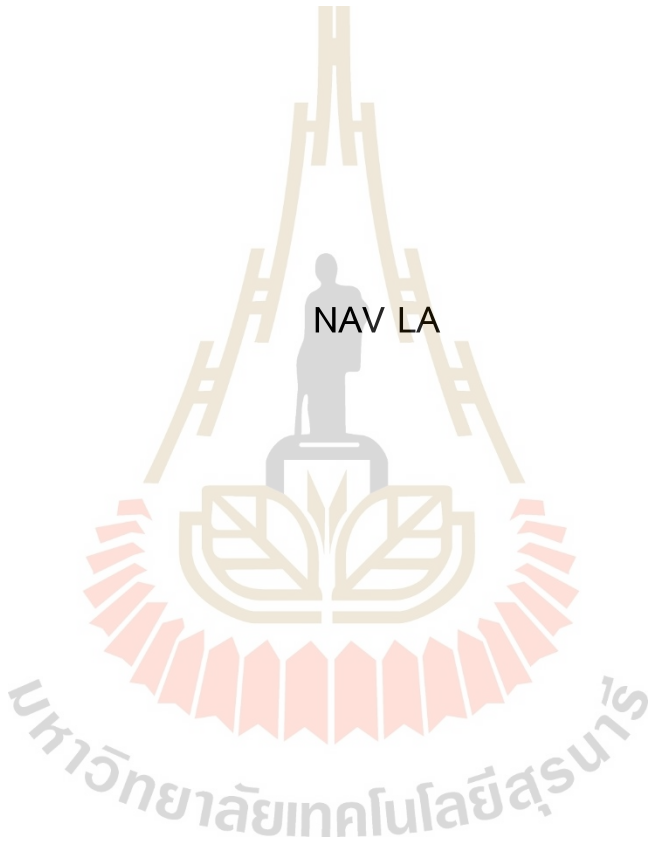
DISCRIMINATION OF HUMAN CARCINOGENIC LIVER FLUKE-LIKE EGGS FROM CAMBODIA USING PARASITOLOGICAL AND MOLECULAR METHODS

NAV LA



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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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นายนาว ลา: การวินิจฉัยไข่พยาธิใบไม้ตับก่อโรคมะเร็งท่อน้ำดี ด้วยวิธีทางปรสิตวิทยาและชีวโมเลกุลในประเทศกัมพูชา (DISCRIMINATION OF HUMAN CARCINOGENIC LIVER FLUKE-LIKE EGGS FROM CAMBODIA USING PARASITOLOGICAL AND MOLECULAR METHODS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ แพทย์ญิงชวัลญ์ญา รัตนพิทูลย์, 174 หน้า.

คำสำคัญ: ออพิสทอร์คิสวิเวอร์รินิ/ ฟอร์มาลินเอทิลอะซิเตท/ ปฏิกิริยาลูกโซ่โพลีเมอเรส/ โรคมะเร็งท่อน้ำดี/ ไฟโลจีเนติกทรี/ ประเทศกัมพูชา

ออพิสทอร์คิสวิเวอร์รินิเป็นพยาธิใบไม้ตับ ที่พบในพื้นที่เอเชียตะวันออกเฉียงใต้และมีความสัมพันธ์ในการเกิดโรคมะเร็งท่อน้ำดี ซึ่งเป็นปัญหาที่สำคัญทางสาธารณสุขในปัจจุบัน ดังนั้นการตรวจวินิจฉัยโรคอย่างแม่นยำและรวดเร็วจึงเป็นสิ่งสำคัญสำหรับการควบคุมการติดเชื้อพยาธิชนิดนี้ได้อย่างมีประสิทธิภาพ ในขณะเดียวกันการตรวจภายใต้กล้องจุลทรรศน์ยังคงเป็นวิธีการหลักสำหรับการตรวจวินิจฉัย แต่วิธีการนี้ยังมีข้อจำกัดของความไวหากมีการติดเชื้อในระดับที่ต่ำ ดังนั้นการวิจัยนี้ จึงมีการประยุกต์ใช้วิธีการของปฏิกิริยาลูกโซ่โพลีเมอเรส [polymerase chain reaction (PCR)] สำหรับตรวจสอบยีน internal transcribed spacer 2 (ITS2) รวมทั้งยีน cytochrome c oxidase subunit 1 (*COX1*) และ ยีน NADH nicotinamide adenine dinucleotide subunit 1 (*NAD1*) เพื่อแยกชนิดย่อยทางพันธุกรรม อีกทั้งยังใช้การตรวจวินิจฉัยของวิธีความเข้มข้นของฟอร์มาลินเอทิลอะซิเตท [formalin-ethyl acetate concentration technique (FECT)] ควบคู่ด้วย วิธีการข้างต้นเป็นการตรวจการติดเชื้อพยาธิออพิสทอร์คิสวิเวอร์รินิ ในตัวอย่างอุจจาระคน นอกจากนี้ยังมีการศึกษาด้วยการใช้เอนไซม์ตัดจำเพาะชนิด *Fau*1 เพื่อจำแนกความแตกต่างของพันธุกรรมในพยาธิออพิสทอร์คิสวิเวอร์รินิระหว่างประเทศไทยและประเทศกัมพูชา

การสำรวจความชุกของการติดเชื้อพยาธิออพิสทอร์คิสวิเวอร์รินิ ใช้วิธีการวิเคราะห์ข้อมูลแบบสองตัวแปรและหลายตัวแปรด้วยโปรแกรมซอฟต์แวร์ SPSS เวอร์ชั่น 23.0 ผลการวิเคราะห์พบว่าอัตราการติดเชื้อปรสิตในลำไส้เท่ากับ 15.91% (จำนวนอุจจาระ 377 ตัวอย่าง) จากการตรวจวินิจฉัยด้วยวิธี FECT พบการติดเชื้อพยาธิออพิสทอร์คิสวิเวอร์รินิ มากที่สุด (5.57%) ตามด้วยพยาธิปากขอ (4.24%), โปรโตซัวกลุ่มอะมีบา (*Entamoeba coli*) (3.97%), พยาธิไส้เดือน (*Ascaris lumbricoides*) (1.06%), พยาธิตืดแคระ (*Hymenolepis nana*) (1.06%), กลุ่มพยาธิตืด (*Taenia* spp.) (0.53%) และพยาธิแส้ม้า (*Trichuris trichiura*) (0.53%) ตามลำดับ ส่วนการตรวจวินิจฉัยด้วยวิธี PCR ของพยาธิออพิสทอร์คิสวิเวอร์รินิ (6.89%) พบสูงกว่าวิธี FECT โดยพบในผู้ชาย (8.92%) สูงกว่าผู้หญิง (5.45%) และกลุ่มอายุน้อย (13.40%) มีความสัมพันธ์กับการติดเชื้อพยาธิออพิสทอร์คิสวิเวอร์รินิ ในบุคคลที่ไม่รู้หนังสือ (8.74%), อาชีพอื่น ๆ (อาชีพที่ไม่เฉพาะเจาะจง) (11.63%), และ

ประชากรที่อาศัยอยู่ในหมู่บ้านตรอเปียง สแร (Trapaing Srae) (9.94%) อำเภอสนัว (Snuol) จังหวัดกระแจะ (Kratie) โดยกลุ่มอายุต่ำกว่า 20 ปี มีความสัมพันธ์กับการติดเชื้อพยาธิ ออพิสทอร์คิสวิเวอร์รินิอย่างมีนัยสำคัญทางสถิติ (P<0.05) มีค่า adjusted OR = 0.601, 95% CI = 0.410-0.882 และ P=0.009

นอกจากนี้สายพันธุกรรมของพยาธิออพิสทอร์คิสวิเวอร์รินิ ในพื้นที่ของ Snuol Kratie มีความแตกต่างของพยาธิออพิสทอร์คิสวิเวอร์รินิที่พบในพื้นที่ของเอเชียตะวันออกเฉียงใต้ รวมถึงภาคตะวันออกเฉียงเหนือของประเทศไทย สปป. ลาว เวียดนาม และกัมพูชาอย่างชัดเจน ในส่วนของการตรวจวินิจฉัยด้วยวิธีการระดับชีวโมเลกุลพบว่ามีความไวสูงสำหรับตรวจพยาธิออพิสทอร์คิสวิเวอร์รินิในตัวอย่างอุจจาระ เมื่อเทียบกับวิธีการตรวจภายใต้กล้องจุลทรรศน์แบบดั้งเดิม ดังนั้นเมื่อพิจารณาถึงผลกระทบด้านสาธารณสุขของการติดเชื้อพยาธิออพิสทอร์คิสวิเวอร์รินิในกัมพูชาแล้ว จึงจำเป็นต้องมีการนำวิธีการตรวจวินิจฉัยระดับโมเลกุลเข้ามาใช้คัดกรองควบคู่กับวิธีทั่วไป โดยเฉพาะอย่างยิ่งในพื้นที่ที่มีความเสี่ยงสูง เพื่อให้ได้มาซึ่งข้อมูลทางระบาดวิทยาที่แม่นยำยิ่งขึ้น นอกจากนี้พยาธิออพิสทอร์คิสวิเวอร์รินิมีระบาดวิทยาในพื้นที่ชนบทใกล้กับแหล่งน้ำจืด จึงจำเป็นต้องมีการเฝ้าระวังเชิงรุกสำหรับการตรวจทางคลินิกที่เกี่ยวข้องกับหน้าที่ตับและมะเร็งท่อน้ำดี รวมทั้งจำเป็นต้องมีการให้ความรู้ด้านสุขภาพเพื่อลดความเสี่ยงให้กับประชาชนในพื้นที่นั้นๆด้วย

สาขาวิชา เวชศาสตร์ปริวรรต ลายมือชื่อนักศึกษา.....

ปีการศึกษา 2566 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

NAV LA: DISCRIMINATION OF HUMAN CARCINOGENIC LIVER FLUKE-LIKE EGGS FROM CAMBODIA USING PARASITOLOGICAL AND MOLECULAR METHODS. THESIS ADVISOR: ASSOC. PROF. SCHAWANYA RATTANAPITOON, M.D. 174 PP.

Keywords: *Opisthorchis viverrini*/ Formalin-ethyl acetate concentration technique/Polymerase chain reaction/ Cholangiocarcinoma/ Phylogenetic tree/ Cambodia

Opisthorchis viverrini, a liver fluke endemic in several Southeast Asian countries and worldwide, poses a significant public health risk due to its association with cholangiocarcinoma. Accurate and early detection is critical for effective disease controlling, while microscopy remains a conventional diagnostic tool, its sensitivity is often compromised, especially in low-intensity infections. The present study aimed to determine *O. viverrini* eggs through fecal samples using formalin-ethyl acetate concentration technique (FECT) and internal transcribed spacer 2 (ITS2) gene-based polymerase chain reaction (PCR), and restriction enzyme *Fau*1 to discriminate the liver fluke-like eggs and cytochrome c oxidase subunit 1 (*COX*1), NADH nicotinamide adenine dinucleotide subunit 1 (*NAD*1) gene mtDNA for genetic sub-type isolation.

Associated prevalence of *O. viverrini* infection was examined within bivariate and multivariate analyses using SPSS version 23.0 software program. Results showed that the infection rate of intestinal parasites was 15.91% (n=377) based on FECT. Among intestinal parasites, *O. viverrini* showed higher frequency (5.57%), followed by Hookworm (4.24%), *Entamoeba coli* (3.97%), *Ascaris lumbricoides* (1.06%), *Hymenolepis nana* (1.06%), *Taenia* spp. (0.53%), and *Trichuris trichiura* (0.53%). Meanwhile, *O. viverrini* positive cases were found to be higher by PCR (6.89%) than by FECT. Both parasitological and molecular methods were analyzed. Infections of *O. viverrini* were found to be higher in men (8.92%) than in women (5.45%), and to be associated more frequently with younger age groups (13.40%), illiteracy (8.74%), participation in other careers (non-specific occupations) (11.63%), and residence in the Trapaing Srae village (9.94%) of the Snuol district, Kratie Province. Age groups under 20

years old were significantly linked with *O. viverrini* infection, with adjusted OR=0.601, 95% CI=0.410-0.882, *p*=0.009 and significant value established at (*P*<0.05).

