การทดสอบการติดเชื้อของฟรานซิสเซลล่า โนอาทูเนนซิส ซับสปีชีส์โอเรียลทาลลิส
สายพันธุ์ VMCU-FNO131 ในปลานิลแดง

นายเหงวียน เวียด เวือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2557

EXPERIMENTAL INFECTION OF *FRANCISELLA NOATUNENSIS* SUBSP. *ORIENTALIS* STRAIN VMCU-FNO131 IN RED TILAPIA (*OREOCHROMIS* SP.)

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for the Degree of Master of Science Program in Veterinary Pathobiology
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Thesis Title	EXPERIMENTAL INFECTION OF *FRANCISELLA NOATUNENSIS* SUBSP. *ORIENTALIS* STRAIN VMCU-FNO131 IN RED TILAPIA (*OREOCHROMIS* SP.)
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เหงวียน เวียด เวือง : การทดสอบการติดเชื้อของฟรานซิสเซลล่า โนอาทูเนนซิส ซับสปีชีส์โอเรียลทาลลิสสายพันธุ์ VMCU-FNO131 ในปลานิลแดง (EXPERIMENTAL INFECTION OF *FRANCISELLA NOATUNENSIS* SUBSP. *ORIENTALIS* STRAIN VMCU-FNO131 IN RED TILAPIA (*OREOCHROMIS* SP.)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร. นพดล พิฆารัตน์, ดร. แสงจันทร์ เสนาปิ่น, 41 หน้า.

ตั้งแต่ปี ค.ศ. 2013 มีการระบาดของโรคอุบัติใหม่หลายครั้งในปลานิลแดง (*Oreochromis* sp.) ซึ่งมีลักษณะของก้อนแกรนูโลม่าสีขาวเล็กๆ (multiple white granulomas) กระจายอยู่ในอวัยวะภายในของปลานิลแดงที่เพาะเลี้ยงในบางจังหวัดของประเทศไทย แต่อย่างไรก็ตามยังไม่มีการตรวจสอบหาสาเหตุของโรคดังกล่าว การศึกษานี้จึงมีวัตถุประสงค์เพื่อตรวจพิสูจน์สาเหตุของโรคติดเชื้อนี้ให้สมบูรณ์โดย ใช้หลักการของ Koch (Koch's postulates) ปลานิลแดงป่วยจำนวน 10 ตัว ถูกเก็บมาจากฟาร์มที่มีการระบาดของโรค 2 ฟาร์ม เพื่อนำมาทำการแยกเชื้อแบคทีเรีย ทำการศึกษาเนื้อเยื่อ และทำการวินิจฉัยหาเชื้อฟรานซีสเซลล่า (*Franciscella* sp.) ที่เป็นสาเหตุโดยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส (Polymerase Chain Reaction, PCR) ตัวอย่างทั้งหมดที่นำมาตรวจให้ผลบวกกับฟรานซีสเซลล่าด้วย PCR ที่มีความจำเพาะในระดับจีนัส (genus-specific PCR) นอกจากนี้ได้นำเชื้อฟรานซีสเซลล่าที่แยกได้จากไตของปลาป่วยจำนวน 1 ไอโซเลท ใช้ชื่อว่าสายพันธุ์ VMCU-FNO131 ไปตรวจพิสูจน์สปีชีส์ด้วยการเพิ่มจำนวนยีน 16S rRNA ด้วย PCR และนำไปทำการหาลำดับนิวคลีโอไทด์ ผลการเปรียบเทียบลำดับนิวคลีโอไทด์ที่ได้กับลำดับนิวคลีโอไทด์ของเชื้อโรค ต่าง ๆ ที่ถูกเก็บไว้ใน GenBank database ด้วยวิธี BLAST พบว่าเชื้อที่แยกได้มีลำดับของนิวคลีโอไทด์ของ 16S rRNA เหมือนกับฟรานซีสเซลล่า โนอาทูเนนซีส ซับสปีชีส์โอเรียลทาลลิส (*Franciscella noatunesis* subsp. *orientalis*) ถึง 100 % จากนั้นนำเชื้อนี้ไปทำการฉีดเข้าช่องท้องของปลานิลแดงทดลองใน ระยะ fingerling ที่แข็งแรงดีจำนวน 2 กลุ่ม โดยทำการฉีดเชื้อจำนวน 1.08×10^3 cfu ต่อตัวปลา ให้ปลากลุ่มที่ 1 และ ฉีดเชื้อจำนวน 1.08×10^5 cfu ต่อตัวปลา ให้ปลากลุ่มที่ 2 ผลการทดลองปรากฏว่าอัตราการตายสะสม (accumulative mortality) ของปลาในกลุ่มที่ 1 ที่มีค่าเฉลี่ยที่ 86.7 ± 23 % ถูกตรวจสอบได้ภายใน 16 วันหลังการฉีดเชื้อ อย่างไรก็ตามในปลากลุ่ม 2 อัตราการตายสะสมค่าเดียวกันนี้ถูกตรวจสอบได้ภายใน 5 วันหลังการฉีดเชื้อ ลักษณะอาการของปลาป่วยและการเปลี่ยนแปลงทางพยาธิวิทยาของเนื้อเยื่อพบว่าปลา ป่วยมีก้อนแกรนูโลม่าอยู่ที่อวัยวะภายในหลายอวัยวะ ทั้งนี้จากผลการทดลองสรุปได้ว่าค่าลีทอลโดส 50 % (LD_{50}) ของเชื้อสายพันธุ์ VMCU-FNO131 ในปลานิลแดงคือ 2.88×10^5 cfu ต่อตัวปลา ณ วันที่ 12 หลังจากการฉีดเชื้อเข้าช่องท้อง ในทางกลับกันไม่สามารถคำนวณค่า LD_{50} สำหรับการทดลองแบบจุ่มปลาไว้กับเชื้อ (immersion trial) ได้ แต่พบว่าเชื้อที่ขนาด 7×10^6 cfu ต่อ 1 มิลลิลิตรของน้ำที่ใช้แช่ตัวปลาสามารถทำให้เกิดอัตราการตายสะสมได้ที่ 25 % ภายใน 21 วันภายหลังการแช่

ภาควิชา พยาธิวิทยา
สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์
ปีการศึกษา 2557

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5675330431 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: CAUSATIVE AGENT, EXPERIMENTAL INFECTION, FRANCISELLOSIS, 41 VIRULENCE

NGUYEN VIET VUONG: EXPERIMENTAL INFECTION OF *FRANCISELLA NOATUNENSIS* SUBSP. *ORIENTALIS* STRAIN VMCU-FNO131 IN RED TILAPIA (*OREOCHROMIS* SP.). ADVISOR: ASST. PROF. DR. CHANNARONG RODKHUM, CO-ADVISOR: ASSOC. PROF. DR. NOPADON PIRARAT, DR. SAENGCHAN SENAPIN, pp.

Since 2013, several outbreaks of an emerging disease characterized by multiple white granulomas in internal organs have occurred in cultured red tilapia (*Oreochromis* sp.) in some provinces in Thailand. The etiological agent of this disease in Thailand, however, has not yet been determined. The objective of the present study was to identify the causative agent of this infectious disease by fulfilling Koch's postulates. Ten diseased red tilapia were collected from two affected farms for bacterial isolation, histological studies, and polymerase chain reaction (PCR) diagnosis for the suspected pathogen, *Francisella* sp.. All fish samples were positive for the *Francisella* genus-specific PCR. One bacterial strain, designated VMCU-FNO131, was successfully recovered from the kidney of diseased fish. Species identification of this strain was established by amplification and nucleotide sequencing of the 16S rRNA gene. BLAST analysis revealed 100 % identity to *Francisella noatunensis* subsp. *orientalis* strains available in the GenBank database. Subsequently, two groups of healthy red tilapia fingerlings were challenged with 1.08×10^3 CFU per fish and 1.08×10^5 CFU per fish by intraperitoneal injection. The accumulative mortality was 86.7 ± 23 % at 5 days post-injection (dpi). On the contrary, fish injected with 1.08×10^3 CFU per fish had died much slower, but the same accumulative mortality was observed at 16 dpi. Clinical signs and histopathological manifestations of typical granulomas were found in multiple organs of both the naturally and experimentally infected fish. Moreover, lethal dose 50 % (LD_{50}) of VMCU-FNO131 in red tilapia was 2.88×10^5 CFU per fish at days 12 via intraperitoneal injection. In contrast, LD_{50} value could not calculate in immersion trial, and the dose 7×10^6 CFU mL^{-1} has just caused 25 % cumulative mortality within 21 days.

Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....

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LIST OF ABBRIVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CFU	Colony forming unit
CHAB	Cystine heart agar + 10 % sheep blood
Fno	*Francisella noatunensis* subsp. *orientalis*
H&E	Hematoxylin and Eosin
I.P	Intraperitoneal injection
LD_{50}	Median lethal dose
min	minute
mL	millilitre
MTSB	Modified tryptic soy agar
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rpm	revolutions per minute
s	second
µL	microliter

CHAPTER I

INTRODUCTION

1. Importance and rationale

Tilapia (*Oreochromis* spp.) is described as “aquatic chicken” because of worldwide distribution and its similarly commercial value to chicken. The world production of tilapia has been increasing annually and made it become one of the most important cultured fish in over the world (Fitzsimmons K et al., 2011). 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). However, intensifying production of commercial tilapia farms with high density has been threatened by outbreaks of severe infectious diseases. Among the most common and serious diseases were reported in cultured tilapia, bacterial diseases such as: columnaris, edwardsiellosis, streptococcosis, and francisellosis were significantly affected tilapia cultured system (Austin B and Austin D, 2013).

