รายงานวิจัยฉบับสมบูรณ์

ทุนส่งเสริมนักวิจัยรุ่นใหม่ในสถาบันอุดมศึกษา

โครงการ: บทบาทของการติดเชื้อเฮลิโคแบคเตอร์สปีชี่ส์ในโรคโอพิสทอร์เชียซีสวิเวอร์รินี่ที่สัมพันธ์กับโรคมะเร็งท่อน้ำดี

โดย ผศ.ดร.พรทิพย์ ปิ่นละออ และคณะฯ

28 ธันวาคม 2558

สัญญาเลขที่ TRG5680032

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สนับสนุนโดยสานักงานกองทุนสนับสนุนการวิจัย และ
มหาวิทยาลัยขอนแก่น

กิตติกรรมประกาศ

รายงานการวิจัยเรื่อง "*บทบาทของการติดเชื้อเฮลิโคแบคเตอร์สปีชี่ส์ในโรคโอพิสทอร์เชียซีสวิเวอร์รินี่ที่สัมพันธ์กับโรคมะเร็งท่อน้ำดี*" เป็นโครงการวิจัยต่อเนื่องสองปี โดยได้รับเงินทุนอุดหนุนการวิจัยร่วมกันจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว) สำนักงานคณะกรรมการการอุดมศึกษา (สกอ) และเงินอุดหนุนการวิจัยมหาวิทยาลัยขอนแก่น ข้าพเจ้าขอขอบคุณแหล่งทุนทุกๆแหล่งทุนที่ให้การสนับสนุนการวิจัยในครั้งนี้ ขอขอบคุณนางสาวอัปสรสวรรค์ อิทธิแต้ตระกูล และนางสาวรุ่งทิวา แดงตาโคตร นักศึกษาระดับป.โท สาขาชีวเวชศาสตร์ และสาขาเทคนิคการแพทย์ ซึ่งเป็นผู้ดำเนินการวิจัยหลักภายใต้การดูแลของข้าพเจ้า ขอขอบคุณศูนย์เครื่องมือกลาง มหาวิทยาลัยขอนแก่น ฝ่ายวิจัย คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น และภาควิชาปรสิตวิทยา คณะแพทยศาสตร์มหาวิทยาลัยขอนแก่น ที่ได้จัดสรรสถานที่และสนับสนุนวัสดุอุปกรณ์และเครื่องมือต่างๆในการทำวิจัยครั้งนี้ ขอขอบคุณหน่วยสัตว์ทดลอง คณะแพทยศาสตร์ และศูนย์วิจัยพยาธิใบไม้ตับและมะเร็งท่อน้ำดี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ที่ช่วยสนับสนุนในด้านการใช้เครื่องมือต่างๆ และสุดท้ายข้าพเจ้าขอขอบคุณกรมวิทยาศาสตร์การแพทย์ ที่ได้อนุเคราะห์เชื้อ *Helicobacter pylori* virulent strain สำหรับเป็นเชื้อมาตรฐานในการศึกษาในครั้งนี้ จนทำให้การศึกษาวิจัยสำเร็จลุล่วงไปได้ด้วยดี

พรทิพย์ ปิ่นละออ
หัวหน้าโครงการวิจัย

บทคัดย่อภาษาไทย

ระหัสโครงการ : TRG5680032

ชื่อโครงการ : บทบาทของการติดเชื้อเฮลิโคแบคเตอร์สปีชี่ส์ในโรคโอพิสทอร์เชียซีสวิเวอร์รินี่ที่สัมพันธ์กับโรคมะเร็งท่อน้ำดี

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ระยะเวลาโครงการ : 2 ปี ตั้งแต่ 15 มิถุนายน 2556 ถึง 14 มิถุนายน 2558

การติดพยาธิใบไม้ตับ (*Opisthorchis viverrini*) และแบคทีเรียรวมทั้งเชื้อเฮลิโคแบคเตอร์สปีชี่เป็นปัจจัยเสี่ยงต่อการเกิดโรคมะเร็งท่อน้ำดี อย่างไรก็ตามก็ยังไม่ทราบบทบาทการติดเชื้อร่วมกันว่ามันเกี่ยวข้องกับการเป็นปัจจัยเสี่ยงมะเร็งท่อน้ำดีได้อย่างไร การศึกษานี้มีวัตถุประสงค์เพื่อ 1) วินิจฉัยชนิดของการติดเชื้อแบคทีเรียในโรคพยาธิใบไม้ตับเรื้อรังในหนูแฮมสเตอร์ และเพื่อ 2) ตรวจหาผลของการติดเชื้อร่วมกันของ *Helicobacter pylori* และพยาธิใบไม้ตับ (*O. viverrini*) ต่อการเปลี่ยนแปลงของระบบทางเดินน้ำดี (1) แบคทีเรียลจีโนมิกดีเอ็นเอได้ถูกตรวจในตับหนูแฮมสเตอร์ที่ติดพยาธิใบไม้ตับ หลังการติดพยาธิที่ 8, 12 และ 15 เดือน โดยวิธีเมตาจีโนมิกส์ ผลการทดลองพบว่าแบคทีเรียที่พบในหนูกลุ่มที่ติดพยาธิเรื้อรัง เชื้อที่พบส่วนใหญ่คือ *Escherichia coli* (10.18%), *Streptococcus luteciae* (10.76%), *Bifidobacterium* spp. (0.58%) และ *Fusobacterium* spp. (13.81%) ซึ่งพบทั้งชนิดและจำนวนมากกว่าหนูปกติ นอกจากนี้ยังตรวจพบจีโนมิกส์แบคทีเรีย *Helicobacter pylori* และ *Helicobacter* spp. ร้อยละ 0.17 และ 0.82 ตามลำดับ และการพบเชื้อ *H. pylori* ในตับได้ถูกตรวจยืนยันด้วยวิธีอิมมูโนฮีสโตเคมี (2) เชื้อ *H. pylori* ได้ถูกติดเชื้อร่วมกับการติดพยาธิใบไม้ตับ แล้วตรวจหาดีเอ็นเอของ *H. pylori* ในกระเพาะ ถุงน้ำดี และตับ สัมพันธ์กับการเปลี่ยนแปลงของพยาธิสภาพของระบบทางเดินน้ำดี ได้แก่ พังผืดรอบท่อน้ำดี เซลล์อักเสบ และการอักเสบของท่อน้ำดี เปรียบเทียบกับกลุ่มที่ติดเชื้อ *H. pylori* หรือ *O. viverrini* เพียงอย่างเดียว ผลการทดลองพบว่าตรวจพบดีเอ็นเอของเชื้อ *H. pylori* ในกลุ่มหนูปกติ กลุ่มที่ติดเชื้อแบคทีเรีย กลุ่มที่ติดพยาธิ หรือกลุ่มที่ติดเชื้อร่วมกัน ในกระเพาะคือร้อยละ 20, 40, 50 และ 62.5 พบในถุงน้ำดีคือร้อยละ 0, 0, 12.50 และ 12.50 และพบในตับคือร้อยละ 0, 40, 25 และ 50 ตามลำดับ ในหนูกลุ่มที่ติดเชื้อร่วมกันโดยเฉพาะในหนูตัวที่ตรวจพบดีเอ็นเอของ *H. pylori* ในเนื้อตับ ตรวจพบการอักเสบของท่อน้ำดี เซลล์อักเสบ และพังผืดรอบท่อน้ำดี เพิ่มมากขึ้นเมื่อเปรียบเทียบกับหนูกลุ่มอื่นๆ ซึ่งคล้ายกับผลของการตรวจระดับเอ็นไซม์ ALT และ AST ในซีรัม สรุป การติดเชื้อ *O. viverrini* ส่งเสริมการเจริญของแบคทีเรียเพิ่มขึ้นในตับและเมื่อติดเชื้อร่วมกับ *H. pylori* เพิ่มความรุนแรงของโรคระบบทางเดินน้ำดีในหนูทดลอง ซึ่งอาจมีประโยชน์ในการรักษาต่อไป

คำสำคัญ: พยาธิใบไม้ตับออพิสทอคีสไวเวอร์รินี่, การติดเชื้อเฮลิโคแบคเตอร์สปีชี่ส์, พังผืดรอบท่อน้ำดี, การวิเคราะห์เมตาจีโนมิกส์, โรคพยาธิใบไม้ตับเรื้อรัง

Abstract

Project Code : TRG5680032

Project Title : Role of *Helicobacter* spp. infection in opisthorchiasis *viverrini*-associated cholangiocarcinoma

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Project Period : 2 years, from 15 June 2013 to 14 June 2015

Infection with *Opisthorchis viverrini* and bacteria including *Helicobacter* spp. are risk factor for cholangiocarcinoma (CCA). However, their role in co-infection contribution risk to CCA is unclear. The aim of this study was to i) identify *Helicobacter* spp. and other bacterial infection in chronic opisthorchiasis in hamsters, and ii) investigate the effect of co-infection of *Opisthorchis viverrini* (OV) and *H. pylori* (HP) on the alteration of hepatobiliary system. (1) Bacterial genomic DNA was investigated in hamster liver infected with *O. viverrini* at 8, 12 and 15 months post-infection using metagenomics analysis. The results revealed that in chronic OV-infected group, the most common of bacteria were *Escherichia coli* (10.18%), *Streptococcus luteciae* (10.76%), *Bifidobacterium* spp. (0.58%), *Fusobacterium* spp (13.81%), which were higher in the population and the frequently of bacteria spp. compared to the normal group. Genomic DNA of *Helicobacter pylori* (0.17%) and *Helicobacter* spp. (0.82%) were also found and the presence of *H. pylori* in the liver was confirmed by immunohistochemistry. (2) *H. pylori* was co-infected with *O. viverrini*, genomic DNA of *H. pylori* was investigated in the gastric, gall bladder and liver in relation to the histopathological changes i.e., periductal fibrosis and cholangitis compared to either *H. pylori* or *O. viverrini* infection alone. The results revealed that identification of *H. pylori* DNA in the normal, HP, OV and HP+OV groups for the gastric were 20%, 40%, 50% and 62.5%; in the gall bladder were 0%, 0%, 12.50% and 12.50%; and in the liver were 0%, 40%, 25% and 50%, respectively. In HP+OV groups, especially in *H. pylori* DNA positive in liver, the histopathological changes including cholangitis, inflammatory cells and periductal fibrosis were higher than in other groups, which was similar to the level of ALT and AST in the serum. Indeed, *O. viverrini* infection enhances the population of bacterial growth in the liver and in co-infection with *H. pylori* increases the severity of hepatobiliary diseases in experimental opisthorchiasis which may be useful for a therapeutic approach outcome.

Keywords: *Opisthorchis viverrini*, *Helicobacter* species infection, Periductal fibrosis, Metagenomic analysis, Chronic opisthorchiasis

Executive summary

Opisthorchiasis caused by *Opisthorchis viverrini* (*O. viverrini*) infection is a major health problem in Thailand. An approximately 6 million people (or around 9.4%) are infected with this carcinogenic parasite. The prevalence of *O. viverrini* infection is the highest in northeastern Thailand and is correlated with a high incidence rate of CCA. Liver cancer including CCA is a leading cause of death in Thailand with an approximately 28,000 cases per year (around 80 cases per day), but CCA is rare in the western countries. Several epidemiological studies and research on animal model supported the linkage between *O. viverrini* infection and CCA. Based on these findings, many control programs for opisthorchiasis have been operating in the endemic communities. The prevalence of *O. viverrini* infection tends to decline from 35.6% in 1988 to 9.4% in 2001. However, the incidence of CCA is still high with 115 and 52.7 cases per 100,000 population for males and females, respectively. According to the surgeons (personal communication), most of the CCA patients nowadays didn't have any liver fluke insides the biliary tree. From these evidences, we assume that there are not only *O. viverrini*-caused CCA but others unknown causative agents such as bacteria may also participate in cholangiocarcinogenesis. This idea is supported by a recently finding demonstrated that Helicobacter pylori (*H. pylori*) DNA was identified in CCA patients in Thailand. Recently, seropositivity to *H. bilis* and *H. pylori* is a high risk factor of developing CCA in a population in Thailand. Bacterial cholangitis due to Helicobacter spp. infection is associated with the histopathological changes such as inflammation, chronic hepatitis, hepatic dysplasia, fibrosis and biliary hyperplasia in normal hamsters. In addition, several aerobic bacteria have also been identified in hepatobiliary tract of human and animal. These aerobic bacteria that commonly cause cholangitis are in genus *Escherichia*, *Klebsiella*, *Enterococcus*, *Enterobacter*, and *Pseudomonas*. The histopathological features due to bacteria infection are similar to *O. vivverri* infection in animal and human. Therefore, it remains unclear whether other causative agent such as aerobic and anaerobic bacteria (i.e. *Helicobacter* spp.) involving in *O. viverrini*-induced CCA development. The outcome of the study may be useful for the development of a new therapeutic approach to reduce the incidence of CCA.

The research contents

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1. Objectives

1.1 To identify *Helicobacter* spp. and other bacterial infection in chronic opisthorchiasis in hamsters using metagenomics analysis.

1.2 To investigate the effect of co-infection of *H. pylori* and *O. viverrini* infections on the alteration of hepatobiliary diseases in hamsters model.

2. Research methodology

2.1 Experimental design

Experiment 1 Study in Syrian golden hamster

Twenty-six male Syrian golden hamsters (*Mesocricetus auratus*) were obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University and aged between 4-6 weeks were used in this study and were house under conventional condition and given water *ad libitum*. The Animal Ethics Committee of Khon Kaen University (AEKKU 63/2556) approved this study. Hamsters were divided into 2 groups.

Table 1 Animal model for experiment 1.

Animal groups	Period time (No. of each group)		
	8 months	12 months	15 months
Group 1 : Normal hamsters (control group)	5	-	7
Group 2 : Normal hamsters + *O. viverrini*	5	1	8

Experiment 2 Study in Syrian golden hamster

Forty male Syrian golden hamsters (obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University) aged between 4-6 weeks were used in this study. The animals were divided into 4 groups.

Table 2 Animal model for experiment 2.

Animal groups	**Period time (6 month)** **(No. of each group)**
Group 1 : Normal hamsters (control group)	10
Group 2 : Normal hamsters + *H. pylori* (Hp group)	10
Group 3 : Normal hamsters + *O. viverrini* (Ov group)	10
Group 4 : Normal hamsters+ *H. pylori* + *O. viverrini* (Hp+Ov group)	10
Total	40

- For control group, hamsters were inoculated orally with phosphate buffer saline (PBS) alone.
- For the *O. viverrini*-infected group, hamsters were infected with 50 metacercaria of *O. viverrini* by oral inoculation.
- For the *H. pylori*-infected group, hamsters were infected with 0.5 ml of *H. pylori* virulent strain containing 10^9 CFU/ml in phosphate buffer saline (PBS) by oral inoculation.
- For the *H. pylori* + *O. viverrini*-infected group, hamsters were infected with 50 metacercaria of *O. viverrini* after infected by *H. pylori* for 1 week and 1 month after that, hamsters were re-infected with *H. pylori* and sacrificed at 6 months post infection as shown in figure 1.

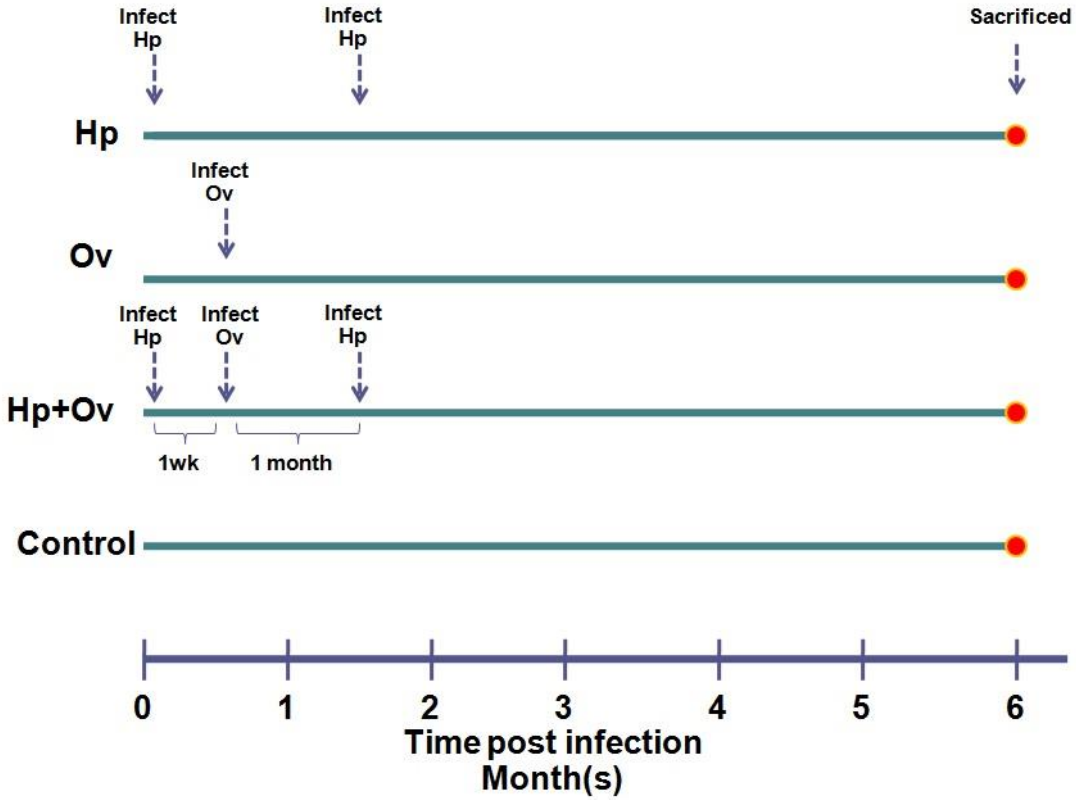


Figure 1 Experimental study in Syrian golden hamster. Hp = *Helicobacter pylori*, Ov = *Opisthorchis viverrini*, Hp + Ov = *H. pylori* plus *O. viverrini*.

2.2 Preparation of *O. viverrini* metacercariae

For experiment 1 and experiment 2, the *O. viverrini* metacercaria were isolated from the naturally infected cyprinoid fishes by artificial pepsin digestion. The cyprinoid fishes were collected from endemic areas. The fish was minced in electric blender in 0.25% Pepsin A (BDH, USA) solution. Fish: pepsin A solution is 1:3 by volume. The mixture was incubated at 37 °C in continuous stirring water bath for 1 hour followed by straining through a set of four sieves 650, 300, 250 and 106 micrometers apertures, respectively. The remainder on the 106 µm apertures were washed with NSS (0.85% NaCl) and strained through 250 micrometers apertures. Finally, the filtrated mixture was washed several times with NSS in a sedimentation jar until the supernatant is clear. The supernatant was poured off and the sediments are taken to examine for the *O. viverrini* metacercaria, an infective stage, under a dissecting microscope.

2.3 Preparation of *Helicobacter pylori*

The *H. pylori* (LMG 8775 DMST 20165 type strain) were used in experimental 2. *H. pylori* were grown on 10% sheep blood agar and incubated at 37 ˚C for 48-72 hours under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2). The bacterial were grown on the plates, and tested for catalase, oxidase, urease and gram strain. Next, *H. pylori* were sub-cultured in brucella broth with 10% fetal bovine serum and incubated at 37°C for 24 hours under microaerobic conditions in incubator shaker. Then, *H. pylori* was suspended in phosphate-buffered saline (PBS) to an optical density at 600 nm of 1.000 OD (~10^9 colony-forming units) and then was tested for urease, catalase, and oxidase test.