Furthermore, there is no *O. viverrini* of Snuol Kratie grouping in the same reference cluster as the commonly found *O. viverrini*, indicating that the *O. viverrini* in this area of Kratie Province is clearly distinct from common *O. viverrini* found throughout Southeast Asia, including northeastern Thailand, Lao PDR, Vietnam, and Cambodia. Molecular methods, particularly PCR, demonstrates that higher sensitivity in detecting *O. viverrini*-like eggs in fecal specimens compared to traditional microscopy. Given the public health implications of *O. viverrini* infection in Cambodia, there is a pressing need to adopt and integrate molecular diagnosis into the routine screening process, especially in high-risk areas to ensure more accurate epidemiological data and better-targeted interventions. Therefore, *O. viverrini* is distributed in rural areas near freshwater reservoirs, where active surveillance, clinical examination related hepatobiliary, and cholangiocarcinoma and health education are needed.

Translational Medicine Program
Academic Year 2023

Student's Signature.....
Advisor's Signature.....
Co-Advisor's Signature.....

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Nav La

CONTENTS

Page

ABSTRACT (THAI) I

ABSTRACT (ENGLISH) III

ACKNOWLEDGMENT V

TABLE OF CONTENTS VI

LIST OF TABLES X

LIST OF FIGURES XI

LIST OF ABBREVIATIONS XIII

CHAPTER

I **INTRODUCTION** 1

1.1 Thesis title 1

1.2 Rational and background 1

1.3 Research Hypothesis 4

1.4 Research Objective 5

1.5 Scope and Limitations of Study 5

1.6 Contribution 6

1.7 Expected Results. 7

II **LITERATURE REVIEWS** 8

2.1 Minute Intestinal Flukes 8

2.1.1 *Haplorchis taichui* in Humans and Fishes 9

2.1.2 Morphologies 9

2.2 History and Taxonomy of liver flukes 10

2.2.1 Opisthorchiidae 10

2.2.2 *Clonorchis sinensis* 11

2.2.3 *Opisthorchis viverrini* 11

2.2.4 *Opisthorchis felineus* 12

2.2.5 *Opisthorchis lobatus* 12

CONTENTS (Continued)

Page

2.3 Morphologies 12
2.3.1 Adult worms of liver fluke 12
2.3.2 Eggs of liver fluke 13
2.3.3 Metacercariae of liver fluke 14
2.4 Epidemiology of liver fluke Infections 15
2.4.1 Prevalence 15
2.4.2 Status of Liver fluke infection in Cambodia 15
2.5 Geographical of Liver Fluke Distribution 17
2.6 Life Cycle and Transmission of Liver Fluke 19
2.6.1 Life Cycle *Opisthorchis* 19
2.6.2 Transmission 21
2.7 Clinical Features of Sign and Symptoms 23
2.7.1 Sign and Symptoms 23
2.7.2 Clinical Features 25
2.8 Risk Factors of Liver Fluke 26
2.9 Pathology and Pathogenesis of Liver Flukes 27
2.10 Diagnosis of Opisthorchiasis and Clonorchiasis 29
2.10.1 Parasitological Techniques 30
2.10.2 Molecular methods 34
2.11 Treatment 41
2.12 Prevention 42
2.13 Conceptual Framework 43
III **RESEARCH METHODOLOGY** 44
3.1 Study design 44
3.2 Population and sample size 46
3.3 Sample and sampling 46
3.4 Materials 47
3.4.1 Protocol 47

CONTENTS (Continued)

Page

3.4.2 Specimen....48
3.5 Methods....48
3.5.1 Parasitological technique....48
3.5.2 Molecular techniques....50
3.6 Statistical analysis....58
3.7 Ethical statement....58
IV RESULTS AND DISCUSSION....59
4.1 Parasitological detection....59
4.1.1 Morphology of *O. viverrini* by using FECT based on light microscopic....59
4.1.2 Morphology of intestinal parasites....60
4.1.3 Intensity of intestinal parasite infection....60
4.1.4 Prevalence of intestinal parasite infection....61
4.1.5 Prevalence of helminths and protozoa infection....64
4.1.6 Prevalence of IPIs association with variables....70
4.2 Molecular Identifying....77
4.2.1 Prevalence of *O. viverrini* infection by using ITS2-PCR....77
4.2.2 PCR-restriction fragment length polymorphism restriction *Fau*1....85
4.2.3 Genetic diversity using *COX*1....88
4.2.4 Genetic diversity using *NAD*1....89
4.2.5 Sequences and alignment....91
4.2.6 Phylogenetic trees based on the sequences of ITS2....94
4.2.7 Phylogenetic trees based on the sequences of *NAD*1....97
V CONCLUSION AND RECOMMENDATION....100
5.1 Conclusion....100
5.2 Recommendation....101
REFERENCES....102
APPENDICES....142

CONTENTS (Continued)

Page

APPENDICES A-DNA EXTRACTION INSTRUCTION 143
APPENDICES B-PCR PROCEDURES 146
APPENDICES C-SEQUENCES RESULTS 150
APPENDICES D-CONFIRMING RESULTS WITH GENBANK 155
APPENDICES E-RAW DATA 157
APPENDICES F-SUT IBC 172
CURRICULUM VITAE 173

LIST OF TABLES

Table **Page**

4.1 The intensity egg per gram (EPG) of intestinal parasite 61

4.2 Detection of IPIs using quantitative concentration FECT (*n*=377).....63

4.3 The co-relationship between helminths and protozoa (*n*=377) 65

4.4 The prevalence of IPIs associated with multivariable (*n*=377) 68

4.5 The prevalence of *O. viverrini* infection by using PCR-ITS2 (n=377) 72

4.6 The prevalence of *O. viverrini* infection by using PCR-ITS2 (n=377) 67

LIST OF FIGURES

Figures **Page**

2.1 The adult worms with eggs of human liver flukes and MIF 10
2.2 The adult worm of human liver flukes 13
2.3 The human liver flukes' eggs of adult worms 14
2.4 The fish-borne trematode metacercariae detection in freshwater cyprinoid fishes 14
2.5 Map of the Greater Mekong Subregion 19
2.6 Life cycle of liver fluke, *O. vivierrini* and *C. sinensis* 21
2.7 Ultrasonography of chronic opithorchiasis 26
2.8 Pathways pathogenesis of liver fluke-induced cholangiocarcinoma (CCA) 29
2.9 Direct smear technique adapted from WHO 32
2.10 Operating steps of the Kato-Katz technique 33
2.11 The intestinal transcribed spacer (ITS) region of nuclear ribosomal gene arrays (above) and the *trnl* region of chloroplast DNA (below) 37
2.12 DNA barcoding identifies the following a, b, and c 41
2.13 Conceptual Framework 43
3.14 Flow chat of study design 45
3.15 Sorting of 377 fecal sample for laboratory performance 47
3.16 Observing and identifying the parasite through fecal samples 47
3.17 Institution and the map of study area 48
3.18 The formalin ethyl-acetate concentration technique 49
3.19 Light microscopic in PDRC, SUT and graphic 50
3.20 The graphic of DNA gene extraction through fecal samples 51
3.21 G-STORM thermocycler in PDRC, Institute of Medicine, SUT 53
3.22 PCR products under 1.5% agarose gel electrophoresis 55
3.23 The gel electrophoresis and imaging documentary 56
4.24 Morphological of *O. viverrini* under light microscopic 59

LIST OF FIGURES (Continued)

Figures **Page**

4.25 Morphological of intestinal parasites' egg under light microscopic 60
4.26 Diagram of IPIs eggs positive prevalence using FECT differentiated by light microscope 72
4.27 PCR products of *O.viverini* amplifying based on internal transcribed spacer 2 (ITS2) 78
4.28 PCR-restriction fragment length polymorphism products of *O. viverrini* 86
4.29 PCR-RFLP products of *O. viverrini* digested by enzyme *Fau*1 88
4.30 PCR-product of *O. viverrini*'s using *COX*1 gene 89
4.31 PCR-product of *O. viverrini*'s using *NAD*1 gene 90
4.32 The graphic view alignment of *O. viverrini*'s ITS2 gene product with sample sequence of the difference countries in the region 93
4.33 Cluster diagram results from a neighbor-joining analysis of ITS2 sequences of Opisthorchiid liver flukes and MIF (also see Table 9) after excluding all sequence gap positions 96
4.34 Cluster diagram results from a neighbor-joining analysis of *NAD*1 sequences of Opisthorchiid liver flukes and MIF (also see Table 10) after excluding all sequence gap positions 99

LIST OF ABBREVIATIONS

95% CI	= 95% Confident interval
AOR	= Adjusted odds ration
APF	= advance periductal fibrosis
ATP6	= Adenosine triphosphate-6
BLAST	= Basic Local Alignment Search Tool
C. sinensis	= *Clonorchis sinensis*
CCA	= Cholangiocarcinoma
Cm	= Centimeter
COR	= Crude odds ratio
COX1	= Cytochrome c oxidase subunit 1
Cyt b	= Cytochrome b
DALY	= Disability-adjusted life years
DNA	= Deoxyribonucleic acid
EC	= Ethic committee
ELISA	= Enzyme-linked immunosorbent assays
EPG	= Egg per gram
ES	= Excretory/secretory
Fau1	= 1-(2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl) uracil
FE	= Formalin-ether concentration
FECT	= Formalin ethyl-acetate concentration technique
g	= gram
GMS	= Greater Mekong Subregion
IBC	= International building code
IBM	= *International Business Machines*
IGS	= Intergenic spacer
IL-6	= Interleukin-6
ITS2	= Internal transcribed spacer subunit 2

LIST OF ABBREVIATIONS (Continued)

Lao PDR	= Lao People's Democratic Republic
MEGAS	= Molecular Evolutionary Genetics Analysis
MIF	= Minutes intestinal fluke
Min	= Minute
mL	= Millimeter
mm	= Millimeter
MNAS	= Maestrosafe nucleic acid stain
mtDNA	= Mitochondria DNA
*NAD*1	= NADH nicotinamide adenine dinucleotide subunit 1
NAD4L	= NADH dehydrogenase
NCBI	= National center for biotechnology information
NO	= Nitric oxide
O. felineus	= *Opisthorchis felineus*
O. Lobatus	= *Opisthorchis lobatus*
O. viverrini	= *Opisthorchis viverrini*
O&P	= Ova and parasite
PCR	= Polymerase chain reaction
PDRC	= Parasitic disease research center
rDNA	= Ribosomal DNA
RFLP	= Restriction fragment length polymorphism
ROI	= Reactive oxygen intermediates
rRNA	= Ribosomal RNA
SPSS	= Statistical package for the social sciences
S. mansoni	= *Schistosoma mansoni*
STHs	= Soil-transmitted helminths
SUT	= Suranaree University of Technology
TAE	= Tris-acetate-EDTA
TLRs	= Toll-like receptors
trnaL	= Transfer RNA Leucine

LIST OF ABBREVIATIONS (Continued)

µL	= Microliter
µm	= Micrometer
WHO	= World health organization
WWF	= Worldwide Fund

CHAPTER I

INTRODUCTION

1.1 Thesis title

DISCRIMINATION OF HUMAN CARCINOGENIC LIVER FLUKE-LIKE EGGS FROM CAMBODIA USING PARASITOLOGICAL AND MOLECULAR METHODS

1.2 Rational and background

Opisthorchis viverrini (*O. viverrini*), commonly referred to the Southeast Asian liver fluke, is a well-documented public health issue in several countries of the Greater Mekong Subregion (GMS), particularly Thailand, Lao PDR, Cambodia, Vietnam, and Myanmar. This parasitic trematode primarily colonizes human bile ducts, resulting to chronic infections, which able further precipitate a range of hepatic complication, and developing to the most severe being cholangiocarcinoma (CCA) a malignant tumor of the bile ducts (Kaewkes et al., 1991). The central for controlling the spread and impact of this parasitic infection is accurate diagnosis and subsequent medical intervention. The liver fluke has a complex life cycle, making it crucial to advise people against ingesting inadequately cooked freshwater fish products. Although it is an uncommon occurrence, acute infections that caused jaundice due to bile duct blockage have also been known to cause cholecystitis, periductal fibrosis, relapsing cholangitis, and cholelithiasis (Elkins et al., 1990; Pungpak et al., 1985; Sripa, 2021). Technology and methods are frequently developed to minimize infection rates in communities through control, treatment, and prevention. However, liver fluke infections have been associated with CCA as a risk factor (Fried & Abruzzi, 2010). The liver fluke, human food borne Trematode prevalence 601 millions are went through to be at high risk for *Clonorchis sinensis* (*C. sinensis*), 67.30 millions of *O. viverrini*, and 12.50 millions of

Opisthorchis felineus (*O. felineus*) (Keiser & Utzinger, 2005) and the eggs morphologies were existed about 70 species worldwide (Chai et al., 2007). According to the most recent estimates, three major liver fluke species causes infections in approximately 45 million people, of which 10 million are parasitized by *O. viverrini* and cause 26,000 fatalities yearly (Smout et al., 2011). Four nations in Southeast Asia have been identified as having the highest rates of the liver fluke infectious prevalence, and total of 12.9 million people are believed to have been infected by *O. viverrini* in 2018 (Zhao et al., 2021a).

