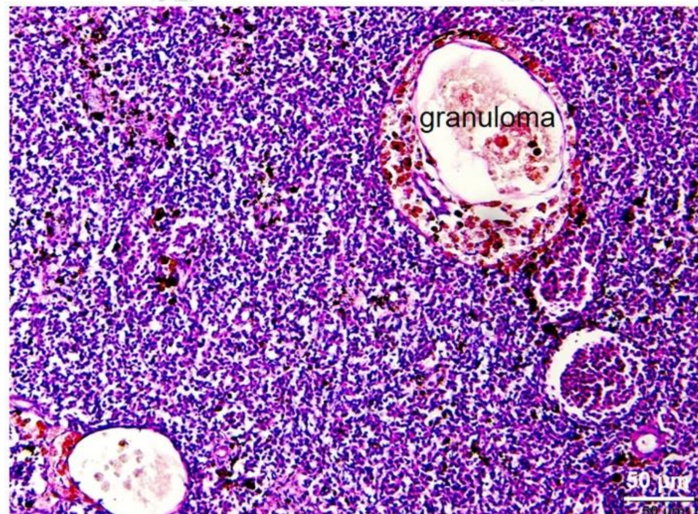

Figure 10 Photomicrograph of H&E stained section of the spleen of subclinically infected broodfish showed presence of granulomas.

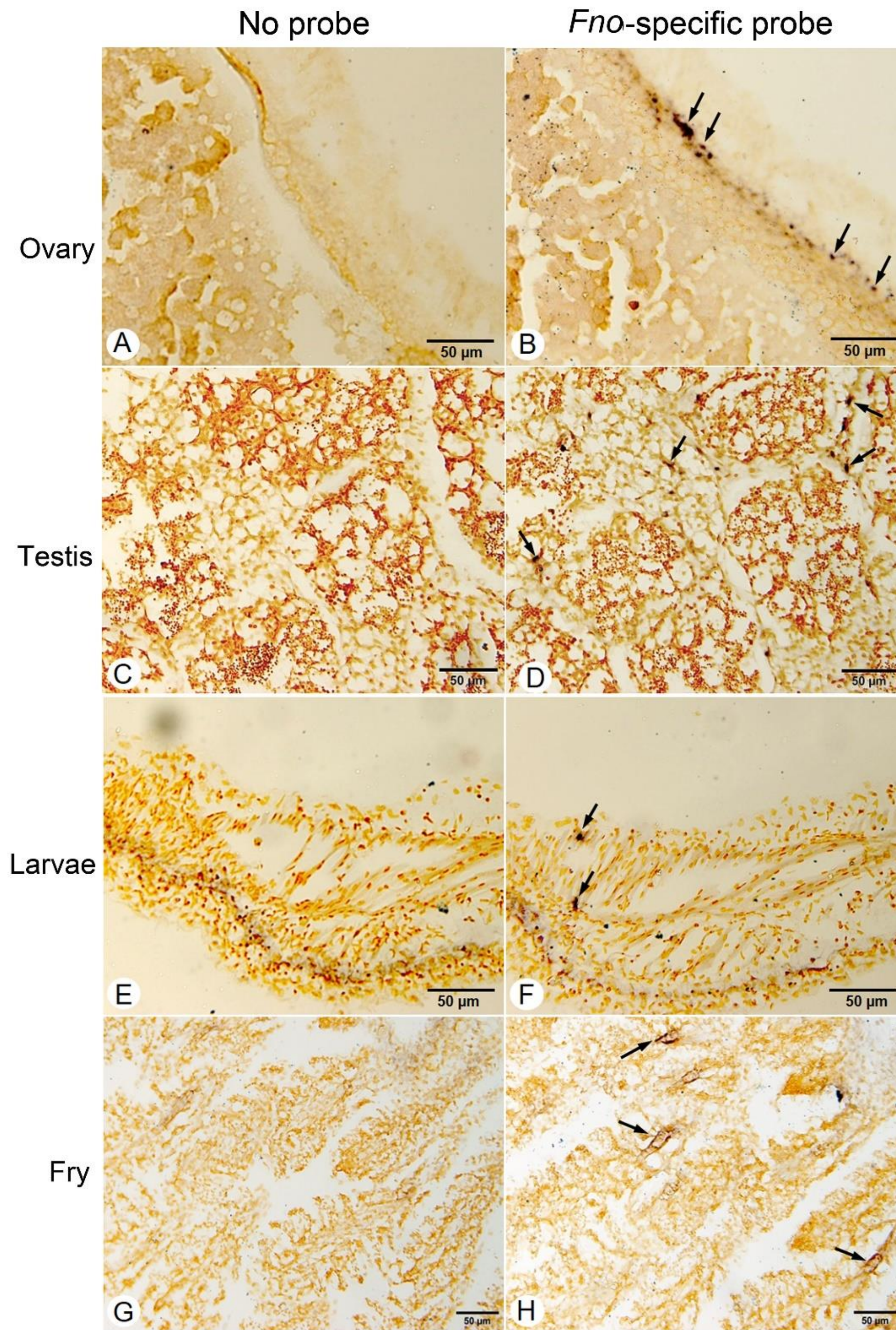

Figure 11 Photomicrographs of ISH results of the reproductive organs (A-D) of broodfish and representative different development stages of their progeny (E-H). Arrows indicated reactive signals of ISH in oocyte membrane (B), in different locations of the testis (C), yolk-sac larvae (F) and gill filaments of 30-day old fry (H). Consecutive sections without probe are shown on the left panel.

Table 3 Detection of F. orientalis from broodstock and different developmental stages of their offspring using specific PCR, in situ hybridization (ISH), and granulomas pathology (G).

Family	Reproductive organs						Development stages								
	Ovary			Testes			Fertilized eggs			Yolk-sac larvae			5 and 30-day old fry		
	PCR	ISH	G	PCR	ISH	G	PCR	ISH	G	PCR	ISH	G	PCR	ISH	G
1	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
2	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
3	+	N	N	+	N	N	+	N	N	+	N	N	+	N	N
Control	-	N	N	-	N	N	-	N	N	-	N	N	-	N	N

(+), positive; (-), negative; N, not determined.

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3.4 Discussion

In nature, as a mouthbrooder fish, female tilapia incubates eggs in their mouth until hatching. During the intensive aquaculture practices, artificial incubation and hatching of fish embryos in water recirculation systems significantly supports large scale production of tilapia fry. Subclinical infection with infectious agent(s) is of concern not only for the health of the broodstock themselves but also for possible pathogen transmission to their fry. Potential vertical transmission of *F. orientalis* and other tilapia bacterial pathogens including *Shewanella putrefaciens, Streptococcus agalactiae,* and *S. iniae* was previously reported from healthy red tilapia broodstock without clinical symptoms (Pradeep et al., 2017; Pradeep et al., 2016). In the mentioned studies,

broodfish from hatcheries with history of bacterial infections were used for *in vitro* fertilization. Interesting, even though not all pairs of the parents were *F. orientalis* positive, all their progeny at late stage i.e. 30-day old fry were tested positive for *F. orientalis* by LAMP detection. Additionally, concurrent transmission of *S. putrefaciens* was co-investigated together with *F. orientalis* in the same sample sets (Pradeep et al., 2017). The present study investigated a single transmission of *F. orientalis* using experimentally infected broodfish. Consequently, the presence of *F. orientalis* in the reproductive organs of the brooders and their offspring was confirmed by a combination of PCR and ISH assays. Note that this experiment was set up to mimic current practice in tilapia hatcheries where fertilized eggs were collected from the mouth of female broodfish for incubation. Therefore, transmission of *F. orientalis* from the infected broodfish to their offspring might take place in either reproductive organ (direct vertical transmission) or during incubation in the mouth of broodfish (indirect vertical transmission). Despite the fact that truly vertical transmission requires further investigation, this study suggests that by using *F. orientalis* subclinically infected tilapia mouthbrooders for fry production, their offspring will be likely infected with this pathogen through either direct or indirect vertical transmission.

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This work also supported other studies (Mauel et al., 2007; Ortega et al., 2016; Pradeep et al., 2017; Soto et al., 2013) that showed that *F. orientalis* could be detected in reproductive organs and/or gametes of infected tilapia, apart from spleen, kidney, and liver, the main target organs (Nguyen et al., 2016; Ramírez-Paredes et al., 2017). Thus, non-lethal sampling of eggs and semen from broodstock might be practical for monitoring this pathogen in tilapia hatcheries thereby allowing selection of the specific pathogen free (SPF) broodfish for fry production.

In conclusion, this study revealed that with the current practice in tilapia hatcheries, *F. orientalis* is likely transmitted from subclinically infected broodstock of hybrid red tilapia to their progeny. *F. orientalis* could be found in reproductive organs of

broodfish and different development stages including embryo, yolk-sac larvae and fry fish. The results also implied that SPF broodfish should be considered for production of *F. orientalis*-free fry.

CHAPTER 4

THE DESTINY OF *FRANCISELLA ORIENTALIS* UPTAKEN BY MOSQUITO LARVAE AND EATEN BY HYBRID RED TILAPIA

Abstract

Mosquito larvae has been considered as a reservoir of Francisella bacteria and plays an important role in transmission of francisellosis in mammal. The aim of this study was to investigate whether mosquito larvae acquire *Francisella* orientalis and transmit disease to healthy hybrid red tilapia at 25°C and 30°C. The bacterium was successfully recovered from mosquito larvae cohabitated with infected fish and exposed to 0.895×10^{7} CFU mL^{-1} at 25°C and 30°C after 24 h. Hybrid red tilapia fed by infected pupae showed typical granulomas resembling for francisellosis infection in kidney, spleen samples. The histopathological changes were more severe in experiment fish maintained at 25°C compared to those in 30°C. In line with histopathology, specific PCR assays were positive in all fish cultured at 25°C while 8/10 positive samples were seen in 30°C group. This is the first study indicates that mosquito larvae *Aedes aegypti* are available to acquire and transmit *F. orientalis* to healthy red tilapia. These finding suggest mosquito larvae might be an important environment reservoir for *F. orientalis.*

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4.1 Introduction

Members of the genus Francisella are characterized as aerobic, facultatively intracellular, small coccobacilli, Gram-negative bacteria. In this genus, *Francisella tularensis* is a well know agent of tularemia disease in a wide range of terrestrial animals and human (Foley and Nieto, 2010; Sjösted, 2005). *Francisella noatunensis* consists of two subspecies *F. noatunensis* subsp. *noatunensis* (*Fnn*) and *F. noatunensis* subsp. *orientalis* cause serious disease effecting various coldwater and warmwater fish species respectively, both wild and cultured with worldwide distribution (Birkbeck et al., 2011; Colquhoun and Duodu, 2011). Recently, *F. noatunensis* subsp. *orientalis* was reclassed as *F. orientalis* sp. nov. (Ramirez-Paredes et al., 2020). The bacterium, *F. orientalis* is extremely infectious to tilapia fingerlings (*Oreochromis* sp.), as 23 bacterial cells can cause disease (Soto et al., 2009a). In tilapia, the disease can develop as an acute infection causing up to 95% mortalities, or as a chronic disease with varying mortality levels. The infected fish shows non-specific clinical signs such as exophthalmia, pale skin, erratic swimming, and multifocal granulomatous lesions in kidney, spleen, liver) (Birkbeck et al., 2011; Colquhoun and Duodu, 2011). The disease can be transmitted horizontally via direct contact between healthy and infected fish, contaminated water (Hsieh et al., 2006; Soto et al., 2013) and vertically from subclinically infected broodstock to their progeny (Nguyen et al., 2019a; Pradeep et al., 2017). Nguyen *et al* (219) indicated that *F. orientalis* persists through the development stages of hybrid red tilapia (fertilized eggs, yolk-sac larvae, 5 days old fry and fingerling). Nevertheless, the pathogenic mechanisms that underlie its remarkable persistent in reproductive organs and transmission in offspring still unknown. Another common cultured fish species that share habitat with tilapia such as striped catfish (*Pangasianodon hypophthalmus*), common carp (*Cyprius carpio*), sunfish (*Lepomis gibbosus*) are not susceptible to francisellosis but could be the disease reservoir (Dong et al., 2016b; Lewisch et al.,

2016). Therefore, transmission between cultured tilapia and wild fish should be concerned.