2.4 Animals infection

Metacercariae : For experiment 1 and experiment 2, fifty metacercariae of *O. viverrini* were given per hamster via gastric or stomach intubation. A gastric tube was gently inserted through the oral cavity into the esophagus until reaching a stomach. A syringe contained 50 metacercariae in approximately 1 ml of NSS connecting to a blunted end needle was then carefully delivered.

H. pylori **:** For experiment 2, hamsters were infected with 0.5 ml *H. pylori* suspended in PBS to an optical density at 600 nm of 1.000 OD (~10^9 colony-forming units). A gastric tube was gently inserted through the oral cavity into the esophagus until reaching a stomach.

2.5 Animals scarification

Hamster in each experiment group was sacrificed at an experimental design according the guideline for the euthanasia of animal under ether anesthesia.

For experiment 1, After 8, 12 and 15 months the period of experiment, hamsters were anesthetized with ether and then liver tissues and worms were collected. For metagenomic analysis and cultivation, the liver and worm were immediately collected in thioglycollate broth supplemented with 20% fetal bovine serum (enhanced aerobic & anaerobic bacterial growth) and brucella broth supplemented with 10% fetal bovine serum + 3.5 mM H_2O_2 (enhanced *Helicobacter* species growth) for amplified with

prokaryotic 16S rDNA (V3-V4 region) and genus specific of *Helicobacter* by polymerase chain reaction (PCR) respectively and then the livers were immediately treated with liquid nitrogen and then store at -20°C until analysis. For histopathologycal study and immunohistochemistry, livers were fixed in 10% buffered formalin.

For experiment 2, four ml of blood samples were taken from plexus and put in test tube. After that, they were centrifuged at 3000 rpm for 10 minutes at 4°C, then plasma were separated and kept at -20°C until assayed for analysis. Gastric, liver and gallbladder were collected in 10% buffer formalin and in test tube which were snapped by liquid nitrogen. The samples were kept at -20°C**.**

2.6 Bacterial cultivation

For experiment 1 of aerobic bacterial identification: After the end of experiment, liver were collected immediately in thioglycollate supplemented with 20% fetal bovine serum broth with sterile technique. Liver was excised and homogenated with sterile buffer and incubated at 37°C. After thioglycollate broth turbid (at least 3 days), bacteria were identified based on traditional cultivation method. Several biochemical tests were used to identify genus and species specific.

2.7 DNA extraction

For experiment 1: DNA was extracted from the livers tissue and/or cultured specimens by using a High Pure PCR template penetration kit (Qiagen, Germany).

For cultured specimens, bacteria isolated from the liver and worm were enhanced bacterial growth in thioglycollate broth supplemented with 20% fetal bovine serum and brucella broth supplemented with 10% fetal bovine serum + 3.5 mM H_2O_2 and centrifuged at 12,000 g for 25 minutes at 4°C ,then discard supernatant. The bacterial pellet was washed with sterile PBS buffer and bacterial DNA was extracted with a QiAmp Tissue kit (Qiagen, Germany).

For experiment 2 : gastric, liver and gallbladder were cut intimately into 25 mg and place on a 1.5 ml microcentrifuge tube. Then, 180 µl ATL and 20 µl proteinase K were added and mixed by votexing before it was incubated at 56˚C until completely lysed. After that, 200 µl buffer AL and 200µl absolute ethanol were added and mix by votexing again. The mixture was moved into column. Following by centrifuge at 8,000

rpm for 1 minute and discard the flow-through. Then, the spin column was transferred to a new centrifuge tube. The DNA was eluted by adding 200 µl buffer AE and incubated for 1 minute at room temperature. Finally, it was centrifuged at 8,000 rpm for 1 minute. DNA yield was collected and measured concentration by using Nanodrop.

2.8 PCR assay

DNA extracted from **experiment 1** was amplified by using *Helicobacter* genus-specific 16S rDNA primers, *H. pylori* ureA primer and v3-v416S rDNA prokaryotic primers. DNA extracted from **experiment 2** was amplified by *Helicobacter* genus-specific 16S rDNA primers and *H. pylori* ureA primer as shown in table 3.

Table 3 List of primers and conditions of PCR reaction used in this study.

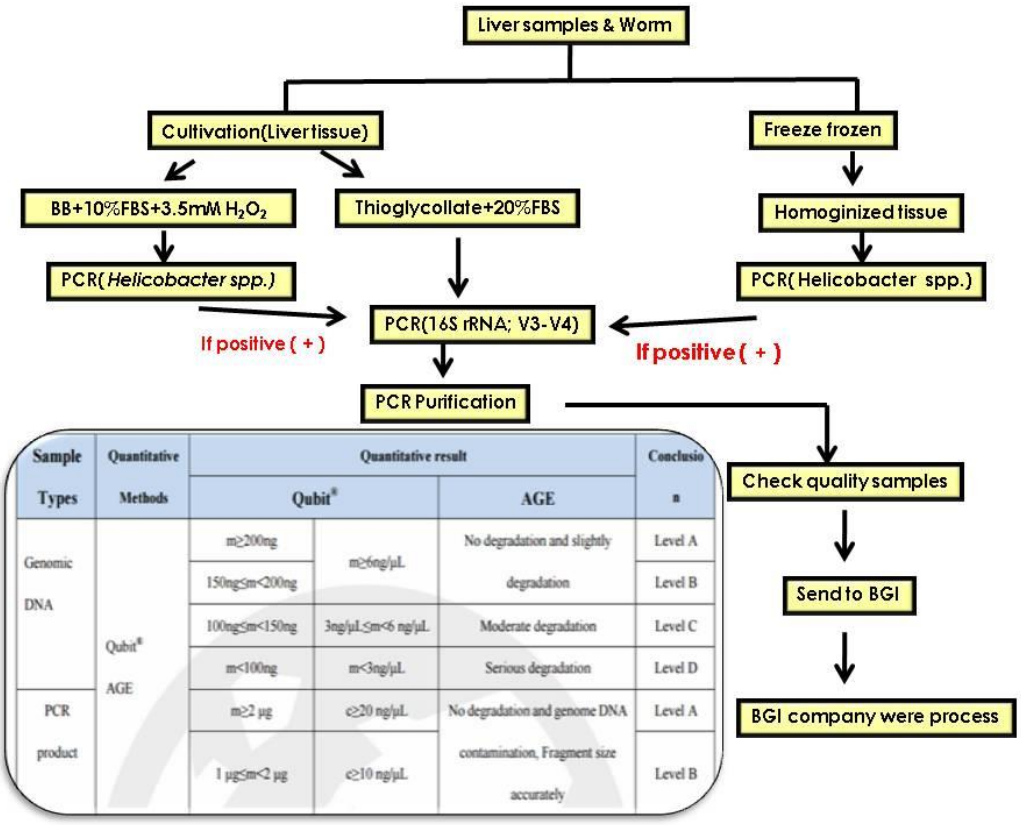
Sequence(5'-3')	Organism	Gene	Cycling conditions	Amplicon size (bp)
5'CCTACGGGNGGCWGCAG3' 5'TACNVGGGTATCTAATCC3'	Prokaryote bacteria	16S rDNA (V3-V4)	94°C 5 min, 94°C 40 sec, 52.8°C 30 sec, 72°C 2 min, 35 cycles, 72°C 10 min	459
5'GCTATGACGGGTATCC3' 5'GATTTTACCCCTACACCA3'	*Helicobacter* genus	16S rDNA	94°C 5 min, 94°C 1 min, 57°C 1.5 min, 72°C 1 min, 35 cycles, 72°C 7 min	411

5′AGTTCCTGGTGAGTTCTTAA3′ 5′AACCACGCTCTTTAGCTCTGTC3′	*H. pylori*	*ureA*	94°C 2 min, 94°C 30 sec, 55.7°C 30 sec, 72°C 1 min, 40 cycles, 72°C 5 min	350

2.9 Metagenomic analysis

2.9.1 Bacterial cultivation and preparation samples for metagenomic analysis

The liver tissue and bacterial isolation in thioglycollate supplemented with 20% fetal bovine serum broth and brucella broth supplemented with 10% fetal bovine serum + 3.5 mM H_2O_2 for enhanced bacterial growth. After that, bacterial genomic DNA were extracted when bacteria growth at least 3 days of cultivation method and then the V3-V4 regions16S rDNA gene was amplified by using specific primer. After that, the PCR product was purified by using clean up PCR purification kit (GeneJET PCR Purification kit, Thermo Scientific). After purification part, DNA was checked quality of samples including concentration and no degradation of DNA by running in gel electrophoresis. Finally, PCR products were sent to the BGI company for processing the next generation sequencing (NGS) was shown in figure 2.



Sample Types	Quantitative Methods	Quantitative result			Conclusion
		Qubit®		AGE	
Genomic DNA	Qubit® AGE	m≥200ng	m≥6ng/μL	No degradation and slightly degradation	Level A
		150ng≤m<200ng			Level B
		100ng≤m<150ng	3ng/μL≤m<6 ng/μL	Moderate degradation	Level C
		m<100ng	m<3ng/μL	Serious degradation	Level D
PCR product		m≥2 μg	c≥20 ng/μL	No degradation and genome DNA contamination, Fragment size accurately	Level A
		1 μg≤m<2 μg	c≥10 ng/μL		Level B

Figure 2 Work flow of PCR technique for metagenomic analysis.

2.9.2 Metagenomic analysis coupled with next generation sequencing using V3-V4 hypervariable region of prokaryotic 16S rDNA

PCR products were sequenced by next generation sequencing (Illumina HiSeq/MiSeq platform) for the metagenomic study in chronic opisthorchiasis. After that, the tags or sequences were clustered to OTU(Operational Taxonomics Unit) using scripts of software USEARCH(v7.0.1090). Then OTU representative sequences were classified to taxonomic levels using Ribosomal Database Project(RDP) Classifier v.2.2 trained on the Greengenes database(0.8 confidence values as cutoff). The final result, OTUs were analyzed a data and represented in terms of presence/absence, abundance, or phylogenetic diversity

2.10 Histopathological studied by H&E staining

To study cholangitis inflammatory cells, the section were incubated at 60 ˚C for 30 minutes and deparaffinized in xylene 5 minutes (three times) to remove the paraffin wax. Then, the sections were rehydrated with absolute alcohol for 3 minutes (two times),

95% alcohol for 3 minutes (two times), 80% alcohol for 3 minutes (two times), finally they were washes by rinsing in the tap water for 1 minute. After that, the sections were stained with Mayer's hematoxylin for 5 minutes and wash in running tap water for 1 minute. Fallowing, tissues were destained in acid alcohol (1% acid in 70% alcohol), washed in running tap water and stained in blue in saturated lithium carbonate for 3-4 seconds. Next, tissues were washed again for 10-20 minutes and stained with eosin solution for 15-20 seconds. The sections were dehydrated and mounted. The appropriate result for nuclei was stained blue and cytoplasm, collagen fiber was stained in red color as shown in Experiment 1 and 2 results.

2.11 Immunohistochemistry for *H. pylori*

H. pylori in gastric and liver section were assessed by immunostaining, the paraffin sections were deparaffinized in xylene and rehydrated in descending gradations of ethanol. Then, samples were autoclaved at 110˚C for 10 minutes in citrate buffer (pH 6.0) for antigen retrieval. Next, the sections were blocked of endogenous peroxidase with 3% H_2O_2 for 30 minutes at room temperature and then were incubated with primary antibody (rabbit polyclonal to somatic antigens of the whole *H. pylori* organism, Cat no: ab 7788) at 4˚C for overnight. Next, the sections were incubated with secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) at room temperature for 1 hour. DAB solution (3', 3'-diaminobenzidine) (0.02%) in1xPhosphate buffer saline (PBS) and 0.01% H_2O_2 was used as a chromogenic substrate. Sections were counterstained with Mayer's hematoxylin. The stained sections were examined using a microscope (Experiment 1 and 2).

2.12 Picrosireus red staining for liver fibrosis

Liver fibrosis was stained using Picrosirius red kit according to manufactures instruction. Briefly, the liver sections were deparafinized and rehydrated, then stained with hematoxylin and follow by the series of reagent for picrosirius red staining. After that, the sections were dehydrated and mounted by mounting media (Experiment 2).

Grading fibrosis, liver fibrosis was graded into 4 grading as following criteria: **Grade 0**: no fibrosis, **Grade 1**: mild fibrous expansion of some portal area, **Grade 2**: moderate fibrous expansion of most portal areas with short fibrous septa, **Grade 3**: severe fibrous expansion of most portal areas with occasional portal to portal bridging, **Grade 4**: more severe fibrous expansion of most portal areas with marked bridging.

2.13 Biochemical assay

Serum aspartate transferase (AST), alanine transferase (ALT), and alkaline phosphatase (ALP), the indicators of liver and bile duct injury, were measured by an automated spectrophotometer (automate RA100) using a commercial kit (Thermo Trace Ltd., Melbourne, Australia) (Experiment 2).

2.14 Statistical analysis

When experiments included only 2 two groups, Mann-Whitney *U* test for fibrosis score grading and Chi-squared test for cholangitis grading were used in this study .When the experiments design included more than two groups, statistical differences were determined by analysis of variance. Results were express as mean±SD for data in each group. $P<0.05$ was considered significant. All statistical analyses were performed using the SPSS version 19 statistical program.

3. Results

3.1 Results of Experiment 1

3.1.1 Bacterial isolation

The result of aerobic bacteria cultivation in liver samples from chronic *O. viverrini*-infected was identified including *Streptococcus group D non Enterococci , Enterobacter* spp., *Escherichia coli, Streptococcus pyogenes (group A), Klebsiella pneumonia.* However, microaerophillic condition for *Helicobacter* spp. cultivation, the bacteria isolation was no grown from hamster liver specimen after incubation time at least 7 days.

3.1.2 Detection of genus-specific of *Helicobacter* and 16S rDNA(V3-V4 region) prokaryotic by PCR from liver samples and cultured specimen

	PCR (V3-V4) thio_specimen	PCR(genus_Helicobacter)BB_specimen	PCR(genus_Helicobcter) Frozen specimen
Normal 8 M (L)	0	0	0
Normal 15 M (L)	85%	0	0
OV-infected 8 M (L)	0	0	40%
OV-infected 12 M (L)	0	0	100%
OV-infected 15 M (L)	100%	0	0
Worm 15 M	100%	0	0

Figure 3 Identification of bacterial genomic DNA from liver tissue and cultured specimen by PCR.

3.1.3 Identification of bacterial genomic DNA by metagenomic analysis

3.1.3.1 Genus-level

The distribution of bacterial genomic DNA isolation at genus-specific level in hamster liver is shown in Figure 4. In normal group, the relative abundance of genus-level of bacterial DNA were *Acidaminococcus* (7.66%), *Aggregatibacter* (0.85%), *Clostridium* (8.24%), *Lactobacillus* (78.32%), *Megasphaera* (0.64%), *Streptococcus* (2%),

Veillonella (0.14%), Unclassified (1.83%), and Others (0.3%). The most frequent of bacterial DNA was *Lactobacillus*, *Acidaminococcus* and *Clostridium*. In chronic OV-infected group, the distribution of bacterial genomic DNA were *Aggregatibacter* (3.34%), *Bifidobacterium* (0.58%), *Escherichia* (10.19%), *Fusobacterium* (13.81%), *Clostridium* (0.58%), *Helicobacter* (0.99%), *Lactobacillus* (24.83%), *Streptococcus* (10.77%), *Veillonella* (1.29%), Unclassified (33.24%), and Others (0.92%). In adult worm, two genus of *Aggregatibacter* (39.65%), *Lactobacillus* (60.29%) DNA was identified (Figure 4). Notably, there was more relative abundance of bacterial population and variety of bacterial DNA in genus-level in chronic OV-infected group than in normal and worm group.

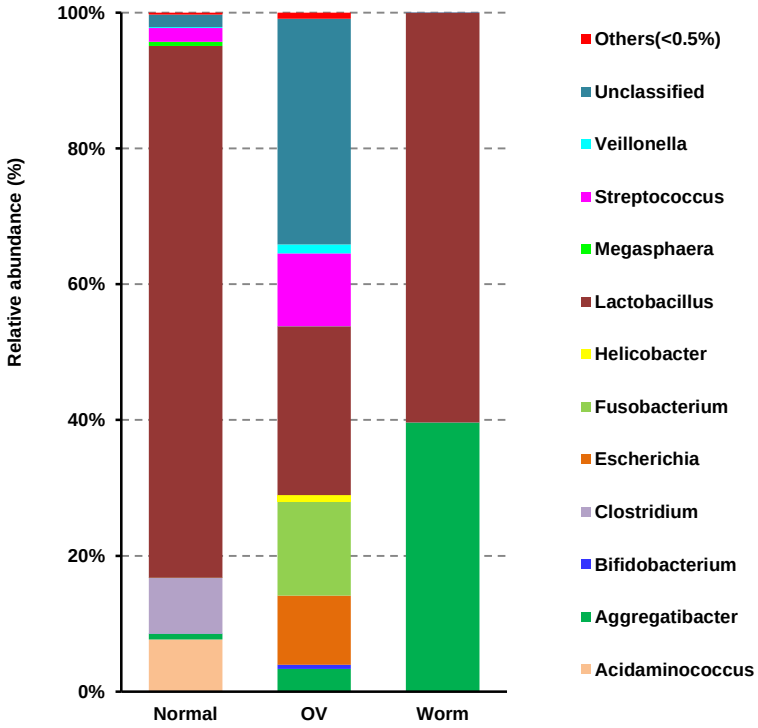


Figure 4 The population and distribution of bacterial DNA at genus-level in hamster liver and worm samples. The species of which genus abundance is less than 0.5% in all samples were classified into 'others' . Normal : Normal group, OV : Chronic *O. viverrini*-infected group , Worm : *O. viverrini* adult

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3.1.3.2 Species-level

The relative abundance of bacterial DNA at species-level is shown in Figure 5. In normal group, bacterial genomic DNA of *Aggregatibacter pneumotropica* (0.84%),

Lactobacillus agilis (18.23%), *Lactobacillus coleohominis* (1.26%), *Lactobacillus reuteri* (21.55%), *Lactobacillus salivarius* (0.02%), *Streptococcus luteciae* (0.08%), *Veillonella dispar* (0.1%) were identified in liver tissues. In chronic OV-infected group, relative abundance of bacterial DNA were *Aggregatibacter pneumotropica* (3.33%), *Escherichia coli* (10.18%), *Helicobacter pylori* (0.17%), *Helicobacter* spp. (0.82%), *Lactobacillus agilis* (3.02%), *Lactobacillus coleohominis* (0.56%), *Lactobacillus reuteri* (4.16%), *Lactobacillus salivarius* (5.85%), *Streptococcus luteciae* (10.76%), *Bifidobacterium* spp. (0.58%), *Fusobacterium* spp. (13.81%), *Veillonella dispar* (1.09%), and Unclassified (45.36%). In adult worm, three species of *Aggregatibacter pneumotropica* (39.66%), *Lactobacillus reuteri* (3.44%), *Lactobacillus salivarius* (56.85%) of bacterial DNA were identided. Among normal, infected liver and adult worm samples, there were difference in bacterial species and the relative abundance of bacteria population, which showed the highest number of bacterial species in chronic OV-infected group.