Francisellosis is an emerging disease, caused by *Fracisella noatunensis* subsp. *orientalis* (*Fno*), a member of genus Francisella. It has been reported as a highly lethal pathogen in various warm water fish species from several continents. Particularly, tilapia has been considered as the most susceptible host resulting in high mortality up to 95% (Colquhoun et al., 2011). Recently, there is an increasing report of new susceptible species with francisellosis in different regions. However the information of francisellosis disease from Southeast Asia region is limited.

In recent years, several disease outbreaks were recorded in cultured red tilapia in floating cage system in Thailand. Diseased fish exhibited the clinical signs of numerous white nodules in internal organs (kidney, spleen, intestine, gills) resembling to francisellosis (Chanagun et al., 2014). However there is no published report on the etiological agent of francisellosis-like disease in cultured red tilapia in Thailand as

well as virulence of the bacterial pathogen associated with the infection in the fish. The objectives of the present study are to determine the causative agent of francisellosis-like disease in red tilapia and to investigate its pathogenicity virulence of the causative agent in red tilapia model. This study provides scientific information about the causative agent of francisellosis-like disease in red tilapia in Thailand and characteristics of its causative agent in red tilapia that might be useful for further research involving disease prevention and treatment.

2. Research questions

- What is the causative agent of francisellosis-like disease in cultured red tilapia (*Oreochromis* sp.) in Thailand?
- Does *Francisella noatunensis* subsp. *orientalis* strain VMCU-FNO131 have high virulence to red tilapia (*Oreochromis* sp.)?

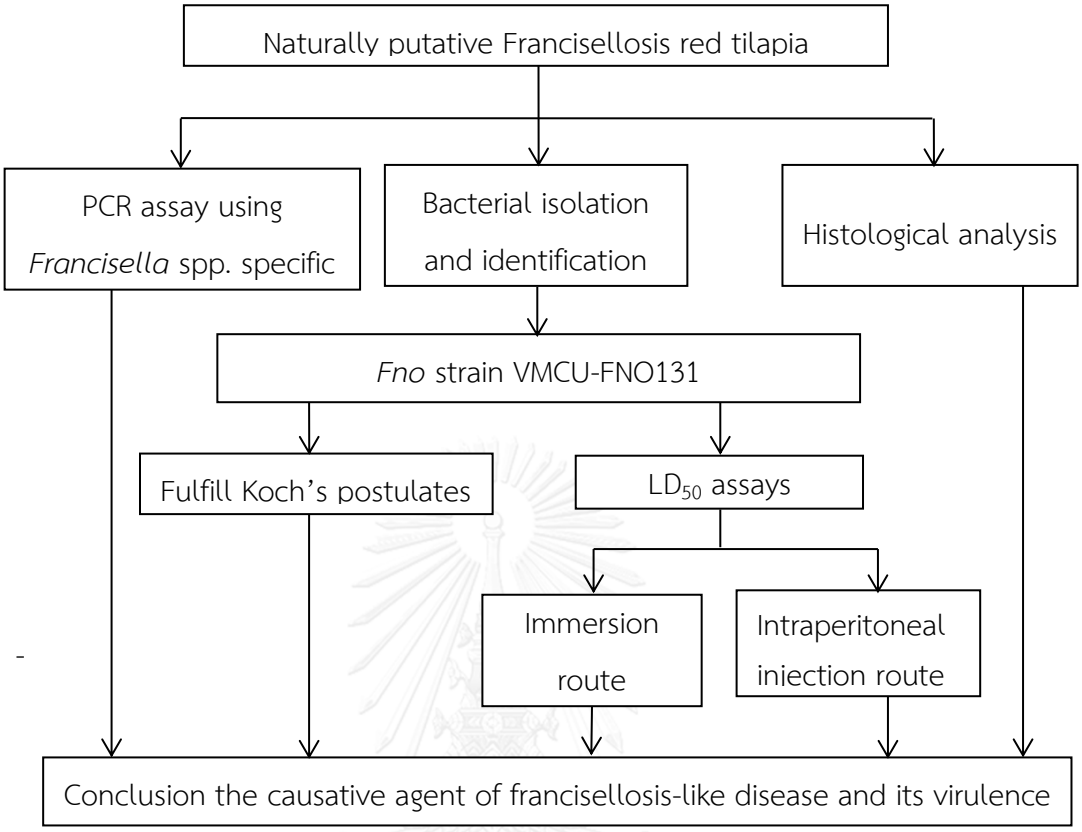
3. Hypothesis

- *Francisella noatunensis* subsp. *orientalis* strain VMCU-FNO131 is the causative agent of putative francisellosis in cultured red tilapia in Thailand.
- *Francisella noatunensis* subsp. *orientalis* strain VMCU-FNO131 has a high virulence to red tilapia.

4. Objectives

- To identify the causative agent of francisellosis-like disease in cultured red tilapia (*Oreochromis* sp.) in Thailand.
- To evaluate infection of *Francisella noatunensis* subsp. *orientalis* strain VMCU-FNO131 in red tilapia (*Oreochromis* sp.).

5. Conceptual framework



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CHAPTER II

LITERATURE REVIEW

1. Francisellosis in tilapia

Francisellosis in fish is not truly emerging disease. It was firstly occurred in cultured tilapia in Taiwan in 1994 and referred to *Rickettsia*-like organism (RLO) (Chern and Chao, 1994). The disease was then reported in both freshwater and marine fish as *Piscirickettsia*-like syndrome (PRS) (Mauel and Miller, 2002). Subsequently, the recent successes in isolation and molecular methods proved that most of PRS cases caused by *Francisella* spp. (Birkbeck et al., 2011; Colquhoun and Duodu, 2011). Recently, *Fno* and *Francisella noatunensis* subsp. *noatunensis* (*Fnn*) have been identified as aetiological agents of Franciscellosis in warm and cold water fish, respectively (Mikalsen and Colquhoun, 2009; Ottem et al., 2009). *Fno* belongs to genus *Francisella*, family *Francisellaceae*, order *Thiotrichales*. The bacteria is a Gram negative, facultative intracellular, non-motile, pleomorphic coccoid, catalase positive and cytochrome oxidase negative (Birkbeck et al., 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*) (Johansson et al., 2015).

Up to date, various affected fish species which distributed in different continents have been reported as susceptible hosts of *Fno* including tilapia, *Oreochromis* spp. in Taiwan (Chen et al., 1994), the United States (Hawaii, California, Florida, Texas and mid-western states) (Mauel et al., 2003; Mauel et al., 2005; Soto et al., 2011), Costa Rica (Soto et al., 2009a), Indonesia (Ottem et al., 2009), UK (Jeffery et al., 2010), Brazil (Leal et al., 2014); threeline grunt (*Parapristipoma trilineatum*) in Japan (Kamaishi et al., 2005); hybrid striped bass, *Morone chrysops* x *M. saxtilis* in the United States (Ostland et al., 2006); ornamental cichlid (11 different species) in Taiwan (Hsieh et al., 2007); Indo-Pacific reef fish (6 different species) imported into

the United States (Camus et al., 2013) (Figure 1). While *Fnn* has been reported in Atlantic cod, *Gadus morhua* L. in southwest Norway, west coast of Sweden, southern North Sea, Celtic Sea (Alfjorden A et al., 2006; Olsen et al., 2006; Zerihun M.A. et al., 2011; Ruane et al., 2015); Atlantic salmon, *Salmo salar* L. in Chile (Birkbeck et al., 2007) (Fig. 1). Particularly, tilapia and cod have been well known as the most susceptibility host to *Fno* and *Fnn,* respectively (Colquhoun and Duodu, 2011).

Figure 1 Geographical distribution of piscine-associated francisellosis (black dots: *F. noatunensis* subsp. *orientalis* and red dots: *F. noatunensis* subsp. *noatunensis*).

Both subspecies of Francisella can cause multiple white nodules in the several internal organs, such as kidney, spleen, liver, intestine, and gill. Infected fish also exhibits nonspecific clinical signs including exophthalmia, pale body colour, and abnormal swimming (Colquhoun and Duodu, 2011). Histologic features of the disease reveals the significant pathological changes in head kidney and spleen. These organs exhibit numerous granulomas which were typified by necrotic cores with large numbers of macrophages containing small pleomorphic cocco-bacilli (Birkbeck et al., 2011; Colquhoun and Duodu, 2011).

It is believed that water temperature may play a critical role in the outbreak of francisellosis in tilapia. Mauel *et al.* (2003a) reported that wild and cultured tilapia on the island of Oahu, Hawaii were more susceptible to francisellosis disease in the

water temperature ranging from 21.5 to 26.3 $^{\circ}$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 $^{\circ}$C. The research of Soto *et al.* (2012) had also revealed similar finding, francisellosis occurred more severely with higher bacterial concentrations in spleen and accumulated mortality was increased when rearing at 25 $^{\circ}$C, comparing with those rearing at 30 $^{\circ}$C. Moreover the water temperature, 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. Disease was most likely happen during the transportation of the fish especially when turbidity and water temperature were fluctuated. (Mauel et al., 2003; Mauel et al., 2007)

Isolation of *Fno* from infected tilapia has been challenging by its fastidious nature, contamination by other opportunistic bacteria and antibiotic treatment. It is well know 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., 2009c). From the infected tilapia, *Fno* 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 *Fno* 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., 2009c).

2. Bacterial pathogenicity

The previous research of Soto et al (2009) has proved that *Fno* has a high virulence in the Nile tilapia fingerlings. The 50% lethal dose, (LD_{50}) was 1.2 x 10^{3} CFU per fish via intraperitoneal injection and 2.3 x 10^{7} CFU mL^{-1} tank water by immersion at 40 days post challenge. The minimum dose that can produce mortality is twenty three bacteria via intraperitoneal injection and 2.3 x 10^{1} CFU mL^{-1} via immersion challenge (Soto et al., 2009a).