The northern and northeastern Thailand, the frequency of *O. viverrini* infection maintained at over 3.3 million people in 2014 (Wongsaroj et al., 2014), Infection cases were 99% asymptomatic, while the severity varied in the remaining instances. Regarding to the very recently study to evaluate the ranking of *O. viverrini* infection in northeastern in Preah Vihear and Stung Treng Province the emerging prevalence (59.8%) was reported to be highest ever in these provinces, Cambodia (Jung et al., 2023). According to data on *O. viverrini* infection rates, Kandal Province had a greater prevalence (43.60%) (Myamoto et al., 2014) and 4.60% in Kratie Province (Shon et al., 2012) were widespread in Cambodia.

Previously, the traditional diagnosis of *O. viverrini* has been reliant on parasitological techniques based on microscopic examination of stool samples to observes the eggs of the parasite. The morphology of the egg suggests poorly separated tiny trematode and Minutes intestinal fluke (MIF) mix infections (Sripa et al., 2007). The formalin-ethyl acetate concentration technique (FECT) has been used to diagnose parasites in mild infection, although it has reduced sensitivity and difficulty identifying eggs that resemble flukes, the gold standard read under light microscopic (Kaewkes et al., 1991; Sithithaworn & Haswell, 2003). Therefore, these traditional techniques often grapple with limitations such as reduced sensitivity, especially in cases with low parasitic loads or when the eggs are morphologically similar to other intestinal flukes, which lead to potential underreporting of cases or misdiagnoses of profound implications for individual and public health.

Amid these challenges, the specify of modern diagnostics has been gained towards molecular methods, which hold the pledge of increased sensitivity and

specificity. In earlier research, PCR-based techniques for the identification and differentiation of *Opisthorchis*-like eggs were created. Repetitive nucleotide sequence, ribosomal DNA, microsatellites, internal transcribed spacer (ITS), and mitochondrial genes are some of the target genes for these molecular techniques (Thaenkham et al., 2007, Duenngai et al., 2008, Parvathi et al., 2008, Umesha et al., 2008, Sato et al., 2009, Traub et al., 2009). The molecular method has shown to have greater specificity and sensitivity when identifying with existed of *O. viverrini* DNA (Wongratanacheewin et al., 2001; Wongratanacheewin et al., 2002). In previous study was reported of molecular characteristics of recovery *O. viverrini* in the patient using the NADH dehydrogenase subunit 1 (*nad*1) in the population in Kratie Province (Sohn et al., 2012). However, the previously described PCR approaches (Sato et al., 2009; Saijuntha et al., 2008) shown a high sensitivity only in situations with more than 1000 eggs per gram (EPG). The PCR test still has to be improved in order to detect *O. viverrini* in fecal samples. In addition, for detection, PCR techniques have also been used to analyze *O. viverrini*'s genetic variation, particularly those that target mitochondrial genes like cytochrome c oxidase (*cox*1) and NADH dehydrogenase (*nad*1) genes (Le et al., 2006; Saijuntha et al., 2008). On other hand, the mitochondrial DNA (mtDNA) genes marker is extending to identify genetic diversity of liver flukes' sub-type in different regions through the Mekong River including Thailand, Lao PDR, Cambodia, and Vietnam (Thaenkham et al., 2010). However, *nad*1-based PCR was utilized revealing homologous nucleotide sequences (97-99%). ITS2 sequence has more specificity than cytochrome c oxidase subunit 1 (*cox*1), with 0.86% and 3.03% nucleotide divergent between *O. viverrini* and *Opisthorchis lobatus* (Thaenkham et al., 2011). The molecular methods were used to study in the form of identifying the genus population and sub-type of adult flukes, *O. viverrini* by some researchers in Cambodia (Thaenkhm et al., 2010; Sohn et al., 2012). *O. viverrini* infection is rich of biodiversity and also notable diseases burden with its unique view to explore, validate, and potentially integrate these advanced molecular diagnostic tools in Cambodia.

This study aimed to determine Opisthorchis-like eggs in fecal specimens from Cambodia using FECT and to explore the efficacy and reliability of molecular methods in discriminating and identifying *O. viverrini*-like eggs by internal transcribed spacer

subunit 2 (ITS2) region and restriction enzyme *Fau*1, however genetic diversity subtype by using *COX*1 and *NAD*1 amplifying based polymerase chain reaction (PCR) between mother and offspring.

1.3 Research hypothesis

The application of molecular diagnostic methods PCR was demonstrated a higher sensitivity and specificity for the identifying of liver fluke-like eggs in fecal specimens from resident of Snuol district, Kratie Province, compared to traditional parasitological methods including microscopic examination. Researchers have been using molecular techniques to detect and identify *O. viverrini* infections all over the world, but particularly in Southeast Asia, including Thailand, Vietnam, and Lao PDR. These techniques take advantage of the variety of genes found in different species of liver flukes to control and minimize infection in humans. The molecular, diversity species, and subspecies analysis of *O. viverrini* DNA was still a sticking point, though, because it needed to show how common molecular diagnosis-based PCR was in order to support Cambodia's genetic separation. The studying from other region where liver fluke endemic has shown the quality of molecular method over traditional technique in term of accuracy and reliability.

Justification of sensitivity and specificity, the molecular methods are known to offer greater results, especially in cases where parasitic load is low, which is potentially to identify even fluke quantities of DNA or RNA of the target organism. Moreover, this method is highly specific, targeting unique gene sequences of the liver fluke, and minimizing the chance of cross-reactivity or confusion with other intestinal-like eggs.

Dietary and cultural practices, the prevalence of *O. viverrini* infections is often dominant in areas where consumption of raw or freshwater fish product is common, which might be a higher incidence of infection. However, not all infection will have a high worm burden, resulting to cases that might be missed diagnosed by microscopy but identified by molecular methods.

Molecular advancements in molecular biology provide robust, accurate, and relevance rapid methods for the identifying of pathogens, including parasites like liver

flukes. These advancements should be important beneficial in areas like Kratie Province, where accurate epidemiological data is essential for public health interventions. The proposed hypothesis is rooted in the potential advantages molecular diagnostics bring to address in areas with potential endemicity where traditional techniques might have limitation. In addition, these hypothesis guidelines were directed focus on exploring the diagnostic modalities and establishing the initial benefits of molecular methods in Kratie Province.

1.4 Research objective

This study was aimed to determine *O. viverrini* eggs in fecal specimens using formalin-ethyl acetate concentration (FECT) and to explore the efficacy and reliability of molecular methods in identifying using internal transcribed spacer 2 (ITS2) region based on polymerase chain reaction (PCR) methods, and restriction enzyme *Fau*1 to discriminate the liver fluke-like eggs and cytochrome c oxidase subunit 1 (*COX*1), NADH nicotinamide adenine dinucleotide subunit 1 (*NAD*1) due to genetic diversity subspecies isolation.

1.5 Scope and limitations of study

This research scope is providing insights into the true burden of liver fluke infections including geographical focus, diagnostic methods, analysis, strain differentiation, and infection assessment in the Snuol district, Kratie Province and the potential advantages of molecular diagnostics in detecting these infections more accurately. The study emphasizes the parasitological technique and application of molecular methods, predominantly PCR-based assays, to differentiate of liver fluke-like eggs. Moreover, molecular methods not just only detect but also differentiate between different strain or species of liver flukes, providing insight into the specific types of prevalence in Kratie Province, Cambodia.

The limitation of this study is due to the insufficient on infrastructure of laboratory facility where the molecular methods require specialized skill and inability of trained personnel to conduct and interpret molecular tests in this area. Sample collection and preservation for molecular methods often demand stringent, storage,

and transportation protocols, which can compromise the DNA quality, affecting test outcomes. Infarct of cost implications on this method can be costlier than parasitological techniques and budget constraint should be limit the number of sample or the frequency of testing. On the other hand, external validity of results obtained from Snuol district, Kratie Province does not use to be generalized for all of Cambodia or other regions, even if they have similar dietary or culture practices. Importantly, false positive/negatives while molecular methods are sensitive, no test is infallible and a risk of false positive or negatives, albeit lower than parasitological techniques. Interference of stool samples, the primary samples type for such studies, can have various substances that might be inhibited molecular reactions.

Nevertheless, while the adoption of molecular methods for discrimination of liver fluke-like eggs in Kratie Province offers propitious depth and accuracy, it is not without its challenges. The scope and potential limitations aids in better study design, interpretation of results, and the formulation of recommendations recognizing. Therefore, this study was performed within 12 months under Bioethics committee approval to certify for supporting in the laboratory experiment.

1.6 Contribution

The author and co-authors participated in the performance of this thesis project study over the course of the research procedure. To undertake the laboratory work for this thesis research, the BioEthic was accredited by the BioEthic committee (IBC-65-05) and undertook a biosafety and biosecurity training course by BIOTEC of Suranaree University of Technology. Moreover, the number sample on this project was used of fecal specimens in PDRC (sample bank), which approved by the human ethic committee of Health office Nakhon Ratchasima number EC: NRPH013. This investigation was carried out using human feces from Cambodia that were preserved at the Parasitic Disease Research Center (PDRC), Institute of Medicine, Suranaree University of Technology. As a result, the information from this study project will be useful for health science research on the incidence of *O. viverrini* and other intestinal parasite infections in Cambodia.

1.7 Expected results

This study was provided an updated prevalence rate, increased detection with molecular methods, variability in diagnostic accuracy, stain or species differentiation, quantitative analysis, risk factors and demographics, validation of molecular protocols, and capacity and training insights of *O. viverrini* infections in Kratie Province, which might vary across different districts or communities.

This work used molecular diagnostic and alignment to identify the available sequences that could be linked with existing sequences from NCBI GenBank to disclose the diversity information of species and subspecies of *O. viverrini* from Cambodia. However, data on mtDNA markers for molecular epidemiology and population studies of the genetics of liver flukes were supplied by the *O. viverrini* gene research, along with recommendations for prevention, molecular diagnosis, and management of opisthorchiasis.

Overall, the expected results were showcase both the strengths and potential weakness of parasitological and molecular methods in the specific context of Kratie Province accompanied with comprehensive data to foundational for inform medical health decisions in the region.

CHAPTER II

LITERATURE REVIEWS

2.1 Minute Intestinal Flukes

Minute intestinal flukes (MIF) are commonly occurred in mix infection with liver fluke, *O. viverrini* infection, especially *Haplorchis taichui* (*H. taichui*), *Haplorchis pumilio* (*H. pumilio*), *Haplorchis yokogawai* (*H. yokogawai*), *Prosthodendrium molenkampi* (*P. molenkampi*), and *Phaneropsolus bonnie* (*P. bonnei*). Hepatic and intestinal fluke species that are common in Southeast Asia, including Thailand, Vietnam, and Lao PDR, have been classified (Chai et al., 2009), which varied by locality (Chai et al., 2005). However, the prevalence of Ov/MIF infection of a countrywide survey by Korean researcher in Cambodia was confirmed (Miyamoto et al., 2014; Yong et al., 2015; Yong et al., 2014). The human MIF infection was reported commonly such as species *Haplorchis spp*. and lecithodendriids (*P. molenkampi* and *P. bonnei*) in Takeo Province, Cambodia (Yong et al., 2012). However, the liver flukes not often excreted from the bile duct into feces (Keiser & Utzinger, 2009; Wijit et al., 2013) and the worms can be classified between *O. viverrini* and MIFs (Buathong et al., 2017; Tesana et al., 1991), in which concentration techniques detection was limited to distinguish. Moreover, there was a study of screening tests using stool examination to differentiate the prevalence of *Opisthochis*-like eggs by the national strategic plan control liver fluke infection and Cholangiocarcinoma in Thailand. To mention of *O. viverrini* and MIF from the specimens using molecular techniques in further testing, which was good improvement to discriminate between *O. viverrini* and MIF infection (Buathong et al., 2020). Therefore, the human infection MIF such as *Haplorchis spp*., and lecithodendriids (*P. molenkampi* and *P. bonnie*) were reported in Takeo province, Cambodia (Touch et al., 2009).