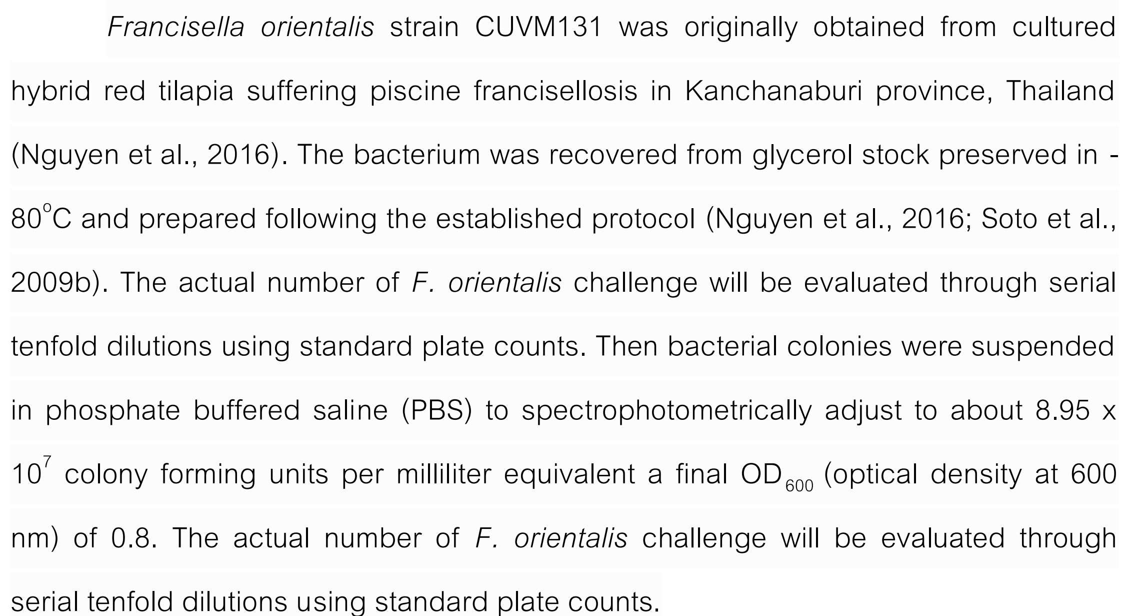
In previous studies, water temperature was concerned as a key factor in outbreaks of francisellosis at tilapia farms. Observation from natural disease case showed that francisellosis caused higher mortality for culture fish at a water temperature of 25°C or less than those reared at 30 °C (Mauel et al., 2003; Soto et al., 2012; Soto et al., 2014). Soto *et al* (2012) provide evidence that culturable *F. orientalis* remaining for longer periods of time with higher numbers in fresh water at 20 °C comparing with 25 and 30°C. Moreover, the bacterial virulence reduced after incubation in water microcosms for 24 h and become non-infective after 2 days in the absence of the fish host (Soto et al., 2012).

In aspect of vector transmission of piscine francisellosis, and how the bacterium persists between outbreaks is still less information. Although several studies reported that many crustacean parasites were concerned as vector transmission of pathogenic agents in fish. Laboratory studies have proved that several groups of parasite (ciliate protozoa, *Argulus* spp, *Caligus* spp., salmon louse) are ability act as vectors of both bacterial and viral infection in aquatic animal (AHNE, 1985; Novak et al., 2016; Xu et al., 2012b). *F. tularensis* subsp. *holartica*, a causative agent of tularemia in mammals, is associated with water environment. Mosquitoes (*Aedes aegypti* and *Anopheles gambiae*) were considered to be the major vectors of the bacterium *Francisella tularensis* in United States, several European countries (Eliasson et al., 2002; Read et al., 2008). Many research studies have demonstrated the presence of *Francisella* genes in all stages of mosquitoes infected with *Francisella* species (Backman et al., 2015; Lundstrom et al., 2011; Thelaus et al., 2014). It is noted that *F. tularensis* subsp. *holarctica* was transstadially maintained from orally infected larvae to adult mosquitoes and that 25% of the adults exposed as larvae were positive for the presence of *F. tularensis*-specific sequences for at least 2 weeks (Thelaus et al.,

2014). On another hand, the mosquito larvae are naturally distribution in tilapia culture area that francisellosis outbreak suggesting potential interaction and thus contribution to the ecological cycle of the disease. There lacks information of mosquito larvae in transmission of aquatic pathogens. The aim of this study was to investigate whether mosquito larvae acquire the bacterium and transmit the disease to healthy hybrid red tilapia.

4.2 Materials and methods

4.2.1 Bacterial preparation



Francisella orientalis strain CUVM131 was originally obtained from cultured hybrid red tilapia suffering piscine francisellosis in Kanchanaburi province, Thailand (Nguyen et al., 2016). The bacterium was recovered from glycerol stock preserved in -80°C and prepared following the established protocol (Nguyen et al., 2016; Soto et al., 2009b). The actual number of *F. orientalis* challenge will be evaluated through serial tenfold dilutions using standard plate counts. Then bacterial colonies were suspended in phosphate buffered saline (PBS) to spectrophotometrically adjust to about 8.95 x 10^7 colony forming units per milliliter equivalent a final OD_{600} (optical density at 600 nm) of 0.8. The actual number of *F. orientalis* challenge will be evaluated through serial tenfold dilutions using standard plate counts.

4.2.2 Experiment fish

All protocols described in this study involving handle fish were approved by by the Biosafety Committee (approval no. IBC 1831055) and Animal Ethics Committee (approval no. CU-ACUP 1931007) of Chulalongkorn University. Healthy hybrid red tilapia *Oreochromis* sp. (n = 100, 17.9 g) were bought from a hatchery in Samut Songkhram province, Thailand and acclimated for two weeks at the FID RU, Fish infectious Diseases Research Unit, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The fish were reared in the 500 L tanks containing chlorine-free

well water with strong aeration and cotton filter. Water quality parameters (pH, total ammonia, DO) were measured three times per week by a commercial test kits (Aqua-VBC, Thailand) during the trials. Fish were fed at a rate of 2% body weight per day with a commercial tilapia feed containing 35% crude protein (Chareon Pokphand, CP, Thailand). Prior to the experiment, 10 random fish were diagnosed as negative for *F. orientalis* infection especially by *F. orientalis*-specific semi nested PCR and bacterial culture as previously established protocol (Dong et al., 2016b; Nguyen et al., 2016).

4.2.3 Exposure of mosquito larvae to *F. orientalis*

Eggs of the tropical mosquito *Aedes aegypti*, kindly provided by Assoc. Prof. Dr. Sonthaya Tiawsirisup (Paraitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University), will be hatched in sterile deionized water (SDW). To examine whether mosquito larvae are able to uptake *F. orientalis*, mosquito larvae were exposed to the bacteria by two trials. In trial 1, approximately one thousand mosquito larvae (second instar) were divided into two groups. In experiment group, 500 larvae were immersed in 1 L SDW containing *F. orientalis* at a concentration of 0.895 x 10^7 CFU mL^{-1} for 1 h with light aeration. After that the larvae were washed in 1 L distill water (three times) before transfer to culture tray containing 1L SDW at 25°C and 30°C. In control group, mosquito larvae were immersed in SDW with the same behavior (Supl Figure 1). The larvae were fed by powdered feed for early fry tilapia (CP, Thailand) one daily. The photoperiod was 12 h light and 12 h dark. Cultured water was changed 90% by SDW every two days. Individual pool of mosquito larvae (n = 15 x 3 replicates per sample time) and 0.1 mL water samples in each culture tray were collected at each 24 h time period from 0 – 4 days post-immersion in all treatments for bacterial culture and specific PCR detection (Figure 11A.).

To compare the persistence of *F. orientalis* in distilled water without mosquito larvae, the bacteria were diluted in distilled water to reach density of 0.895 x 10^7 CFU mL^{-1}, then, the bacteria solution was maintained at 25°C and 30°C for 4 days. The

volume of 1 mL of culture water at different temperature (3 replicates per sample time) was collected at each 24 h. The sample was centrifuged for 5 minutes at 11000 rpm, the 0.1 mL sediment was spread on CHA to test culturable of *F. orientalis*.

In trial 2, francisellosis fish (n = 45) were obtained by intraperitoneal injection with *F. orientalis* at sublethal dose 10^4 CFU mL^{-1} for five days to get chronic diseased fish. To ensure challenged fish infected with *F. orientalis*, five representative fish were tested by semi-nested PCR assay as mention in chapter II (Supl Figure 2). Then the infected fish were cultured in two tanks (20 fish per tank) at 25°C and 30°C. Third instar of *A. aegypti* larvae were cultured in trays placed in the fish tanks at 25°C and 30°C to reach to pupa stage as illustrated in (Supl Figure. 3). Subsequently, the mosquito larvae and water were randomly collected from each culture tray by sterile plastic Pasteur pipette 4 ml (BRAND® pipette, Merck) for *F. orientalis* detection by PCR and microbial culture (Nguyen et al., 2019c). Each tank contained individual air stones and cotton filter. The filters were replaced every two days and water parameters (pH, ppm nitrite, ppm total ammonia) were checked daily during experiment period.

4.2.4 Experimental transmission of *F. orientalis* to hybrid red tilapia via mosquito larvae

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In order to evaluate whether mosquitoes are able to transmit *F. orientalis* to susceptible host, the experimental design is illustrated in **Figure 11B**. Mosquito larvae (third instar) were cohabited with francisellosis hybrid red tilapia and collected as mention above. The larvae were removed from the culture tray by sterile plastic Pasteur pipette 4 ml (BRAND® pipette, Merck), and rinsed two time in 1000 mL distilled water prior to being used for feeding healthy fish. Healthy hybrid red tilapia (n = 40) were divided in two groups (two tanks per each group). Experiment group was fed with infected mosquito larvae (30 larvae per fish) while control group was fed with the same number of non-infected larvae (Supl Figure 4). Fish were observed clinical signs and recorded mortalities twice daily and maintain until 21 days. This time period

allowed for *F. orientalis* causes significantly pathological changes in susceptible host. At the end of experiment, all of fish were killed by clove oil (40 mg/mL) and collected for *F. orientalis* culture, specific PCR assay and histopathological analysis.

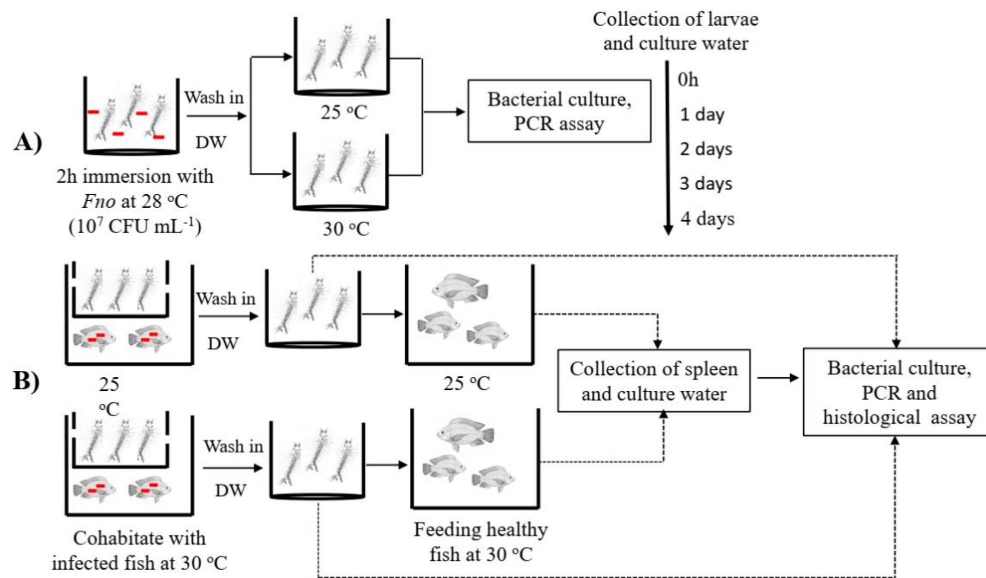

Figure 12 A) Experimental model for investigating the uptake and persistent of *F. orientalis* in the mosquito. B) Experiment to investigate the ability of the mosquito larvae A. aegypti to transmit *F. orientalis* to red tilapia. Mosquito larvae (second instar) were cohabitated with francisellosis diseased red tilapia. Pupa were washed by distilled water and were later used as food for healthy red tilapia at different groups.