On the other hand, the bacteria is able to survive in both sea and freshwater microcosm for eight days in 20-30 ^{0}C and the number of bacteria maintained at 20 and 25 ^{0}C are significantly higher than those maintained at 30 ^{o}C. Bacteria can maintain its pathogenicity for three days when culture the bacteria in freshwater microcosms (Soto and Revan, 2012).

CHAPTER III

MATERIALS AND METHODS

1. History of diseased fish

In September 2013, the infected red tilapia was collected from two commercial farms located in Kanchanaburi province where the disease outbreaks resembling francisellosis-like symptoms. Moribund fish exhibited exophthalmia, pale body color, abnormal swimming, numerous white nodules on gills and internal organs (spleen, kidney, liver and intestine) (Figure 2 A). According to the farmer, cumulative losses of the cultured fish were 50 to 60%. Five diseased fish from each farm were collected and rapidly transported to the laboratory for bacterial isolation. Although DNA extracted from spleen of all infected fish were positive with PCR assay using *Francisella* genus specific primer (Forsman et al., 1994) (Figure 3 A), but only one putative *Francisella* sp. isolate, designated VMCU-FNO131, was successfully recovered from kidney of the diseased fish. This isolate was used in this study.

2. Bacterial identification

2.1. Bacterial isolation

The spleen and kidney tissues obtained from naturally infected red tilapia were streaked directly on cysteine heart agar added 10% sheep blood and with or without Polymixin B 100 units mL^{-1} (CHAB) (Soto et al., 2009c). Bacteria were incubated at 28 $^{\circ}C$ for 5 days. Colonies observed from primary isolation were sub-cultured under the same condition to obtain pure colony. Bacterial colonies fulfilled primary test (Gram negative, non-motile, pleomorphic coccoid, catalase positive, cytochrome oxidase negative) were subjected to identification as *Francisella* spp. using *Francisella* genus-specific PCR as mention below. The isolate was then suspended in tryptic soy broth containing 1% D-glucose and 0.2% cysteine (MTSB) overnight at 29 $^{\circ}C$ in 175 rpm shaker incubator, and bacteria was preserved at -80 $^{\circ}C$ in the broth media containing 20% glycerol for later use.

2.2. DNA preparation for molecular analysis

Extraction of total DNA from tissues of naturally diseased fish and experimentally infected fish were performed using the DNeasy Blood and Tissue kit© (Qiagen) as recommended by the manufacture. Genomic DNA of bacterial isolates was extracted by suspending one colony of pure bacterial culture in 50 µL nuclease-free water. The suspension was boiled for 10 min, cooled in ice immediately for 10 min, finally centrifuged for 5 min at 9000 rpm. A quantity of 2 µL of supernatant was used as template DNA for polymerase chain reaction (PCR) amplification.

2.3. *Francisella noatunensis* subsp. *orientalis* identification

Francisella genus-specific PCR was applied in the present study for the purpose of *Francisella* genus confirmation. The PCR was conducted using *Franciscella* genus-specific primer targeting 16S rRNA gene (F11, 5´-TAC CAG TTG GAA ACG ACT GT-3´ and F5, 5´-CCT TTT TGA GTT TCG CTCC-3´) (Forsman et al., 1994). DNA template extracted from the kidney of healthy fish was used as negative controls. PCR conditions were performed as follows: PCR reaction mixture (25 µL) was composed of 12.5 µL PCR GoTaq® Green Master Mix (Promega, USA), 1 µL of each PCR primer (0.2 µM), 4 µL DNA template (100-500 ng genomic DNA), and 6.5 µL nuclease-free water. Cycling conditions were followed: an initial denaturation at 94 $^{\circ}$C for 3 min; 35 cycles of 30 s at 94 $^{\circ}$C (denaturation), 60 s at 60 $^{\circ}$C (annealing), and 60 s at 72 $^{\circ}$C (extension); 5 min at 72 $^{\circ}$C (final extension); 4 $^{\circ}$C indefinitely. The PCR product was electrophoresed in 1 % agarose gel in TBE buffer 0.5X at 100V, 120 mA for 45 min and stained with Red Safe DNA gel stain (Thermo Fisher Scientific). Electrophoresis result was detected under UV light of gel documentation system (Vilber Lourmat, France).

Bacteria isolate that gave positive result to *Francisella* genus-specific PCR assay, was then confirmed as *Francisella noatunensis* subsp. *orientalis* based on nucleotide identity of 16S rRNA gene. Universal primers (F1, 5'-GAG TTT GAT CCT GGC TCAG-3' and R13, 5'-AGA AAG GAG GTG ATC CAG CC-3') were used for amplification of 16S rRNA according to (Dorsch and Stackebrandt, 1992). PCR reaction mixture (25 µL) was composed of 12.5 µL PCR GoTaq® Green Master Mix (Promega, USA), 1 µL of each

PCR primer (0.2 µM), 2 µL DNA template (100-500 ng genomic DNA), and 8.5 µL nuclease-free water. Thermo cycling conditions were consisted of an initial denaturation at 94 $^{\circ}$C for 30 s; followed by 30 cycles of 30 s at 94 $^{\circ}$C, 60 s at 58 $^{\circ}$C , and 90 s at 72 $^{\circ}$C; 7 min at 72 $^{\circ}$C (final extension); 4 $^{\circ}$C indefinitely. PCR products were purified with Wizard® SV Gel and PCR clean-up system (Promega, USA) as described by the manufacturer and submitted for nucleotide sequencing (1st BASE Pte Ltd) using the same primer pair. The sequences were compared to 16S rRNA sequences of *Francisella* species available in GenBank using Nucleotide BLAST tool (http://www.ncbi.nlm.nih.gov).

2. 4. Experimental fish and water quality management

The healthy fingerling red tilapia, *Oreochromis* sp. (*n* = 280) were obtained from commercial hatchery and then acclimatized for 2 weeks before challenge test. During acclimatization period, kidney and spleen were harvested from 10 randomly selected fish, followed by PCR using *Francisella* genus specific primer and bacterial isolation using CHAB in order to confirm that the fish were not infected with bacteria. The gills and mucus of fish were also checked up for parasite infection by standard wet mount technique.

Fish were fed daily with a commercial pellets fish food sized 5 mm (Charoen Pokphand (CP), Thailand). Water temperature, dissolved oxygen (DO), pH, and ammonia in aquariums had been monitored by commercial test kits (Aqua-VBC, Thailand) three times per week.

3. Experimental challenge of red tilapia with strain VMCU-FNO131

3.1. Bacterial growth condition

The strain *F. noatunensis* subsp*. orientalis* VMCU-FNO131 recovered from diseased red tilapia rearing in freshwater cages in Kanchanaburi province was used in the present study. The bacterium was grown in cysteine heart agar with 10% sheep blood (CHAB) as described above. A full loop of bacteria was suspended in 5 ml phosphate buffered saline (PBS) and 100 µl of the suspension was spread on CHAB and inoculated at 28 °C for 24 hr. The bacteria were then harvested and suspend in

PBS to spectrophotometrically adjust to about 7 x 10^8 colony forming units per milliliter equivalent a final OD_{600} (optical density at 600 nm) of ∞. Subsequently, the bacterial concentration was serially diluted tenfold in PBS. Absolute bacterial numbers were determined by placing 50 µL drops of each dilution on CHAB and counting the colonies after 4 days of incubation at 28 $^{\circ}$C.

3.2. Experimental challenge to confirm strain VMCU-FNO131 was the causative agent of francisellosis in red tilapia in Thailand

To fulfill Koch's postulates, forty five fingerling red tilapia *Oreochromis* sp. (9 ± 1.2 g in weight) were divided into three experimental groups (five fish per tank with triplicate). In order to observe clinical signs in chronic case which occurs at low challenge dose, two experimental groups were intraperitoneally injected (i.p) with 0.1 mL of 1.08 x 10^6 and 1.08 x 10^4 CFU mL^{-1} of bacterial suspension, respectively. Fish in control groups were treated in a similar manner, but received 0.1 mL of sterile MTSB instead of bacterial suspension. After injection the fish were placed in different tanks with aeration. The fish was monitored for 21 days after challenge. Dead and survivor fish from each group were subjected to clinical, bacteriological examination as mention above. Four fish in each group (20%) were collected for histology and PCR assay.

3.3. Virulence assay of strain VMCU-FNO131 in red tilapia

To evaluate the virulence of strain VMCU-FNO131, fingerling red tilapia were challenged with the bacteria by immersion and intraperitoneal injection methods to determine LD_{50}. In case of injection route, one hundred red tilapia (19.5 ± 0.9 g in weight) were divided in 10 tanks containing 10 fish per each that were distributed into five groups. Four challenge groups received 0.1 ml bacterial suspension at dose 2.88 x 10^6, 2.88 x 10^5, 2.88 x 10^4, 2.88 x 10^3 CFU mL^{-1} by intraperitoneal injection route and control group was injected with sterile PBS. After injection the fish in different groups were cultured in different clean, 50 L water tank with aeration.

Regarding to immersion challenge, number of fish and groups were the same as in injection challenge protocol. Fish in five challenge groups were immersed in

different doses of 7×10^6, 2.88×10^5, 2.88×10^4, 2.88×10^3 CFU mL^{-1} of tank water for 2h with aeration. After immersion period, fish were moved and cultured in their respected clean, 50 L water tank with aeration.

After challenge, the fish were observed daily for 21 days. Clinical signs and mortality were recorded. Dead and survivor fish in each group were subjected to bacteriological, histological and PCR examination as mention above. The spleen, kidney, liver, gills, and intestinal tissues were collected for histopathology. Suspected *Francisella* sp. isolates from experimental fish and spleen tissues of challenge and control groups were used for PCR assay to confirm Francisealla infection. The LD_{50} value was calculated by the trimmed Spearman-Karber method (Hamilton et al., 1977).