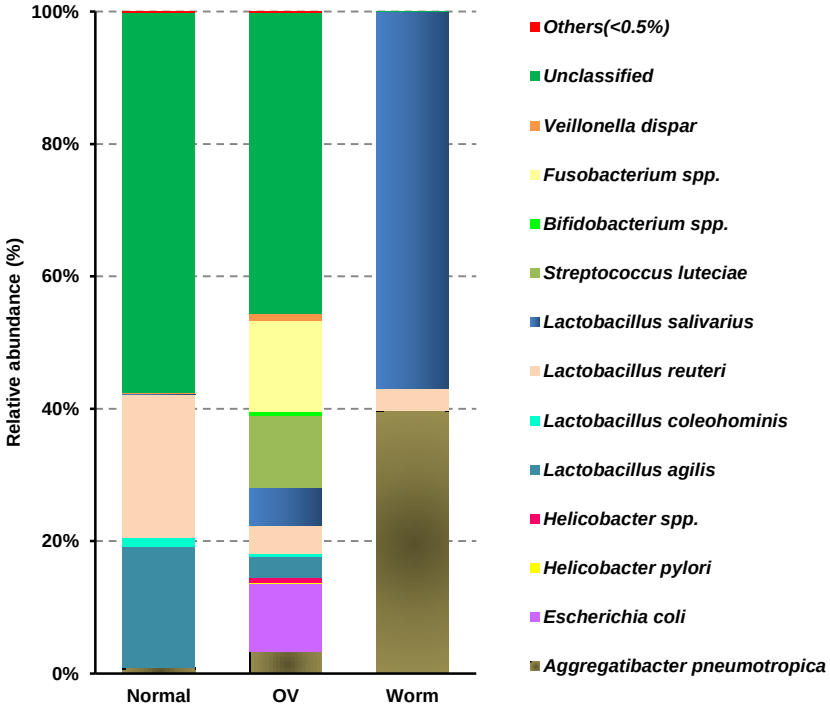

Figure 5 The population and distribution of bacterial DNA at species-level in hamster liver and worm samples. The species of which species abundance is less than 0.5% in all samples were classified into 'others'. Normal : Normal group, OV : Chronic *O. viverrini*-infected group , Worm : *O. viverrini* adult.

3.1.4 Isolation of bacterial DNA in host and parasite

In order to identify the co-evolution of bacteria species between host-parasite interplay, venn diagram was constructed in Figure 6. Identification of bacteria species between normal liver and worm were *A. pneumotropica* and *L. reuteri*, while *L. salivarius* were identified in worm only. *L. agilis, L. coleohominis*, *S. luteciae*, *A. pneumotropica, L. reuteri* were isolated from normal group and OV-infected group, suggesting that these bacteria are normal flora in hepatobiliary system. The growth of bacteria diversity including *E. coli*, *H. pylori*, *Helicobacter* spp., *Bifidobacterium* spp., *Fusobacterium* spp., *V. dispar*, and *L. salivarius* were found only in infected group, suggesting that *O. viverrini* infection increases influx of gut and other site of bacteria population growth. *A. pneumotropica, L. salivarius* and *L. reuteri* were identified from worm and OV-infected group and no bacteria was found only in worm, implying that worm might be infected during reside in the hepatobiliary system of the host. Notably, *A. pneumotropica* and *L. reuteri* were found in among three groups, suggesting that these two bacteria are normal flora in hepatobiliary tract of hamster. Moreover, *E. coli*, *H. pylori*, *Helicobacter* spp., *Bifidobacterium* spp., *Fusobacterium* spp., and *V. dispar* were identified only in OV-group, suggesting that *O. viverrini* infection might be enhanced these bacterial growth from other sites such as gastrointestinal lumen.

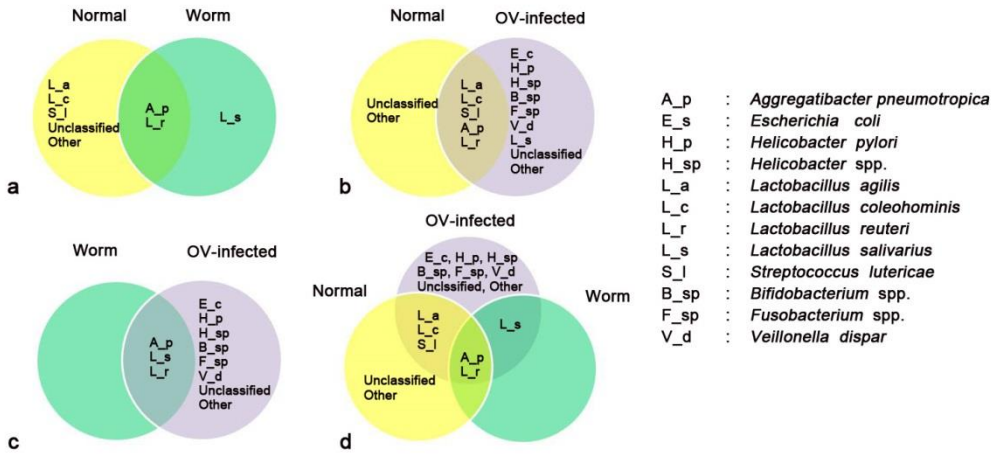

Figure 6 Venn diagram of identified bacterial species among difference groups. (a) Normal group and Worm group. (b) Normal group and OV-infected group. (c) Worm group and OV-infected group. (d) Among three groups. Different color is presented in different samples or groups. The overlapping area represents bacteria species commonly present in the counterpart group.

Figure 7 showed the co-evolution of bacterial growth at the genus-level in the difference groups according to time-post infection. The genus-level of bacteria was closely similarity at 8, 12 and 15 months post-infection but was different in bacteria population when compared to the other groups. *Aggregatibacter, Lactobacillus* and unclassified were co-evolution among three groups. *Fusobacterium*, *Escherichia* and *Bifidobacterium* were relative abundance according to time-post of *O. viverrini* infection which were higher abundance in OV-group than in normal group. In addition, *Megasphaera, Clostridium,* and *Acidaminococcus* were high relative abundance in normal but were low abundance in OV-infected and in worm, suggesting that *O. viverrini* infection causes environmental changes leading to affect on these bacteria growth.

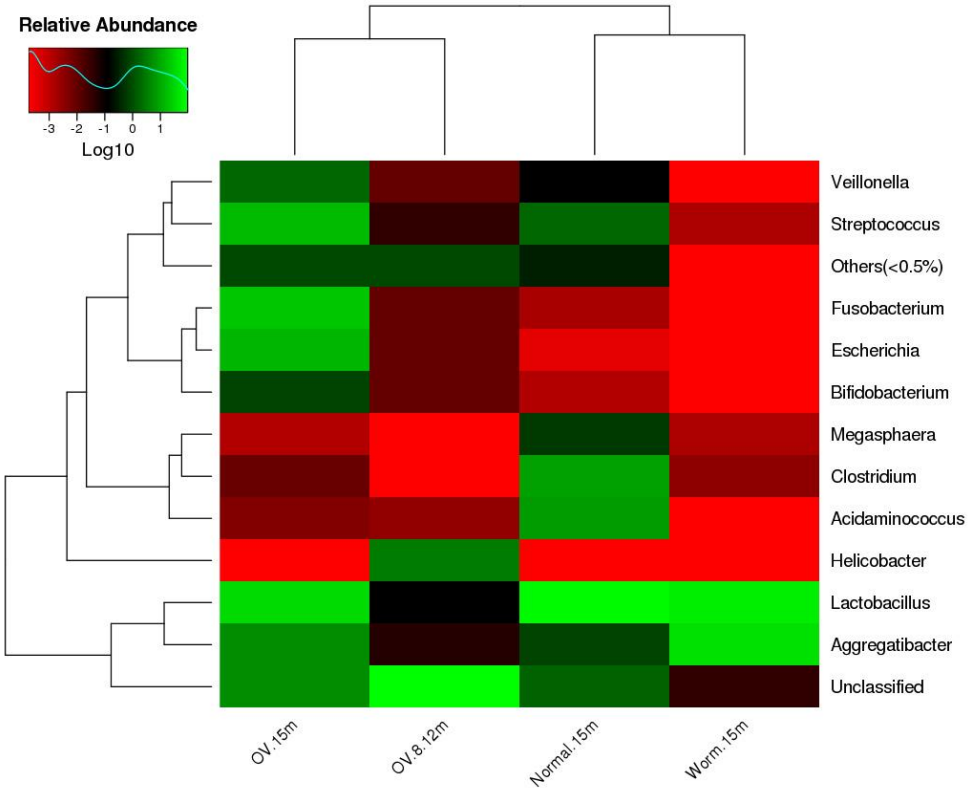

Figure 7 Heat map of identified bacteria at genus-level in hamster liver and parasite. Longitudinal clustering indicates the similarity of all species among different samples, and the horizontal clustering indicates the similarity of certain species among different samples. The closer distance is the shorter of the branch length and the more similar the species composition is between the samples. Normal. 15 m: Normal group, OV.15m: *O. viverrini*-infected group at 15 months, OV.8.12m : *O. viverrini*-infected groups at 8 and 12 months, Worm 15 m: worm obtained from *O. viverrini*-infected for 15 months.

3.1.5 The Evolution of bacteria between group

Figure 8 showed a phylogenetic tree analysis of identified bacteria based on the nucleotide sequences of the V3-V4 hypervariable region of prokaryotic 16S rDNA isolated from liver and worm. There were three routes of evolutionary relationships among various biological species of bacterial identification in hamster liver and in worm. These consisted of 6 phylum including *Actinobacteria*, *Bacteroidetes, Cyanobacteria*, *Firmicutes*, *Fusobacterium* and *Proteobacteria*. The relationship between taxonomy and phylogenic tree of 42 genus from 6 phylum is shown in Figure 8.

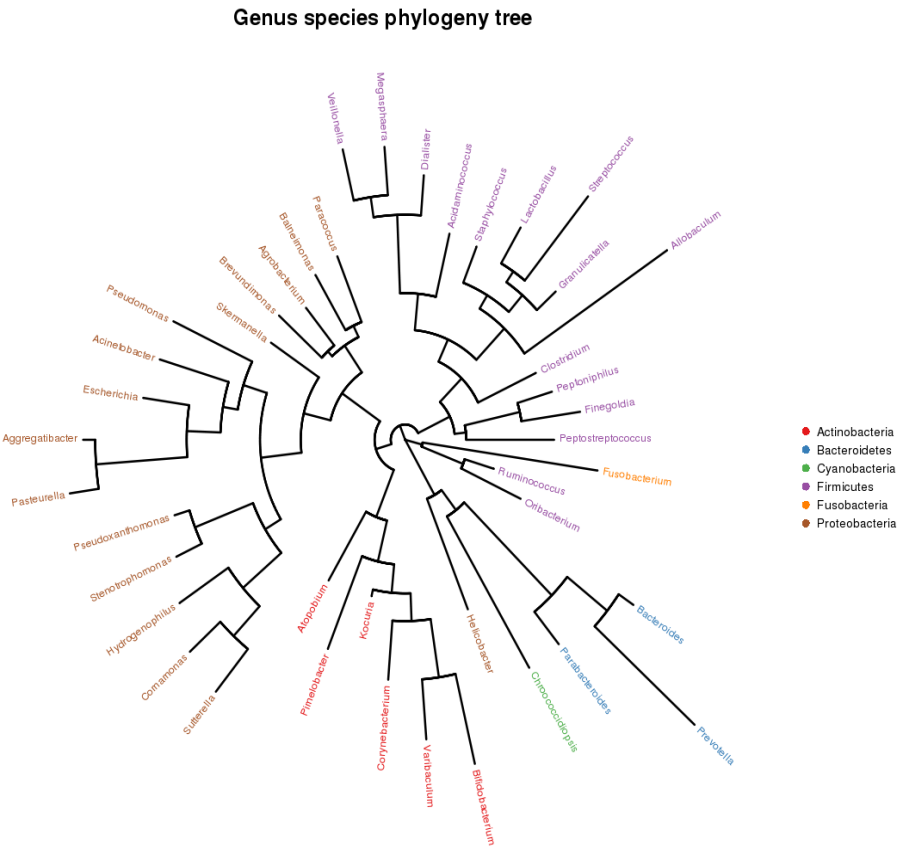

Figure 8 Phylogenetic tree of identified bacteria based on the nucleotide sequences of the V3-V4 hypervariable region of prokaryotic 16S rDNA. The same Phylum is shown as the same color.

3.1.6 Detection of specie-specific of *Helicobacter pylori* (ureA gene) by PCR from liver tissue

In order to confirm the metagenomic analysis, specie-specific of *H. pylori* (ureA gene) was analyzed by PCR using specific primer. All the 3 *Helicobacter* genus positive samples from OV-infected group were analyzed for the presence of *ureA* gene. One sample gave positive amplification for *ureA gene* which was supported to metagenomics result. The amplified product of *ureA* gene is represented in Figure 9.

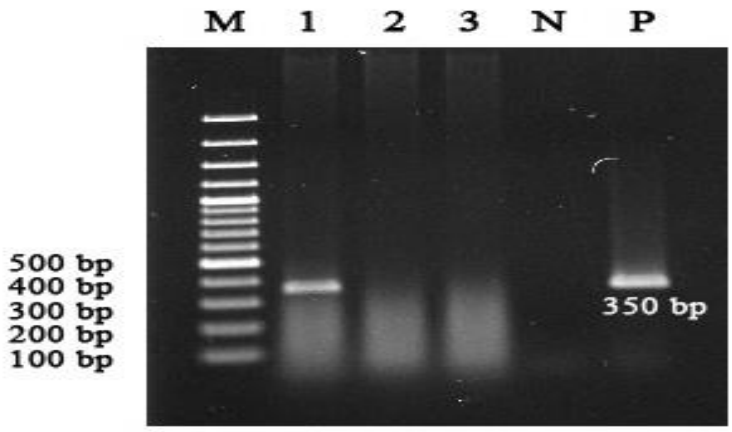

Figure 9 Detection of *Helicobacter pylori* (*ureA* gene) from liver frozen specimen by PCR technique. Product size was 350 bp. M: 100-bp molecular weight Marker, P: Positive control, N: Negative control, Lane 1-3: positive with genus specific of *Helicobacter* from three OV-infected hamsters. Lane 1 showed positive of both genus and species specific for *Helicobacter* and *Helicobacter pylori*. Lane 2 and 3, positive with genus specific of *Helicobacter* from OV-infected hamsters at 8 months post-infection, but was negative results for *H. pylori* (*ureA* gene).

3.1.7 Specific detection of *Helicobacter pylori* infection using Immunohistochemetry technique

In order to localize of *H. pylori* in liver tissue, we performed by immunohistochemical stain using specific antibody against to *H. pylori*. The immunoreactive staining was observed in the hepatocytes, sinusoids, epithelial cell of large bile duct and inside the *O. viverrini* worm. In addition, normal liver didn't show any signal of immunoreactivity (Figure 10).

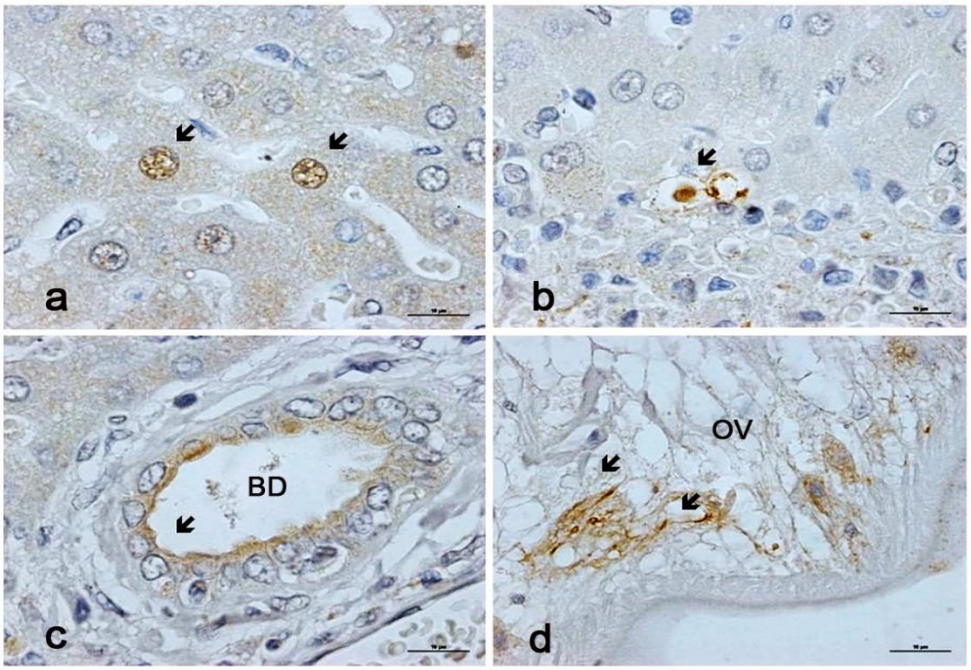

Figure 10 Immunohistochemical localization of *Helicobacter pylori* infection in hamster liver-infected with *O. viverrini*. Immunoreactive staining for *H. pylori* presents in brown color (arrow) of (a) hepatocytes, (b) sinusoid, (c) bile duct and (d) *O.viverrini* worm. Original magnification, x1000.

3.1.8 Histopathological study

Liver tissue was stained by hematoxylin & eosin staining (Figure 11). Cholangitis grading was defined by the accumulation of inflammation cells, especially polymorphonuclear cells including neutrophil and eosinophil around large and small bile ducts. In liver tissue of OV-infected group, the accumulation of cholangitis grading was significantly observed higher than in normal group ($P<0.05$).

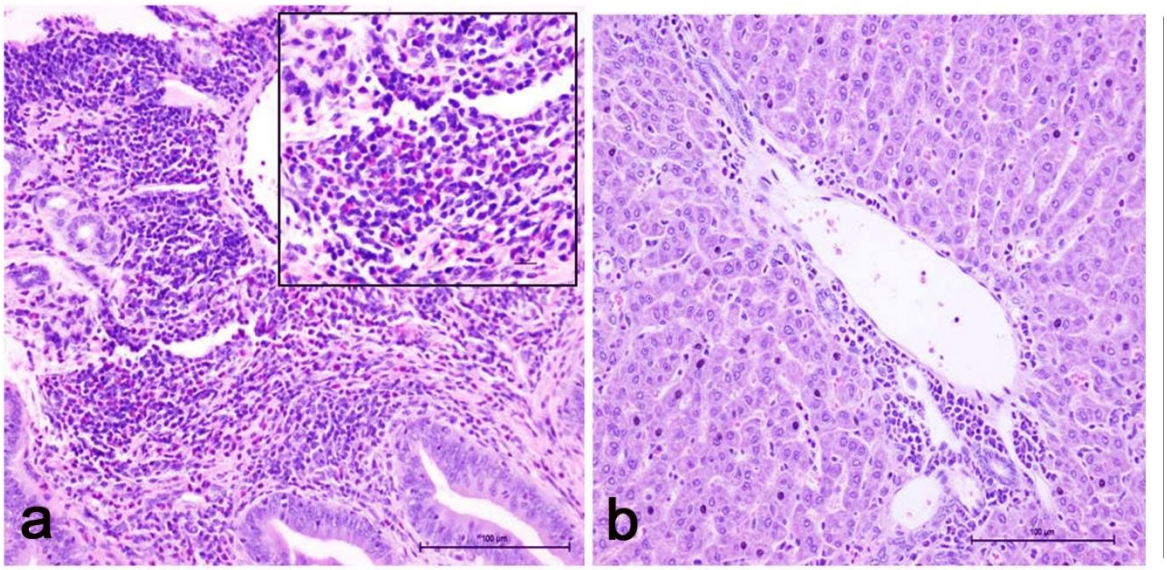

Figure 11 Histopathology of hamster liver tissue (H&E stains). (a) neutrophilic, eosinophilic and mixed inflammation cell surrounding bile duct and hepatic portal vein in liver tissue of chronic OV-infected group. (b) normal hamster liver tissue. Original magnification, x200.