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4.2.5 *Francisella orientalis* detection

For bacterial isolation, the larvae were removed from the culture tray and washed two times in distilled water (DW), then deep in ice for 10 minutes. Pull samples of mosquito larvae (n = 15, 3 replicates) were ground in 1 ml phosphate buffered saline (PBS) and suspended into PBS to produce various ten-fold dilution (10^{-1} – 10^{-5}). After that, 0.1 ml of the suspension was spread on MCHA (cystine heart agar supplied 10% sheep blood, Polymyxin B 100 units mL^{-1} and ampicillin 50 µg mL^{-1}) and

incubated at 28°C for 5 days. The water sample (0.1 mL) from culture tray and cohabitated tank was collected as mention above and placed on MCHA. Subsequently the suspected colonies for *F. orientalis* were subjected to DNA extraction and confirm by semi-nested PCR as the result of part I.

For DNA extraction, the pull individual mosquito larvae (n = 15) collected from immersion trial with *F. orientalis* and cohabitation with infected fish were ground in Tris-EDTA (TE) buffer (20 – 100 µl) and boiled for 10 min. Subsequently, the samples were subjected to extract DNA using Wizard® Genomic DNA Purification kit (Promega, US) according to the manufacturer's instructions. In addition, total DNA of spleen tissues sample of cohabitation experiment fish and *F. orientalis*-infected fish were also extracted by Wizard® Genomic DNA Purification kit (Promega, US). To extract DNA from water sample, initially, 100 mL of culture water per sample was pre-filtered through 11 µm Whatman® qualitative filter paper, grade 1(Merck, Germany). The aliquots of each culture water were re-filtered through non-sterile mixed cellulose esters (MCE) membrane filter, pore size 0.45 µm (© Thomas Scientific, US). For each 5g feces sample was suspended in 95 ml distill water before applying the same filter protocol as culture water. The filter membrane of each sample was subjected to DNA extraction using Wizard® Genomic DNA Purification kit (Promega, USA) as recommended by the manufacture. The DNA was eluted in nuclear free water and quantified using the NanoDrop spectrophotometer (Thermo Scientific) and stored at -40°C. The newly design semi-nested PCR that mention in part I was used for assay *F. orientalis* in different development stage of mosquito larvae. DNA of *F. orientalis* strain VMCU131 and spleen of healthy fish were used as positive and negative controls.

4.2.6 Histopathological assays

Fish tissues (spleen and head kidney) of experimental and control fish that collected as mention above, were used for histopathology assessment. In brief, the samples were fixed in 10% neutral buffered formalin overnight and then placed in 70%

ethanol. Afterwards the tissues were dehydrated by incubating in increasing concentrations of ethanol (70–100%) and then transferred to xylene. The tissues were infiltrated and embedded in paraffin. The paraffin-embedded tissues were cut at 5 µm thickness and the sections were picked up onto HistoGrip (Zymed, San Francisco, CA, USA) coated glass slides. The paraffin sections were deparaffinized with xylene and then rehydrated in alcohol series and distilled water. The sections were stained with hematoxylin and eosin (H&E) for histopathological examination.

4.3 Results

4.3.1 Maintenance and uptake of *F. orientalis* by mosquito larvae

The mosquito larvae samples were collected by sterile plastic pipette and wash 3 times in distilled water to avoid contaminate from culture water. *F. orientalis* was re-isolated from pool individual larvae samples (3 replicates per time) exposed to 0.895 x 10^7 CFU mL^{-1} at 25°C and 30°C after 24 h (Figure 12A). The colonies were confirmed by *F. orientalis* specific PCR. While none of them was seen on MCHA from control group (Figure 12B). The bacterium was not able to recover in distilled water containing 0.895 x 10^7 CFU mL^{-1} since 48 h. Even thought, the bacterium was not observed on MCHA since 48 h post immersion, the specific PCR detections of *F. orientalis* in mosquito larvae were positive until 96 h post immersion (Figure 13). Water samples from container used for rearing mosquito were negative for *F. orientalis* specific PCR (Figure 13). The results indicated that mosquito larvae were able to uptake *F. orientalis* by immersion and the bacteria were presented until 96 h post challenge. In cohabitation trial with infected fish, *F. orientalis* specifiec semi-nested PCR assays shown the present of *F. orientalis* in culture water samples and mosquito larvae at both 25°C and 30°C temperature conditions (Figure 14). In addition, the bacterium was successfully recovered from cohabitated mosquito (Figure 13). Taken together, the results shown an evident of mosquito larvae *A. aegypti* acquired *F. orientalis* via immersion or cohabitation.

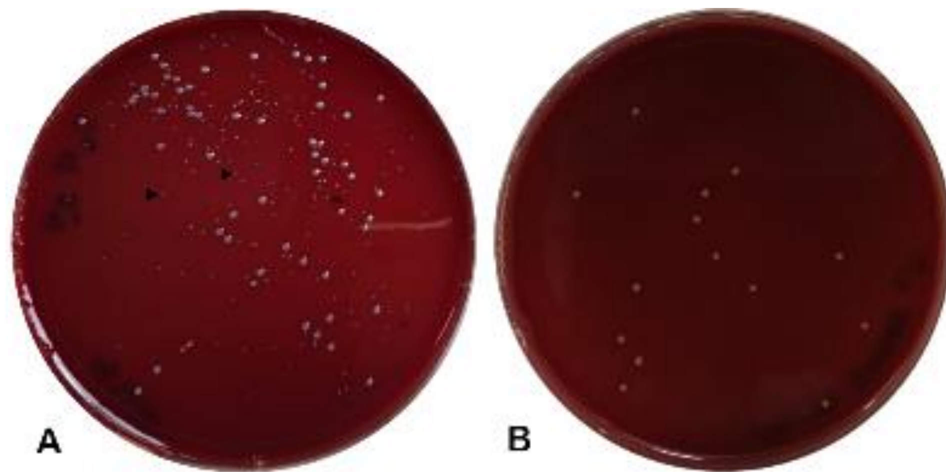

Figure 13 A) *F. orientalis* (head arrow) on MCHA plate recovered from mosquito larvae in immersion and cohabitation trials. B) Contaminated colonies seen on MCHA in control groups.

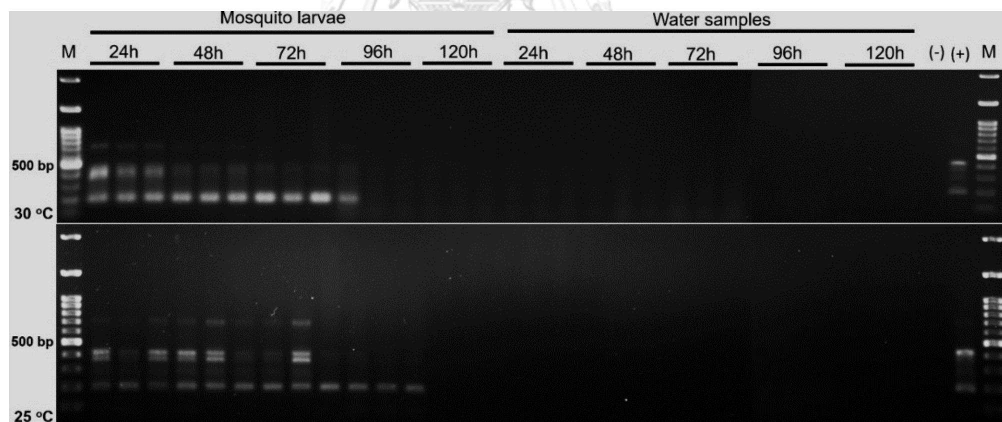

Figure 14 Positive specific semi-nested PCR detection indicated the presence of *F. orientalis* DNA immersion mosquito larvae until 96h post challenge at both 25°C and 30°C, but not in culture water, M: DNA marker, (-): no template control, (+): positive control.

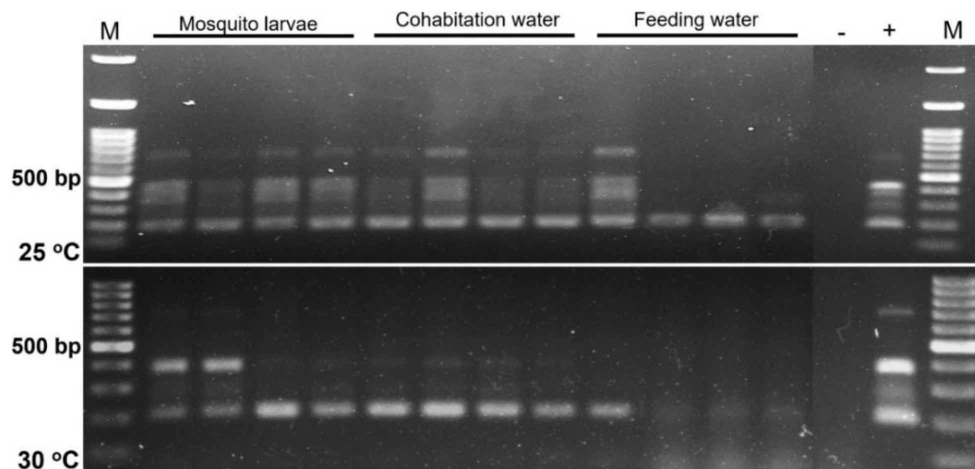

Figure 15 Semi-nested PCR amplification of *F. orientalis* in mosquito larvae cohabitated with infected fish, water samples of cohabitation tanks, water samples from fish tanks fed by infected mosquito larvae at 25°C and 30°C. **M**: DNA marker, **-** : negative control, **+** : positive control.

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4.3.2 Transmision of *F. orientalis* to healthy hybrid red tilapia by feeding infected mosquito larvae

Hybrid red tilapia fed by infected pupae did not reveal external and internal pathological signs resembling for francisellosis infection during the 21 days post challenge (Figure 15). However, typical granulomas caused by *F. orientalis* infection were observed in kidney, spleen of experiment fish in both 25 °C and 30 °C groups. No remarkable histopathological change was seen in control group (Figure 15). No dead fish was recorded and the bacteria *F. orientalis* was not re-isolated from challenged fish at the end of experiment. To further detect *F. orientalis*, a semi-nested PCR was conducted with all challenged fish and culture water samples. All fish cultured at 25 °C were positive with *F. orientalis* specific PCR assay, while 8/10 positive samples were seen in 30 °C group (Figure 16). The culture water samples of both groups were also positive with PCR assay. However, the water samples in 25 °C showed stronger positive than 30 °C group (Figure 14). Fish and water samples of control group were negative

with PCR detection (Figure 17). It could be concluded that mosquito larvae can transmit directly *F. orientalis* to healthy red tilapia via feeding.

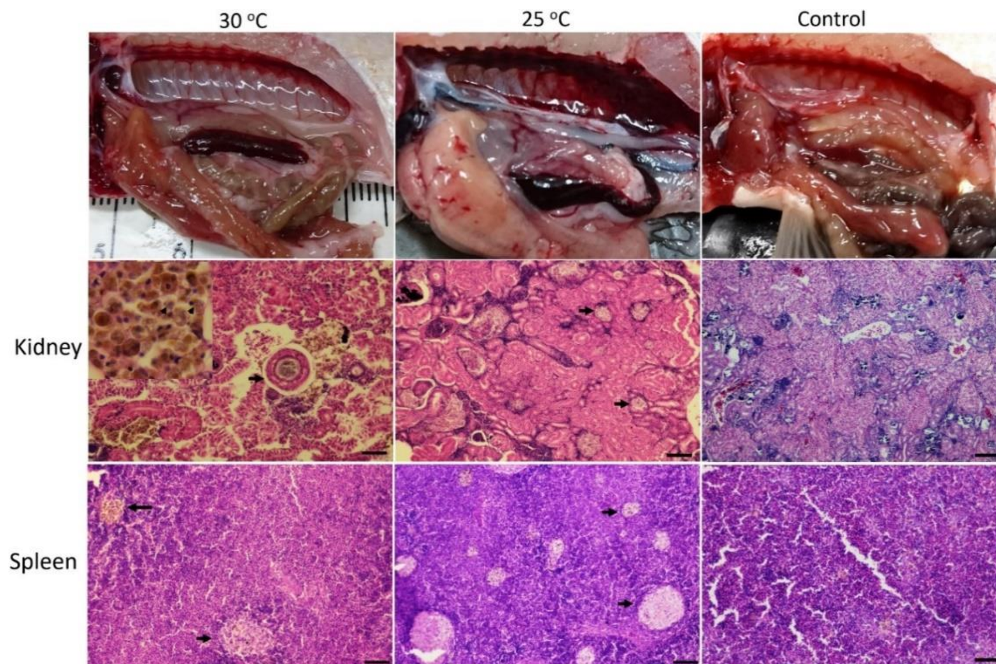

Figure 16 No white nodules correlating to francisellosis presented in all groups. Multiple granulomas (arrow) found in kidney, spleen of challenged fish culture at 25°C and 30°C. High magnification showed various small cocci bacteria (arrow) in granuloma. No remarkable change was found in the same organs of control fish.