4. Histolopathogical analysis

Histopathological changes of the natural infected and experimental challenged fish were described by histological examination. The spleen, kidney, liver, intestines, gill of the infected fish were fixed in 10% neutral buffered formalin overnight, then transferred to 70% ethanol. The target tissues were dehydrated via a graded series of alcohol for eight hours, cleared via series xylene for three hours, infiltrated with series paraffin for three hours, finally embedded in block of paraffin. Sections were cut at 3-5 µm and mounted onto glass slides before staining with Hematoxylin and Eosin (H&E) by modified (Luna, 1968) method and examined under light microscope. The average number of granulomas presented in anterior kidney, spleen and liver of each fish under 10X microscopic field was used to evaluate the severity of the disease in each group. The level of disease was ranked as severe (>20), moderate (7-20), mild (1-6).

Head kidney and spleen smears of naturally diseased and experimentally diseased fish were air-dried and fixed in absolute methanol for 10 min. The slides were then stained by the Wright-Giemsa and Gram methods and observed under microscopic with high magnification (100X). Moreover a piece of the fresh spleen or

kidney tissue was also pressed on a microscope slide by a cover slip. Subsequently the slides were observed under microscopic to search the presence of granuloma.

5 Data analysis

Sequencing results were contig using CLUSTALX 2.0 software and eliminated nucleotide sequences with noise signal. The identities of the obtained sequences were confirmed using BLASTN program (MA et al., 2007).

Phylogenetic tree was generated using neighbour-joining method of 1000 bootstrap. The evolutionary relationship was computed using the p-distance method after discarding gaps and unidentified nucleotides (complete deletion option) in Molecular Evolutionary Genetics Analysis (MEGA 5.2.2) program (Tamura et al., 2011).

Results from experiment challenge were analysed using Statistical Package for the Social Sciences (SPSS, IBM version 20.0) software. Firstly, density and date were analysed using construction of two factors regressive model to conduct analysis of variance (ANOVA). When the overall test indicated significance, pairwise comparisons were calculated using Post Hoc Test. For the mortality studies the percentage mortalities were transformed with a log transformation to normalize data. Difference with $p \leq 0.05$ was considered statistically significant (Soto et al., 2009c).

CHAPTER IV

RESULTS

1. Bacterial identification

All DNA samples extracted from mix the internal organs (spleen, liver, kidney) of natural infected fish were positive with PCR assay using *Francisella* genus primer, amplification for 16s rRNA gene (Figure 2), and one random PCR product was selected to sequencing. A 1009 bp was obtained and submitted to GenBank (accession number KJ925052). This sequence has a 100% sequence identity with sequences of *Francisella noatunensis* subsp. *orientalis* in the GenBank database.

One strain designated as VMCU-FNO131 recovered from natural infected red tilapia was positive with PCR assay using *Francisella* genus primer (Figure 3). The strain was confirmed as *Francisella noatunensis* subsp. *orientalis* based on 16s rRNA gene nucleotide identity to previously published sequences in the GenBank database. A 1393 bp sequence was obtained and deposited in GenBank under accession number KJ841935. This sequence has 100% nucleotide sequence identity to 16S rRNA fragment obtained from an infected tissue (accession number KJ925052) mentioned above and 16S rRNA of *Francisella noatunensis* subsp. *orientalis* sequences available in GenBank.

The strain VMCU-FNO131 was growth on cysteine heart agar supplemented with 10% sheep blood and Polymixin B 100 units mL^{-1} but not able to grow on tryptic soy agar (TSA) with 5% sheep blood or TSA with 1% D-glucose, 0.1% cystine and 10% sheep blood (MTSA); however other organisms including *Streptococcus* spp, *Aeromonas* spp. were isolated. Bacterial colony was gray or pale yellow, smooth, convex and mucoid, at 3 - 5 days after inoculation at 29 ^{0}C (Figure 4 A). The bacterium was Gram-negative, pleomorphic coccoid, facultative intracellular, non-motile, catalase positive, cytochrome oxidase negative and required cysteine for growth (Figure 4 B).

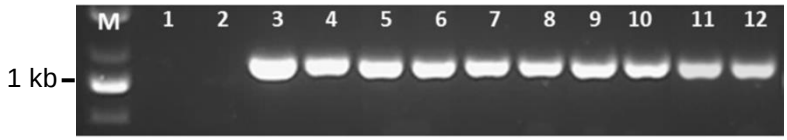
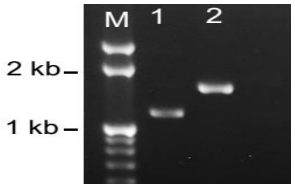


Figure 2: PCR diagnosis of *Francisella* sp. from infected red tilapia collected from the farms using genus-specific primers. Lanes: M: 1 kb ladder; 1-2: DNA obtained from healthy fish; 3-12: DNA from affected fish.



M: DNA marker
1: *Francisella* genus specific primer
2: universal primer.

Figure 3: PCR amplification of strain VMCU-FNO131 recovered from naturally infected red tilapia.

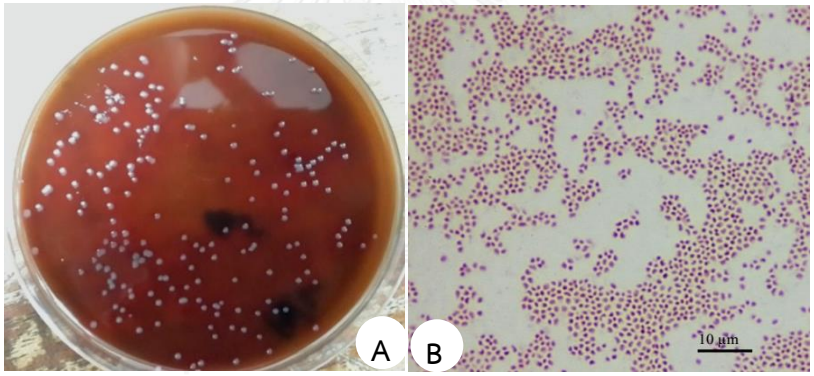


Figure 4: A) Colonies of *Fno* were grey and convex on CHA after 5 days post - inoculation. B) Gram negative polymorphic coco-bacilli VMCU-FNO131 strain.

The 16S rRNA sequences of two sub-species *F. noatunensis* subsp. *orientalis* (GenBank accession nos. FJ217163, DQ007455, EU672884, and NR_112536) and *F. noatunensis* subsp. *noatunensis* (GenBank accession nos. NR_043696, EF490217, AM403242, and EF685354) representing pathogens from war and cold water fish, respectively, were selected for sequence analysis. The 16S rRNA sequences of *F. halioticida* (GenBank accession no. AB449247) and *F. tularensis* subsp. *tularensis* (GenBank accession no. AY968225) were used as outgroups for the phylogenetic reconstruction. The phylogenetic tree revealed two clusters (I and II) with a bootstrap value of 76 (Figure 5). The cluster I contained four previously published sequences of

F. noatunensis subsp. *orientalis* isolated from warm water fish in different geographical locations, such as tilapia in Taiwan, Indonesia and Costa Rica, threeline grunt in Japan, and the sequence of the strain VMCU-FNO131 (Figure 5). The cluster II contained four previously published sequences of *F. noatunensis* subsp. *noatunensis*, which were originated from cold water fish Norwegian Atlantic cod and Chilean Atlantic salmon (Figure 5). According to all these results, the strain VMCU-FNO131 isolated from red tilapia (*Oreochromis* sp.) in Thailand was identified as *F. noatunensis* subsp. *orientalis*.

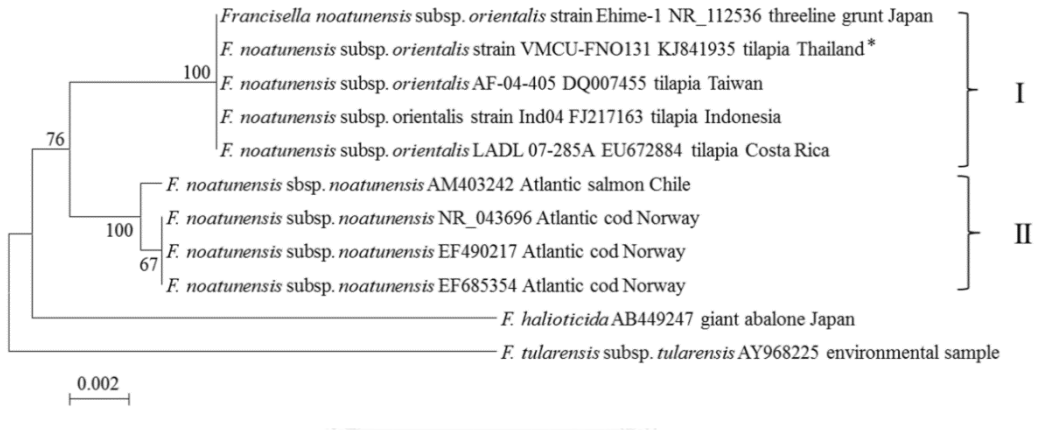


Figure 5: Phylogenetic tree was constructed based on alignment of 1380 bp of 16S rRNA sequences (position 91-1479 of *E. coli* 16S rRNA) from *Francisella* species. Sequence from *F. noatunensis* subsp. *orientalis* isolated from the red tilapia in Thailand is marked by an asterisk. Numbers at branch nodes indicate bootstrap confidence values in percentage.