3.2 Results of Experiment 2

3.2.1 Detection of *H. pylori* by PCR technique

3.2.1.1 In normal group

- Gastric: Positive 1 sample (20%)
- Liver: All of samples were negative (0%)
- Gallbladder: All of samples were negative (0%)

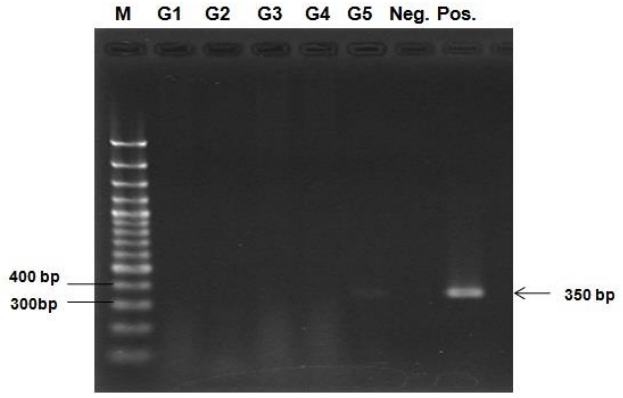

Figure 12 *H. pylori* DNA positive (G5) for ureA in gastric normal hamsters. G = gastric, G1-G5 = gastric from hamster 1-5, M = marker DNA ladder, Pos = positive control, Neg = negative control.

3.2.1.2 In *H. pylori*-infected group

- Gastric: Positive 2 samples (40%)
- Liver: Positive 2 samples (40%)
- Gallbladder: All of sample were negative (0%)

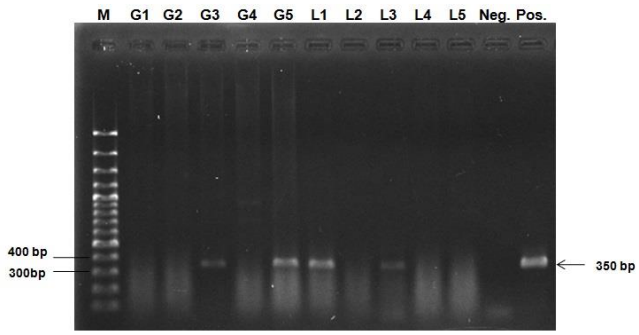

Figure 13 *H. pylori* DNA positive for *ureA* gene in gastric (G3 and G5) and liver (L1 and L3) in *H. pylori*-infected hamsters. G = gastric, L = Liver. G1-G5 = gastric from hamster 1-5, L1-L5 = liver from hamster liver 1-5, M = marker DNA ladder, Pos = positive control, Neg = negative control.

3.2.1.3 In *O. viverrini*-infected group

- Gastric: Positive 4 samples (50%)
- Liver: Positive 2 samples (25%)
- Gallbladder: Positive 1 sample (12.5%)

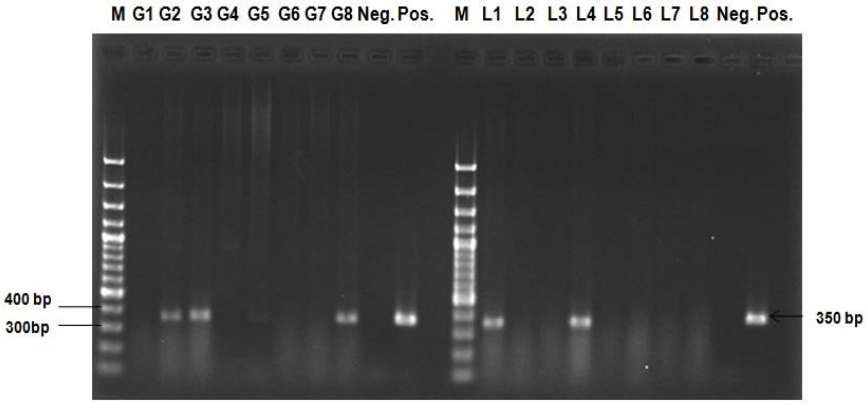

Figure 14 *H. pylori* DNA positive for *ureA* gene in gastric (G2, G3, G5 and G8 6) and liver (L1 and L4) in *O. viverrini*-infected hamsters.

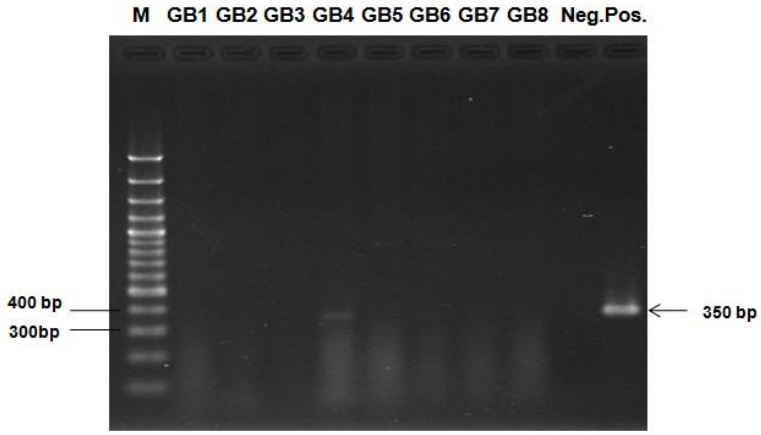

Figure 15 *H. pylori* DNA positive for *ureA* gene in gallbladder (GB4) in *O. viverrini*-infected hamsters. GB = gall bladder.

3.2.1.4 In *H. pylori* +*O. viverrini*-infected group

- Gastric: Positive 5 samples (62.5%)
- Liver: Positive 4 samples (50%)
- Gallbladder: Positive 1 sample (12.5%)

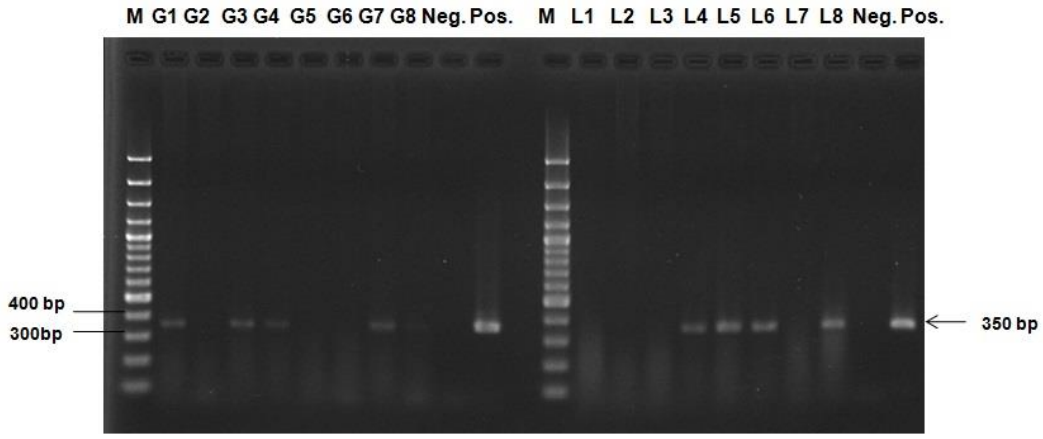

Figure 16 *H. pylori* DNA positive for *ureA* gene in gastric (G1, G3, G4, G7 and G8) and liver (L4, L5, L6, and L8) in *H. pylori+O. viverrini*-infected hamsters. Abbreviations are similar to Fig.13 Legend.

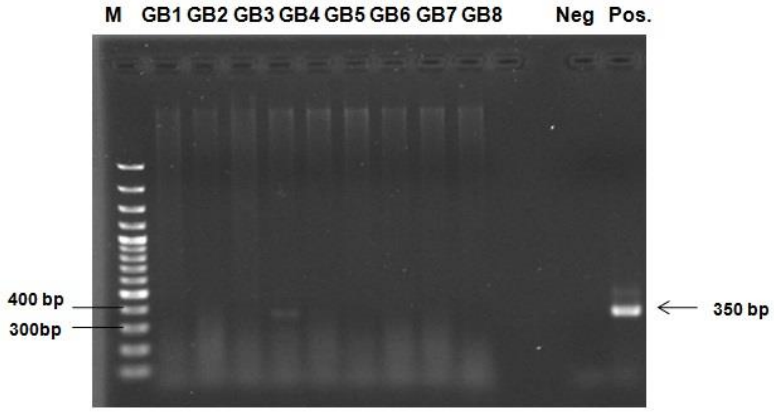

Figure 17 *H. pylori* DNA positive for *urea* gene in gallbladder (GB4) in *H. pylori+O. viverrini*-infected hamsters. GB = gall bladder, other abbreviations are similar to Fig.13 Legend.

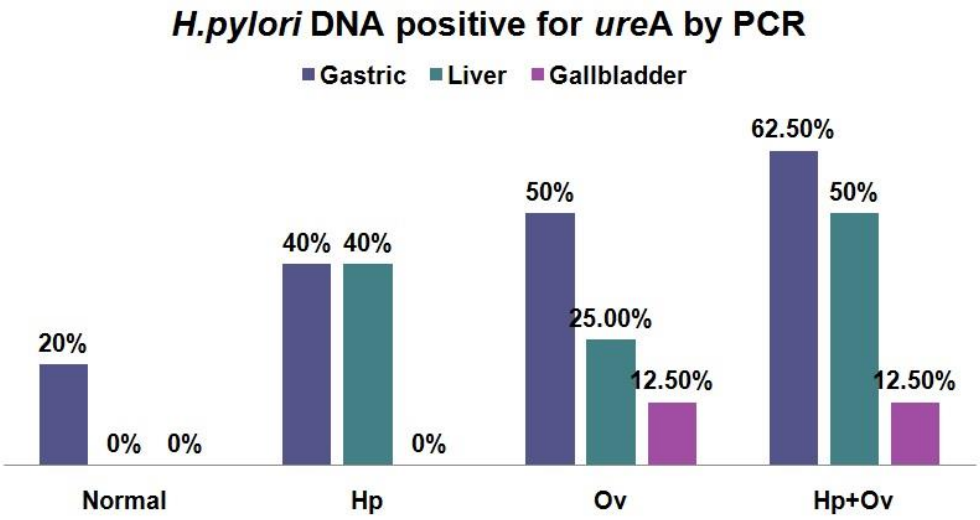

Figure 18 *H. pylori* DNA positive in gastric, liver and gallbladder samples. Identification of *H. pylori* DNA was performed for ureA by PCR in 1) normal, 2) Hp (*H. pylori*-infected group), 3) Ov (*O. viverrini*-infected group), and 4) Hp+Ov (*H. pylori* +*O. viverrini*-infected group).

3.2.2 Detection of *H. pylori* by immunohistochemistry

For normal group: all of samples were negative for *H. pylori*

For Hp-infected group: 1 gastric sample and 1 liver sample were positive for *H. pylori*.

For Ov-infected group: all of samples were negative for *H. pylori*

For Hp+Ov- infected group: 4 gastric samples and 1 liver sample were positive for *H. pylori*.

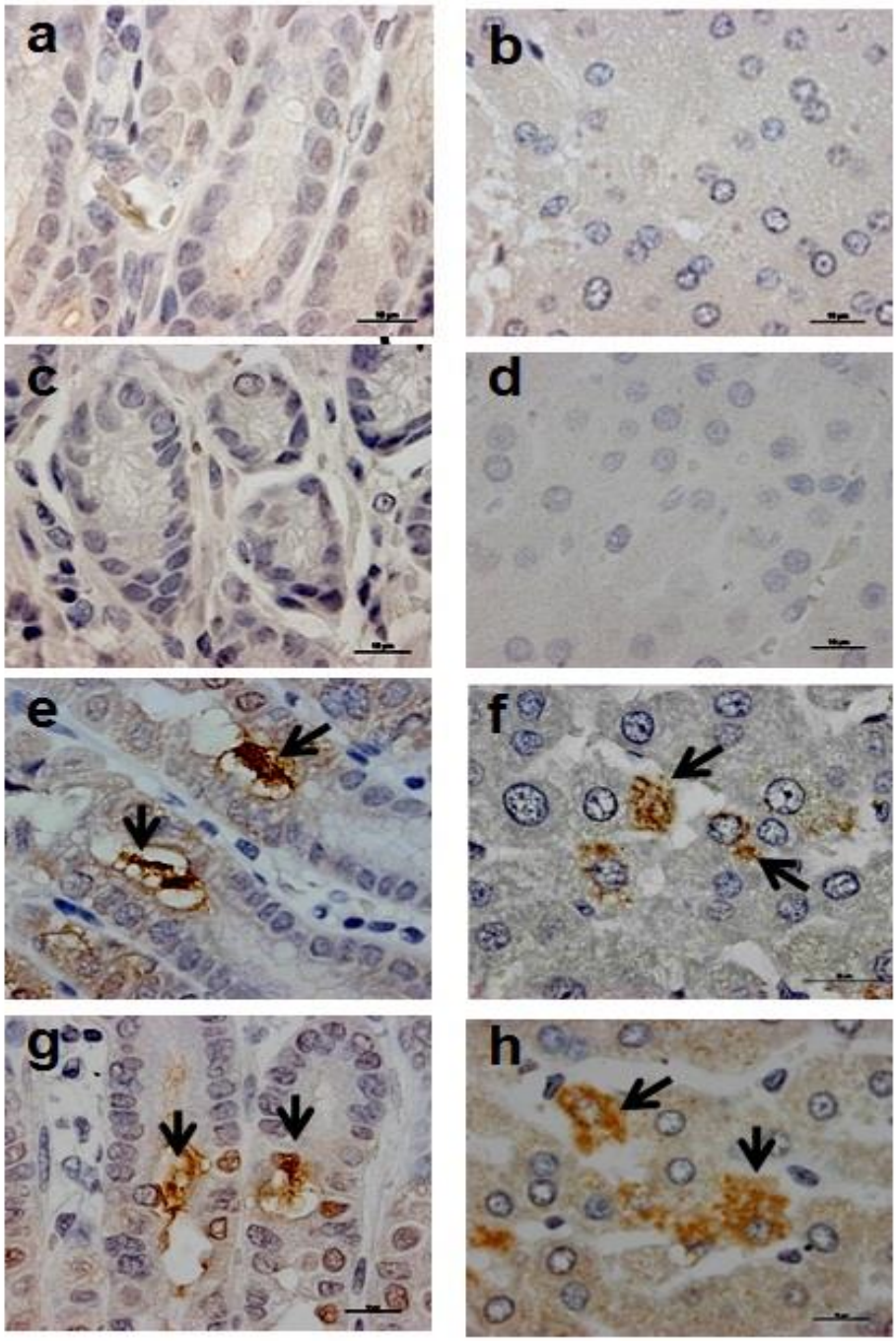

Figure 19 Demonstration of *H. pylori* positive in the stomach and liver tissue by immunohistochemistry using antibody against to *H. pylori*. Normal hamsters have no *H. pylori* colonized in gastric (a) and liver tissue (b). Ov-infected hamsters have no *H. pylori* colonized in gastric (c) and in liver tissue (d). Colonization of gastric tissue by *H. pylori* was observed in a gastric pit of Hp-infected hamster (e) and in Hp+Ov-infected hamsters (g). *H. pylori* in liver tissue was observed in Hp-infected hamster (f) and in Hp+Ov-infected hamsters (h). Scale bar, 10 μm. arrow is indicated positive area.

3.2.3 Histopathological study

3.2.3.1 Cholangitis and inflammatory cells

- Experimental groups that were infected by *H. pylori* virulence strain had gastric lesion as shown in figure 20.
- In the liver of Ov-infected group and Hp+Ov-infected group showed many of inflammatory cells around the bile duct shown in figure 5. Moreover, in Hp+Ov-infected group was found cholangitis and inflammatory cells more than Ov-infected group shown in figure 21.

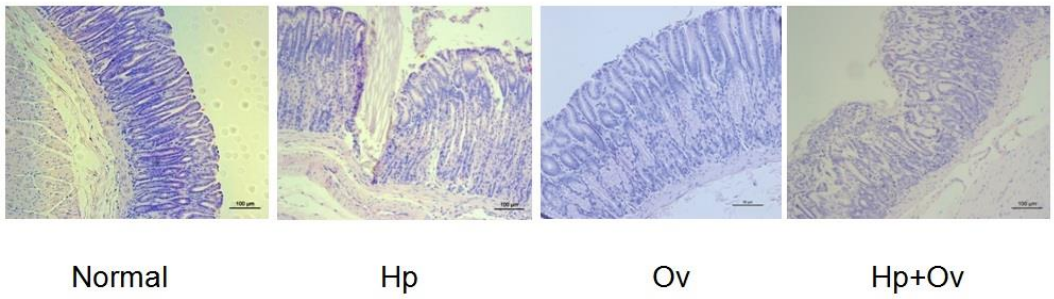

Figure 20 Experimental groups that were infected by *H. pylori* virulence strain showed gastric lesion (Hp group and Hp+Ov group). Hp = *H. pylori*-infected, Ov = *O. viverrini*-infected, Hp+Ov = *H. pylori* plus *O. viverrini*-infected.

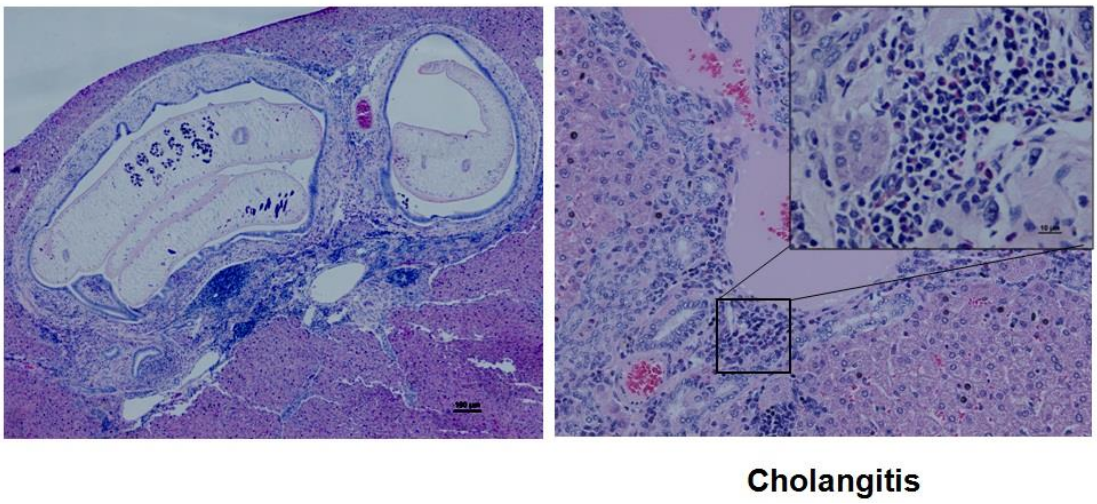

Figure 21 Demonstration of inflammatory cell around the bile duct and cholangitis.