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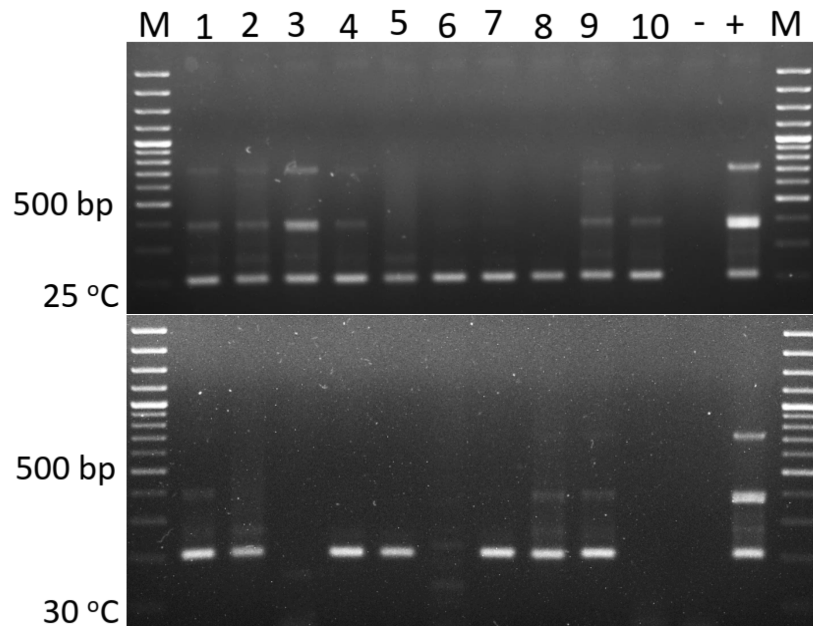

Figure 17 PCR detection of *F. orientalis* in hybrid red tilapia were fed by infected mosquito larvae cultured at 25°C and 30°C, M: DNA marker, - : negative control, + : positive control.

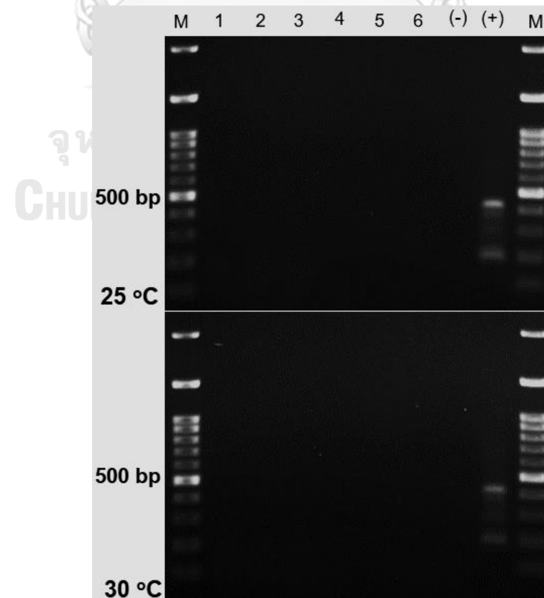

Figure 18 Negative semi-nested PCR amplification of *F. orientalis* in hybrid red tilapia in control group, M: DNA marker, - : negative control, + : positive control.

4.4 Discussions

The results indicated that *F. orientalis* was re-isolated from mosquito larvae *A. aegypti* immersed and cohabited with infected fish. In addition, the water samples of mosquito culture container were negative with *F. orientalis* by PCR assays. It could be conclusion that mosquito larvae truly acquired *F. orientalis* via immersion and cohabitation challenge. However, the bacterium was only re-isolated from mosquito after 24 h post challenge. Even thought, DNA of *F. orientalis* was continuously detected in mosquito till 96 h post immersion by specific PCR. Remarkably, mosquito was negative with *F. orientalis* PCR assay after 120 h post immersion. It suggested that *F. orientalis* may not replicate and persist in mosquito larvae for longer 24 h. In addition, the bacterium was not re-isolated in sterile distill water inoculating 0.895 x 10^7 CFU mL^{-1} at both 25°C and 30°C conditions. It was reported that fish *Francisella* was not able to growth in the absence of added nutrients. No bacterial colony was observed after 48 and 96 h in the filtered water microcosms suspended 10^9 CFU mL^{-1} incubated at 30°C and 25°C respectively (Duodu and Colquhoun, 2010; Soto and Revan, 2012). Since the bacterium was only recovered after 24 h and lack of DNA detection after 120 h post immersion, we suppose that *F. orientalis* may not growth after acquisition by mosquito larvae. In this study, we cannot evaluate the number and localization of *F. orientalis* in mosquito larvae as well. Further studies of the interaction and persistence mechanism of the bacteria within the mosquito body are needed to confirm how *F. orientalis* is transmitted by mosquito.

Healthy fish fed by infected mosquito larvae did not exhibit clinical signs resembling for francisellosis. But the histopathological changes in survivors showed typical granulomas caused by *F. orientalis* (Figure 15). Presence of *F. orientalis* was confirmed by semi-nested PCR using specific primers (Figure 16). In addition, the culture water samples were also positive with PCR assays. The result indicated that mosquito larvae can transmit *F. orientalis* to healthy fish via feeding. In nature, mosquito

larvae are known to feed on bacteria and organic matter. Its ability to uptake *F. orientalis* in culture water is not surprising. As mosquito serve as a food for fish so viable *F. orientalis* within the larvae upon consumption by fish could serve as a mechanism transmission of francisellosis in nature. In this study we were not able to quantify *F. orientalis* in mosquito larvae during immersion and cohabitation challenge by culture method. Therefore, the number of bacteria in mosquito used to feed fish was not detectable. Since *F. orientalis* is well known as a highly fastidious bacteria that grows slowly and often cover by other bacteria concurrent present in sample. The culture method has reported as low sensitivity and limitation diagnosis tool for this pathogen (Assis et al., 2017; Ramírez-Paredes et al., 2017).

It was noted that the challenged fish in 25°C group revealed higher number of granulomas and more severe histopathological changes than those in 30°C group (Figure 15). PCR screening showed 100% positive in 25°C group while only 70% (n = 10) was seen in 30°C group. The results are in line with previous studies as francisellosis caused higher mortality and splenic bacterial load in fish cultured at 25°C compared to 30°C. Moreover, infected fish maintained at 30°C reduced mortality and development of clinical signs (Soto et al., 2014; Soto and Revan, 2012).

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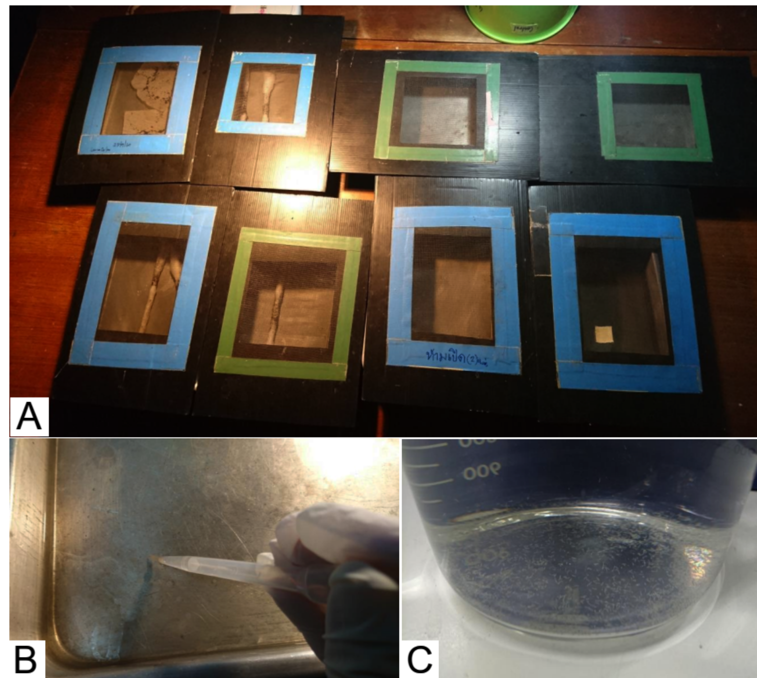
In conclusion, this is the first study indicates that mosquito larvae are available to acquire and transmit *F. orientalis* to healthy red tilapia. The transmission of francisellosis via feeding infected mosquito larvae occur more severe at 25°C compares to 30°C. The bacterium was re-isolated from cohabitated larvae with infected fish. These finding suggest mosquito larvae might be an important environment reservoir for *F. orientalis.* Future studies in persistence mechanism of *F. orientalis* in mosquito larvae will be crucial to understand how the bacterium persists between outbreaks.

Supplement pictures



Supl Figure 1: A) *A. aegypti* eggs were hatched in sterile tray containing 1 L distilled water. B) Mosquito larvae were collected by sterile plastic Pasteur pipette. C) Washing mosquito larvae after collection.

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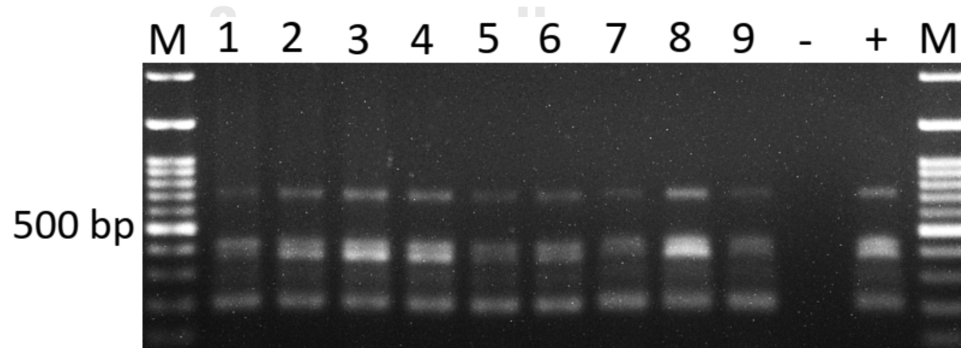
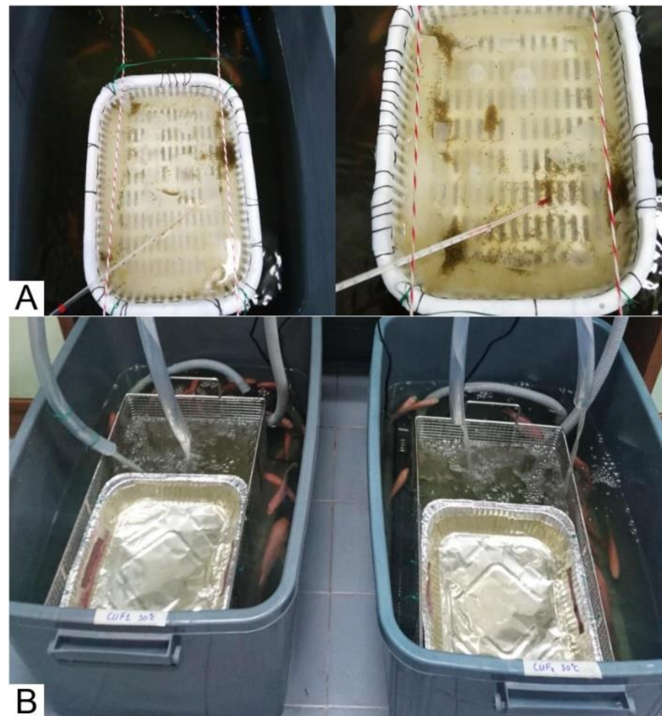
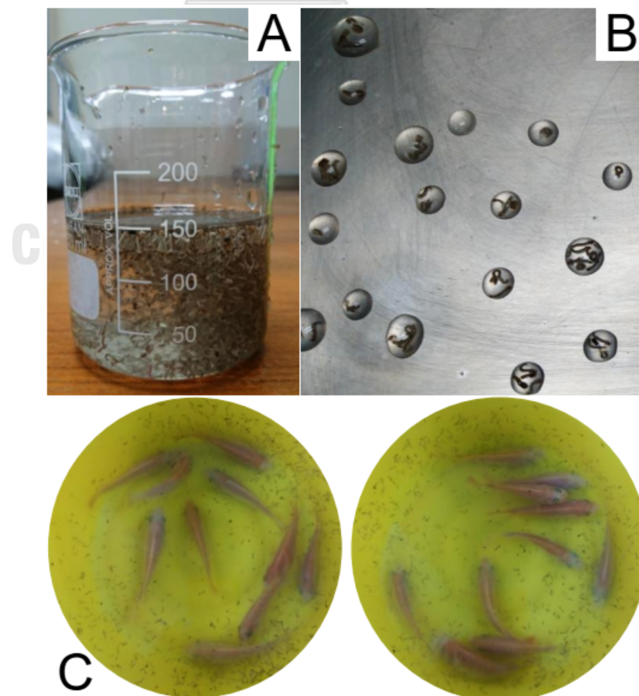


Supl Figure 2: Positive *F. orientalis* PCR detection of hybrid red tilapia cohabited with mosquito larvae.