2. Experimental challenge to confirm strain VMCU-FNO131 was the causative agent of francisellosis in red tilapia in Thailand

Experiment infection of red tilapia (9 ± 1.2 g in weight) with 1.08 x 10^5 CFU of *Fno* via i.p injection caused acute death. The accumulative mortality was 86.7 ± 23% at 5 days post-injection (dpi). On the contrary, fish injected with 1.08 x 10^3 CFU per fish had died much slower, but the same accumulative mortality of 86.7 ± 23% was observed at 16 dpi (Figure 6). While no dead fish was found in control group during experimental period. Water quality had monitored daily within acceptable ranges for cultured tilapia in fresh water (total ammonia reading: 0.4 ± 0.1 mgL^{-1}, dissolved

oxygen: 6 mgL^{-1} at pH of 7, temperature: 26.1 ± 0.3 ^{0}C) during the experiment (Popma and Masser, 1999).

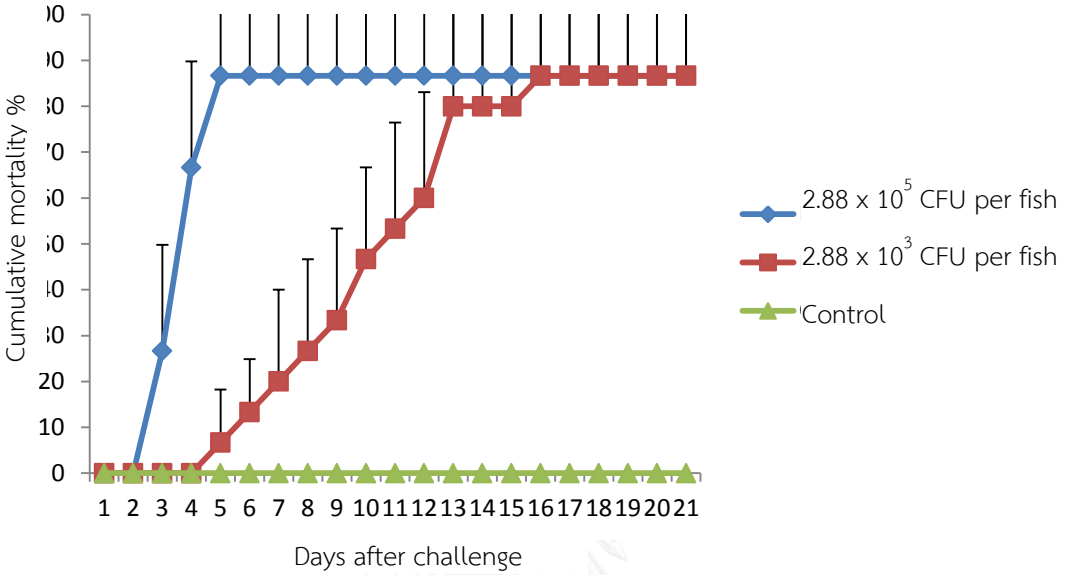


Figure 6: Cumulative mortality of red tilapia fingerling challenged by VMCU-FNO131 via i.p

Clinical signs were observed in both challenged groups which were to those occurred in naturally infected fish. Generally, challenged fish did not show abnormal symptom, several fish showed pale body colour and loss of appetite. The internal organs were enlarged and white nodules were seen on kidney, spleen, intestines, liver and gills (Figure 7 A, B). For the control group, all fish were alive and expressed no abnormal symptom (Figure 7 C).

At the end of experiment, the survived fish did not show white nodules in internal organs, but anterior kidney and spleen squash of them exhibited numerous granulomas microscopically (Figure 8 A) while none was found in control fish (Figure 8 B). Large amount of small, intra- and extracellular pleomorphic cocco-bacilli bacteria were also found in these tissue impression stained with Giemsa and Gram (Figure 9)

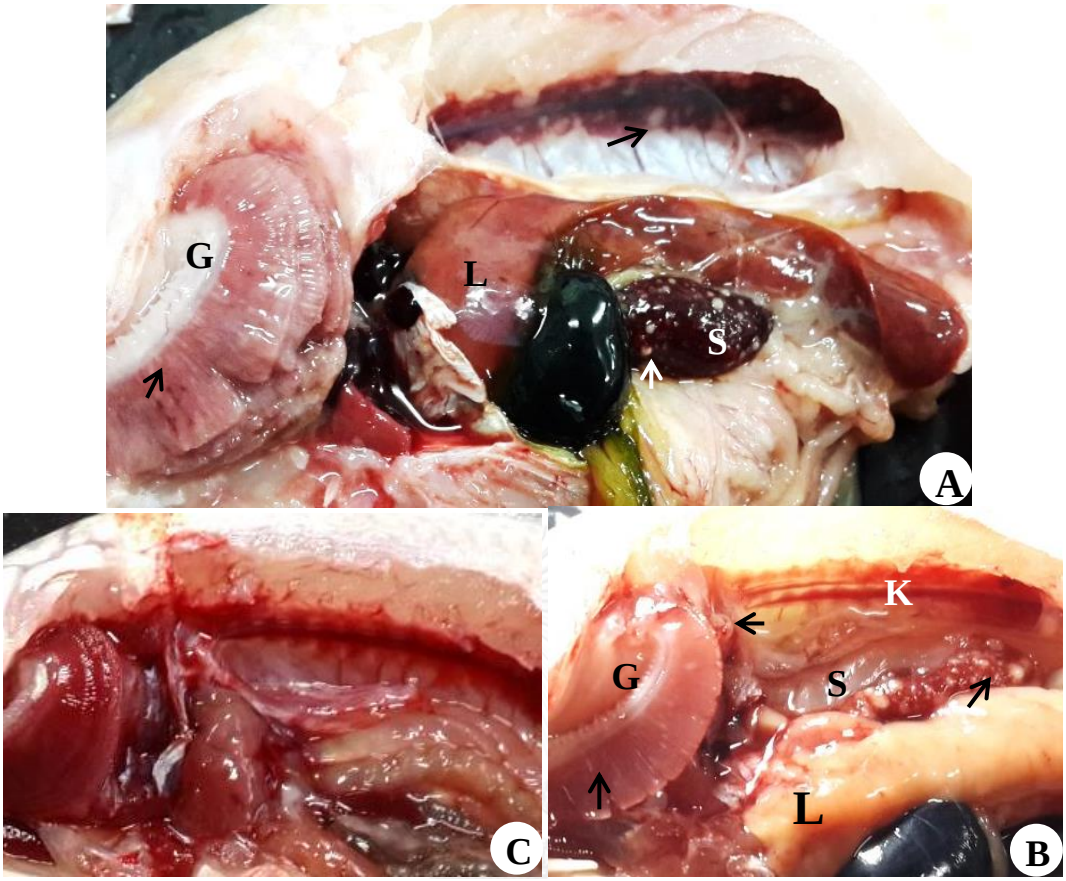


Figure 7: White nodules (arrow) were presented in various internal organs (S: spleen, L: liver, K: kidney and G: gill) of red tilapia infected with *Fno*. A) Natural infected fish. B) Challenged fish. C) Control fish

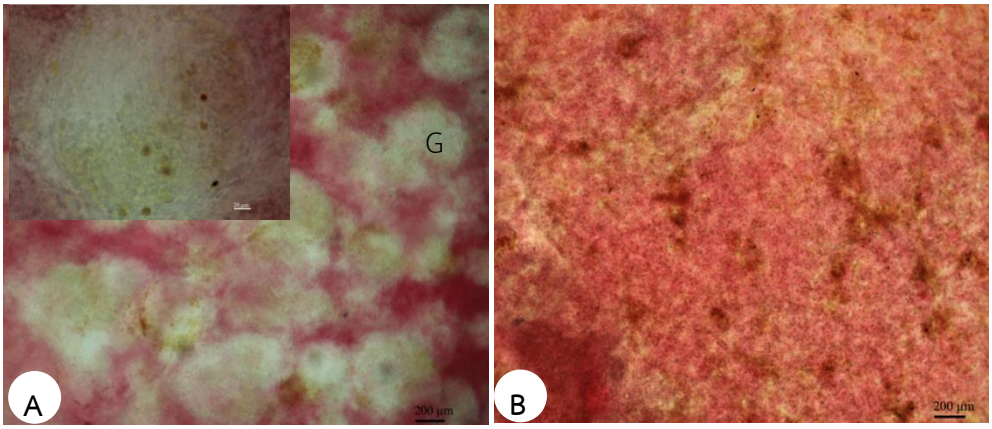


Figure 2: A piece of wet mount of the fresh spleen tissue. A) Granulomas formation (G) in infected red tilapia and granuloma in high magnificent. B) Normal tissue from control fish.

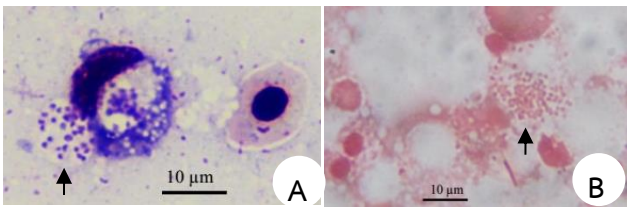


Figure 3: Impression smears of anterior kidneys stained with Giemsa (A) and Gram (B) revealed numerous gram negative coco-bacilli bacteria inside and outside macrophage.