3.2.3.2 Periductal fibrosis

Periductal fibrosis was stained by Picrosirius red staining. The data was presented as the mean ± SD. Non-parametric Mann-Whitney *U* test was used to compare the grading score.*P<0.05 compared to normal group, †P< 0.05 compared to *H. pylori* -infected group. ‡P< 0.05 compared to Ov-infected group. The result was shown in table 4 and figure 22. Grading of score of fibrosis increased in the order of normal, *H. pylori*-infected, *O. viverrini*-infected and in *H. pylori*+*O. viverrini,* respectively. The most severity of fibrosis was observed in *H. pylori*+*O. viverrini* –infected group.

Table 4 Grade of periductal fibrosis in normal hamsters, *H. pylori*-infected hamsters, O. viverrini-infected hamsters and in *H. pylori*+*O. viverrini* –infected hamsters.

Experimental group	Fibrosis score (Mean±SD)
Normal	0.17±0.19
Hp	1.07 ±0.36*
Ov	2.79 ±0.43*,†
Hp+Ov	3.29 ±0.60*,†

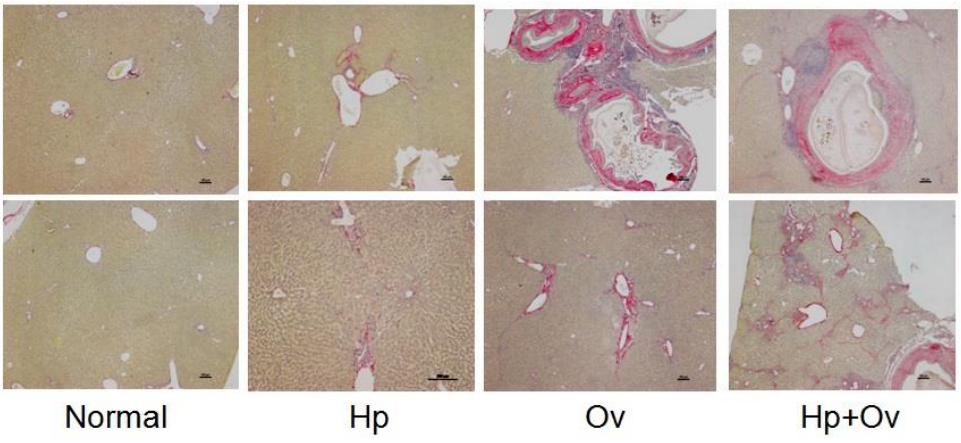

Figure 22 Demonstration of fibrosis in liver tissue by using Picrosirius red staining. Scale bar, 100 µm. Fibrosis was stained in red color.

3.2.4 Biochemical analyses

- There are no significant of ALT, AST or ALP between each group. However, some hamster that have high level of ALT, AST or ALP have *H. pylori* DNA positive in liver and severe periductal fibrosis as well.
- The data presented as the mean±SD. using analysis of variance (one-way ANOVA) was shown in table 5 and figure 23.

Table 5 The effects of infection of *H. pylori* and *O. viverrini* on biochemical tests in experimental animals**.**

Experimental group	**ALT(U/L)**	**AST(U/L)**	**ALP(U/L)**
Normal	41.60 ±2.07	47.2±4.32	55.00 ±16.54
Hp	52.4 ±39.16	60.8 ±28.93	54.4 ±11.65
Ov	57.63±17.32	59.75±12.22	58.75 ±3.88
Hp+Ov	74.13±46.08	68.75±17.96	55.63±18.63

***P*< 0.05 when compare to normal group**

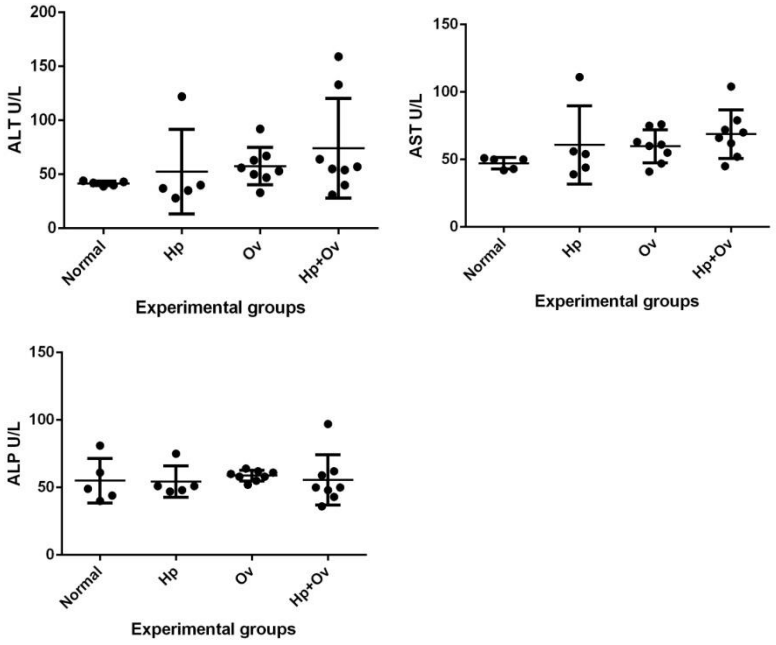

Figure 23 The effects of infection of *H. pylori* and *O. viverrini* on biochemical tests in experimental animals.

4. Conclusion and discussion

The aim of this research to investigate 1) To identify *Helicobacter* spp. and other bacterial infection in chronic opisthorchiasis using hamsters model, and 2) To investigate the effect of co-infection of *O. viverrini* and *H. pylori* on the alteration of hepatobiliary diseases (HBD) in hamsters model. The result of metagenomic study in chronic opisthorchiasis revealed that metagenomic analysis of 16S rDNA (V3-V4 region) sequences was successfully amplified of hamster liver. Thus, this technique is an extremely powerful tool in a high throughput sequencing technology for identification of taxa of bacteria. Metagenomic analysis results was supported and confirmed by the presence of *H. pylori* DNA positive by PCR and *Helicobacter pylori* antigen in liver tissue by immunohistichemical technique. These results suggest that all of bacterial infection may be had a relationship between host-parasite interaction. *O. viverrini* infection might change the microenvironment and enhances bacteria growth in hepatobiliary system such as Enterobacericeae, anaerobic bacteria and *Helicobacter* species, especially *H. pylori* after post- infection. Increase bacteria growth including *H. pylori* due to chronic infection might synergistically induce immune response and cause the alteration of hepatobiliary system contribution to exacerbate of opisthorchiasis-associated HBD. The outcome of these findings in an animal model will be investigated in chronic opisthorchiasis patient and in cholangiocarcinma patient, which may be useful for a new therapeutic approach in opisthorchiasis-associated HBDs including CCA in the future.

Accordingly, *O. viverrini* infection enhances *H. pylori* growth in HBD in experiment I, which might be involved to induce the severity of opisthorchiasis. In order to clarify whether the role of *H. pylori* and *O. viverrini* co-infection causes HBD, *H. pylori* virulence strain was co-infected with *O. viverrini* in hamster model. The results revealed that the combination of *H. pylori* and *O. viverrini* enhanced bacteria growth in the liver and caused the more severity of HBD such as inflammation reaction, cholangitis and periductal fibrosis than that of single infection alone. Although average of fibrosis score between *O. viverrini*-infected group and co-infected group were not significant differences, we found severe periductal fibrosis and high level of ALT and AST, the indicators of liver injury, in some case of co-infected hamsters. Increase of *H. pylori* in liver after co-infection with liver fluke might be explained by (i) *O. viverini* might carry *H. pylori* colonized in gastric into hepatobiliary system directly or (ii) physical obstruction of

the bile ducts caused by *O. viverini* infection leading to influx of *H. pylori* growth from other sites such as gastric and gastro-hepatobiliary system, and (iii) *O. viverrini* might be changed microenvironment in hepatobiliary system to enrich of bacteria growth. These results indicated that co-infection between *H. pylori* virulence strain and *O. viverrini* may be enhanced periductal fibrosis, a relative risk condition for CCA development. The outcome of this study may be useful for a therapeutic approach.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

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1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

จัดเตรียมบทความ (first draft) แล้วจำนวน 1 บทความ คือ

1) Upsornsawan Itthitaetrakool, Porntip Pinlaor*, Somchai Pinlaor, Rungtiwa Dangtakot, Chariya Chomvarin, Arunee Sunga, Puangrat Yongvanit. *Opisthorchis viverrini* infection enhances bacterial population growth in hamsters liver by metagenomic analysis. Will be submitted in ***Infection genetic and evolution*** (IF= 3.015) in the year 2016.

2) กำลังจัดเตรียมบทความ อีกจำนวน 1 บทความ คือ

Rungtiwa Dangtakot, Porntip Pinlaor*, Somchai Pinlaor, Chariya Chomvarin, Upsornsawan Itthitaetrakool, Arunee Sunga, Puangrat Yongvanit. *Helicobacter pylori* infection increases hepatobiliary diseases in experimental opisthorchiasis viverrini. Will be submitted in ***Journal of Clinical Microbiology*** (IF= 3.99) in the year 2016.

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)

-

- เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)

-

- เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

มีการร่วมกันของกลุ่มติดเชื้อแบคทีเรียและเชื้อปรสิต ในการศึกษา co-infection ต่อโรคระบบทางเดินน้ำดี รวมทั้งโรคมะเร็งท่อน้ำดี ซึ่งได้รับทุนอุดหนุนวิจัย จากมหาวิทยาลัยขอนแก่น ต่อเนื่อง 3 ปี ตั้งแต่ ปี 2559 ถึง ปี 2561 แล้ว

- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

1. ใน ระดับป.ตรี ใช้สำหรับประกอบการศึกษาโครงงานวิจัยของ นักศึกษาเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น

1.1) ปีการศึกษา 2556 โครงงานวิจัย ของนักศึกษา

นายศุภณัฐ ชลมาตร์ และ นายปิยมิตร สุวัฒน์ ศึกษาเรื่อง *การวินิจฉัยเชื้อแอโรบิคและเฮลิโคแบคเตอร์แบคทีเรียในหนูแฮมสเตอร์ที่ติดพยาธิใบไม้ในตับ*

1.2) ปีการศึกษา 2557 โครงงานวิจัย ของนักศึกษา

นางสาวนิศากร คงเสถียร และ นางสาวเบญจวรรณ ขิ้งชัยภูมิ ศึกษาเรื่อง *การเพิ่มการเจริญเติบโตของเชื้อ Helicobacter pylori ในบรูเซลล่าบรอสด้วยไฮโดรเจนเปอร์ออกไซด์และฟีทรัลโบวายซีรัม*

1.3) ปีการศึกษา 2558 โครงงานวิจัย ของนักศึกษา

นางสาววิภาวี แตงสี และนางสิรภัถร แสงนวล ศึกษาเรื่อง *การสำรวจการปนเปื้อนของเชื้อ Helicobacter pylori ในกระเพาะและเพี้ยของวัว ในจังหวัดขอนแก่น*

2. ในระดับบัณฑิตศึกษา ป.โท

2.1) นางสาวอัปสรสวรรค์ อิทธิแต้ตระกูล นักศึกษาระดับ ป.โท สาขาชีวเวชศาสตร์ บัณฑิตวิทยาลัย มหาวิทยาลัยขอนแก่น

2.2) นางสาวรุ่งทิวา แดงตาโคตร นักศึกษาระดับ ป.โท สาขาเทคนิคการแพทย์ คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

3.1 เสนอผลงานประชุมในระดับชาติ แบบบรรยายโดยนางสาวอัปสรสวรรค์ อิทธิแต้ตระกูล เรื่อง “การศึกษาข้อมูลเมต่จีโนมของแบคทีเรียในโรคโอพิสทอร์คิเอซีสเรื้อรังในหนูแฮมสเตอร์” ในการประชุมวิชาการ “จาก CASCAP สู่ท้าทายไทย” ณ โรงแรมพลูแมนขอนแก่นราชาออคิด อำเภอเมือง จังหวัดขอนแก่น ในวันที่ 24-25 ธันวาคม พ.ศ. 2558

3.2 เสนอผลงานประชุมในระดับชาติ แบบบรรยายโดยนางสาวรุ่งทิวา แดงตาโคตร เรื่อง “*การติดเชื้อร่วมกันของ Helicobacter pylori และ Opisthorchis viverrini ที่สัมพันธ์กับการเกิดพังผืดรอบท่อน้ำดีในหนูแฮมสเตอร์*” ในการประชุมวิชาการ “จาก CASCAP สู่ท้าทายไทย” ณ โรงแรมพลูแมนขอนแก่นราชาออคิด อำเภอเมือง จังหวัดขอนแก่น ในวันที่ 24-25 ธันวาคม พ.ศ. 2558

ลงนาม .....

(ผู้ช่วยศาสตราจารย์พรทิพย์ ปิ่นละออ)

(หัวหน้าโครงการวิจัยผู้รับทุน)

ลงนาม .....

(ศาสตราจารย์พวงรัตน์ ยงวณิชย์)

(นักวิจัยที่ปรึกษา)

03-01-59-Infection genetic and evolution (IF= 3.015)

Chronic *Opisthorchis viverrini* infection promotes *Helicobacter pylori* growth and enhances microbiome in the liver

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Abbreviations: OV, *Opisthorchis viverrini*; CCA, cholangiocarcinoma; HBD, hepatobiliary disease; PCR, polymerase chain reaction; rDNA, ribosomal nucleic acid; OTU, Operational Taxonomics Unit; RDP, Ribosomal Database Project

ABSTRACT

Opisthorchiasis is caused by *Opisthorchis viverrini* (OV) infection, which adult worm resides in the biliary system and induces inflammation of bile ducts, leading to hepatobiliary disease (HBD) including cholangiocarcinoma. Beside a carcinogenic liver fluke, bacterial infection might participate to promote in opisthorchiasis-associated HBD. To identify *Helicobacter* spp. and other bacterial infection in chronic opisthorchiasis in hamsters (at 8, 12 and 15 months), bacterial genomic DNA from the liver and worm were investigated by many approaches including cultivation bacteria, PCR for *Helicobacter* spp. and a high-throughput next-generation sequencing based on the nucleotide sequences of the V3-V4 hypervariable region of prokaryotic 16S rDNA. For metagenomic analysis, of 855,046 sequences, 417,953 with useable reads were assignable to 155 operational taxonomy units (OTUs), 6 phyla and 24 genera of bacteria. In chronic OV-infected group, the most common relative abundance sequences of *Fusobacterium* spp. (13.81%), *Streptococcus luteciae* (10.76%), *Escherichia coli* (10.18%), and *Bifidobacterium* spp. (0.58) were detected in the liver, which had difference of bacteria diversity from normal group. Furthermore, *Helicobacter pylori* (0.17%) and *Helicobacter* spp. (0.82%) were also identified in the liver of chronic opisthorchiasis, but didn't in normal liver. The finding of *H. pylori* in the liver was confirmed by PCR and immunohistochemistry in relation to the histopathological changes. Cholangitis grading score was significantly increased in chronic OV-infected group (P 0.05). In conclusion, chronic *O. viverrini* infection promotes *H. pylori* growth and modifies other

microbiome in the liver, which together participates in enhancing of immune response-mediated the hepatobiliary diseases. The study may be useful for a new therapeutic approach and prevention of opisthorchiasis-associated HBD to reduce CCA incidence.

Keywords: *Opisthorchis viverrini*, bacteria, *Helicobacter*, *prokaryotic 16S rDNA*, cholangitis, metagenomics, next generation sequencing

1. Introduction

Opisthorchiasis is caused by *Opisthorchis viverrini* infection which remains a major health problem in the Greater Mekong Subregion, including Thailand, Laos, Vietnam and Cambodia. A food-born disease is a high prevalence, especially in the northeastern Thailand, where estimates that 6 million people are currently infected with this carcinogenic parasite (IARC, 2012; Sithithaworn et al., 2012). Humans acquire the infection by eating undercooked fish, which are contaminated with infective stage metacercaria. After infection, metacercaria excysts in the duodenum and the juvenile worm migrates into the hepatobiliary system. At the biliary tree, the parasite matures over 4 weeks into adult stage and then it lays egg pass through the feces. Egg is ingested by *Bithynia* snail, undergoes transformation and multiplication to release many cercariae that penetrate the skin of freshwater cyprinid fish.

In acute infection, parasites induce inflammation and proliferation of bile duct epithelium, which produces clinical silent or asymtomatic. In chronic infection, a consequence of histopathological changes leading to many hepatobiliary diseases (HBD) including cholangitis, periductal fibrosis, cholecystitis, obstructive jaundice and cholangiocarcinoma (CCA) are seen in a severe of opisthorchiasis patients (IARC, 2012; Sripa et al., 2012). Extensive experimental and epidemiological studies have been strongly supported the closer of liver fluke connection to CCA. Epidemiological study revealed that

only in heavy infection, approximately 10% of opisthorchiasis patients have contribution risk factor for CCA development (Mairiang et al., 1993). The incidence rate of CCA is correlated with a high prevalence of *O. viverrini* infection (Sripa et al., 2012) which its incidence is still high with 115 and 52.7 cases per 100,000 populations for males and females, respectively (Landis et al., 1998).

Although a precise mechanism of opisthorchiasis-associated CCA is not known, the pathogenesis of infection with a carcinogenic liver fluke contribution to CCA is likely multifactorial factors including, a diet rich in nitrosamine contamination, chronic inflammation of bile duct and parasite secretes molecules etc., (IARC, 2012; Sripa et al., 2012). The histopathological changes are observed in the consequence of inflammation, bile duct hyperplasia, periductal fibrosis, advanced fibrosis, HBD leading to CCA (Sripa et al., 2012). As a consequence, periductal fibrosis after long-term liver fluke infection or partial obstruction by flukes may reflect on the bile flow and bile compositions, leading to enhance bacteria growth (Orlicek et al., 1993).

Although bacteria and parasites including liver flukes are recognized to participate in cholangitis (Carpenter, 1998); however, there are a few information of *O. viverrini* infection on the alteration of liver and HB tract microbiome of the host. Recently, human gut microbiome (Schloissnig et al., 2013) and experimental opisthorchiasis in the colon (Plieskatt et al., 2013) are recently reported. Moreover, several *Helicobacter* species was identified from the gallbladders of Syrian hamsters in association with cholangiofibrosis and centrilobular pancreatitis (Franklin et al., 1996) as well as in precancerous lesion before CCA development (Sirica, 2012). We therefore hypothesize that there are not only *O. viverrini*-caused CCA but also other unknown causative agents such as bacteria may participate in CCA genesis. This idea is supported by a recent finding demonstrating that *Helicobacter pylori* was identified in CCA tissue patients in Thailand (Boonyanugomol et al., 2012).