Supl Figure 3: Cohabitation mosquito larvae with infected fish at 25 °C (A) and 30 °C (B).



Supl Figure 4: A) *A. aegypti* pupae stage. B) Number of pupae was evaluated by drop method. C) Hybrid red tilapia were fed by infected pupae.

CHAPTER 5

SYNERGISTIC INFECTION OF THE ECTOPARASITE *ICHTHYOPHTHIRIUS MULTIFILIIS* AND THE INTRACELLULAR BACTERIUM *FRANCISELLA ORIENTALIS* IN HYBRID RED TILAPIA (*OREOCHROMIS* SP.)

Abstract

Francisella orientalis and *Ichthyophthirius multifiliis* (*Ich*) are deadly infectious pathogens in farmed tilapia, particularly during cold season when the water temperature drops to under 25°C. We hypothesized that infection of the ectoparasite *Ich* might enhance susceptibility of hybrid red tilapia (*Oreochromis* sp.) to the facultative intracellular bacterium *F. orientalis*. To prove the hypothesis, the experiment was designed as follows. Hybrid red tilapia naturally infected by *Ich* at 9 ± 6 theronts/fish gills and 4 ± 3 theronts/fish skin were distributed into 5 distinct groups exposed to different concentrations of *F. orientalis*. In parallel, the same number of *Ich*-free tilapia were challenged to only *F. orientalis* in the same manner. The results showed that cumulative mortality in the *F. orientalis* single infection with 2.88×10^{6} CFU mL^{-1} of water was 25 ± 7%, whereas 100% mortality was found in the coinfection treatment at dose of 1.93×10^{5} CFU mL^{-1} of water. No mortality was observed in both control groups (*Ich*-infected and *Ich*-free fish). The coinfected fish revealed typical clinical signs and histopathological manifestations of francisellosis and ichthyophthiriasis. This study revealed synergistic effect of the *Ich* and *F. orientalis* infection in hybrid red tilapia leading to the exacerbated mortality. Thus, farming management of fish to be free from the *Ich* ectoparasite might reduce risk of francisellosis and probably other bacterial diseases in farmed tilapia.

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5.1 Introduction

Tilapias are currently the most popular freshwater fish farmed in over 120 countries and average production growth increased by 11% over the past three decades (FAO, 2019a). Tilapia global aquaculture production was the fourth place in the top 10 aquaculture species in term of both quantity and value (FAO, 2019b). However intensively cultured fish is threatened by the devastation of infectious diseases. The bacterium *F. orientalis*, a causative agent of piscine francisellosis emerges as one of major pathogens which causes mass mortalities in not only tilapia but also various warm water fish species with worldwide distribution (Birkbeck et al., 2011; Colquhoun and Duodu, 2011). *F. orientalis* causes acute to chronic infections and typically affects the spleen, kidney, liver with noticeable clinical signs of multiple white spots (Dong et al., 2016a; Ramírez-Paredes et al., 2017). Recent studies revealed that the bacterium was able to transmit from sub-clinically broodstock to their offspring, suggesting vertical transmission route (Nguyen et al., 2019b; Pradeep et al., 2017).

Ichthyophthiriasis (also known as white spot disease) is probably the most dangerous parasite disease in wild and culture freshwater fish caused by protozoan *Ichthyophthirius multifiliis* (*Ich*) (Jørgensen, 2017; Matthews, 2005; Ventura and Paperna, 1985). In many cases, light infections generally cause little damage to the skin tissues so that do not lead to fish mortality. However, higher infection level of ectoparasites can cause severe damage on fish population especially, under the high stocking densities in intensive farms. *Ich* is an obligate ectoparasite and attach itself to the skin and gills of the host. The typical signs of ichthyophthiriasis is multiple white spots on the skin, fin and gills of fish (Matthews, 2005). Its life cycle has three developmental stages including a reproductive stage (tomocyst), an infective stage (theront) and a parasitic stage (trophont) (Ventura and Paperna, 1985; Wei et al., 2013).

In nature, a single host normally infected by multiple pathogens (Kotob et al., 2017). Cases of coinfection by bacteria and parasite, including *Streptococcus iniae* and

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Ich (Xu et al., 2009), *Edwardsiella ictaluri* and *Ich* (Xu et al., 2012c), *S. iniae* and *Gyrodactylus niloticus* (Xu et al., 2007) has been reported in fish. Moreover, the evidence of a low present of parasite such as *Dactylogyrus* spp., *Trichodina* spp. and *Apisoma* spp. on francisellosis infected fish have been reported in Thailand, United States, Costa Rica, Mexico (Jantrakajorn and Wongtavatchai, 2016; Ortega et al., 2016; Soto et al., 2011; Soto et al., 2009b). However, the interaction of *F. orientalis* and parasite in the host and their possible impact on disease outbreak has not been explored. Outbreak of francisellosis and ichthyophthiriasis commonly occurs in young tilapia (fingerlings and juveniles) and strongly relation to rearing water temperature lower than 25°C (Colquhoun and Duodu, 2011; Shoemaker et al., 2006; Soto et al., 2012; Wei et al., 2013), suggesting possibility of realistic confections. However, under stressful of intensive husbandry and poor environment conditions, those disease can occur in atypical temperatures and affect in all life stages of fish (Colquhoun and Duodu, 2011; Pradeep et al., 2017; Wei et al., 2013). In Thailand, the concurrent infection of *Ich* and *F. orientalis* was observed in several hybrid red tilapia farms at temperature around 28°C. Up-to-date, there is no report about the association of the two pathogens in farmed tilapia. Therefore, this study aims to investigate whether infection of the ectoparasite (*Ich*) enhance susceptibility of tilapia to *F. orientalis* at atypical temperature, 28°C.

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5.2 Materials and Methods

5.2.1 Populations of *Ich*-infected and *Ich*-free fish

The juvenile hybrid red tilapia (*Oreochromis niloticus* × *O. mossambicus*) used in two experimental challenges were purchased from different tilapia farms in Nakhon Pathom Province, Thailand. In trial I, the fish (8.2 ± 0.4 cm in length) were from a commercial farm which had a suspected history of concurrent infection of francisellosis and ichthyophthiriasis based on typical clinical signs. In trial II, the fish (8.5 ± 0.5 cm in length) were selected from disease-free stock held at the other local hatchery. Wet

mount examination indicated that fish in trial I were naturally infected with *Ich* (9 ± 6 parasites/fish gills; 4 ± 3 parasites/fish skin) while fish in trial II were free of *Ich*.

The fish were acclimatized separately in two different 1000 L tanks for 10 days. Fish were fed at a rate of 5% body weight per day with commercial tilapia pellets containing 30% crude protein (CP, Thailand). During the trial, water parameters were measured by commercial test kits (Aqua-One, Australia) and in the optimum range for culturing tilapia (pH: 7 ± 0.5, dissolved oxygen (DO): 6.5 ± 0.3, temperature: 27 ± 0.4^{o}C).

Before the challenge, 8 fish from each group were examined for parasite infection by wet mount method and bacterial infection by microbiological culture. In order to isolate *F. orientalis* and common bacteria causing infectious disease in fish, spleen samples of each fish were streaked on tryptic soy agar and cysteine heart agar supplemented with 10% sheep blood (CHAB). Gill mucus samples were plated onto Anacker and Ordal agar for isolation of *Flavobacterium* spp. (Roberts, 2005). All fish were negative for fish pathogenic bacteria.

5.2.2 Parasite examination and identification

The number of *Ich* trophonts infection was determined by wet mount method from previous description (Xu et al., 2000). Initially, fish were anaesthetized by an overdose of clove oil (Arowana Stabilizer, Thailand), 100 mg L^{-1}. Mucus sample was prepared by scraping body surface including skin and fins of each fish. Gill filaments from one side of each fish were clipped and placed on Petri dishes. The subsamples were compressed by cover slip and observed under microscope (40x) magnification. The trophont was characterized by horseshoe-shaped macronucleus, spherical cell which is surrounded by a thick layer of cilia (Matthews, 2005).

Further identification of the parasite was conducted based on the ciliated protozoa 18S rDNA sequence. One set of specific primers Ich-F 5'-AAC CAA ACT CGG CCT TCA CT-3' and Ich-R 5'-TGT CTT GCG CTA CGT GAG TT-3' targeting 18S rDNA

was designed in this study using Primer3 software (v.0.4.0) (Untergasser et al., 2012). Total genomic DNA was extracted from approximately 50 mg of gill mucus using the DNeasy Blood and Tissue kit (Qiagen). The PCR mixtures (25 µL) contained 12.5 µL of PCR GoTaq® Green Master Mix (Promega, USA), 1 µL of each primer (0.4 µM), 4 µL of DNA template, and 6.5 µL of nuclear-free water. The optimal cycling conditions were as follows: an initial denaturation at 94°C for 3 min; 35 cycles of amplification at 94°C for 30 s, annealing at 50°C for 40 s, and extension at 72°C for 40 s; final extension at 72°C for 5 min. Representative of one amplification product (513 bp) was purified by Favorgen Gel/PCR purification kit. Purified DNA amplicons were submitted for DNA sequencing at the 1st BASE Pte Ltd. (Malaysia). The similarity between the *Ich* sequences and other available sequences at GenBank was carried out using nucleotide BLAST program. Phylogenetic tree was constructed by Neighbor-Joining method of MEGA 6.0 software (Tamura et al., 2013) with bootstraps value of 1000 replicates.

5.2.3 *F. orientalis* bacterial preparation

The bacterial strain *Francisella orientalis* VMCU-FNO131 isolated from a diseased hybrid red tilapia (Nguyen et al., 2016) was used in this study. The bacterium was recovered from stock modified tryptic soy broth supplemented with 1% D-glucose, 0.2% cysteine and 2% bovine hemoglobin (MTSB; BD, Oxford, UK) added 20% glycerol at -80°C using cysteine heart agar supplemented with 10% sheep blood (CHAB; BD, Oxford, UK) and incubated at 28°C for 3 days (Soto et al., 2009b). A loopful of bacterial colony was then inoculated in MTSB at 28°C in a shaking incubator overnight. Bacterial suspension was adjusted spectrophotometrically to OD_{600} = 0.8 (~10^{8} CFU mL^{-1}) and the accurate bacterial density (colony forming units per millilitre CFU mL^{-1}) was calculated through serial of tenfold dilution using plate counting method (Soto et al., 2009b).