2.1.1 *Haplorchis taichui* in humans and fishes

Heterophyid intestinal fluke is typical species and common cause in human (Giboda et al., 1991) including Haplorchis spp. (*H. taichui*, *H. pumilio*, and *H. yokogawai*) and lecithodendriids (*Prosthodendrium molenkampi* and *Phaneropsolus bonnei*) were discovered of minute intestinal flukes among Laotian population (Chai et al., 2005; Chai et al., 2007; Chai et al., 2009; Chai et al., 2013; Chai et al., 2010; Sayasone et al., 2009). However, in recently study by Korean research was found that there are mixed infection of *O. viverrini* and *H. taichui* from the volunteers in Preah Vihear Province as the first reported of *H. taichui* in Cambodia (Jung et al., 2023).

2.1.2 Morphologies

The minute's egg and morphological are like one another that is difficulty to identify in human feces. In overall the eggs were ovoid, pyriform, or elliptical shape with the average size 21-35 x 12-21 µm and the morphology of some egg is very similar from one to another. The shape of *C. sinensis*, *P. summa*, *S. falcatus*, and *S. fuscata* eggs are pyriform, whereas *G. seoi* eggs were elliptical (Lee et al., 2012). Thus, there were 6 kinds of eggs have measured and observed the morphological of different among 11 species named *Metagonimus yokogawai*, *Metagonimus miyatai*, *Metagonimus takahashii*, *Heterophyes nocens*, *Heterophyopsis continua*, *Stellantchasmus falcatus*, *Stictodora fuscata*, *Stictodora lari*, *Pygidiopsis summa*, *Gymnophalloides seoi*, and *Acanthotrema felis* (Cho et al., 2010; Lee et al., 1984) (Figure 2).

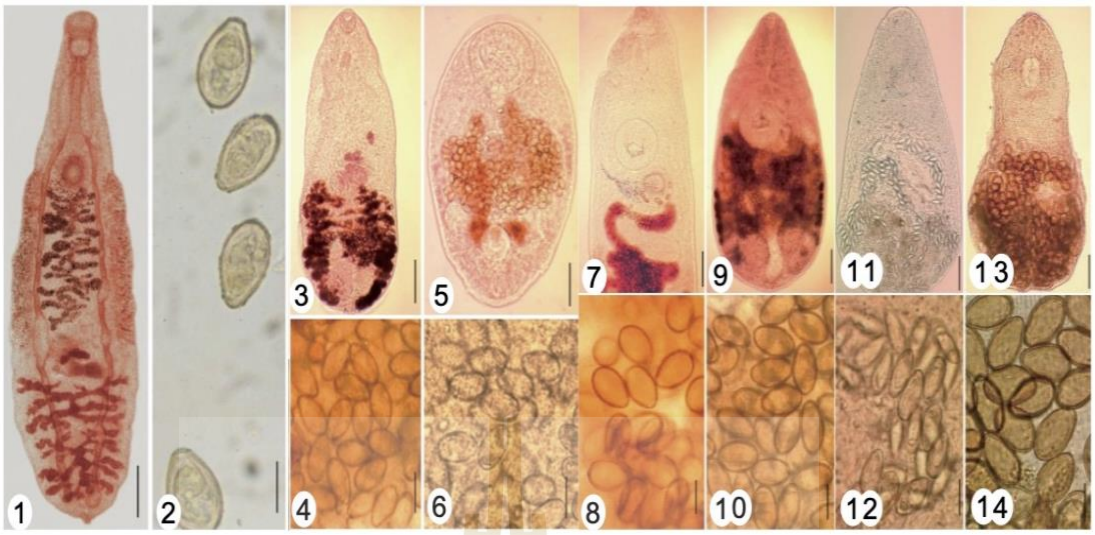

Figure 2.1 The adult worms with eggs of liver fluke and MIFs. (1-2) *C. sinensis* and eggs, (3-4) *P. summa* and eggs, (5-6) *G. seoi* and eggs, (7-8) *H. continua* and eggs, (9-10) *H. nocens* and eggs, (11-12) *S. falcatus* and eggs, (13-14) *S. fuscata* and eggs under light microscopy (Lee et al., 2012).

2.2 History and taxonomy of Liver Flukes

Liver fluke or food borne trematode is the neglected tropical disease that occurred in human liver or hepatobiliary pathogen, which is frequently in southeast Asia and Europe. The *Clonorchis sinensis* (*C. sinensis*) and *O. viverrini* are genera of subfamily opisthorchiidae that commonly found in human hepatobiliary infection, whereas *Opisthorchis felineus* (*O. felineus*) is the pathogen infected found in Europe across eastern Sibaria, which was not recognized as the carcinogen. However, *C. sinensis* from East Asia and *O. viverrini* from the Lower Mekong region, these liver flukes are associated with human CCA, which was classified as class one carcinogen by the international Agency for research on cancer a part of world health organization (Humans, 2012). Therefore, both are pathogen cause of chronic liver diseases inducing hepatobiliary inflammation particularly periductal fibrosis that shown by ultrasound detection.

2.2.1 Opisthorchiidae

The members of opisthorchiidae such as *C. sinensis*, *O. felineus*, *Everanorchis ophidiarum*, and *Gomtia piscicola* were found in the bile duct, liver, and gall bladder

of birds, mammals and digestive tract of reptiles and teleost's. In 18S, ITS-1, ITS2 and *COX*1 sequencing have used to distinguished *C. sinensis* from Korea and China, which indicated sequencing were similarity at the DNA gene level (Lee & Huh, 2004). The study in Northern Europe of morphometrical and molecular differentiated of Pseudamphistomum truncatum metacercariae of fishes and adults mink by ITS2 rDNA sequencing that classified the Pseudamphistomosis verifying of the nucleotide similarities with *C. sinensis* and *O. viverrini* were suggested for close relationship from Asia (Skov et al., 2008). Another research of the development PCR method for ITS2 rDNA from adult flukes and eggs in infected humans in Thailand. They found that molecular methods analyzed by ITS, *COX*1, and *NAD*1 were moderated conserved among isolated diversity species of *opisthorchis* family. This analysis has verified the tool to evaluate the infection and molecular epidemiology examination of parasites (Huang et al., 2012).

2.2.2 *Clonorchis sinensis*

August 21st, 1875, by J. F. P. McConnell has described of *C. sinensis* in postmortem specimens collected of the bile duct from a Chinese seaman corpse in Calcutta, India. On September 18th the same year, T. S. Cobbold was published in the Lancet that indicated the trematodes with unclear a new species named *Distoma sinensis* from McConnell's description. However, Looss was revised this liver fluke as *C. sinensis*, the type of species for the genus (Looss, 1907).

2.2.3 *Opisthorchis viverrini*

The initially discovery of *O. viverrini* was described of the biliary ducts of a fishing cat called *Prionailurus viverrinius*, which was kept in the Zoological Gardens at the Paris Natural History Museum (Poirier, 1886). 20 years later, there was discovered initially from human supplied by W. F. J. Kerr in Chiang Mai north of Thailand, which was described by Leiper (Leiper, 1915). Moreover, the total 230 adult male prisoner were examined found *O. viverrini* infections that published in parasitological by Kerr in 1916. There were 74% of their examined found in Laotians who lived a rest of their lives in Chiang Mai and very few Thias specimens group (Prommas, 1927).

2.2.4 *Opisthorchis felineus*

The originally paper of Kerr was first identified the *O. felineus* that was confused between *O. viverrini*, in which was identified by Leiper. However, a decade later Kerr's paper was first case reported of *O. felineus* infection in northeast Thailand at Roi-et, while there were highest prevalence (25, 15%) in Thakhek and Vientiane, Lao PDR by Bedier and Chesneau (Bedier & Chesneau, 1929). Moreover, *O. felineus* has been first described as subfamily Opisthorchiidae by Gurlt, and he was confused with *Distomum conus* Creplin 1825 (Erhardt et al., 1962). Therefore, *O. felineus* was isolated from cats as a valid species by Rivolta (Rivolta, 1884), and later moved from *Distomum felineus* to *Opisthorchis* by Blanchard in 1895. The type genus of Opisthorchiidae family in human (Looss, 1907), and *O. felineus* was first described by Vinogradoff in Siberia (Vinogradoff, 1892).

2.2.5 *Opisthorchis lobatus*

A new species was known as *O. lobatus* found in freshwater fish that has been added into the family of Opisthorchiidae, which was not well understood of it role in human, and its originated found in Lao PDR (Thaenkham et al., 2011).

2.3 Morphologies

2.3.1 Adult worms of Liver Fluke

Human liver fluke is dorso-ventrally flattened, thin, lancet-shaped, and transparent. The *O. felineus* adult worms are flat, leaf or lanceolate shaped, elongated, measured generally 7.12 mm in length and 1.5-2.5 mm wide (Figure 3A) (Pozio et al., 2014). The adult worms of *O. viverrini* are flat, leaf or lanceolate, elongated, shaped generally 5.5-10 millimeter (mm) in length and wide 0.8-1.6 mm (Figure 3B) (Pozio et al., 2013). The morphology of the *C. sinensis* adult worms are elongated, flat, leaf or lanceolate generally 8-15 mm in length and 1.5-4.0 mm wide (Figure 3C). The body of *O. lobatus* is thin, oval elongate, and transparent with measurement anterior end to posterior end broad 4 (4-6) mm long and 0.9 (0.7-1.1) mm wide (Figure 3D) (Thaenkham et al., 2011). However, even though all these liver flukes are quite similar in sized, but there were particularly different having highly branched testis (Scholtz, 2008). Its suckers existed in both oral and ventral suckers with approximately one-fifth of body

length at the anterior. The deeply lobed and diagonal consisted of two testes located at posterior extremity (Figure 3A, B, C). However, the structure of *O. viverrini* has shaped similarly with *C. sinensis* (8-15 mm in length, and 1.5-4.0 mm wide) (Chai et al., 2005) but the size mainly differences between these parasites. The seminal vesical are slightly coiled a bit long, and ejaculatory duct bound to end of duct. The genital pore is situated in front of cirrus sac and ventral sucker. The multilobate of the ovary is placed in front of the anterior testes nearby the seminal receptacle and Laurer's canal. Thus, the vitellaria comprised a lot of follicles. Additionally, their eggs are yellowish-brown, oval, and have a tubercle-like knob at the opercular end.

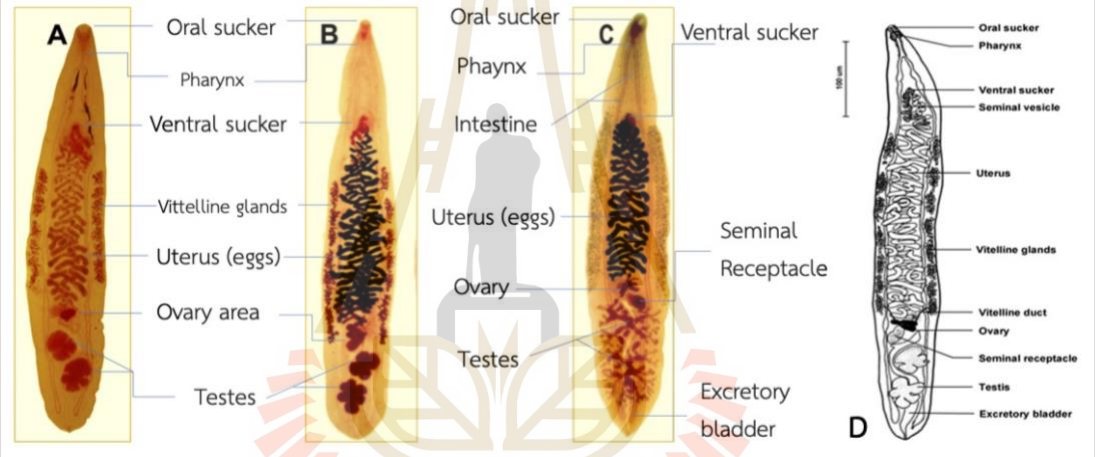

Figure 2.2 The adult worm of human liver flukes. (A) *Opisthorchis felineus*, (B) *Opithorchis vivverini*, and (C) *Clonorchis sinensis*, *O. lobatus* (Thaenkham et al., 2011; Murrell et al., 2019).