In infected fish, histological change was formation of granulomas in various organs such as liver, intestine, gills. Especially head kidney and spleen where the most ruin and largest number of granulomas were observed. The granuloma containing necrotic core surrounded by a large number of macrophages and fibrosis (Figure 10 B), where numerous small pleomorphic cocco-bacilli bacteria was engulfed in cytoplasmic vacuole of macrophages (Figure 10 C). In the spleen, normal lymphoid tissues were replaced by multifocal to confluent granulomas inflammatory infiltrates (Figure 10 A). Numerous inflammatory cells were deposited in haematopoietic tissues of anterior and renal tissues of posterior kidney. In severe case, blood vessels were necrotic and resulting in fibrin thrombi, and individual to coalescing granulomas (Figure 11 A). Liver and intestine lesions were relatively mild, with small number of granulomas. In some case, Liver tissue showed severe multifocal hepatocellular necrosis; fibrin thrombi and congestion in sinusoids were observed in blood vessels (Figure 12 A). Lesions observed in intestines and stomach were mainly in submucosa and lamina propria. In the most case, infiltration of chronic inflammation cells and granulomas formation were presented commonly (Figure 12 C). On gills of infected fish, primary and secondary lamella were moderate to severe hyperplasia and fusion. Granulomas and mixed inflammatory cells were occasionally presented at the base of gill filaments (Figure 13 A). In contrast granulomas formation was not presented in tissues of internal organs of control fish. None abnormal histological change was observed in these tissues (Figure 10 D; 11 B; 12 B, D; 13 B).

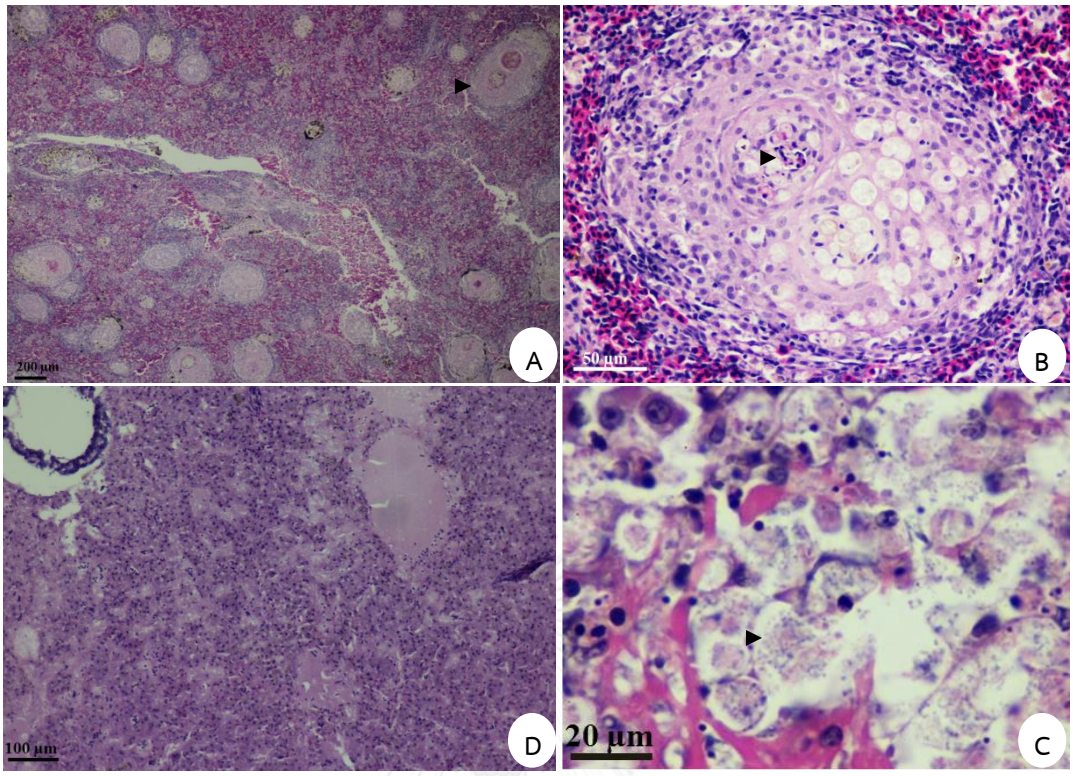


Figure 4: Haematoxylin and eosin stained sections of the spleen of infected red tilapia show multiple granulomatous lesions (head arrow) (A). Typical granuloma contains necrotic material (head arrow) surrounded by epithelioid macrophages, enclosed by fibrous capsule (B). *Fno* was intra and extracellular at granuloma with high magnificent (C). Normal spleen of control fish revealed parenchyma and white and red pump (D).

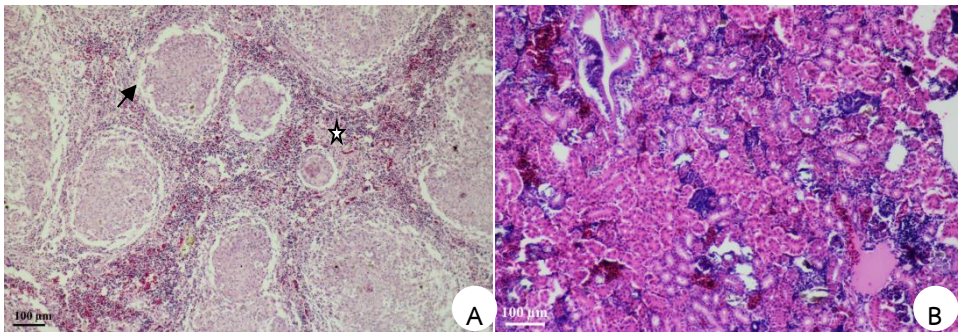


Figure 5: A) Histological lesions in kidney of red tilapia challenged with *Fno* displayed granulomas (arrow), inflammatory cells replaced haemopoietic tissue (asterisk). B) Kidney of control fish exhibited normal renal tubules and haematopoietic tissues. H&E stain.

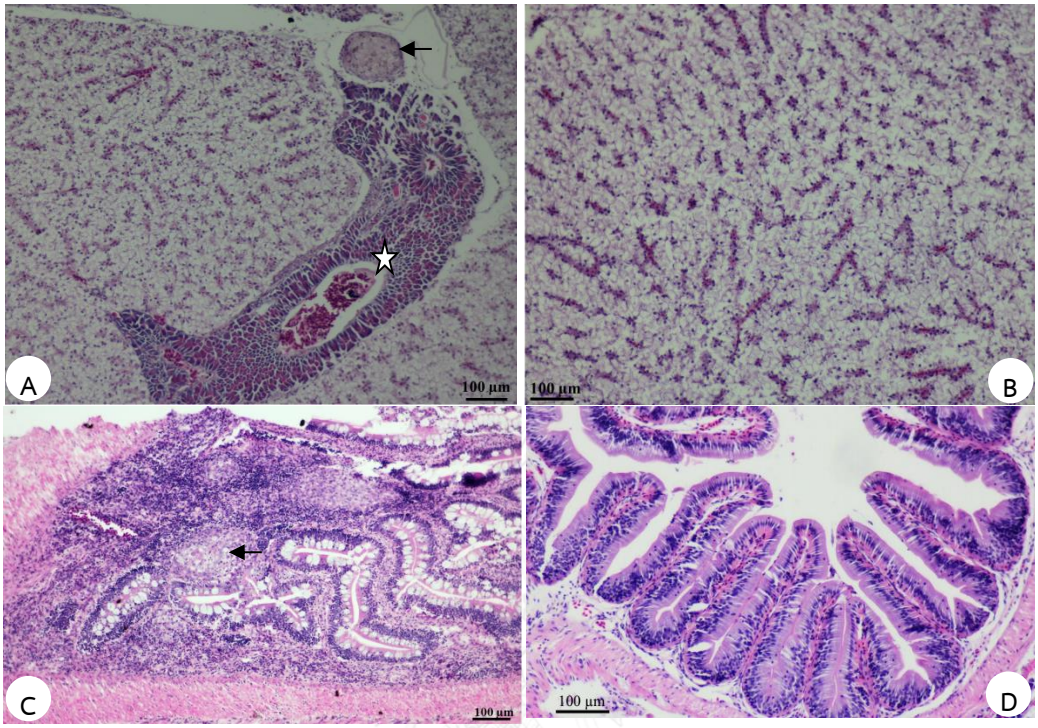


Figure 6: A) Liver of infected fish showed granuloma formation (arrow) and severe vasculitis with fibrin thrombus (asterisk). B) Normal liver tissue of control fish. C) Infiltration of inflammatory cells and granulomas in mucosa of intestine (arrow). D) Normal intestine. H&E stain.

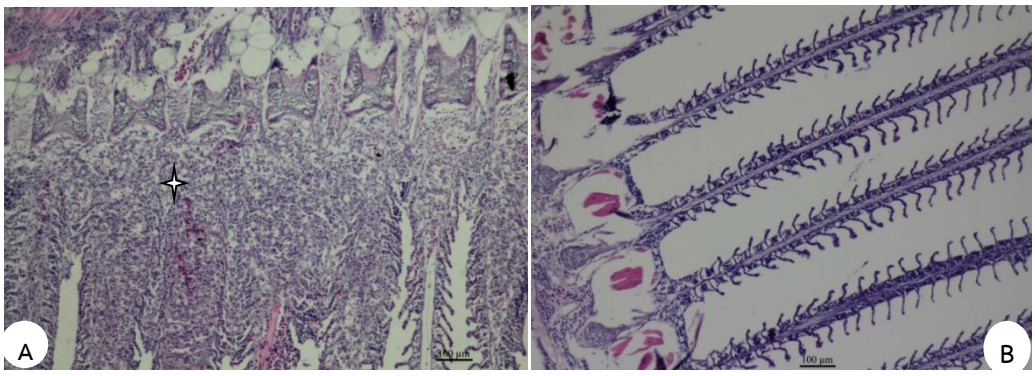


Figure 7: A) Primary and secondary gill lamella of infected red tilapia presented hyperplasia and fusion, and granulomas inflammation at basal of gill arch. B) Normal gill of control fish. H&E stain.