5.2.4 Experimental challenges

In the first experiment, 100 hybrid red tilapia (8.2 ± 0.4 cm in length) naturally infected with Ich were divided into 5 groups. Four groups of fish were immersed with the bacterial strain VMCU-FNO131 at 1.93 x 10^{6}, 1.93 x 10^{5}, 1.93 x 10^{4}, 1.93 x 10^{3} CFU mL^{-1} of tank water for 30 min before being distributed into different tanks with 10 fish per tank, 2 replicates per group. The non-*F. orientalis* challenge control group was immersed with culture medium without bacteria in the same manner. In the second experiment, healthy Ich-free hybrid red tilapia (8.5 ± 0.5 cm in length) were immersed with the bacterium at 2.88 x 10^{6}, 2.88 x 10^{5}, 2.88 x 10^{4}, 2.88 x 10^{3} CFU mL^{-1} of tank water, and non-infected control immersed with culture medium for 30 min. Following each challenge exposure, 20 fish of each group were delivered into duplicate tanks containing 50 L treated water with aeration (10 fish/tank). Clinical signs and mortality of the fish was recorded twice daily for duration of 19 days. Totally 10 fish representative from each group (both moribund and survivor fish from *F. orientalis* alone, low doses of coinfection (10^{4}, 10^{3} CFU mL^{-1}); only moribund fish from high doses coinfection (10^{6}, 10^{5} CFU mL^{-1}); survivors of control groups) were subjected to investigation of the parasite by wet mount (see Section 2.2), *F. orientalis* by specific PCR assays (see Section 2.5) and histopathological analyses (see Section 2.5). When the coinfection fish showed clearly visible "white spots" for 7-10 days post-challenge (dpc), the moribund fish were sampled to determine the number of parasite infections per fish. The remaining fish at the end of the experiment were euthanized with a lethal dose of clove oil 0.1 g L^{-1} (Arowana Stabilizer, Thailand) before sampling. All fish were handled and treated according to the protocol approved by the Biosafety Committee (approval no. IBC 1831055) and Animal Ethics Committee (approval no. CU-ACUP 1931007) of Chulalongkorn University.

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5.2.5 Investigation of *Ich* and *F. orientalis* infection by PCR and histopathological assays

Total genomic DNA from gill mucus and spleen tissues were extracted using the DNeasy Blood and Tissue kit (Qiagen). The DNA from mucus was subjected to *Ich* PCR assays based on the ciliated protozoa 18S rDNA primers mentioned above. The DNA from spleen was screened for the presence of *F. orientalis* by PCR using *F. orientalis*-specific primer (FnoF1 5'-GGC GTA ACT CCT TTT AGC TTC C-3' and FnoR1 5'- TTA GAG GAG CTT GGA AAA GCA-3') (Dong et al., 2016a). The PCR conditions were performed following previous study (Dong et al., 2016a). A PCR reaction contained 12.5 µL of Master Mix (Go-Taq®Green, Promega USA); 4 µL of DNA template (150–200 ng); and 0.5 µL of both forward and reverse primers (0.4 µL); ultrapure water to final volume of 25 µL. PCR conditions were performed as follows: an initial denaturation step of 3 min at 94°C; 35 cycles of amplification at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 5 min. Genomic DNA of the isolate VMCU-FNO131 (accession no. KJ841935) was used as a template for positive control and nuclease-free water as the negative control. For the histopathological investigation, spleen, gill, skin samples of moribund and survivor fish were preserved in 10% buffered formalin. Subsequently, the samples were embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (Olympus BX51, Tokyo, Japan) equipped with a digital camera (AxioCam MRc digital camera, Carl Zeiss, Göttingen, Germany) (Jantrakajorn and Wongtavatchai, 2016).

5.2.6 Statistical analysis

All data were performed using SPSS software version 22 (IBM Corporation). Significant difference between percentages of mortality in each experiment groups were analyzed using one-way analysis of variance (ANOVA), general linear models, and post-hoc Tukey HSD test. Differences were considered significant when $P < 0.05$.

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5.3 Results

5.3.1 Identification of *I. multifiliis* from naturally infected fish population

Ichthyophthirius multifiliis trophonts which are characterized by a horseshoe-shaped macronucleus, were identified on gill and skin by wet mount preparation (Figure 18). Furthermore, the parasite was also identified by partial sequence (488 bp consensus sequence) of 18S rDNA fragment. It was shown that the 18S rDNA sequences of a representative amplicon indicated 100% identity with *I. multifiliis* sequences available in GenBank. Phylogenetic analysis of 18S rDNA gene placed the parasite in the same group with *I. multifiliis*, supported by bootstrap value of 100% (Figure 19). The 488 bp-18S rDNA partial sequence of *Ich* collected in this study was deposited into the GenBank under accession number KX988000.

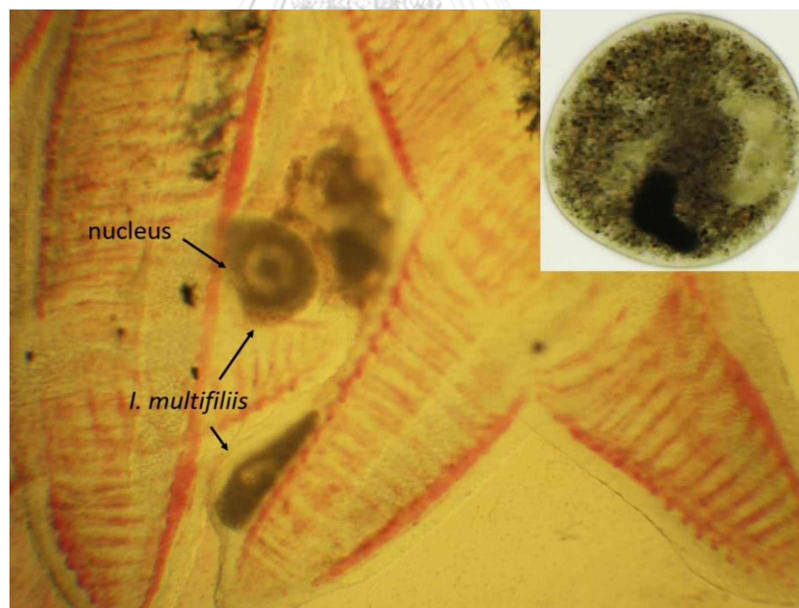

Figure 19 Mature trophont of *Ichthyophthirius multifiliis* and its horseshoe-shaped macronucleus (high magnification) observed in fresh-mounted smear from the gill filaments of hybrid red tilapia (*Orochromis* sp.).

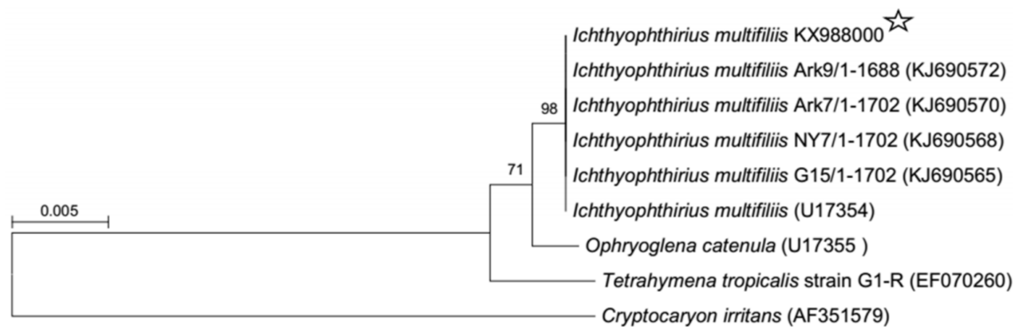

Figure 20 Neighbor-joining tree (right) based on alignment of 488 bp-18S rDNA sequences derived from *I. multifiliis* in this study (marked by star) with that of other *I. multifiliis* obtained from GenBank. The partial 18S rDNA sequence of *Cryptocaryon irritans* was used as an outgroup. Bootstraps of 1000 replicates were performed, and percentage bootstrap values are shown at each branch point. The scale bar represents the nucleotide substitutions per site.

5.3.2 Without Ich, single *F. orientalis* immersion exposure resulted in zero to low mortality in tilapia

In this experiment, a population of Ich-free hybrid red tilapia was used for immersion challenge with different doses of *F. orientalis*. At a high dose of 2.88 x 10^6 CFU mL^{-1} of tank water, 30% fish mortality was obtained at the end of experiment (19 dpc) (Figure 20A). No mortality was found from fish treated with the three lower doses and the control group (Figure 20A). The moribund fish from the high dose exhibited clinical signs of francisellosis (Supl Figure 5).

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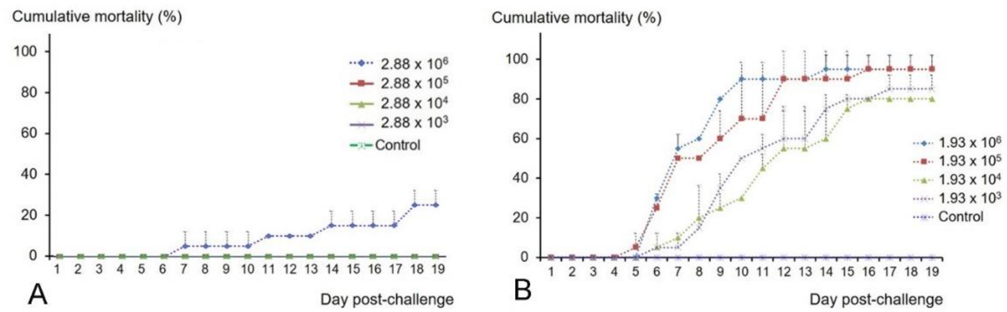
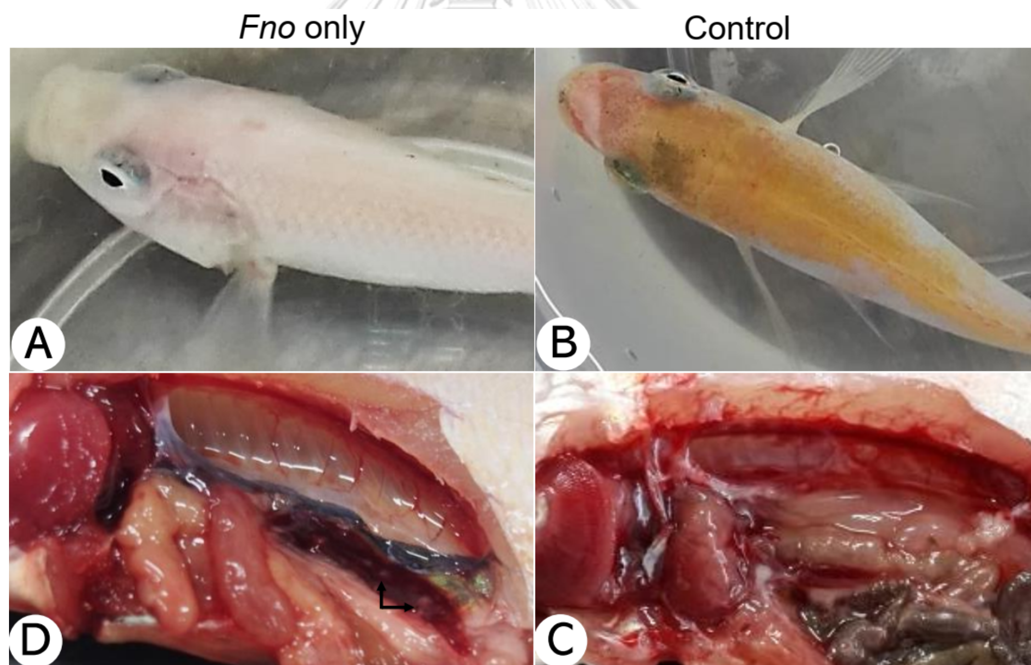


Figure 21 Cumulative mortality of healthy Ich-free hybrid red tilapia (A) and (B) naturally Ich-infected hybrid red tilapia immersed with different doses of *F. orientalis*. Each treatment was performed in duplicates with 10 fish/group. Upper half of the SD bar is shown.



Supl Figure 5: Moribund fish infected with *F. orientalis* alone showed white pale skin colour (A) and multiple white nodules in spleen (arrow) (D). Uninfected control fish displayed normal skin colour (B) and internal organs (C).

5.3.3 Primary *Ich*-infection does not kill tilapia while secondary infection with *F. orientalis* does

In this study, a population of Ich-infected hybrid red tilapia was used for immersion challenge with different doses of *F. orientalis*. *Ich*-infected fish exhibited high cumulative mortality, ranging from 80 to 100% when immersed with *F. orientalis* at low (1.93×10^{3} CFU mL^{-1}) to high doses (1.93×10^{6} CFU mL^{-1}) (Figure 20B). Mortality of high dose challenged group started at 5 dpc and rose very rapidly until it reached 100% at 10 dpc. No dead fish were observed in *Ich*-infected fish control group without *F. orientalis* challenge. Comparison of cumulative mortality between the Ich-free fish and Ich-infected fish after exposed to *F. orientalis* by immersion was statistically significant ($P < 0.05$).