97 Moreover, the seropositivity against to *H. pylori* has been reported to its association with
98 biliary inflammation and proliferation in CCA in Thai population (Boonyanugomol et al.,
99 2012).

100 In order to identify microbiome in environment niche at a sterile site, several methods
101 have been used to identify bacterial infection such as traditional cultivation technique, PCR
102 technique, restriction fragment length polymorphism analysis, sequencing technique, and
103 metagenomic analysis, etc. (Bragg and Tyson, 2014; Lee et al., 2015). Although cultivation is
104 gold standard method for diagnosis of bacteria; however, a microaerophilic bacterium, such
105 as *Helicobacter* bacteria is difficult to culture, so molecular technique is a method of choice
106 which it can increase the sensitivity and specificity than cultivation technique. Recently, a
107 high throughput next generation sequencing to metagenomic analysis is a power tool for
108 identification and classify of various types bacteria in environment samples by amplicon of
109 16S rDNA sequences (V3-V4 regions) (Bhatia et al., 2015; Lim et al., 2014; Schneeberger et
110 al., 2015).

111 The present study aims to identify *Helicobacter* spp. infection and other aerobic
112 bacteria in chronic opisthorchiasis in the liver, a sterile site in hamster model, we therefore
113 performed many techniques including cultivation for aerobic bacteria, PCR for *Helicobacter*
114 spp. and using metagenomic analysis. Our results revealed an enhancing of bacterial diversity
115 and discovered of *H. pylori* in the liver of chronic opisthorchiasis. The outcome of the study
116 may provide novel information of co-infection between bacteria and *O. viverrini* in
117 hepatobiliary system.

118

119 **2. Materials and methods**

120 *2.1 Experimental animals*

Twenty-six male Syrian golden hamsters (*Mesocricetus auratus*) were obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University and aged between 4-6 weeks were used in this study and were house under conventional condition and given water *ad libitum*. The Animal Ethics Committee of Khon Kaen University (AEKKU 63/2556) approved this study. To induce a chronic condition, hamsters were rearing more than 8 months (8, 12 and 15 months). Animals were divided into 2 groups: 1) normal hamster (Normal, n = 12, 5 animals at 8 months and 7 animals at 15 months), and 2) chronic *O. viverrini*-infected group (OV, n = 14, 5 animals at 8 months, 1 animal at 12 month and 8 animals at 15 months). In OV group, hamsters were infected with single 50 *O. viverrini* metacercaria by oral inoculation and they were anesthetized with ether at 8, 12 and 15 months post-infection.

O. viverrini metacercaria were isolated from the naturally infected cyprinoid fishes by artificial pepsin digestion. The cyprinoid fishes were collected from endemic areas. The fish was minced in electric blender in 0.25% Pepsin A (BDH, USA) solution. Fish : pepsin A solution was 1:3 by volume. The mixture was incubated at 37 °C in continuous stirring water bath for 1 hour followed by straining through a set of four sieves 650, 300, 250 and 106 micrometers apertures, respectively. The remainder on the 106 μm apertures were washed by NSS (0.85% NaCl) and strained through 250 micrometers apertures. Finally, the filtrated mixture was washed several times with NSS in a sedimentation jar until the supernatant was clear. The supernatant was poured off and the sediments are taken to examine for the *O. viverrini* metacercaria, an infective stage, under a dissecting microscope.

2.2 Specimen collection

Hamsters were anesthetized with ether and then liver tissues and worms were immediately collected and snapped frozen in liquid nitrogen and then store at -20°C until

analysis. The second part of liver was immediately collected in thioglycollate broth supplemented with 20% fetal bovine serum (for enhancing aerobic & anaerobic bacterial growth) and in brucella broth supplemented with 10% fetal bovine serum + 3.5 mM H_2O_2 (for enhancing *Helicobacter* species growth). The remained one was fixed in 10% buffered formalin for histopathological study and immunohistochemistry.

In addition, only complete adult worms were randomly collected from gallbladder and biliary system. One worm was collected from each infected hamster and 5-pooled worms were used for gDNA extraction.

2.3 DNA extraction

Genomic DNA (gDNA) was extracted directly from individual livers tissue or worm by using a QiAmp Tissue kit (Qiagen, Germany) according to the manufacturer's protocol. In addition, to ensure for bacterial growth, DNA isolation was also extracted from after cultivation specimens in thioglycollate broth supplemented with 20% fetal bovine serum for 3 days. Concentration, purity and integrity of the gDNA were determined by spectrophotometry (Nanodrop 1000; NanoDrop Technologies, Washington, DE, USA). The gDNAs were stored at 80 °C for further study.

In order to identify *Helicobacter* bacteria in liver and worm, genomic DNA was extracted from frozen liver or after cultivation specimens in brucella broth supplemented with 10% fetal bovine serum + 3.5 mM H_2O_2 for 7 days by using a QiAmp Tissue kit (Qiagen, Germany). Next, gDNA was amplified for *Helicobacter* spp. by specific primers (C97 and C98) and PCR positive for *Helicobacter* was used for amplification of prokaryotic 16S rDNA (V3-V4 region).

gDNA was also extracted from pooled 5 complete adult worms obtained from 5-infected hamsters at 15 months post-infection by using a QiAmp Tissue kit (Qiagen, Germany). The gDNAs was stored at 80 °C for further study.

2.4 Bacterial cultivation

To identify aerobic bacteria, liver tissues were also collected immediately in thioglycollate supplemented with 20% fetal bovine serum broth with sterile technique. Liver was excised and homogenated with sterile buffer and incubated at 37°C until broth was begun turbid. After thioglycollate broth turbid (at least 3 days), bacteria were plated on Blood agar, chocolate agar and Mac Conky's agar. After bacteria growth, several biochemical tests were used to identify genus and species specific.

2.5 PCR analyses

In order to identify for genus-specific of *Helicobacter*, after DNA isolation from frozen liver tissue and cultured specimens (brucella broth), PCR was performed by using genus-specific primers (C97&C98) (Fox et al., 2009). *Helicobacter pylori* DNA (LMG 8775 DMST 20165 type strain was obtained from the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand and was used as a positive control. PCR amplification was performed with a thermal cycler and an Expand high-fidelity PCR system (Bio Rad C100^{TM} Thermal cycle). Each reaction mixture (20 µl) was contained 1× Expand high-fidelity buffer, 1U Platinum *Taq* DNA polymerase, a 5 µM concentration of primers, a 10 mM concentration of each deoxyribonucleotide triphosphate, and 50 mM $MgCl_2$, as described elsewhere. Amplification condition was carried out as protocol (Table 1).

In order to identify for species-specific of *Helicobacter pylori*, the positive liver samples with *Helicobacter* genus PCR was further analyzed for the presence of *H. pylori* with *ureA* gene primer. This gene was used for PCR as per the mentioned protocol in Table 1.

192 In addition, primers used and PCR condition for prokaryotic 16S rDNA (V3-V4
193 region) was presented in Table 1. PCR positive for 16S rDNA (V3-V4) from individual
194 samples was pooled to serve as normal, OV-infected and worm groups and stored at 80 °C
195 until analysis.
196 *2.6 Metagenomic analysis coupled with next generation sequencing using V3-V4*
197 *hypervariable region of prokaryotic 16S rDNA*
198 In order to demonstrate of all bacterial genomes in chronic opisthorchiasis hamsters,
199 metagenomics coupled with next generation sequencing analysis was performed from gDNA
200 isolated from frozen livers tissue and culture specimens. Then, the V3-V4 region of
201 prokaryotic 16S rDNA was amplified using Pro341F and Pro802R primer for prokaryotes
202 and protocol as mentioned in Table 1. After amplification, the PCR products were purified
203 individually by using clean up PCR purification kit (GeneJET PCR Purification kit, Thermo
204 Scientific). After DNA purification, DNA was checked in a quality of samples including
205 concentration and no degradation of DNA by running in gel electrophoresis. Finally, the PCR
206 product was sequenced by using next generation sequencing (Illumina HiSeq/MiSeq
207 platform) for the metagenomic study. Next, the tags or sequences were clustered to
208 Operational Taxonomics Unit (OTUs) using scripts of software USEARCH (v7.0.1090).
209 Then OTUs representative sequences were classified to taxonomic levels using Ribosomal
210 Database Project (RDP) Classifier v.2.2 trained on the Greengenes database (0.8 confidence
211 values as cutoff). The final result, OTUs were analyzed and a data representation in terms of
212 presence/absence, abundance, or phylogenetic diversity (Morgan et al., 2013).

213 *2.7 Analysis of community patterns, taxonomic identification, and diversity analysis*

214 The tags or sequences were assembled into OUTs with an identified threshold of $\geq 97\%$
215 sequence similarity (Schloss et al., 2009; Wang et al., 2007) by using UPARSE. Chimeras were

filtered out tags by using UCHIME (v4.2.40). All of tags were grouped to each OTUs representative sequences by using USEARCH GLOBAL, and then the tags were calculated the number of tags in each sample and summarized to OTUs abundance. Then, OTUs representative sequences were classified to taxonomic levels by using Ribosomal Database Project (RDP) Naive Bayesian Classifier v.2.2 (Schloss et al., 2009; Wang et al., 2007). Accordingly, mother (Schloss et al., 2009) was used to examine the alpha and beta diversity of the microbial communities by using Mother (v1.31.2) and QIIME (v1.80), respectively. A data mining for 16S rRNA data sets was further investigated by suing rarefaction cures, principal coordinates analysis (PCoA), multivariate and regression analysis and analysis of variance.

2.8 Heat map analysis

Heat map is a graphical representation of data where the individual values contained in a matrix which represented as different colors. Heat maps were generated using the g-plots of software R (v3.0.3) and analyzed data based on the relative abundance of each species in each sample in genus- and species-levels. To minimize the differences degree of the relative abundance value, the values were all log transformed.

2.9 Phylogenetic analysis

The sequences were aligned against the Silva core set (Silva_108_core_aligned_seqs) using PyNAST by 'align_seqs.py'. A representative OTU phylogenetic tree was constructed using the QIIME (v1.80) built-in scripts including the fast tree method for tree construction. The tag with highest abundance of each genus was chosen as the corresponding genus representative sequences, and genus level phylogenetic tree was obtained by the same way of OTU phylogenetic tree. The phylogeny tree was generated image by software R (v3.0.3).

2.10 Histopathological study

Paraffin section was used for H&E staining. Slides were incubated at 100°C for 10 minutes and deparaffinized in xylene three times for 3 minutes to remove the paraffin wax. After deparaffinization, slide was hydrated by decreasing concentrations of ethanol. After hydration, slide was stained with Harr's hematoxylin and eosin solution (shaking the slides for all the times). The slides were dehydrated through repeat three times for 3 minutes in each step as following: 70%, 80%, 95% and absolute alcohol, xylene solution, and then mounted. The appropriate result for nuclei was stained blue and cytoplasm was stained red color.

2.11 Immunohistochemistry study

In order to indicate the presence of *Helicobacter pylori* in liver tissue, liver paraffin sections (5-μm thickness) were deparaffinized in xylene and rehydrated in descending gradations of ethanol. To enhance the immunostaining, the sections were placed in citrate buffer (pH 6.0) and autoclave at 110°C for 10 minutes for retrieval antigen. Next, the sections were transferred to 3%H_2O_2 in PBS buffer jar for inhibition of endogenous peroxidase activity. Then, the sections were incubated with 5% fetal bovine serum in PBS for 30 minutes. Then, the sections were incubated with primary antibody (rabbit polyclonal to somatic antigens of the whole *H. pylori* organism; 1:10 dilution) at 4°C for overnight. Finally, slides were incubated with the secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) at room temperature for 1 hour. The stained sections were examined using a microscope.

2.12 Statistical analysis

All statistical analyses were performed using the SPSS version 15 statistical program. Chi-squared test were used for non-parametric data, cholangitis grading. *P* less than 0.05 was considered a significant difference.

3. Results

3.1. Bacterial isolation

Isolation of aerobic bacteria from the liver samples was positive for 100% (5/5) at 8-months post-infection (p.i.), while it was negative (0/5) for normal liver. These bacterial growth in agar plate culture included *Streptococcus group D non Enterococci* (20%), *Enterobacter* spp. (20%), *Escherichia coli* (40%), *Streptococcus pyogenes (group A)* (20%) *and Klebsiella pneumonia* (20%). In contrast, after incubation for at least 7 days, no bacteria growth was observed for microaerophillic condition of *Helicobacter* spp. cultivation from liver tissues at 8 and 15 months p.i.

3.2. Detection of genus-specific of Helicobacter and 16S rDNA(V3-V4 region) prokaryotic by PCR from liver samples

Identification of *Helicobacter* DNA was analyzed with genus of *Helicobacter* primer from 14 liver samples from chronic OV-infected group. Three samples (3/14, 22%) were positive with genus of *Helicobacter* gene in OV-infected group but was negative result in normal liver and in worm. For identification of other bacteria, all of liver samples in chronic OV-infected group was positive (14/14, 100%) for prokaryotic16S rDNA (V3-V4 region). In addition, bacteria were cultured in a sterile site of normal liver to enhance bacterial growth before being amplified by V3/V4 region and were positive 85.71% (6/7, normal at 15 months) in normal group.

3.3. OTUs from 24 genera and 6 phyla of Helicobacter spp. and other bacteria

Genomic DNA isolation from liver and worm samples of OV-infected and normal groups were investigated by metagenomics analysis and the result was classified to taxonomic levels follow as genus-level and species-levels. Of 855,046 sequences, 417,953

with useable reads were assignable to 155 operational taxonomy units (OTUs), 6 phyla and 24 genera of bacteria. OTUs of *Bifidobacterium*, *Escherichia* and *Helicobacter* were read from only in OV-group but not from other group. OTUs of *Fusobacterium*, *Aggregatibacter*, *Streptococcus* and *Veillonella* were increased in OV-group compared to normal control. In contrast, OTUs of *Acidaminococcus*, *Clostridium*, *Lactobacillus*, *Megasphaera* were decreased in OV-group compared to normal control. In OV-group, although the sequence reads was less than that in normal group, but the OTUs was higher, indicating a high genetic diversity among these group (Table 2).

3.4. Chronic O. viverrini infection promotes Helicobacter pylori growth and enhances other microbiota in the liver and hepatobiliary tract

The distribution of bacterial genomic DNA isolation at genus-specific level in hamster liver and hepatobiliary tract (HB) is shown in Fig. 1. In normal group, the relative abundance of genus-level of bacterial sequences included *Lactobacillus* (78.32%), *Clostridium* (8.24%), *Acidaminococcus* (7.66%), *Streptococcus* (2%), *Aggregatibacter* (0.85%), *Megasphaera* (0.64%), *Veillonella* (0.14%), Unclassified (1.83%), and Others (0.3%). The most frequent of relative abundance of sequences was *Lactobacillus*, *Acidaminococcus* and *Clostridium*. In chronic OV-infected group, the distribution of bacterial genomic DNA were *Lactobacillus* (24.83%), *Fusobacterium* (13.81%), *Streptococcus* (10.77%), *Escherichia* (10.19%), *Aggregatibacter* (3.34%), *Veillonella* (1.29%), *Helicobacter* (0.99%), *Bifidobacterium* (0.58%), *Clostridium* (0.58%), Unclassified (33.24%), and Others (0.92%). In adult worm, two genus of *Aggregatibacter* (39.65%), *Lactobacillus* (60.29%) DNA was identified. Notably, there was more relative abundance of genetic bacterial diversity in genus-level in chronic OV-infected group than in normal and worm group (Table 2).

The relative abundance of bacterial DNA at species-level is shown in Fig. 2. In normal group, bacterial genomic DNA of *Lactobacillus reuteri* (21.55%), *Lactobacillus agilis* (18.23%), *Lactobacillus coleohominis* (1.26%), *Aggregatibacter pneumotropica* (0.84%), *Veillonella dispar* (0.1%), *Streptococcus luteciae* (0.08%), *Lactobacillus salivarius* (0.02%), were identified in liver tissues. In chronic OV-infected group, relative abundance of bacterial DNA were *Fusobacterium* spp. (13.81%), *Streptococcus luteciae* (10.76%), *Escherichia coli* (10.18%), *Lactobacillus salivarius* (5.85%), *Lactobacillus reuteri* (4.16%), *Aggregatibacter pneumotropica* (3.33%), *Lactobacillus agilis* (3.02%), *Veillonella dispar* (1.09%), *Helicobacter* spp. (0.82%), *Bifidobacterium* spp. (0.58%), *Lactobacillus coleohominis* (0.56%), *Helicobacter pylori* (0.17%), and Unclassified (45.36%). In adult worm, three species of *Lactobacillus salivarius* (56.85%), *Aggregatibacter pneumotropica* (39.66%), and *Lactobacillus reuteri* (3.44%) of genomic bacteria were identified. Among normal, infected liver and adult worm samples, there were difference in bacterial species and the relative abundance of genetic bacteria population, which showed the highest number of bacterial species in chronic OV-infected group.

Fig. 3 showed the taxonomic clustering of microbiomes based on 16S rRNA sequences from liver and worm according to time-post infection. The genus-level of bacteria was closely similarity at 8, 12 and 15 months post-infection but was different in bacteria population when compared to the other groups. *Aggregatibacter*, *Lactobacillus* and unclassified were co-evolution among three groups. *Fusobacterium*, *Escherichia* and *Bifidobacterium* were relative abundance according to time-post of *O. viverrini* infection which were higher abundance in OV-group than in normal group. In addition, *Megasphaera*, *Clostridium*, and *Acidaminococcus* were high relative abundance in normal but were low abundance in OV-infected and in worm, suggesting that *O. viverrini* infection causes environmental changes leading to affect on these bacteria growth.

335 The community diversity in microbiota of OV-infected liver was analyzed by OTU
336 rank curve, observed species and shannon indicies (Fig.4). The OTU rank curve (Fig.4a),
337 observed species (Fig.4b) represented species richness of microbial community in OV-
338 infected group than normal group. The rarefaction analysis by shannon indices, mean indicies
339 of 1.76, 2.27, 0.43, and 0.80 were presented for normal, OV-infected 15 months, OV-infected
340 8,12 months and adult *O. viverrini*, indicating that the species diversity of microbial
341 immunity in OV-infected 15 months than the other group (Fig. 4c). OTU PCA analysis
342 showed that the differences of OTU composition in different samples of normal, OV-infected
343 at 15 months, and adult *O. viverrini* were closely relationship with aerobic and anaerobic
344 bacteria, and in OV-infected at 8, 12 months were closely relationship with *Helicobacter*
345 species (Fig.4d).

346 *3.5. The Evolution of bacteria between host and parasite*

347 The relationship between taxonomy and phylogenic tree of 42 genera from 6 phyla
348 based on the nucleotide sequences of the V3-V4 hypervariable region of prokaryotic 16S
349 rDNA isolated from liver and worm (Fig.5). There were three routes of evolutionary
350 relationships among various biological species of bacterial identification in hamster liver and
351 in worm. These consisted of 6 phyla including *Firmicutes* (79.338%), *Proteobacteria*
352 (17.134%), *Fusobacterium* (3.187%), *Actinobacteria* (0.188%), *Bacteroidetes* (0.039%) and
353 *Cyanobacteria* (0.006%). The most frequent of bacteria in a phylum was found in the order
354 of *Proteobacteria* (16 genus), *Firmicutes* (15 genus), *Actinobacteria* (6 genus), *Bacteroidetes*
355 (3 genus), and each genus for *Cyanobacteria* and *Fusobacterium*.