2.3.2 Eggs of Liver Fluke

The stage of liver fluke diagnosis when the embryonate egg passed in feces. These liver flukes' eggs are yellowish-brown, oval, and have a tubercle-like knob at the opercular end generally measuring 19-29 by 12-27 micrometer (μm) that monoecious was referred as adult worm (Ash & Oreil, 2007) (Figure 4). Thus, the eggs containing miracidium with average 28μm by 16μm in size when it laid (Chai et al., 2005).

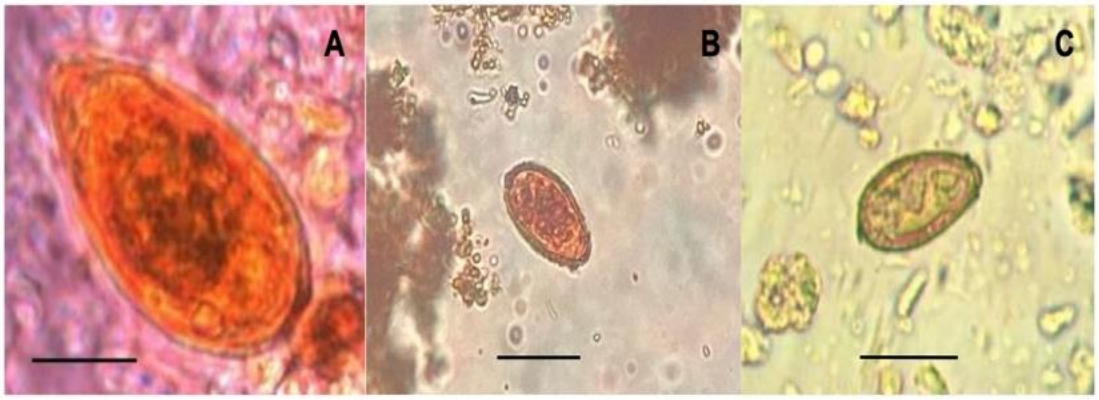

Figure 2.3 The human liver flukes' eggs of adult worms. (A) *C. sinensis*, (B) *O. viverrini*, and (C) *O. felineus* (Chai et al., 2005).

2.3.3 Metacercariae of Liver Fluke

The infected stage transmission of larval from fish to humans and mammals feeding baby was consumed of raw freshwater fish contained metacercaria, which encysted in the second intermediate host tissue. The metacercaria of *O. felineus* is oval generally 0.25-0.30 x 0.19-0.23 in size (Figure 5A). *O. viverrini*'s metacercaria is round and oval with measuring 0.19-0.25 x 0.15-0.22 in size (Figure 5B). The metacercaria of *C. sinensis* is round to oval, with measured 0.13-0.14 x 0.09-0.10 (Figure 5C) (Chai et al., 2005). The encysted metacercaria of *O. lobatus* is oval shape transparent, thin, and single-layered cyst with 150 (100-200) long and 80 (70-90) wide. The metacercaria shape will be changed when excysted 432 (320-489) and 72 (60-80) wide (Figure 5D) (Thaenkham et al., 2011).

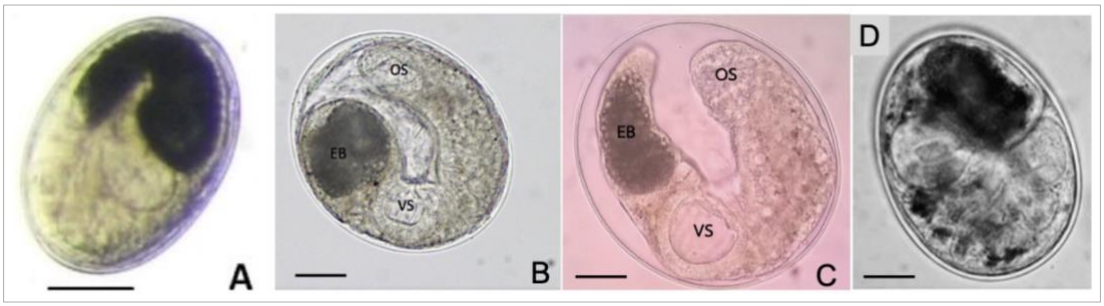

Figure 2.4 The fish-borne trematode metacercariae detected in freshwater cyprinoid fishes: (A) *O. felineus*; (B) *O. viverrini*, (C) *C. sinensis*, and (D) *O. lobatus*. OS (oral sucker), EB (Excretory bladder), and VS (Ventral sucker).

2.4 Epidemiology of Liver Fluke infections

2.4.1 Prevalence

There is trans-nation in southeast Asia countries originated in the Greater Mekong Subregion (GMS). *O. viverrini* infection was initiated found in human within 100 years ago, in Thailand (Leiper, 1915). However, the reports of this fluke behind Thailand were Lao PDR, Cambodia, and Vietnam with opisthorchiasis condition, in which case reports of sporadic in Malaysia, Singapore, and the Philippines (Sripa et al., 2010). Foodborne trematode remains major public health of neglected trematodes diseases (NTDs) in Asia, and highest prevalence of trematodiases, which is world's top 3 Global burden of diseases comprising Thailand, Lao PDR, and China in 2013 (Herricks et al., 2017). The number of liver fluke infections in GMS was estimated over 15-18 million people infected by *O. viverrini* and *C. sinensis* most common seen in China, however there were *O. felineus* Siberian liver fluke also existed in the GMS. Moreover, there are more than half of the world's cases that highly endemic by *O. viverrini* and *C. sinensis* in the region (Sithithaworn et al., 2012). There was reported of *C. sinensis* prevalence in China with 14 millions of people (Brattig et al., 2020), whereas a total 12.39 million people infected by *O. viverrin* such as Thailand (6.71 million), Lao PDR (2.45 million), Vietnam (2.07 million), and Cambodia (1.00 million) (Zhao et al., 2021a). In addition, the prevalence of *O. felineus* revealed 60% of western Siberia (Fedorova et al., 2020). Therefore, the infection throughout the world was 700 million people are estimated at high-risk of these small liver flukes (Keiser & Utzinger, 2005).

2.4.2 Status of Liver fluke infection in Cambodia

Since 1995, *O. viverrini* the Ministry of Public Health of Cambodia claimed that opisthorchiasis was prevalent in Cambodia without providing any details information (WHO, 1995a). In the years 1981–1982, clonorchiasis was discovered in American immigrant Cambodian children who had spent a temporarily of time at a camp for refugees on the Thai–Cambodian border (Parish, 1985). In Kracheh (Kratie) Province between 1994 and 1995, a stool survey for schistosomiasis revealed the presence of an infection with Opisthorchis spp. (Stich et al., 1999). The National Center of Malaria, Parasitology and Entomology (CNM) of Cambodia previously carried out a stool survey in southern Cambodia's Ang Svay Chek Village in Takeo Province and Ampil Village in

Kandal Province. According to the findings, 12.5% of the people had ascites, and 13.4% of residents tested of positive for small trematode eggs (personal communication of the director of Krang Yov Health Center, Kandal Province) (Touch et al., 2009). The national helminth control program launched in 2006. There was conducted as systemic review within 20 years about the *O. viverrini* infection throughout nationwide with the prevalence ranging 0.01% to 47.5% from 3 three provinces in Cambodia (Khieu et al., 2019). However, in recently published of *O. viverrini* infection from two province that showed higher prevalence 59.8% in Preah Vihear and Stung Treng Province was reported by Korean researcher (Jung et al., 2023). Moreover, there was not yet report the opisthorchiasis patient has been diagnosed since the initially research. In the previous report of the Cambodian refugees infected by *C. sinensis* among (2/102 participants), and a few were infected by *O. viverrini* in the United States during 1981-1982s (Parish., 1985). Nevertheless, some studies were confirmed that the number of *O. viverrini*'s egg-positive cases gradually increased among the schoolchildren communities in the Mekong River flood plain (Stich et al., 1999). In particularly province such as Kampong Cham (4.0%) (Lee et al., 2002), Takeo (32%) (Sohn et al., 2011) high infection rates generally ranging 0.2% to 47.5% in 2006-2010 (Miyamoto et al., 2014; Yong et al., 2012). Even though, there were typically of research on *O. viverrini* infection, however the various endemic areas are described contradictory results during 2011 from 60 villages 0.2% of prevalence (Khieu et al., 2014). The study period between 2006 and 2011 were conducted in Kampong Cham and Takeo Province with high prevalence of *O. viverrini*/minutes intestinal fluke (Ov/MIF) ranging 23.8-24%), meanwhile large-scale estimated from 19 provinces among 32,201 schoolchildren was 5.7% of Ov/MIF infections (Yong et al., 2014). In addition, another survey on *O. viverrini* infection was prevalent in Kampong Cham (23.9-28.5%) and Kandal province (20.2%) during 2008-2012 recorded by Japanese parasitologist (Miyamoto et al., 2014). Likewise, there was funded by USAID for nationwide schoolchildren survey on *Opisthorchis* infection with generally prevalence 4.1% in 2013 (Khieu et al., 2019). Therefore, the lower prevalence of human liver fluke infection 0.7% in the northwest Cambodia and Uddor Meanchey Province (Khieu et al., 2019). To conclusion, from a systemic review

estimated 1,000,000 people are infected by *O. viverrini* in Cambodia (Zhao et al., 2021a).

2.5 Geographical of Liver Fluke distribution

The Greater Mekong Subregion (GMS) has covered huge extending of plateaus, massifs, lowlands, floodplains limestone karsts, and deltas, forests (evergreen and semi-evergreen, deciduous, mangroves, dipterocarp, and swamp), and grasslands. The Tonlé Sap (mini freshwater sea) Southeast Asia's largest freshwater lake in Cambodia that habituated by fast-flowing rocky mountain streams and expansive wetlands (Figure 6). There was a study conducted for evaluated 20,000 plant species, 1,300 fish species, and 430 mammalian species that distributed geographic region encapsulates 16 of the World Wide Fund for Nature's (WWF) Global 200 ecoregions, and habitats (WWF, 2012). The principal of parasite domination to humans was reproduced based on their life cycle enable sustainable through variety geography and climate especially in subtropical and tropical monsoon that different between northern and southern regions, Vietnam. The influence from both diverse landscape and climatic ranges were induced higher transmission of liver flukes linking to environments (Wang, 2012). Certainly, showing of both opisthorchiasis and clonorchiasis were declared of mainly manifestation in lowland and floodplains less than 300 meters. Since, *O. viverrini* infection was reported about in 100 years ago in Thailand (Leiper, 1915) and followed by other countries such as Lao PDR, Cambodia, and central Vietnam, which some case reported of sporadic in Malaysia, Singapore, and the Philippines (Sripa et al., 2010). Whereas *C. sinensis* was found from bile duct of a Chines carpenter who died in Calcutta, India in 1874s (Grove, 1990; Lun et al., 2005; Sripa et al., 2017b). Moreover, *C. sinensis* has infected in northeast China, Japan, Taiwan, northern Vietnam, southern Korea (Sripa et al., 2010), and the far eastern part of Russia. In contrast *Opisthorchis felineus* (*O. felineus*) mainly found in western Siberia. However, the evident of human clonorchiasis was revealed in public health problem more than 2,000 years, which was detected of *C. sinensis* eggs in fecal specimens from an individual person in the middle stage in the Warring States Period (475-221 BC) in Hubei (Wu et al., 1996). The Lower Mekong Basin countries not yet reports endemicity of *C. sinensis*, except Vietnam, but

clonorchiasis and opisthorchiasis were identified by PCR in the community from the central region, Thailand (Traub et al., 2009). In contrast, only *O. viverrini* infection was reported in the same province by the several researchers (Buathong et al., 2017; Suwannahitatorn et al., 2013; Suwannahitatorn, 2019). The geographical range circumstance is suitable snail intermediate hosts distribution as other trematodes, liver flukes (Petney et al., 2013), *O. viverrini* is dominated in three *Bithynia* snail species, especially *P. manchouricus* a main intermediate host of *C. sinensis* in all endemic countries, which is infected in other snails in China (Doanh & Nawa, 2016). The geographical of *B. s. goniomphalosis* is based on environmental factors (Pratumchart et al., 2019; Suwannatrai et al., 2011). The appropriate regions for *B. s. goniomphalosis* basically in northeast Thailand (Nithikathkul et al., 2017) and a portion northern part, where highly prevalence of *O. viverrini* infection. The *O. viverrini* was detected commonly in laboratory experience from *B. funiculata* and *B. s. siamensis* more than *B. s. goniomphalosis* species, hence divergent snail species have different to liver fluke infection and cercarial shedding (Chanawong & Waikagul, 1991). In contrast, *O. viverrini* is likely increased more than 6 times higher in *B. s. goniomphalosis* than *B. funiculata* (Dao et al., 2017). Therefore, to control the geographical barriers of snail distributes liver fluke infection is growing international freshwater aquaculture trade resistant to liver fluke from endemic areas to lower the infection prevalence and avoiding liver fluke spoiling in lower endemic countries such as United States and Europe incase consuming insufficiency freshwater fish existing parasite metacercariae (Stauffer et al., 2004; Yossepowitch et al., 2004). In 2007-2008, there was reported of *O. viverrini* metacercariae with typically species of freshwater fish including Hampala dispar and Puntius brevis from the vicinity of Lake 500 the water reservoir, where is boundary of Takeo and Kandal Province, Cambodia. Therefore, highly prevalence of *O. viverrini* infection rate from 2.1% to 66.7% of fish captured in Kandal Province (Touch et al., 2009), evaluation with 600,000 people infected in Cambodia (IARC, 2011).