Natural *Ich*-infected hybrid red tilapia immersion challenged with *F. orientalis* showed mixed-clinical signs of both francisellosis and ichthyophthiriasis. The typical signs of ichthyophthiriasis were white spots on skin and gills (Figure 21A). The formation of multifocal white nodules on spleen, kidney and other organs which resembled an F. orientalis infection were observed on affected fish (Figure 21C). In addition, notable signs of haemorrhage and huge damage on the body surface pale skin and emaciation were also seen in the coinfected fish (Figure 21B), whereas no abnormal sign was found in the *Ich* alone control group (Figure 21 D, E, F).

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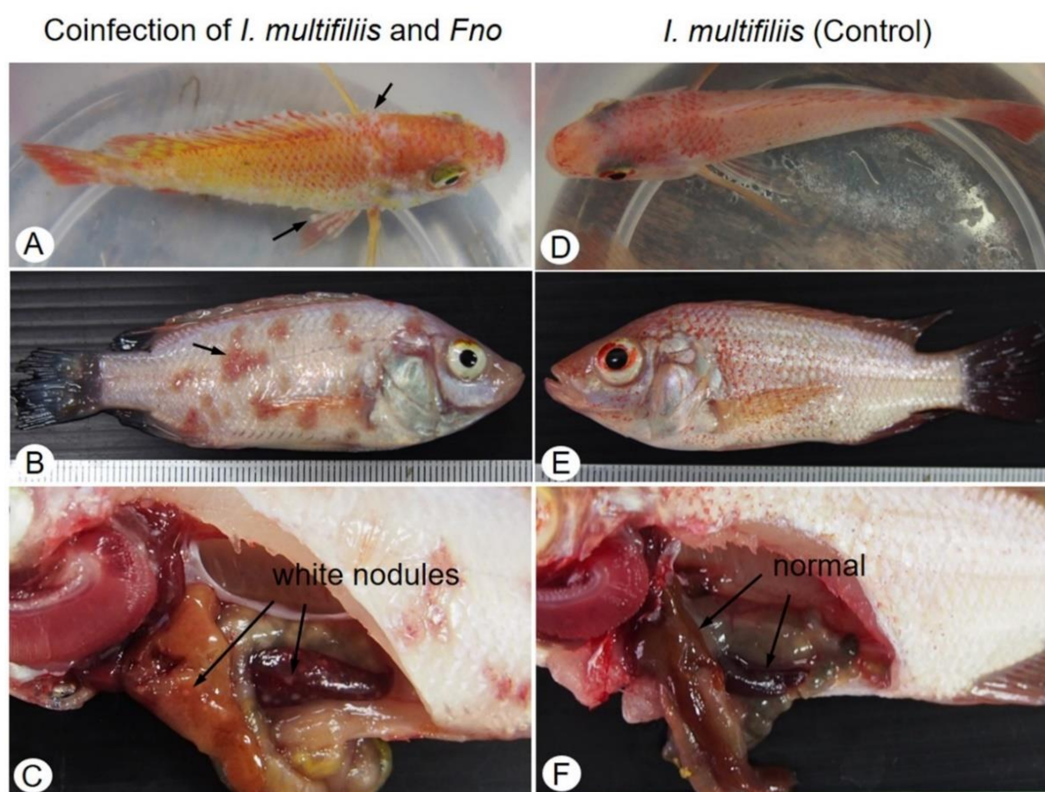

Figure 22 The naturally Ich-infected hybrid red tilapia challenged with *F. orientalis* showed white spots (arrows) on the skin epithelial layers, fins (A) and haemorrhage on skin (B), and white nodules on internal organs resembling francisellosis (C). By contrast, naturally Ich-infected without *F. orientalis* exposure revealed normal appearance externally and internally (D-F).

Table 4 Numbers of *I. multifiliis* counted on naturally Ich-infected hybrid red tilapia challenged with different doses of bacteria F. orientalis at day 7-10 post-challenge.

Group	*F. orientalis* challenge dose (CFU mL^{-1})	Number of *Ich* /fish (before coinfection)		Number of *Ich* /fish (7-10 dpc with *F. orientalis*)	
		Gill	Skin	Gill	Skin
1	1.93×10^6	8.6 ± 6.0 (n=8)	3.9 ± 2.8 (n=8)	56.8 ± 23.8 (n=6)	24.8 ± 19.4 (n=6)
2	1.93×10^5			94.3 ± 92.8 (n=3)	26.0 ± 22.7 (n=3)

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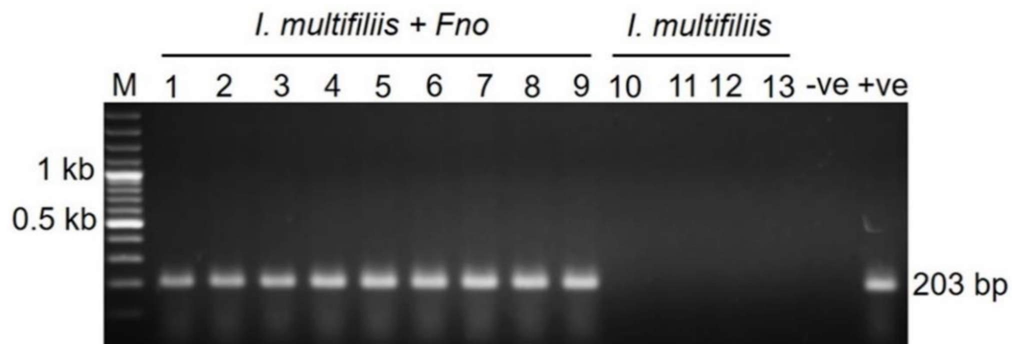

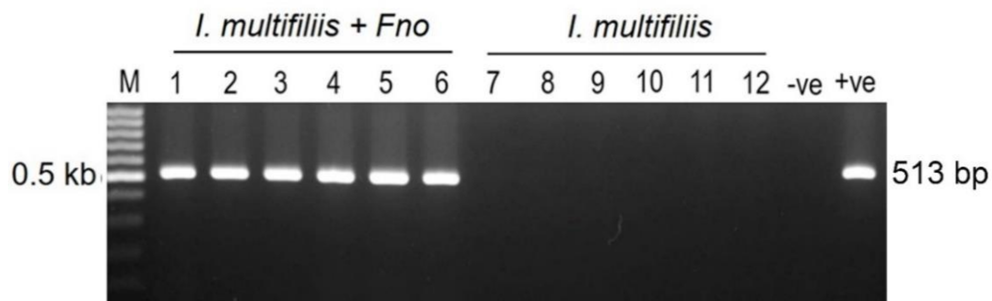

Figure 23 Detection of *F. orientalis* (A) and Ich (B) from *I. multifiliis+F. orientalis* coinfected fish and Ich-infected fish. In (A), *F. orientalis* PCR detection was performed using *F. orientalis*-specific primers and DNA extracted from spleen tissues as template; 1-9, the naturally Ich-infected fish immersed with *F. orientalis*; 10-13, the naturally Ich-infected fish control. –ve, no template control; +ve, positive control using DNA extracted from VMCU-FNO131 isolate. In (B), *I. multifilis* detection was conducted using newly designed primers based on 18S rDNA sequences. DNA extracted from gill and mucus were used as PCR template. 1-6, the moribund coinfection fish; 7-12, the naturally Ich-infected fish control at end of experiment (19 dpc). –ve, no template control; +ve, DNA of *I. multifiliis* previously confirmed by 18S rDNA sequence analysis. Note that different individual fish were used in (A) and (B).

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The clinical sign of white spots on skin was firstly recorded on several coinfected fish in all challenge doses at day 5 and became an obvious signal on all infected fish at day 7. The number of parasites increased sharply in coinfection group at day 7-10 from 8.6 ± 6.0 to 94.3 ± 92.8 on gill and from 3.9 ± 2.8 to 26.0 ± 22.7 on skin (Table 4). Interestingly, no parasites were observed on moribund fish from 12 dpc onward. *F. orientalis* was successfully recovered on CHAB agar plates from spleen tissues of 7 out of 10 moribund coinfected fish, even though all the spleen samples were positive for *F. orientalis* by specific PCR. The *Ich* alone control fish were negative for *F. orientalis* using PCR method. Figure 22A demonstrated the representative test results of 9 and 4 spleen samples from the moribund and survivable coinfected and control fish, respectively. For *Ich* PCR detection, DNA extracted from gill mucus of moribund and survivable coinfected fish and *Ich* alone control fish for 19 dpc were used in PCR assays. The results were shown in Figure 22B indicated the presence of *Ich* in the moribund coinfected samples. However, *Ich* amplicons were not detected from the naturally *Ich*-infected fish control and survivor coinfected fish at the end of the experiment (19 dpc). Representative PCR results of 6 samples each were shown in Figure 22B. It is noted that typical signs of francisellosis and ichthyophthiriasis were not seen in survivor fish from coinfection group, even though positive results of PCR assays for *F. orientalis*.

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Characterizations of histopathological manifestation of both francisellosis and ichthyophthiriasis disease were observed in moribund *Ich*-infected fish exposed to *F. orientalis* during experiment period. Varying degrees of granulomatous lesions which are referred to as francisellosis were found in kidney, spleen, and liver (Figure 23C). Histopathology analysis of gill sections showed presence of *I. multifiliis* at the base of primary filaments (Figure 23A). In addition, hyperplasia, fusion of secondary lamella and shortening of secondary lamella were also observed. The survivor fish of coinfection and *F. orientalis* alone showed only resembling lesions for francisellosis. The naturally *Ich*-infected fish in control group mostly revealed mild hyperplasia of secondary lamella or

normal gill structure (Figure 23B) both before and at the end of experiment. No abnormality was seen on spleen tissue except hyperplasia of white pulps (Figure 23D).

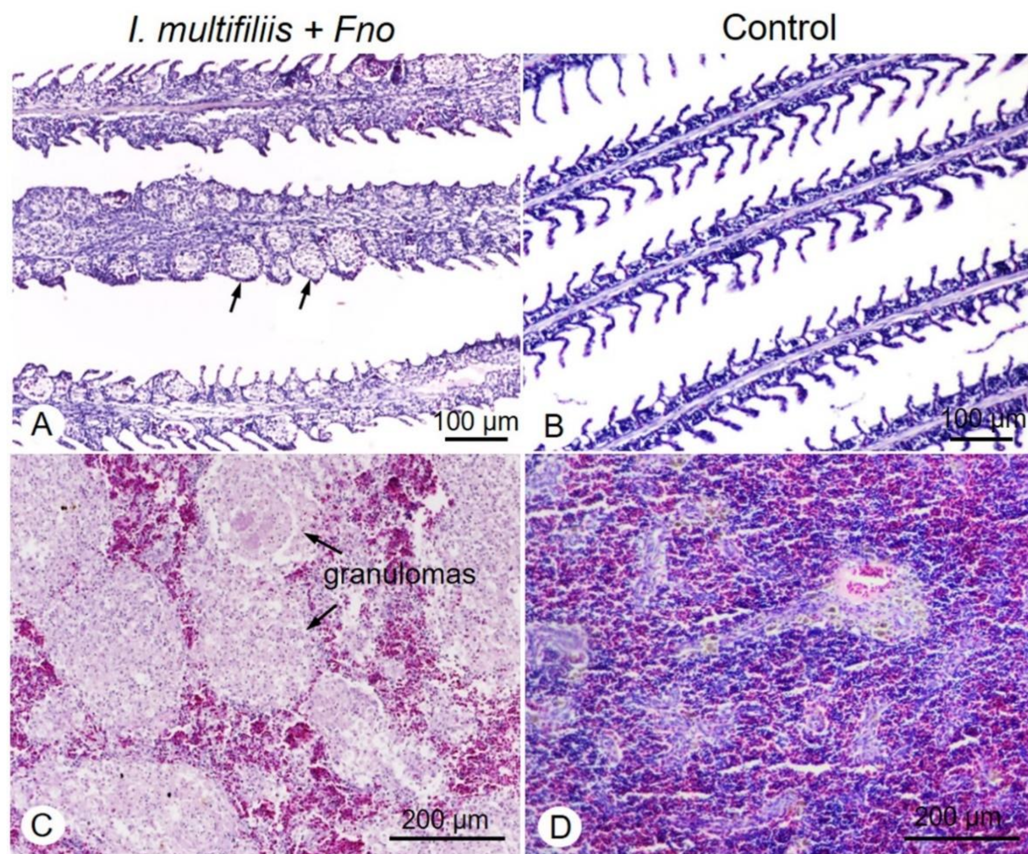

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Figure 24 Naturally Ich-infected hybrid red tilapia immersion challenged with *F. orientalis* showed presence of trophonts (arrow) of *I. multifiliis*, severe consolidation, hyperplasia of secondary lamellae (A) and multiple granulomas in spleen (C). Fish in control group of Ich alone revealed normal gill lamellae (B) and hyperplasia of splenic cells (D).