356 In order to identify the co-evolution of bacteria species between host-parasite
357 interplay, venn diagram was constructed in Fig. 6. Identification of bacteria species between
358 normal liver and worm were *A. pneumotropica* and *L. reuteri*, while *L. salivarius* were

identified in worm only. *L. agilis*, *L. coleohominis*, *S. luteciae*, *A. pneumotropica*, *L. reuteri* were isolated from normal group and OV-infected group, suggesting that these bacteria are normal flora in hepatobiliary system. The growth of bacteria diversity including *E. coli*, *H. pylori*, *Helicobacter* spp., *Bifidobacterium* spp., *Fusobacterium* spp., *V. dispar*, and *L. salivarius* were found only in infected group, suggesting that *O. viverrini* infection increases influx of gut and other site of bacteria population growth. *A. pneumotropica*, *L. salivarius* and *L. reuteri* were identified from worm and OV-infected group, implying that worm might be infected during reside in the hepatobiliary system of the host. Notably, *A. pneumotropica* and *L. reuteri* were found in among three groups, suggesting that these two bacteria are normal flora in hepatobiliary tract of hamster. Moreover, *E. coli*, *H. pylori*, *Helicobacter* spp., *Bifidobacterium* spp., *Fusobacterium* spp., and *V. dispar* were identified only in OV-group, suggesting that *O. viverrini* infection might be enhanced these bacterial growth from other sites such as gastrointestinal lumen.

3.6. Detection of specie-specific of Helicobacter pylori (ureA gene) by PCR from liver tissue

In order to confirm the metagenomic analysis, specie-specific of *H. pylori* (ureA gene) was analyzed by PCR using specific primer. All the 3 *Helicobacter* genus positive samples from OV-infected group were analyzed for the presence of ureA gene. One sample gave positive amplification for ureA gene which was supported to metagenomics result. The amplified product of ureA gene is represented in Fig. 7.

3.7. The presence of Helicobacter pylori infection in tissue and worm by Immunohistochemetry technique

In order to localize of *H. pylori* in liver tissue, we performed by immunohistochemical stain using specific antibody against to *H. pylori*. The immunoreactive staining was observed in the hepatocytes, sinusoids, epithelial cell of large bile duct and

inside the *O. viverrini* worm. In addition, normal liver didn't show any signal of immunoreactivity (Fig. 8).

3.8. Histopathological study

Liver tissue was stained by hematoxylin & eosin staining (Fig. 9). Cholangitis grading was defined by the accumulation of inflammation cells, especially polymorphonuclear cells including neutrophil and eosinophil around large and small bile ducts. Three normal livers were also observed of cholangitis similar to OV-group. In liver tissue of OV-infected group, the accumulation of cholangitis grading was significantly observed higher than in normal group (P 0.05).

4. Discussion

Several bacterial infection in hepatobiliary diseases (HBD) have been previously reported in human (Aviles-Jimenez et al., 2015; Boonyanugomol et al., 2012) and hamster tissues (Fox et al., 2009). Boonyanugomol et al. have identified *H. pylori* in CCA tissue patients (Boonyanugomol et al., 2012). In hamster-infected with *O. viverrini*, typically, bile is a good sample of microenvironment for microbiota discovery in HBD; however, in chronic opisthorchiasis, especially at more than 8 months p.i., it's scarcely to obtain bile, therefore, we used liver instead of bile sample. Moreover, *H. pylori* was rarely identified by direct PCR of infected liver samples (less than 20%) or after cultivation. In order to investigate *Helicobacter* spp. and other microbiota in a sterile site, liver, we used many techniques including cultivation of aerobic bacteria, PCR for *Helicobacter* and metagenomics coupled with next generation sequencing based on nucleotide sequences of the V3-V4 hypervariable region of prokaryotic 16S rDNA. We have successful to identify genomic DNA of *H. pylori* in the liver. In addition, OTUs of genomic bacteria by direct PCR of V3-V4 hypervariable

region of prokaryotic 16S rDNA and after cultivation wasn't significantly different in bacteria diversity of infected liver (Table 2).

Recent finding showed that infection with the *O. viverrini* at 45 days modified intestinal and microbiome in hamsters by using pyrosequencing and amplified V7-V9 hypervariable region of prokaryotic 16S rDNA (Plieskatt et al., 2013). In this study, we applied the high throughput techniques of next generation sequencing (NGS) to the metagenomics study and identified PCR amplicon of 16S rDNA sequences (V3-V4 regions) in a sterile site, liver tissue. Typically, PCR amplicon of 16S rDNA sequences (V3-V4 regions) with the metagenomics technique is typically detected in fresh specimens by direct PCR of target regions of 16S rDNA sequences and could be generated a > 5 million of OTUs reads sequences from the samples(Akinsanya et al., 2015; Herlemann et al., 2011). In this study, 417,953 reads were assignable to 155 OTUs, 6 phyla and 24 genera of bacteria in the liver and worm. After *O. viverrini* infection at 8, 12, and 15 months, the most common of these bacteria were found in phylum of *Proteobacteria* (16 genus), *Firmicutes* (15 genus), *Actinobacteria* (6 genus), *Bacteroidetes* (3 genus), and each genus for *Cyanobacteria* and *Fusobacterium*, respective. The most common relative abundance sequences of *Fusobacterium* spp. (13.81%), *Streptococcus luteciae* (10.76%), *Escherichia coli* (10.18%), and *Bifidobacterium* spp. (0.58) were detected in the liver, which had difference of bacteria diversity from normal group. Furthermore, *Helicobacter pylori* (0.17%) and *Helicobacter* spp. (0.82%) were also identified in the liver of chronic opisthorchiasis, but didn't in normal liver. We hypothesize that *H. pylori* and other microbiota in the liver during chronic opisthorchiasis might be reflected from an obstructive of biliary system from adult worm and influx of bacterial growth from GI tract and gastric leading to participate in HBD. Our finding was supported with a previous observation that *O. viverrini* is a reservoir for species of *Helicobacter* (Deenonpoe et al., 2015) in the same model.

Aggregatibacter pneumotropica, *Lactobacillus reuteri* and *Lactobacillus salivarius* were commonly found in the liver and worm during chronic OV- infected group. These results suggest that worm may carry bacteria growth though the gastro-intestinal system into hepatobiliary system after chronic opisthorchiasis. Because of these bacteria didn't identify in normal liver (Fig.6). *Escherichia coli*, *Helicobacter pylori*, *Helicobacter* spp., *Streptococcus luteciae, Bifidobacterium* spp., and *Fusobacterium* spp., were also found high prevalence in OV-infected group whereas *Helicobacter pylori*, *Helicobacter* spp. and *Veillonella dispar* were rarely seen, but they are obvious in relation to tumor development (Cao and Yu, 2015; Kasai et al., 2016).

Alternatively, the combination of bacteria and parasite infection may synergistic increase the severity of the disease similar to *Wolbachia* bacteria in filarial nematodes associated syndrome in animal (Kramer et al., 2005) and patients (Nambiar et al., 2006). Thus, anti-*Wolbachia* bacteria treatment is a new therapy approach for lymphatic filariasis (Stolk et al., 2005). However, the underlying mechanism of the combination of bacteria and *O. viverrini* infection involving in HBD didn't clearly depict and are required for further study.

The present study demonstrated that *Helicobacter, Enterobacteriaceae, Bacteroides, Lactobacillus* and anaerobic bacteria were also identified in the liver, which are similar from previous study by molecular methods in CCA tissues of patients (Abu Al-Soud et al., 2008). Also, in non-liver fluke endemic areas, bacteria cause most cases of infectious cholangitis in Western countries (Catalano et al., 2009). This suggests the possible mechanism of co-infection with bacteria has been hypothesized. It is assuming that co-infection with bacteria may occur via several mechanisms. These explanations are: (1) as a direct result of the irritating chemical composition of the parasite, parasitic secretions, or eggs; (2) physical obstruction of the bile ducts; (3) induction of formation of biliary stones; and (4) introduction

of bacteria into the biliary system during migration from the duodenum (Carpenter, 1998). After epithelial damage caused by fluke sucker, *Helicobacter* spp. such as *H. pylori* and non-*Helicobacter* may enter into blood and cause cholangitis leading to HBD which finally develop to CCA happening. Similarly, the novel *Helicobacter*, *H. hepaticus* causes chronic active hepatitis, which is a likely candidate for the etiology of hepatocellular tumors in mice (Pace et al., 1989).

Our results are agreement with many previously evidences that several bacterial infections are identified in HBD including aerobic, microaerophillic and anaerobic bacteria. *Enterobacteriaceae* are the most commonly found in aerobic bacteria such as *Escherichia coli*, *Enterobacter* spp. and *Bacteroides* spp. In microaerophillic bacteria, *Helicobacter* spp. is the most interesting, because it has been identified in a variety of the gastrointestinal tract diseases including peptic ulcer, gastric cancer, and inflammatory bowel disease in humans and animals (Andersen, 2001; Fox et al., 2009; Orlicek et al., 1993; Simmons et al., 2000; Zenner, 1999). *H. pyroli* is well known as a causative agent in gastric cancer, which is a well-recognized linkage of infection and cancer (Maeda, 1998). Moreover, several species of *Helicobacter* have been identified in normal hamsters such as *H. hepaticus, H. muridarum, H. bilis, H. rodentium, H. cinaedi, H. mesocricetorum, H. aurati, H. cholecystus* and *H. pylori* (Fox et al., 2009; Patterson et al., 2000; Simmons et al., 2000; Zenner, 1999). Although we couldn't successful to identify these *Helicobacter* spp. in the liver, many unidentified bacteria was discovered, which might belong to these species. Nevertheless, unclassified bacteria in chronic OV-infected group may be involved in chronic opisthorchiasis-related HBD and are required further identification.

5. Conclusion

Next generation sequencing to the metagenomics study and identified PCR amplicon of 16S rDNA sequences (V3-V4 regions) was successful to identify microbiota in a sterile site, liver tissue. *Helicobacter* spp. and *H. pylori* was identified in the liver of chronic opisthorchiasis. Chronic opisthorchiasis enhances bacterial diversity in the liver, which might be related to HBD. The results in this study might provide a basic knowledge and may be useful for a new therapeutic approach in opisthorchiasis-associated hepatobiliary disease and can be reduce incidence of CCA.

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Research Highlights

- Genomic DNA of *Helicobacter pylori* was identified in chronic opisthorchiasis.
- Chronic opisthorchiasis enhances genomic bacteria diversity in the liver.
- Bacteria and liver fluke might have co-evolution during chronic opisthorchiasis.
- *Helicobacter* species and other bacteria may be involved in hepatobiliary diseases.

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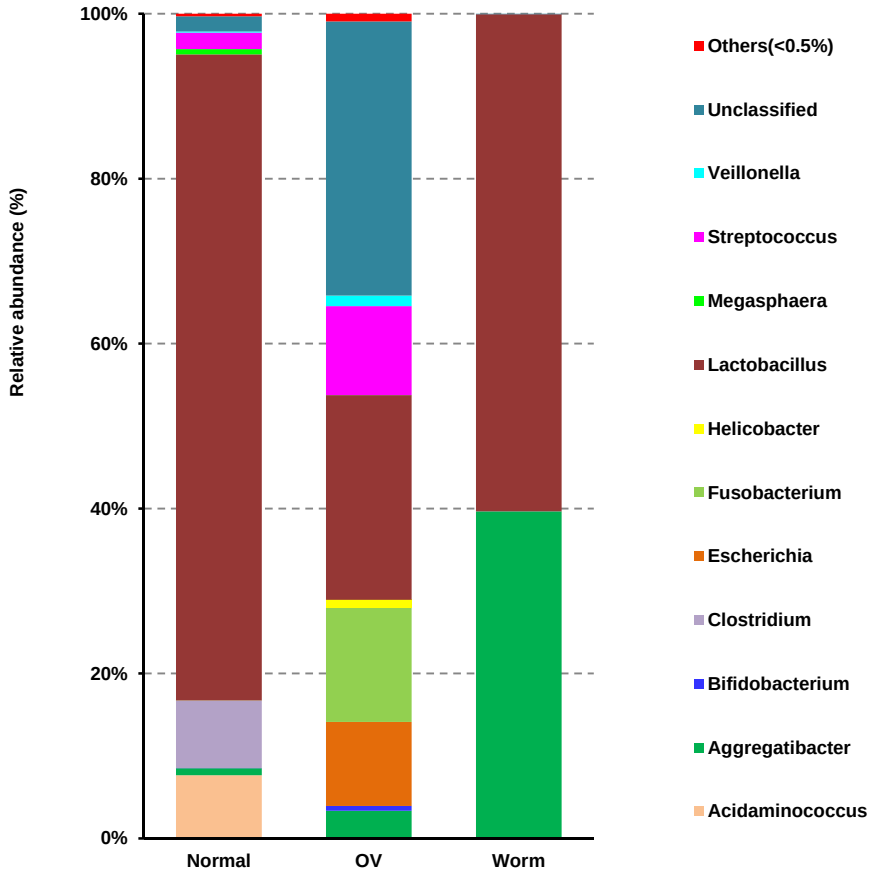

Fig. 1. Distribution and diversity of bacterial DNA at genus-level in hamster liver and worm samples. The species of which genus abundance is less than 0.5% in all samples were classified into 'others'. Normal : Normal group, OV : *O. viverrini*-infected group, Worm : *O. viverrini* adult.

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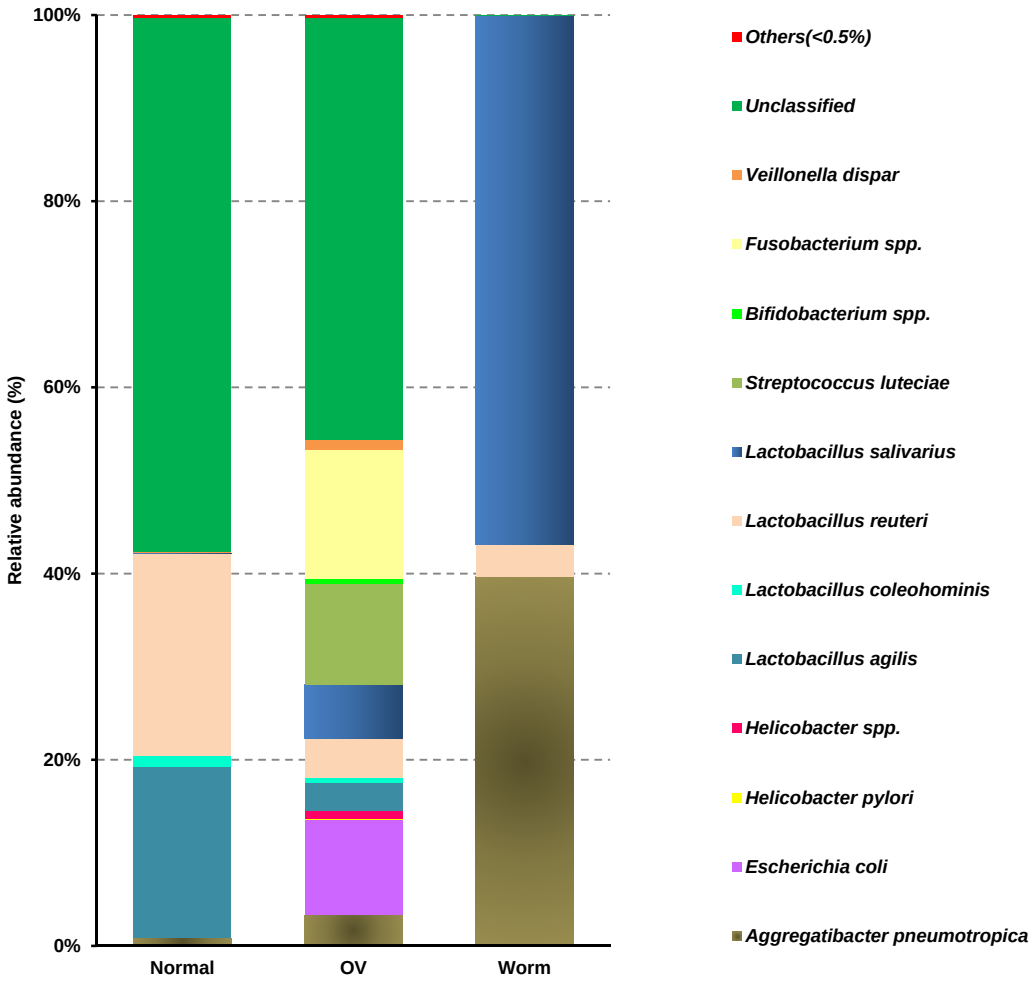

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631 Fig. 2. Distribution and diversity of bacterial DNA at species-level in hamster liver and

632 worm samples. The species of which species abundance is less than 0.5% in all samples were

633 classified into 'others'. Normal : Normal group, OV : *O. viverrini*-infected group, Worm : *O.*

634 *viverrini* adult.

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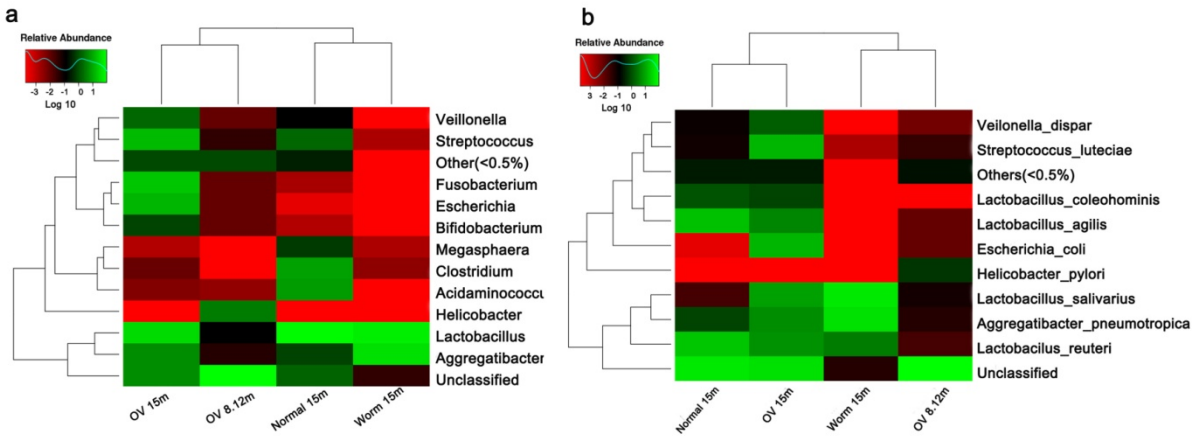


Fig. 3. Heat map of identified bacteria at genus (a) and species (b)-levels in hamster liver and worm. Longitudinal clustering indicates the similarity of all species among different samples, and the horizontal clustering indicates the similarity of certain species among different samples. The closer distance is the shorter of the branch length and the more similar the species composition is between the samples. Normal. 15 m (n = 7): Normal group, OV.15m: *O. viverrini*-infected group at 15 months, OV.8.12m : *O. viverrini*-infected groups at 8 and 12 months, Worm 15 m: worm obtained from *O. viverrini*-infected for 15 months.