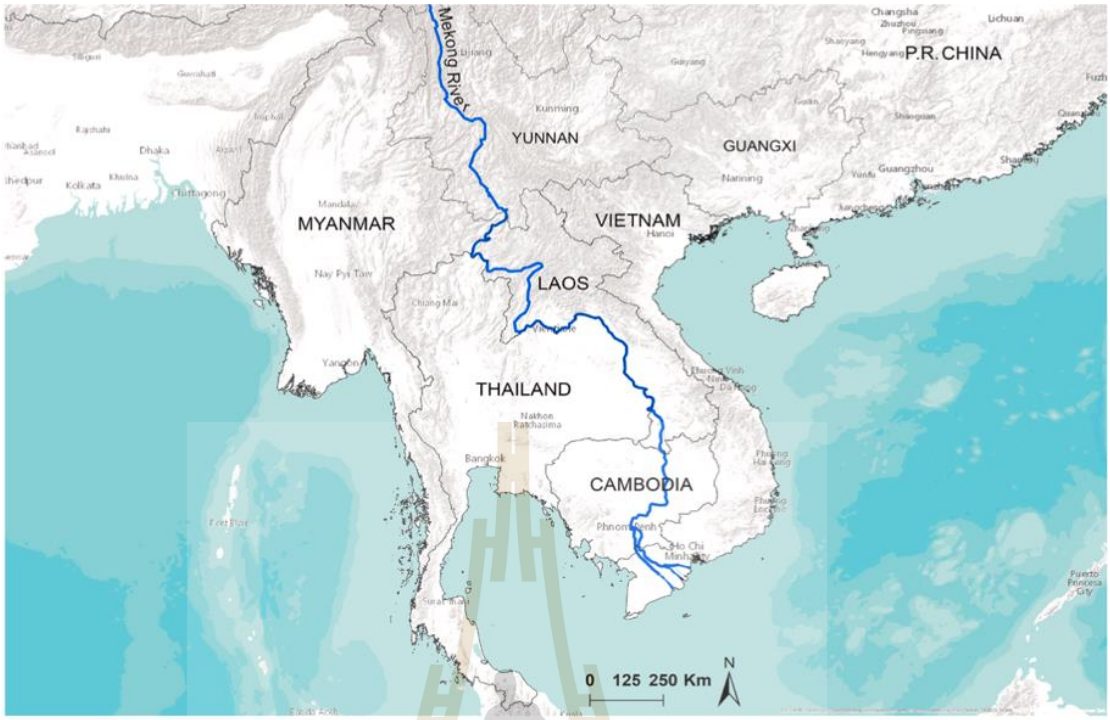

Figure 2.5 Map of the Greater Mekong Subregion. The Mekong River extend over 4,350 km along Southwest highland China to Myanmar, Lao PDR, Thailand, Cambodia, before draining into the South China Sea in Vietnam (Sripa, 2021).

2.6 Life cycle and transmission of Liver Fluke

2.6.1 Life cycle of *Opisthorchis*

Human liver fluke, adult worms have complex life cycle that dominated intra- and extrahepatic biliary system (Figure 7). In concise, the embryonated eggs existing miracidium released from gravid worm have excysted through feces into environment (Δ diagnosis stage) then the miracidia were ingested by snail (first intermediate host: *Bithynia* spp. Or *Parafossarulus* spp.) in the freshwater (Prueksapanich et al., 2018). The snails have dominant in shallow water, rice fields, and wetlands endemic areas, where there was commonly found the snails in water nearby the village highly feces contaminated scatters (Kaewkes et al., 2012; Kim et al., 2016). After miracidia egg were ingested by the snail and then hatched transformed into sporocysts and under sexual production developed a few steps from rediae to cercariae. Free swimming cercariae penetrate in the skin tissue of the 2nd intermediate host (*Cyclocheilichthys* spp., *Puntius* spp., *Hampala dispar*) freshwater fish (Welzel et al., 2006). The cercariae developed

into metacercariae form that penetrated to the muscle or scales of fish, which has abilities to infects human who consumed of raw or insufficiency cyprinoid fish products (Δ infective stage). Moreover, wild fish more likely infected by liver fluke more than the culture fish (Bui et al., 2016). In addition, there were over 70% of cyprinid fish infected by *O. viverrini* in the Lava Lake, Khon Kaen Province, Thailand (Sripa et al., 2015). Humans are the definitive hosts of liver flukes that can be infected by ingestion the raw secondary intermediate hosts or insufficiency freshwater fish products, which has contained metacercariae. There are kind of freshwater fish product depending on food dish customs of consumer from the different nation. In Thais and Laos used to make daily dish from fish including *Koi pla* (raw fish spicy salad) and *Pla som* (short-fermented fish) the most popular dish in northeast Thailand and Lao PDR, *Pra hok* in Cambodia, *Gỏi cá* (raw fish salad), *Gỏi cá nhảy* (eat small alive fish) in Vietnam, *Nyar lay Chin/Nyar lay Chin salad* (raw pickled fishes) in Myanmar, and other raw fish dishes including *Cao yu*, *Ci yan yu*, *Li yu*, *Lan dao yu*, and *Qing zhu yu* in Guangxi, China (Sithithaworn et al., 1991). Therefore, the metacercariae moves from small intestinal to bile within the ampulla of Vater and matured in bile duct and growth as the adult worms within 4 weeks. Finally, adult worms lay eggs around 200 eggs per day and able to survive more than 20 years in human body (Murrell et al., 2019; Prueksapanich et al., 2018). Thus, *C. sinensis* long lifespan in human over 26 years (Attwood & Chou, 1978).

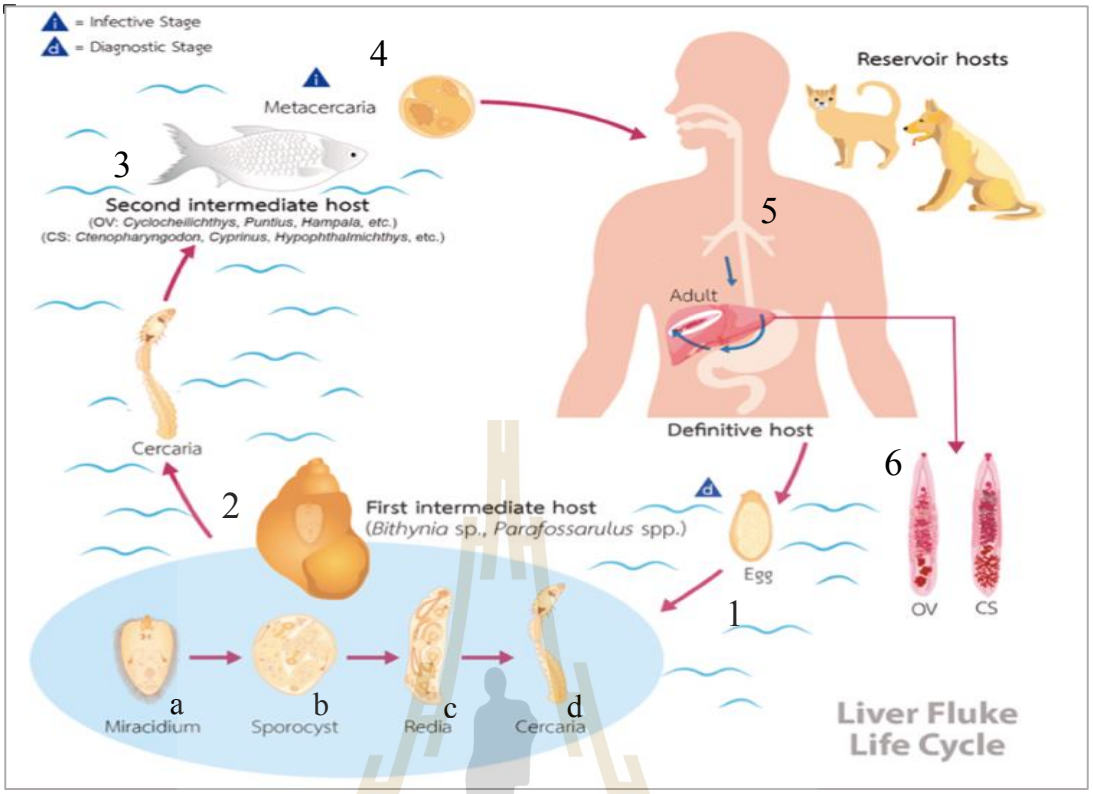

Figure 2.6 Life cycle of liver fluke, *O viverrini* and *C. sinensis*. Embryonated eggs discharged into biliary duct and feces, (1) Eggs are ingested by snail, (2) Each egg free a miracidia (2a) sporocysts (2b), rediae (2c), and cercariae (2d). The cercaria was free from snail after a short period of free-swimming in the water, and then penetrates into the flesh of freshwater fish, encysted as a metacercaria, (3) Humans infected by comsumed undercooked or raw freshwater fish, (4) The metacercaria excysted in duodenum, (5) and ascended to biliary tract through the ampulla of Vater. The maturation to adulthood takes approximately a month (6) (Sripa, 2021).

2.6.2 Transmission

2.6.2.1 Snails

The 1st intermediate hosts of liver flukes are known as key transmission determinants of both *O. viverrini* and *C. sinensis* of various areas in the GMS (Lun et al., 2005; Miyamoto et al., 2014; Petney et al., 2013). *C. sinensis* has eight major freshwater snail host species that belonged to Bithyniidae, Assimineidae, Hydrobiidae, Melaniidae, and Thiaridae families, which *O. viverrini* is dominated to three *Bithynia*

snail species (Lun et al., 2005). There are some susceptible snail species of *C. sinensis* that classified by the researchers in China are Lun et al., Petney et al., and Na et al., (Lun et al., 2005; Na et al., 2020; Petney et al., 2013). Moreover, some of snails were found in all *C. sinensis* endemic countries both China and Vietnam (Doanh et al., 2016; Petney et al., 2013). Whereas some others Bithynia snail species *O. viverrini* is dominated such as *M. tuberculatus* and *Maningila* sp. as the first intermediate hosts were unclear defined (Doanh & Nawa, 2016). In addition, some others snail species are infected by *C. sinensis* in nature that existed in northern Vietnam (Nguyen et al., 2021a; Nguyen et al., 2021b). Interestingly only in Vietnam that mentioned both opisthorchiasis and clonorchiasis are restricted in *P. manchouricus* found in northern and *Bithynia* spp. in central-south part, which has reported of definite human clonorchiasis in Nghe An Province (upper central Vietnam) (Doanh & Nawa, 2016). Likewise, the observing on snail intermediate hosts regions junction between clonorchiasis and opisthorchiasis endemic regions are warranted including ecology of snail dominant was not different. The *Bithynia* spp. is dwell and slow flowing in muddy rivers, artificial and natural ponds, lakes, irrigation cannels, paddy fields, marshes, reservoirs, swamps and fishponds similar to those *C. sinensis* restriction spp. (Petney et al., 2012; Petney et al., 2018; Petney et al., 2013; Suwannatrai et al., 2011; Wang et al., 2015). Therefore, the local habitat ecology was not concerned as key transmission in divergent geographical areas, however less infection prevalence in snail hosts (<2%) (Conlan et al., 2011) both liver flukes. In addition, an infected snail able release > 1,000 cercariae per day (Laoprom et al., 2016; Phongsasakulchoti et al., 2005) and this liver fluke first intermediate hosts in lakes or water bodies enable transmission to second intermediate host in endemic regions (Petney et al., 2018).