5.4 Discussion

Previous studies have demonstrated that coinfection of ectoparasite such as *I. multifiliis* (Xu et al., 2009), *Neoparamoeba perurans* (Crosbie et al., 2012), *Trichodina* sp (Xu et al., 2015), *Dactylogyrus intermedius, Gyrodactylus niloticus* (Zhang et al., 2013) enhance bacterial load in fish and result in significantly higher mortality. *F orientalis* and *I. multifiliis* are two common pathogens of tilapia that share similar optimum temperature (cool water temperature) for disease manifestation. No study has been conducted to demonstrate an important role of *Ich* parasite in coinfection with *F. orientalis*. The present study indicated that the mortalities were significantly higher in naturally *Ich*-infected hybrid red tilapia challenged with *F. orientalis* group when compared to other single infection groups. Our results are consistent with synergistic effect of coinfections of bacteria with parasite on the host in recent studies (Xu et al., 2012a; Xu et al., 2014; Xu et al., 2009). Xu *et al*. (2009) reported that cumulative mortality of tilapia concurrently challenged with *Ich* and *Streptococcus iniae* was significantly higher than that of the fish infected with *S. iniae* alone (Supl Table 1). Mortality of *Ich*-infected catfish exposed to *Edwardsiella ictaluri* was 71.1% while only 26.7% mortality was found in non-infected fish exposed to *E. ictaluri* in the same manner (Shoemaker et al., 2012). Similarly, coinfections of *Ich* and *Flavobacterium columnare* caused 60.4% mortality in hybrid tilapia while single infection of *F. columnare* caused only 29.1% mortality (Xu et al., 2014) (Supl Table 1).

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Supl Table 1: Cumulative mortality (%) of bacterial infection singly and dually with *Ich* in experimental challenged fish.

Bacteria	Single infection	Coinfection with *Ich*	Fish host	Ref.
S. iniae	≤20%	88%	Nile tilapia (*O. niloticus*)	(Xu et al., 2009)
A. hydrophila	22%	80%	Channel catfish (*Ictalurus punctatus*)	(Xu et al., 2012a)
E. ictaluri	26.7%	71.1%		(Xu et al., 2012c)
F. columnare	29.1%	60.4%	Hybrid tilapia (*Oreochromis* spp.)	(Xu et al., 2014)
F. orientalis	25 ± 7%	100%	Hybrid red tilapia (*Oreochromis* sp.)	This study

Soto *et al.* (2013) suggested that gills and skin may act as potential sites of *F. orientalis* entry. However, mortality level of *F. orientalis* infected fish depends on the route of inoculation and dose dependent (Fernandez-Alarcon et al., 2019; Soto et al., 2009b). In this study, the *F. noatunensis* subsp. *orientalis* VMCU-FNO131 was a virulent strain which caused a cumulative mortality of 90% within 19 days post injection at a dose of 1.5 x 10^6 CFU/fish (Nguyen, 2015). In contrast, zero to low mortality (30%) was recorded in immersion experiment at dose of 2.88 x 10^3 to 2.88 x 10^6 CFU/fish respectively. The results were in line with previous study reported that dose required to cause 50% mortality of challenged fish (LD_{50}) by injection and immersion at 20 dpc was 1.8 x 10^4 CFU/fish and 6.9 x 10^7 CFU mL^{-1} respectively (Soto et al., 2009a). This result could be explained by the bacteria inoculated directly into the visceral cavity are not subjected to the fist line defence of fish skin/gills in immersion challenge (Soto et al.,

2009a). It is thus suggested that the high mortality of francisellosis in tilapia may need injury on fish body to open portal entry. *I. multifiliis* is considered as a pathogen of mucosal surfaces (Matthews, 2005). *Ich* infections damage the epithelium of fish gills and skin. It leads to focal necrosis and epithelial rupture at the site of infection (Dickerson and Findly, 2014). As a result, mucus and epidermis in gills and skin, the first line of host defence was damaged, thereby creating the possible route for bacterial invasion and increased susceptibility to bacterial pathogens (Xu et al., 2012a; Xu et al., 2014; Xu et al., 2009). The result of this study suggested that sub-lethal dose of *Ich* infection can enhance susceptibility of the host to *F. orientalis* and realistically, the parasite may play an important role in outbreak of francisellosis in hybrid red tilapia farm.

The present study indicated an exacerbated pathogenesis eliciting more serious clinical signs and pathological lesions of *Ich*-infected fish exposed to *F. orientalis* than in the respective single challenged fish. The moribund fish of coinfected group showed mixed-clinical signs of both francisellosis and ichthyophthiriasis disease with severe damage on skin and lesions in internal organs (Figure 21)., while few white nodules resembling for francisellosis only presented in high immersion dose of 2.88×10^6 CFU/fish *F. orientalis* alone group (Supl Figure 5). There was a strong correlation between the clinical sign, accumulate mortality and histopathological manifestation, wherein the gills and spleen tissues of co-challenged fish were far more severe in the respective tissues of single challenged fish (Figure 23). The disease did not occur in control groups. It is not known how *Ich* and *F. orientalis* interact to worse disease in fish. It was reported that *Edwardsiella ictaluri* appeared on trophonts of *Ich* from skin and gill samples of infected fish. And it was able to survive and replicate in different development stages of *Ich* (Xu et al., 2012b). It is critically important to better understand the potential of *Ich* as a vector of *F. orientalis* in tilapia. There is a lack of information regarding the immune response of tilapia to *Ich*, *F. orientalis* infection alone

and coinfection. Thus, future work should investigate the possible immunosuppressive actions of *F. orientalis* and *Ich* coinfection.

In the hybrid red tilapia farming practice in Thailand, the francisellosis infected fish are often found in cage culture rather than earthen ponds. It could be explained by higher risk of exposure to heterogenous pathogens of fish in the open system of cage culture. The results of this study indicated that secondary infection with *F. orientalis,* following the primary *Ich* infection, caused a synergistic effect and, resulted in apparent symptoms, severe histopathology, significantly increased mortality of the coinfected fish. Therefore, periodic treatment to reduce external parasitic infections, not only *Ich* but also others, might be considered to reduce the risk of severe synergistic coinfection. The future studies focus on conditions contributing to outbreaks of coinfection, mechanisms of synergistic interaction of *Ich* and *F. orientalis* in tilapia will be crucial to develop a suitable and integrated infectious disease management programmes.

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CHAPTER 6

GENERAL DISCUSSION

6.1 Conclusion

Tilapias are currently the most popular freshwater fish farmed in over 120 countries and the rapid expansion of global tilapia aquaculture industry has created ideal circumstances for one of the most importance bacterial infectious disease caused by *Francisella orientlais*. Despite attempts to prevent francisellosis, there is lack of effective control measures. The investigation of vertical transmission, role of other organisms on disease outbreak will be an essential step to develop control measures for *F. orientalis*. This study confirmed transmission of *F. orientalis* from subclinically infected tilapia mouthbrooders to their offspring through the current practice of fry production in tilapia hatcheries. The presence of *F. orientalis* in the reproductive organs of the brooders and their offspring (fertilized eggs, yolk-sac larvae, 5 and 30-day old fry) was confirmed by a combination of PCR and ISH assays. Thus, non-lethal sampling of eggs and semen from broodstock might be practical for monitoring this pathogen in tilapia hatcheries thereby allowing selection of the specific pathogen free (SPF) broodfish for fry production. This study provides a new semi-nested PCR to increase 100 folds sensitivity of *F. orientalis* detection comparing with single PCR. The specific primer was designed base on *a unique hypothetical protein gene sequence* of *F. orientalis* strains. The results implied that with the current practice in tilapia hatcheries, *F. orientalis* is likely transmitted from subclinically infected broodstock of hybrid red tilapia to their progeny. Therefore, SPF broodfish should be considered for production of *F. orientalis*-free fry.

In aspect of vector transmission of piscine francisellosis, and how the bacterium persists between outbreaks is still less information. Mosquitoes (*Aedes aegypti* and

Anopheles gambiae) were considered to be the major vectors of the bacterium *Francisella tularensis* in United States, several European countries (Eliasson et al., 2002; Read et al., 2008). Many research studies have demonstrated the presence of *Francisella* genes in all stages of mosquitoes infected with *Francisella* species (Backman et al., 2015; Lundstrom et al., 2011; Thelaus et al., 2014). On another hand, the mosquito larvae are favourite food of tilapia suggesting potential interaction and thus contribution to the ecological cycle of the disease. In chapter II, to examine whether mosquito larvae could uptake *F. orientalis*, we immersed larvae with *F. orientalis* at dose of 0.895×10^7 CFU mL^{-1} for 1 hour. In parallel, the larvae (third star) were cohabited with infected fish until pupae state. The results indicated larvae *A. aegypti* are available to acquire *F. orientalis*. And the bacterium was re-isolated from immersed larvae at 24 h post challenge and cohabitation larvae. Our data show that *F. orientalis* was re-isolated from immersed larvae at 24 h and cohabited pupae, and DNA screening of the bacteria presented in immersed larvae at 96 h but negative at 144 h exposure. The bacterium was not recovered from inoculated distill water, larvae after 48 h. It suggested that *F. orientalis* could not growth in non-nutrition environment or without suitable host. In feeding trial, water temperature significantly influenced the transmission of francisellosis. Indeed, healthy fish were fed by immersed pupae showed typical granulomas resembling for histopathological changes of francisellosis in spleen, kidney tissues. However, the number granulomas found in fish in 25°C group were significantly higher in those 30°C. In addition, 100% fish in 25°C were positive with PCR detection while only 70% (n = 10) were in 30°C. These findings are in agreement with previous reports indicating that increasing the water temperature from 25 to 30°C prevented the development of clinical signs and mortality in *Francisella* challenged fish (Soto et al., 2012; Soto et al., 2014). Our results indicated mosquito larvae are able to acquire and transmit *F. orientalis* to healthy fish but not support the bacterium growth. It suggested that mosquito larvae could be a mechanical vector of francisellosis in fish.

Several studies showed synergistic effect of ectoparasite on bacterial infection in fish (Crosbie et al., 2012; Xu et al., 2015; Xu et al., 2009; Zhang et al., 2013). There was no report about coinfection of *Ich* parasite and *F. orientalis*, two common fish pathogens in tilapia. The results of this study indicated that secondary infection with *F. orientalis,* following the primary *Ich* infection, caused a synergistic effect and, resulted in apparent symptoms, severe histopathology, significantly increased mortality of the coinfected fish. Our results are consistent with synergistic effect of coinfections of bacteria with parasite on the host in recent studies (Xu et al., 2012a; Xu et al., 2014; Xu et al., 2009). Periodic treatment to reduce external parasitic infections, not only *Ich* but also others, might be considered to reduce the risk of severe synergistic coinfection.

6.2 Recommendations for future research

The results presented in this thesis suggest several research ideas in near future.

- To elucidate the role of cell mediated immunity in the protection of tilapia upon infection with *F. orientalis*.
- Investigate effect of *F. orientalis* on hatching rate, growth performance and immune response of fry to understand its pathogenesis in early development stages of tilapia.
- Elucidate the location and persistence mechanism of *F. orientalis* in mosquito larvae will be crucial to understand how the bacterium persists between outbreaks.
- Investigate the interaction of *F. orientalis* biofilms with common fish parasite such as *I. multifiliis*, *Argulus* sp., *Trichodina* sp. in the persistence and transmission of francisellosis.
- There is a lack of information regarding the immune response of tilapia to *Ich*, *F. orientalis* infection alone and coinfection. Thus, future work should investigate the possible immunosuppressive actions of *F. orientalis* and *Ich* coinfection.

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