During the experiment, *Francisella noatunensis* subsp. *orientalis* was recovered from dead and survivor fish. These isolates were confirmed by PCR analysis using *Francisella* genus primer (Figure 14). However there was only 40% (12/30) success in re-isolation bacteria from infected fish although they were positive with PCR assay (Figure 14B). In contrast, control fish were negative with PCR assay and none bacteria was found.

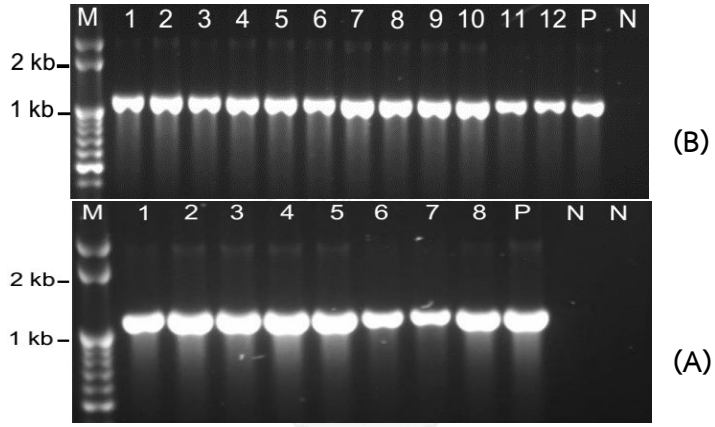


Figure 8: A) PCR assayexperiment red tilapia using *Francisella* sp. genus primer. Lanes M: DNA marker; 1-8: challenge fish; P: positive control; N: control fish. B) PCR amplification of *Fno* isolates recovered from challenge red tilapia. Lans: M: DNA marker; 1-12: *Fno* isolates; P: positive control; N: negative control

3. Virulence assay of strain VMCU-FNO131 in red tilapia

In order to determine median lethal dose (LD_{50}) of red tilapia infected with strain VMCU-FNO131, healthy fingerling red tilapia were challenged by injection and immersion route. Base on the cumulative mortality of red tilapia (Figure 15), for immersion route, only dose 7×10^6 CFU mL^{-1} water caused 25% mortality, for i.p route, percentage of dead fish reached 50% at day 8 and 12 in dose 2.88×10^5, 2.88×10^4 CFU per fish, respectively. Mortality increased gradually in remaining doses and gained 30% at end of trial. No dead fish was observed in control group. Water quality parameters during the experiment were acceptable ranges for cultured tilapia in fresh

water (total ammonia reading: 0.4 ± 0.1 mgl^{-1}, Dissolved Oxygen: 6 mgl^{-1} at pH of 7, temperature: 28.5 ± 0.5 ^{0}C) (Popma and Masser, 1999).

From experiment data, construction of general linear model for two factors time and bacterial density was performed by regression analysis application of Statistical Package for the Social Sciences (SPSS) programme (version 20.0). We used the statistical method to analyse the dose that could cause lethal dose of 50% at day 12 post immersion. The analysis showed that LD_{50} of VMCU-FNO131 strain was $10^{4.}$ CFU per fish at day 12 post i.p (R^2=0.934) (Figure 16).

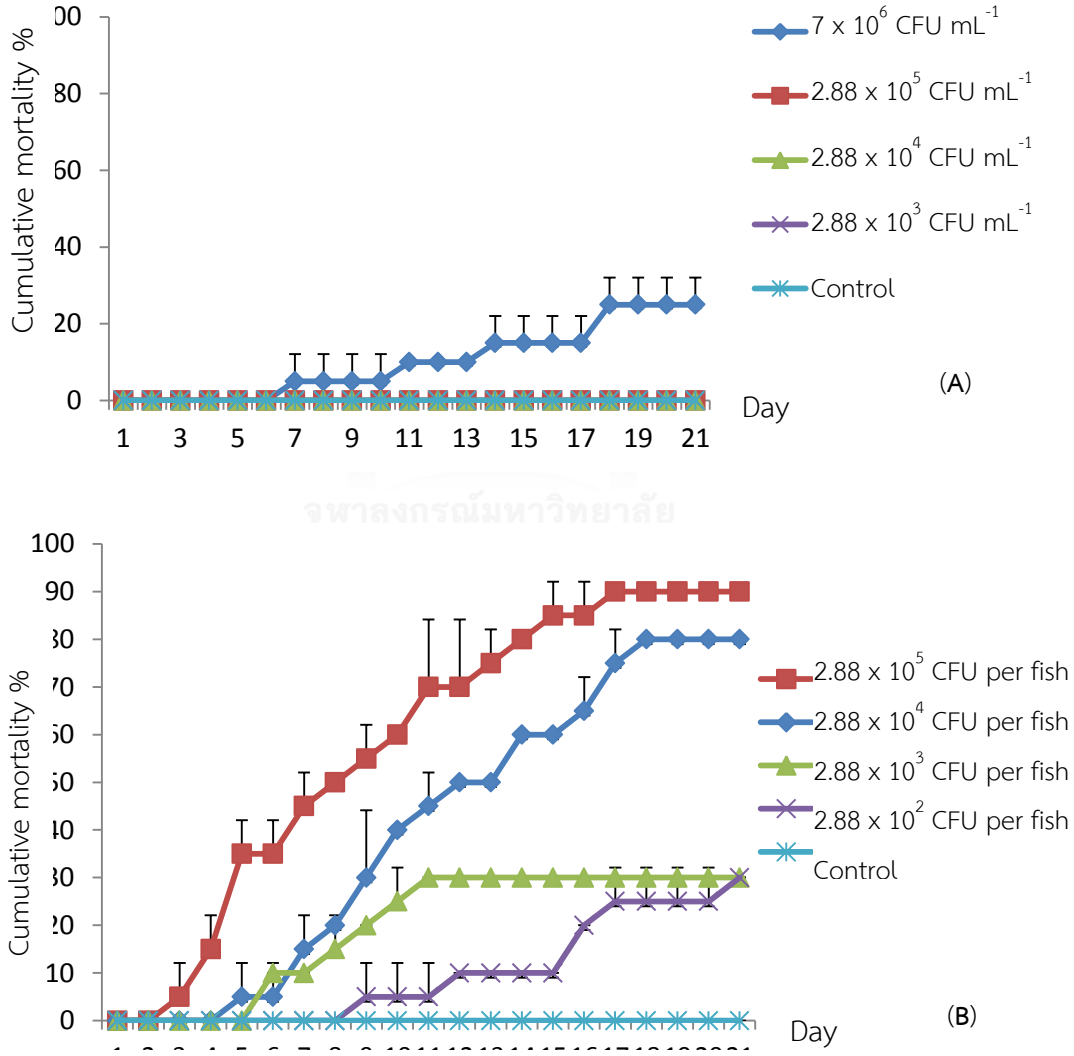
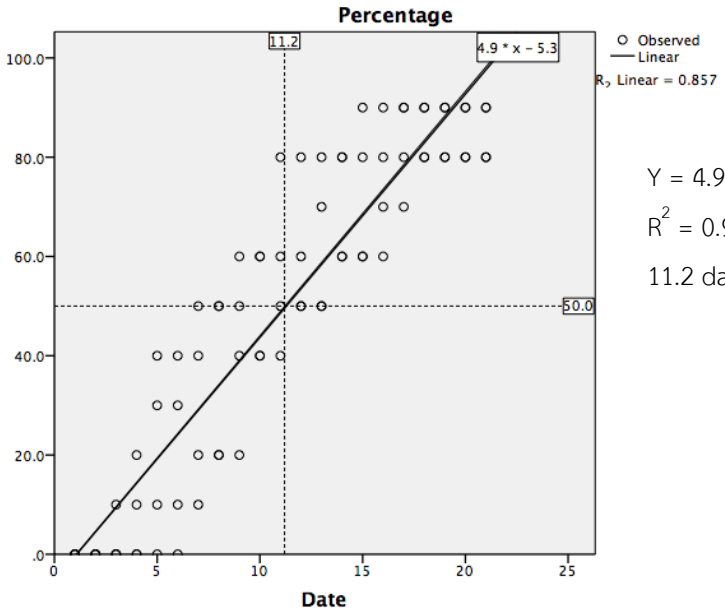


Figure 9 Cumulative mortality of red tilapia during 21 days challenged with different doses of *Fno* strain VMCU-FNO1313 via immersion route (**A**) and i.p route (**B**).



Y = 4.925*a + 17.857*b – 103.464

R^2 = 0.934

11.2 day LD_{50} = $10^{4.5}$ CFU per fish

Figure 10: Evaluation of 11.2 day LD_{50} concentration of bacteria using data from Figure 15 and determined from linear regressive equation of densities of 10^4 and 10^5 CFU per fish.

Regarding symptom, only dead fish challenged with dosage of 10^4 and 10^5 CFU per fish exhibited typical symptoms as natural disease fish as mention above, while survived fish and the fish in other groups did not reveal any notable signs at end of trial. According to pathological changes, spleen tissues squash of all infected fish showed numerous granulomas as Figure 8 A. Granuloma formation of francisellosis were observed in infected fish from all groups as described above, although level of damage was different in each group (Table 1). Despite the positive PCR results in all survived fish, bacteria was be able to re-isolate only from i.p challenged group and with high dose (10^5 and 10^6 CFU mL^{-1}) of immersion (Figure 17).