2.6.2.2 Fishes

The Cyprinids fish family, 2nd intermediate hosts of *O. viverrini* and *C. sinensis* transmission in freshwater (Petney et al., 2013). The scale fish more than 100 fish species were listed as susceptible host of the liver fluke cercariae dwell in the flesh tissue. Unlike the 1st intermediate hosts, fish species was not recorded evidence as susceptible species of both *C. sinensis* and *O. viverrini* or vice versa. However, there were several fish species of *O. viverrini* was detected in southeast Asia and China. The

various categorical of 48 cyprinid species are harbour *O. viverrini* metacercariae, 31 have been reported from Cambodia, Lao PDR, Thailand, and Vietnam. Other 10 in Cambodia, Lao PDR, and Thailand, in which other 9 in various combination of these countries where endemic of *O. viverrini* infections (Petney et al., 2018). Thus, 7 species are found in Singapore, 11 in Myanmar, 14 in China, 21 in Malaysia (Petney et al., 2012).

2.6.2.3 Animal reservoirs

The liver fluke dwell in the reservoir hosts without harmful to the mammalian species, that they were act as the carrier (Tangkawattana & Tangkawattana, 2018). However, there was research on cats, dogs, and pigs with their highest prevalence (70%), (50%), and (27%) of *C. sinensis* infection in southern China (Lin et al., 2011; Petney et al., 2013). Moreover, the cats and dogs are majority hosts reservoirs of *O. viverrini* and *C. sinensis* infection in Thailand, Lao PDR, Cambodia, and Vietnam (Hung et al., 2015; Lin et al., 2011; Nguyen et al., 2018; Xu et al., 2021). The highest prevalence (35%) was found in infected cats, while dogs are lower of *O. viverrini* infection in Thailand (Aunpromma et al., 2012; Enes et al., 2010). Likely there were ranging rates from 33-60% of *O. viverrini* infection in cats in central Vietnam, however there was 2.5% found in non-local cats infected *O. viverrini* from Hanoi slaughterhouse, and *C. sinensis* found in domestic cats (Doanh & Nawa, 2016). Thus, the lower prevalence infection in non-local cats by *O. viverrini* was suggested that the transborder migration from neighboring endemic region posed of risk spreading to non-endemic areas in northern Vietnam (Nguyen et al., 2018). However, there was no report of *O. viverrini* infections were discovered in monkeys, rodents, small residential mammal endemic areas from Thailand (Tangkawattana et al., 2021). Therefore, basically animal reservoirs host acts to free the parasite eggs into water bodies and then life cycle of liver fluke will be done similarity as definitive hosts (Burli et al., 2018).

2.7 Clinical features, sign, and symptoms

2.7.1 Signs and symptoms

Hepatobiliary tract diseases are induced by liver fluke leading to chronic inflammatory diseases that highly of worm burden resulting in bile duct cancer (Pakharukova et al., 2016; Qian et al., 2016; Sithithaworn et al., 2007). However, most

of these clinical signs are mild and asymptomatic, which manifested as jaundice condition of cases, but 99% were asymptomatic (Sripa et al., 2021). Moreover, if the flukes presented lower than 100 worms symptom may not occurring (Armignacco et al., 2008), and presented of the flukes intensities 100-1000 worms whatever might show as acute clinical sign, which were caused indigestion, jaundice, mild fever, epigastric discomfort, anorexia, general malaise, and diarrhea (Chai et al., 2005). Participants in the study underwent a clinical examination and were asked about any clinical symptoms they may have experienced in the two weeks before to the assessment, particularly any symptoms associated with parasite infections. A number of symptoms were recorded such as abdominal pain (64.5%), diarrhea (54.0%), fever (52.6%), right upper quadrant pain (25.0%), vomiting (21.9%), itchy skin (15.8%), nausea (14.9%) and weight loss (11.8%). Clinical signs observed by study physicians included generalized rash (2.6%), pale skin color (2.2%), pale subconjunctiva (2.2%), jaundice (1.8%) and (3.1%) poor general condition. None of the subjects had ascites or hepatomegaly, and just one person had splenomegaly. A statistical analysis (regression analysis with univariable and multivariable variables) revealed none of the clinical symptoms were connected to LTE infection (Bless et al., 2015). The chronic infection if not treated leading to liver enlargement, allergic lesions, congestion of spleen, cholecystitis, bile stone development, and liver cirrhosis. Nevertheless, the severity condition inducing CCA development, however benign hepatobiliary disease including cholangitis, periductal fibrosis (PDF), obstructive jaundice hepatomegaly, cholecystitis, and gallbladder stone were shown in clinical features. In addition, liver fluke infection mortality and morbidity resulting in worsted complication (e.g., hepatic lesions, cholangitis, and CCA), which was occurred in chronic infections with a lot of worm burden that became a highly impact on health status endemicity areas. Recent year evaluation of the clonorchiasis affected on morbidity gained a Disability-adjusted life years (DALY) value 275,370 cases associated with high impact of helminthic disease. Finally, highly endemic *O. felineus* in Western Siberia related with CCA that highest 77% of opisthorchiasis cases and followed by 34.2% cases without opisthorchiasis (Pakharukova et al., 2016). Therefore, human liver flukes, *O. viverrini* infection was not

shown the signs and symptoms for long times of silent period lasts for 25-30 years of the parasite's life span if not treated.

2.7.2 Clinical features

The specific signs and symptoms are appeared in the chronic human opisthorchiasis and clonorchiasis, except an increased frequency of palpable liver, as demonstrated in community-based studies (Rim, 2005; Upatham et al., 1982; Upatham et al., 1984). However, the heavy infection has been shown ordinary results of haematological and biochemical features (Pungpak et al., 1994). In contrast, ultrasonography reveals image features of sludge, gallbladder enlargement, gallstones and poor function in asymptomatic (Choi et al., 2005; Mairiang et al., 1992; Mairiang et al., 1993). The efficacy of praziquantel has resolved the abnormality of gallbladder within one or two years after treatment (Mairiang et al., 1993). In addition, ultrasonographic grading has indication for observing chronic opisthorchiasis more than 30% of cases in advance periductal fibrosis (APF) (Figure 8) (Pungpak et al., 1997). The differentiation of positive APF significant in poor contraction of the gallbladder length before and after meal on *O. viverrini* infections case, and the observing between positive APF and presence of sludge in the gallbladder were similarity features (Sripa et al., 2009). Therefore, sludge and stones from opisthorchiasis and clonorchiasis patient existing fluke eggs (Rim, 2005; Sripa et al., 2004). Opisthorchias and clonorchiasis patient are generally symptomatic appearing right upper quadrant pain, diarrhoea, decreased of appetite, fullness and indigestion. Moreover, there are 5-10% of condition accompanied of weakness or malaise, flatulence or dyspepsia, and abdominal pain, 5% experience of hepatomegaly. Additionally, the intensity of infection is related to clinical symptomatic in opisthorchiasis patients (Upatham et al., 1982; Upatham et al., 1984). The liver fluke infection is enhancement of CCA development with main clinical features. The consumer history of raw freshwater fish revealed the positive serologic result and radiological evidenced of *C. sinensis* infection found to be risk of CCA (Choi et al., 2006). There is prediction of patients associated with CCA clinical manifestation, especially jaundice was accounted for 60% on opisthorchiasis-related CCA hospitalization in northeast Thailand (Mairiang et al., 2003). The clinical manifestation might present as obstructive jaundice, obstructive jaundice with fever, obstructive

jaundice with acute complications including cholangitis, acalculous cholecystitis and generalized bile peritonitis. Non-jaundiced patients might be appeared with dyspeptic pain, anorexia, weight loss, right upper abdominal mass and distant metastasis (Mairiang et al., 2003; Rim, 2005). Therefore, abdominal masses can be single or multiple bulging and palpable that involved in both lobes in form of peripheral or intrahepatic CCA (Uttaravichien et al., 1999).

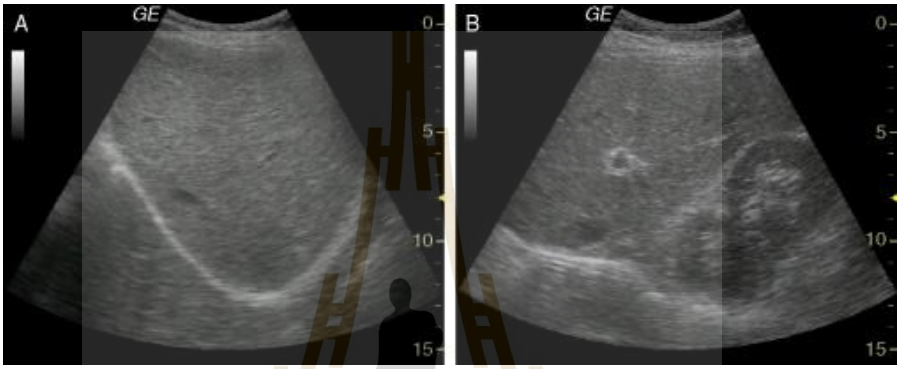

Figure 2.7 Ultrasonography of chronic opisthorchiasis. Showing severe periportal echoes along the intrahepatic biliary trees (B) compared to the normal liver (A). (Courtesy of Dr. E. Mairiang).

2.8 Risk Factors of Liver Fluke

The multiple risk factors related with *O. viverrini* infection such as demographic factors involving of male, age, younger than 24 years, older than 55 years, low level of education, ethnicity, laborer, and farmer (Wongba et al., 2011). However, environmental and geographic factors according to population has lived nearby to the reservoir; the habitation distancing less than 1 kilometer away water source and food resource (Chudthaisong et al., 2015) situated the Mekong corridor where households, pet, dogs or cats, and insanitary. Health behavior factors (persistent smoking, alcohol consumer, consumption of raw freshwater fish, and unsafe disposal of food waste). Moreover, mothers infection by *O. viverrini* and parents or guardians ate raw fish. Medical history and Praziquantel treatment agents (Pengput & Schwartz, 2020). Therefore, other parasitic or viral (hepatic) infection and smoking are important risk factors that caused severe morbidity such as bile duct cancer and co-infection (Forrer et al., 2012).