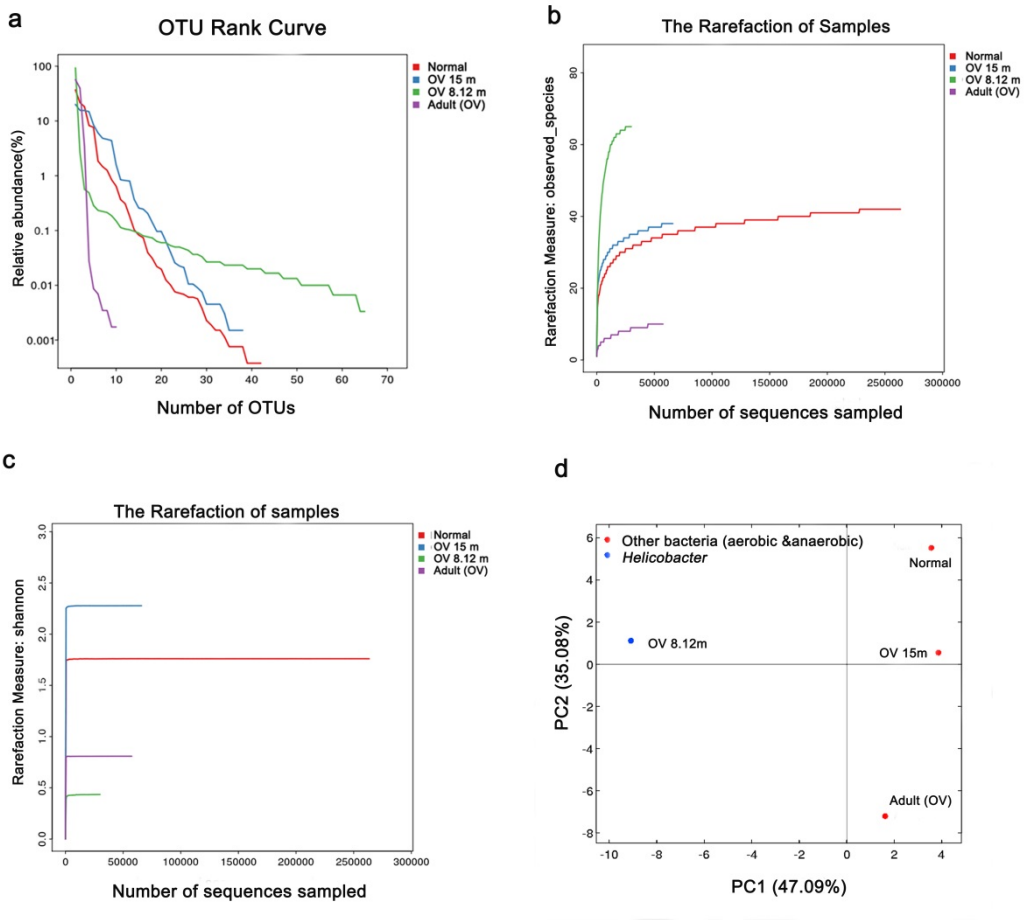
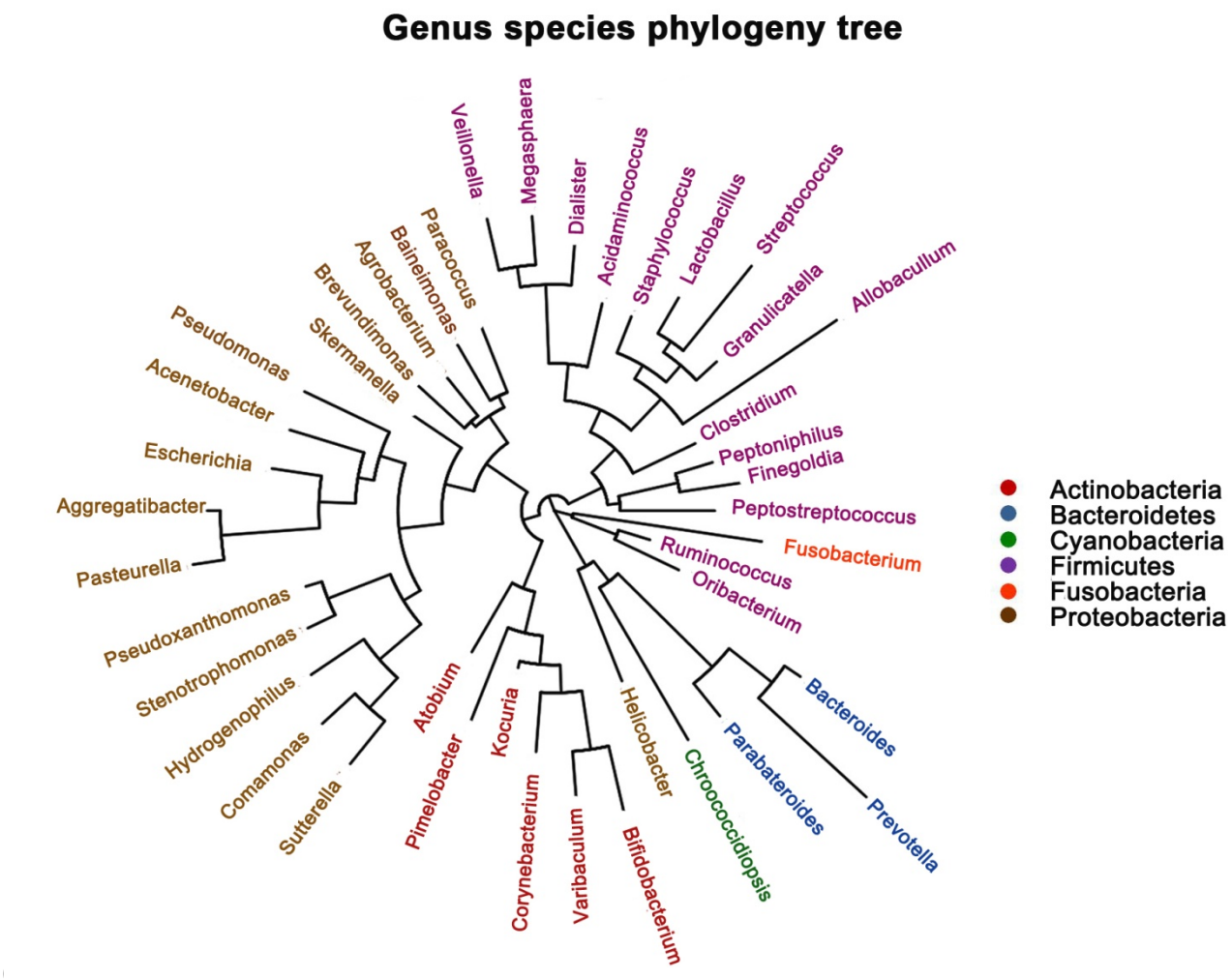


Fig.4. Complexity of microbial diversity in hamster liver and worm samples in chronic opisthorchiasis. (a) OTU rank abundance curve. (b) Rarefaction plot of alpha diversity (observed species indices). (c) Rarefaction plot of alpha diversity (shannon indices). (d) PCA (principle component analysis) displays the difference OTU composition in different samples.



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661 Fig. 5. Phylogenetic tree of identified bacteria associated with chronic opisthorchiasis based
662 on the nucleotide sequences of the V3-V4 hypervariable region of prokaryotic 16S rDNA.
663 The same Phylum is shown as the same color. The evolution distance between genus is closer
664 in shorter branch length.

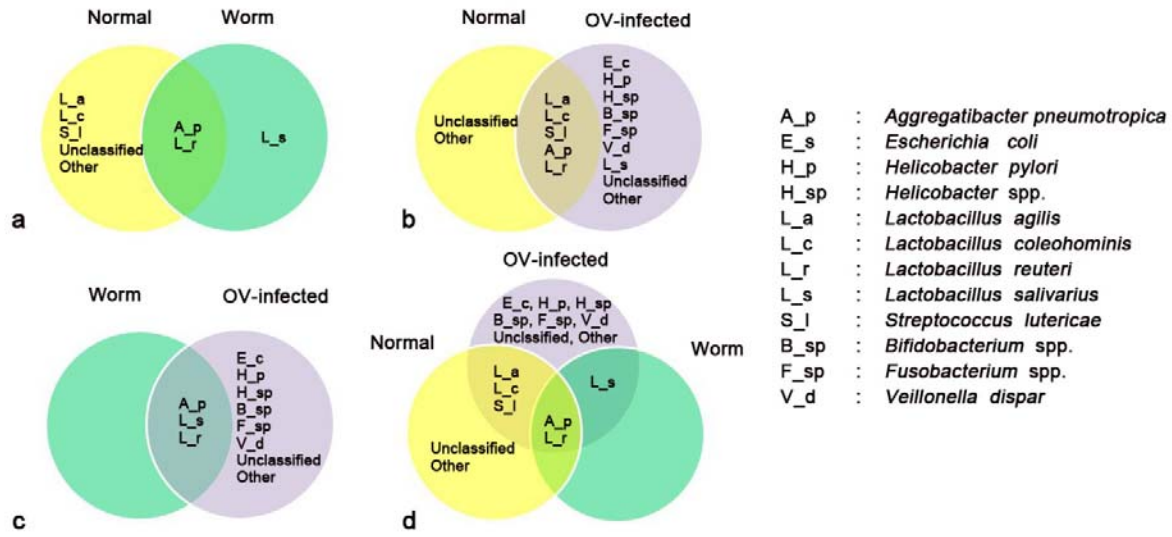

Fig.6. Venn diagram of identified bacterial species among difference groups. (a) Normal group and Worm group. (b) Normal group and OV-infected group. (c) Worm group and OV-infected group. (d) Among three groups. Different color is presented in different samples or groups. The overlapping area represents bacteria species commonly present in the counterpart group.

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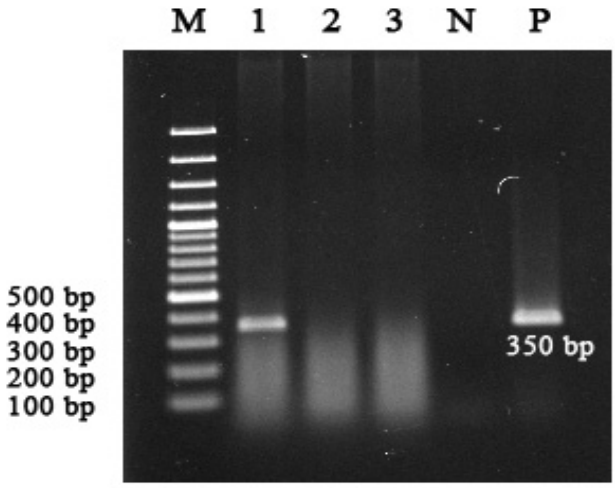

673 Fig.7. Detection of Helicobacter pylori (ureA gene) from liver frozen specimen by PCR
674 technique. Product size was 350 bp. M: 100-bp molecular weight Marker, P: Positive control,
675 N: Negative control, Lane 1-3: positive with genus specific of Helicobacter from three OV-
676 infected hamsters. Lane 1 showed positive of both genus and species specific for
677 Helicobacter and Helicobacter pylori. Lane 2 and 3, positive with genus specific of
678 Helicobacter from OV-infected hamsters at 8 months post-infection, but was negative results
679 for H. pylori (ureA gene).

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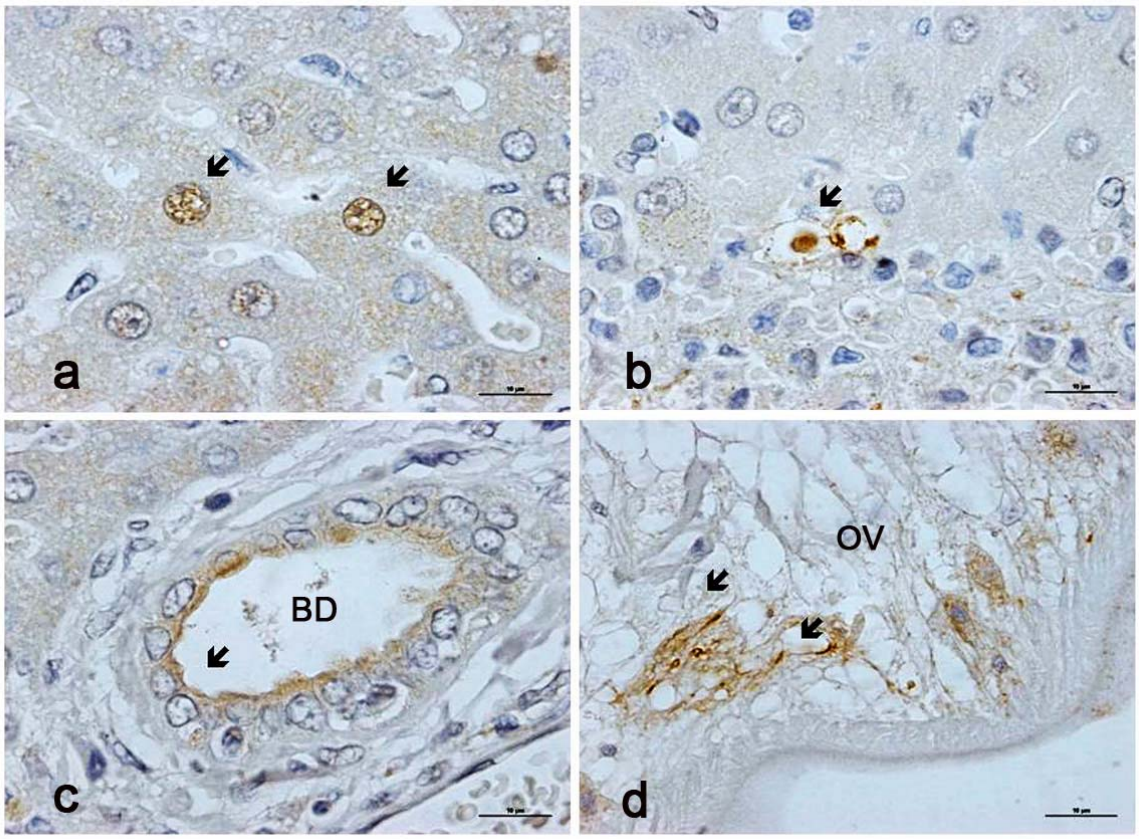

Fig.8. Immunohistochemical localization of *Helicobacter pylori* infection in hamster liver-infected with *O. viverrini*. Immunoreactive staining for *H. pylori* presents in brown color (arrow) of (a) hepatocytes, (b) sinusoid, (c) bile duct and (d) *O.viverrini* worm. Original magnification, x1000.

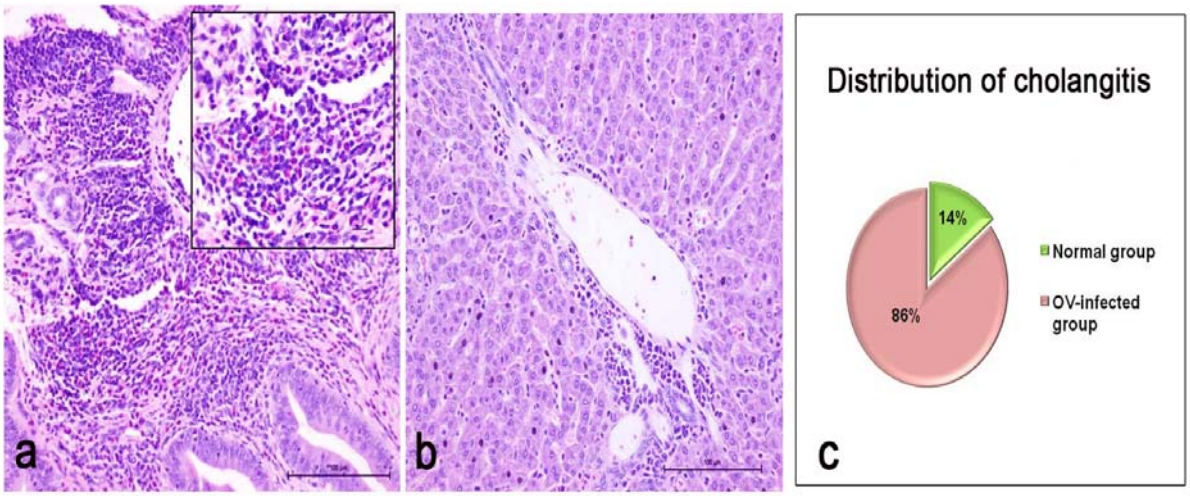

Fig.9. Histopathology of hamster liver tissue (H&E stains). (a) neutrophilic, eosinophilic and mixed inflammation cell surrounding bile duct and hepatic portal vein in liver tissue of chronic OV-infected group. (b) normal hamster liver tissue. Original magnification, x200.

Table 1 List of primers and conditions for PCR reaction used to amplify a particular target of bacteria regions.

Organism	Genes	Primer sequence (5'→3')	Product size (bp)	PCR conditions
Prokaryote bacteria	16S rDNA (V3-V4) Pro341-F Pro802-R	 5'CCTACGGGNGGCWGCAG3' 5'TACNVGGGTATCTAATCC3'	459	94°C 5 mins, 94°C 40 sec, 52.8°C 30 sec, 72°C 2 mins, 35 cycles, 72°C 10 mins
Helicobacter genus	C97-F C98-R	5'GCTATG ACG GGT ATC C3' 5'GATTTT ACC CCT ACA CCA3'	411	94°C 5 mins, 94°C 1 mins, 57°C 1.5 mins, 72°C 1 mins, 35 cycles, 72°C 7 mins
Helicobacter pylori	*ureA*-F *ureA*-R	5'AGTTCCTGGTGAGTTCTTAA3' 5'AACCACGCTCTTTAGCTCTGTC3'	350	94°C 2 mins, 94°C 30 sec, 55.7°C 30 sec, 72°C 1 mins, 40 cycles, 72°C 5 mins

718 **Table 2** Number of read sequences in bacteria at genus-level in hamster liver and worm samples by next generation sequencing

719 (Illumina HiSeq/MiSeq platform) of *V3-V4 hypervariable region of prokaryotic 16S rDNA.*

720

Genus-level	Number of read sequences or tags				
	Normal, Liver		OV-infected, Liver		Adult *O. viverrini*
	Frozen	Thioglycollate	Frozen	Thioglycollate	
Acidaminococcus	ND	20221	1	3	0
Aggregatibacter	ND	2233	15	3205	22853
Bifidobacterium	ND	0	3	560	0
Clostridium	ND	21742	0	6	2
Escherichia	ND	0	3	9820	0
Fusobacterium	ND	5	3	13314	0
Helicobacter	ND	0	962	0	0
Lactobacillus	ND	206694	39	23905	34744
Megasphaera	ND	1704	0	1	1
Streptococcus	ND	5279	11	10378	1
Veillonella	ND	374	3	1244	0
Unclassified	ND	4848	28724	3333	23
Others (<0.5%)	ND	810	262	624	0
Total reads	ND	263910	30026	66393	57624
OTUs number		42	65	38	10

721 ND = Not determined

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