Table 1: Summary of *Fno* VMCU-FNO131 virulence assay in red tilapia

Trial dose	Cumulative mortality (%)	Number (%) of bacteria recovered from dead fish	Number (%) of bacteria recovered from survivors	PCR test for survivors (%)	Mean number of granulomas in 10X microscopic field		
					Head kidney	Spleen	Liver
Intraperitoneal injection (CFU per fish)							
2.88×10^5	90	3 (75)	1 (100)	100	Severe	Severe	Mild
2.88×10^4	80	3 (75)	2 (100)	100	Severe	Severe	Mild
2.88×10^3	30	2 (67)	2 (50)	100	Moderate	Severe	Mild
2.88×10^2	30	2 (67)	-	100	Moderate	Moderate	Mild
Control	0	0	-	-	-	-	-
Immersion (CFU/ml of tank water)							
7×10^6	25	1 (50)	3 (60)	100	Moderate	Severe	Mild
2.88×10^5	0	N/A	2 (40)	100	Moderate	Severe	Mild
2.88×10^4	0	N/A	-	100	Moderate	Moderate	Mild
2.88×10^3	0	N/A	-	100	Moderate	Moderate	Mild
Control	0	N/A	-	-	-	-	-

Notes: -, negative; N/A, not applicable

Mild, 1-6 granuloma; Moderate, 7-20; Severe, > 20

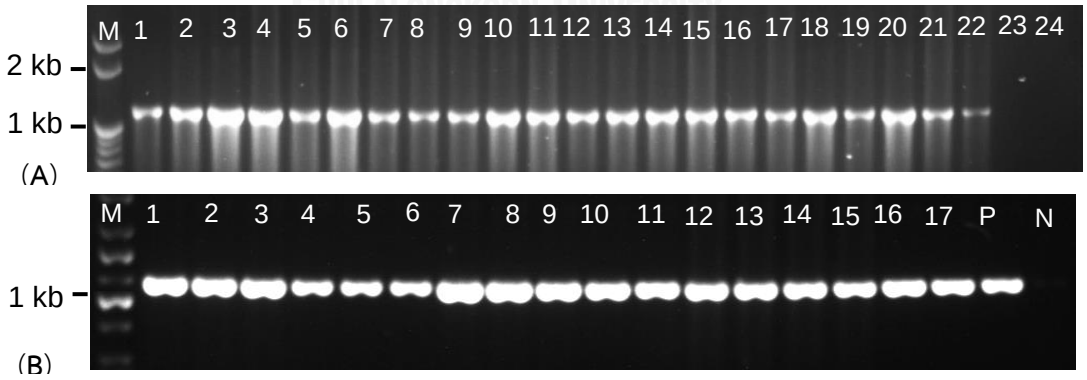


Figure 11 A) PCR detection in challenged red tilapia using *Francisella* genus primer. Lanes: 1-10: infected fish in injection group; 11-21: infected fish in immersion group; P: positive control; N: DNA extracted from control fish. B) PCR amplification of *Fno* recovered from challenged fish. Lanes: M: DNA marker; 1-14: isolates recovered from injection groups; 15-17: isolates recovered from dose 7×10^6 CFU mL^{-1} of immersion groups.

CHAPTER V

DISCUSSION

During last decade, francisellosis in warm water fish caused by *Francisella noatuenensis* subsp. *orientalis* has been prominent as an important emerging disease (Colquhoun and Duodu, 2011). Although the report of this disease is increasing in many continents but the information of it in Southeast Asia is still limited. In Thailand, Francisella bacteria has detected by PCR test in cultured tilapia since 2009 (Visanu et al., 2011; Chanagun et al., 2014). However it has not been proved as the aetiological agent of francisellosis. Due to the present study is the first report confirming that *Francisella noatunensis* subsp *orientalis* is the causative agent of visceral granulomas in red tilapia in Thailand.

Similarly to the description of previous researches, contaminate bacteria such as *Flavobacterium columnare, Aeromonas* sp., *Streptococus* sp. could be isolated from the fish naturally infected with Francisella bacteria (Mauel et al., 2007; Soto et al., 2009c). While the Francisella bacteria grows slowly on cultured media as early visible colonies were observed at day three. There is a potential problem that inoculated plates maybe overgrown with contaminating organisms and produce a false negative.

Despite all of infected fish were positive with PCR assay using *Francisella* genus primer but bacterial isolation was failure in the immersion challenge groups which did not observe dead fish. It agrees with previous research that culturing the bacteria from infected tissues is difficult (Jeffery et al., 2010; Camus et al., 2013). Interestingly, the bacteria usually stick together on inoculate plate. Even more surprisingly the highest number of bacteria calculated on CHAB was 7×10^{8} CFU mL^{-1} when suspending bacteria cells which were harvested immediately on CHAB after incubation at 29 ^{0}C for 24 hours. It demonstrated the fastidious nature of Francisella bacteria.

Another similar finding between present study and the previous reports was infected fish displayed white nodules on various organs, particularly the head kidney,

spleen, sometime on gill, liver, intestine; resulted in granulomas formation in histological change. Moreover large number of bacteria was found in macrophage of infected fish (Hsieh et al., 2006; Mauel et al., 2007; Soto et al., 2009c). It was demonstrated in previous research that *Fno* was able to survive and replicate in macrophage of tilapia prior kidney by in vitro assays. The bacteria was found in vacuole of macrophage at 2 h post infection and increased rapidly the number, some bacteria moved to the cytoplasm at 12 h post infection (Soto et al., 2010). According to Soto *et al.* (2013), the number of *Fno*, as well as number of granulomas in spleen and kidney was significantly higher than other organs after 96 h post infection. Additionally, granuloma formation was first present in spleen and kidney, subsequently in intestine, liver.

In immersion route, the fish exhibited no special clinical signs while gross lesions of enlarged kidney, spleen and liver were appeared but no appearance of white nodules. Comparing with i.p challenge, immersion challenge was able to induce the similar macroscopic and histological lesions. It suggests that survivor fish could expose healthy appearance. However dramatic damage in haematopoietic and lymphoid tissues of spleen and head kidney could make them more susceptible to other common fish disease such as columnaris, streptococcosis. As in nature, we suppose that these fish may become reservoir. Base on the result of study, we propose that presence of granulomas in spleen tissue squash preparation, visualization of bacteria in the impression smear of spleen, head kidney tissues staining Giemsa, combining with typical clinical signs can be considered as presumptive diagnosis. However granuloma can be able to find in other diseases such as Edwardsiellosis, Mycobacteriosis, Nocardiosis, hence non-careful interpretation of granulomatous lesion might leads to misidentification (Wolf and Smith, 1999; Wang et al., 2007; Soto et al., 2012). Therefore, molecular diagnostic method is still necessary for accurate diagnosis of the Franciseallosis.

According to the result of median lethal dose experiment, *Fno* VMCU-FNO131 exhibited high virulence to red tilapia via i.p route. However LD_{50} value could not calculate in immersion trial and the dose 7 x 10^6 CFU mL^{-1} just caused 25%

cumulative mortality on day 21. This result was consistent with previous study on Nile tilapia, Soto *et al.* (2009) found that only 1.2 x 10^4 CFU per fish was capable to induce 50% mortality on day 20 by i. p route while 2.3 x 10^7 CFU mL^{-1} was needed for immersion route at day 40. On another hand, experimental immersion challenge was conducted at temperature 28.5 ± 0.5 ^{0}C. It was reported that tilapia was more susceptible to francisellosis disease in cool temperature (lower 25 ^{0}C) comparing with high temperature (Mauel et al., 2003; Soto and Revan, 2012). Therefore high water temperature could be a reasonable affected to mortality in the trial. Moreover immersion challenge demonstrated that red tilapia can infected *Fno* via waterborne.

In term of gross lesion, in i.p trial, with high dose (2.88 x 10^5 CFU per fish) disease symptoms occurred more acute, since most of dead fish did not reveal clinical sign and white nodules were present in only kidney and spleen. While with low dose (2.88 x 10^3 CFU per fish) displayed a chronic disease showing multifocal white nodules in most these organs as natural cases. However, dead fish in immersion route and survivors did not reveal white nodules in internal organs. It indicated that *Fno* can cause acute to chronic disease with few or non-specific clinical signs. This finding was consistent with description in previous studies by Mauel *et al*. (2007), Soto *et al*. (2009) in tilapia.

In conclusion, the present study had fulfilled the Koch's postulates to confirm that *Francisella noatunensis* subsp. *orientalis* is the causative agent of an emerging disease which resembled francisellosis in cultured red tilapia in Thailand.

Present study provides the bacterium strain *Francisella noatunensis* subsp. *orientalis* VMCU-FNO131 and its virulence information in Thai red tilapia. Moreover, the causative agent of putative francisellosis in red tilapia was declared. All these scientific information will be useful for further investigation to find out treatments and prevention methods to prevent and control this emergent disease in culture tilapia in Thailand.

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APPENDIX

Modified Cystine Heart Agar (Soto et al., 2009)

Beef Heart	10 g
Proteose Peptone	10 g
Dextrose	10 g
Sodium Chloride	5 g
L-Cystine	1 g
Agar	15 g
Sheep blood	100 ml
Distilled water	849 ml

Modified Tryptic Soy Broth (Birkbeck et al., 2011)

Enzymatic Digest of Casein	17 g
Enzymatic Digest of Soybean Meal	3 g
Sodium Chloride	5 g
Dipotassium Phosphate	2.5 g
Dextrose	2.5 g
D-glucose	10 g
Cysteine	2 g
Distilled water	958 ml

Final pH: 7.3 ± 0.2 at 25°C

Preservative formula (Birkbeck et al., 2011)

Sterile glycerol 60%	100 ml
Bacterial culture in Modified Tryptic Soy Broth	900 ml

TBE electrophoresis buffer (10X)

Tris base	108 g
Boric acid	55 g
EDTA (0.5 M)	40 ml
rNase-free H_20	100 ml

Prepare TBE buffer (1X) for gel running buffer. Add DNase-free H_20 to 100 mL (10X) to achieve 1 L (1X). Store for up to 6 month at room temperature

Phosphate buffered saline (1X)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO	1.44 g
KH_2PO_4	0.24 g
Distilled water	990 ml

Final pH: 7.4

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