2.9 Pathology and Pathogenesis of Liver Flukes

Human has infected by consumed raw fermented fish food products, especially from cyprinoid freshwater fish. The principal mechanisms of *O. viverrini* infection leading to massive chronic infection by three pathways: (1) Mechanical damage, (2) Immunopathology, and (3) excretory/secretory (ES) molecules the products from parasites (Figure 9). These three main pathways have indicated the mechanical related to *O. viverrini* infection inducing CCA (Bissell et al., 2001). However, the mechanical damaging known as cause of parasite feeds in the biliary tract by sucker to hook into biliary epithelial cells that growth in the bile duct. The flukes mature caused more lesions, enlargement, and ulceration induced the nitric oxide and oxygen radicals resulting in DNA damage. Immunopathology occurred when the host immune responses of parasites penetration in biliary epithelial that damaged and inflamed by parasite-specific and non-specific plasma interleukin-6 (IL-6). Moreover, the risk of advance periductal fibrosis (APF) of Opisthorchiasis leading hepatobiliary abnormalities changing into CCA (Sripa et al., 2012; Sripa et al., 2009). The bile duct of infected hamster was inflamed within surrounding duct that caused by liver flukes that responded to antigens infiltration cells into periductal region and liver related with presence of fluke antigens in Opisthorchiasis (Sripa et al., 2000). However, Toll-like receptors (TLRs) have recognized and responded to variate molecules pathogens and associated molecular patterns (PAMPs) that merged to activate cellular signaling pathways to induce immune response genes mainly pro-inflammation cytokines (Venugopal et al., 2009). Moreover, TLR signaling assessment of ES *O. viverrini* products stimulated the normal immortalized human cholangiocytes cells line (H69) of *O. viverrini* infected patient in early stage of immunopathology in biliary tract. In addition, the ES products have expressed on receptors and proteins such as LTR4 mRNA, I**K**B-degradation and activated NF-**K**B nuclear translocation (Ninlawan et al., 2010). Thus, IL-6 and IL-8 secreted from cholangiocytes induced expression *O. viverrini* ES products (Sripa et al., 2012; Sripa et al., 2009). The *O. viverrini* ES produced by parasites to survive in long periods within the host environment that secreted and excreted such a soluble proteins and other mediators. These ES products have digested nutrients, host immune

system regulation, and tissue invasion playing of typical roles in the host-parasite interface. The long acting of parasites interaction has changed homeostasis involving a malignant transformation (Pairojkul et al., 1991; Vatanasapt et al., 1998). The cell states altered by the way of investigation if fluke infection as an entrance to genomic instability inducing the difference of growth factors (Schwartz et al., 2002). The most common lesions are epithelial dysplasia, metaplasia with goblet cells are metaplasia, adenomatous hyperplasia (Moore et al., 1988). The demonstration of fluke has fed on biliary epithelium that stimulated damaging through mechanical harm and inflammatory responding to ES proteins of the tegument and excretory pore to the bile, or culture *in vitro*, which highly immunogenic (Mulvenna et al., 2010). Likewise, the metabolic products might toxic or interact within biliary epithelium resulting to induce the immune response. The performing co-cultured between murine fibroblasts (NIH-3T3) and *O. viverrini* proliferate comparing to cells in media alone (physically separated from the worms in Transwell plates), which reported the hyperplasia of Opisthorchiasis-associated biliary epithelial cells (Bhamarapravati et al., 1978; Thuwajit et al., 2004). Therefore, the characterization of transcriptome and ES proteome of *O. viverrini* molecular identification of some liver fluke proteins are revealed (Daorueang et al., 2012; Smout et al., 2009).

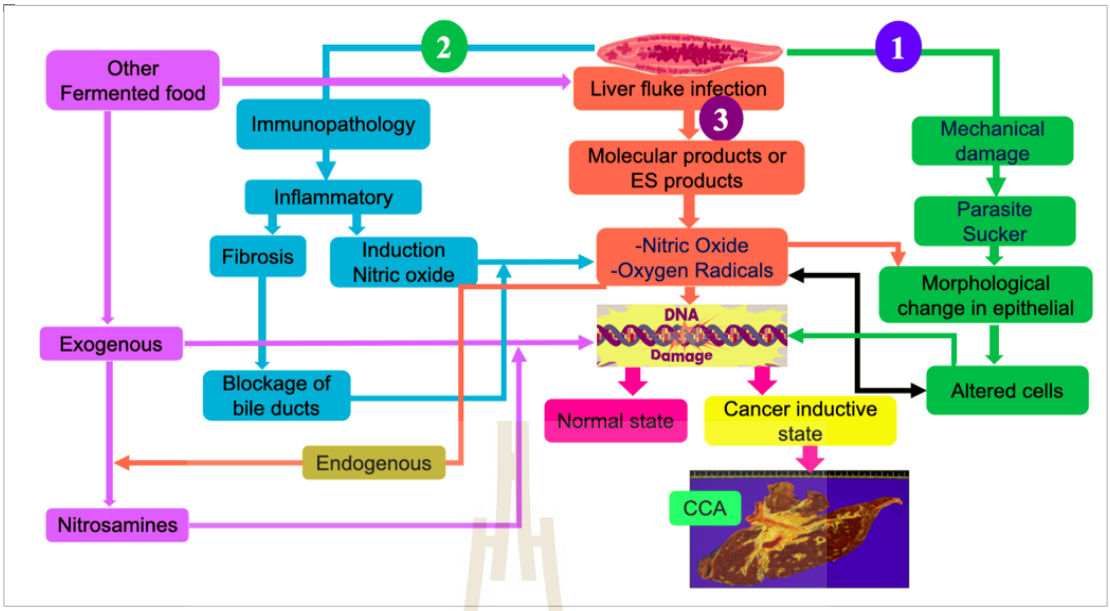

Figure 2.8 Pathways pathogenesis of liver fluke-induced cholangiocarcinoma (CCA). The *O. viverrini* damaged the bile duct epithelial with main three pathways: (1) mechanical damage; (2) immunopathology, (3) direct effects of fluke have secreted proteins to induce the cell proliferation and inhibited cancer prevention pathways (DNA repair/apoptosis). These pathways have converted resulting in genetic lesions that became fixed after successive replicated, leading to malignant transformation of cholangiocytes into CCA (Sripa et al., 2012).

2.10 Diagnosis of Opisthorchiasis and Clonorchiasis

The parasitological examination techniques are widely used as frequency routine diagnosis of opisthorchiassis and clonorchiasis or *O. viverrini* and *C. sinensis* infection of fecal samples. The commonly techniques testing eggs in feces including Kato-Katz thick smear, Stoll's dilution and quantitative formalin ethyl acetate concentration technique, however the sensitivity and accuracy of testing is based on techniques fecal examination and expertise of the microscopist (Bergquist et al., 2009; Elkins et al., 1990; Hong et al., 2003; Viyanant et al., 1983). Moreover, the morphology of eggs of liver flukes are not well differentiated based on microscopic particularly trematodes and minute intestinal flukes that co-endemic in Lao PDR and northern Thailand (Chai et al., 2005; Kaewkes et al., 1991; Radomyos et al., 1994). Additionally,

parasitological techniques examination read under light microscopy has limitation and time consuming. There was new technology invent for immunodiagnostic test *Opisthorchis* and *Clonorchis* infections (Wongratanacheewin et al., 2003). Based to the previous investigation proved that mot antigens of liver flukes are non-specific and antibodies last long after treatment, which revealed normal result and gained by serological test using antigens detection-based isotype-specific antibodies. However, enzyme-linked immunosorbent assays (ELISAs) by monoclonal antibodies against secretory antigens and DNA probes demonstration satisfaction results from fecal antigens (Chaicumpa et al., 1991; Sirisinha et al., 1995). Likewise, PCR-based approaches were given an attention on detecting of egg DNA from fecal specimens for diagnosis of human and animal liver fluke infection effectiveness (Duenngai et al., 2008; Le et al., 2006b; Lovis et al., 2009; Traub et al., 2009; Wongratanacheewin et al., 2001). The study from Medical Biological Union and Institute of Cytology and Genetics, Novosibirsk, and Russia has showed four Opisthorchiidae flukes by Taqman-based real-time PCR with high degree of sensitivity and specificity in fecal specimens from infected hamsters with local strains. Therefore, molecular diagnosis of *O. viverrini* and *C. sinensis* infection have validated similarly results from the studies of *O. felineus* infection (Arimatsu et al., 2012; Port et al., 2014).

2.10.1 Parasitological Techniques

Cholangiocarcinoma is alerted and chemotherapeutic control for decades that revealed a main public health particularly parasitic diseases, which caused by *O. viverrini* infection in the region of GMS (Jongsuksuntigul & Imsomboon, 1998). Moreover, the epidemiology of opisthorchiasis has extended geographical region especially North and Northern Thailand, which is required of control program to implement (Jongsuksuntigul & Imsomboon, 1997; Prakobwong & Suwannatrai, 2020; Sithithaworn et al., 2012). The surveillance and control for screening individuals of opisthorchiasis using conventional parasitological techniques including Kato-thick smear, FECT, Kato-Katz, and simple smear (Jongsuksuntigul & Imsomboon, 2003; Petney et al., 2018).

2.10.1.1 Light microscope examination

Microscopic examination is known as standard to examine parasite, liver fluke eggs in fecal specimens based on parasitological techniques (Bogoch et al., 2016), but

it is difficulties to differentiate between human liver flukes and small intestinal flukes (Sripa et al., 2011). The microscopic ova and parasite examination (O&P) is the traditional technique for parasite observing. Although the O & P test is labor-intensive and requires a high level of specialized knowledge to analyze the result, this testing remains the traditional cornerstone diagnostic for intestinal protozoa. Fecal specimens are collected and examined for detection of the gastrointestinal parasites. Two preparations of each specimen are usually set: one unstained preparation (saline wet mount) and another temporarily stained preparation. Therefore, advantage of the saline preparation is that fast, simple procedure and provides a quick result when positive, and help to detect the characteristic motility of parasites (Holgate et al., 2003).

2.10.1.2 Direct Smear

This test is simple demonstration and not complicated test for intestinal parasite infected subjects passing through feces (Figure 10). The protozoa (trophozoites or cysts) or helminth eggs detected by light microscopy (WHO, 2014). The direct microscopy examination is important to observe the larvae of *Strongyloid stercoralis* (*S. stercoralis*) and differentiate of high concentration of helminth's egg of *Ascaris lumbricoides* (*A. lumbricoides*) infection. The advantage of this test is faster and cheaper, but it is semi-quantitative and rare for control programs utilized. However, there is more range uses to detect protozoan parasites including trophozoites of *Entamoeba histolytica*, *Giardia lamblia*, and rarely *Balantidium coli* infection. The procedure of this thin smear is involved of small quantity of fresh stool add a drop of saline on microscope glass slide and cover glass on the emulsified stool. Finally, the eggs, larvae, trophozoites of parasites are demonstrated under light microscopy and eosin or iodine utilization is essential to detect the cysts, oocytes of intestinal protozoa (Cheesbrough, 2005; WHO, 1994). Therefore, accurate examination result is upon the stool specimens, which is commonly used for field survey of parasitic infection in endemicity arears and common for transcribing the results from the hospital lab register to the lab case report.

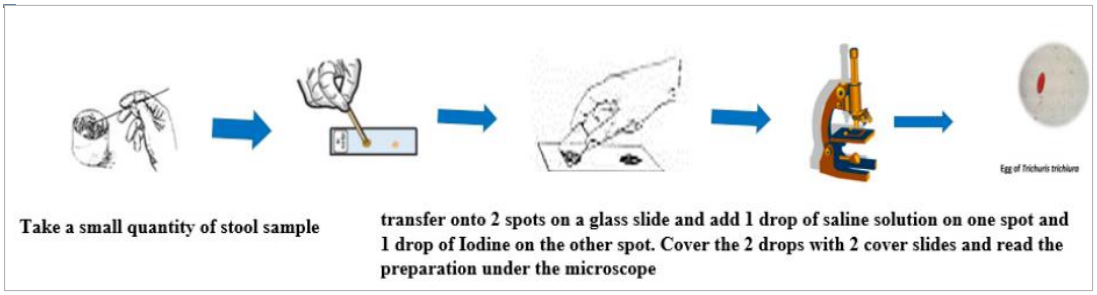

Figure 2.9 Direct smear technique adapted from (WHO, 1994).

2.10.1.3 Kato-Katz

WHO reveals Kato-Katz (KK) technique as the gold standard and essential uses to approach the prevalence and infection intensity of soil-transmitted helminths (STHs). The KK is the standard diagnostic technique to examine the gastrointestinal parasite infection, especially *O. viverrini* and *C. sinensis*. Both cellophane thick smear (Keto or KK) and formalin-ether concentration (FE) techniques were the modalities important used for diagnosis of clonorchiasis that related with heavy infection and serious complication as needed for early diagnosis and treatment is emerged estimate prevalence in East Asia (Figure 11) (Rim, 1986). The KK technique is useful in the field and simple, rapid and cheap that commonly work for mass screening of clonorchiasis, shcistosomiasis, ascariasis, trichuriasis and hookworm infection (Odongo-Aginya et al., 1995). The advantages of the KK has sensitivity of 44.7% for diagnosis clonorchiasis, egg quantification, cost effectiveness and utilized minimal infrastructure through copro-microscopy methods (Chai et al., 1982). The sensitivity of fecal diagnosis based on egg density that measured by EPG standard index for the quantitative detection of helminthiasis. This test is used to stratifies infection intensity by egg count and cut-off values that strained feces specimen about 41.7 mg, 20 mg, or 50 mg upon the template size place on a glass slide (Ebrahim et al., 1997). Therefore, infection burden, clinical damage, and efficacy of anthelmintics have been estimated based on EPG data.

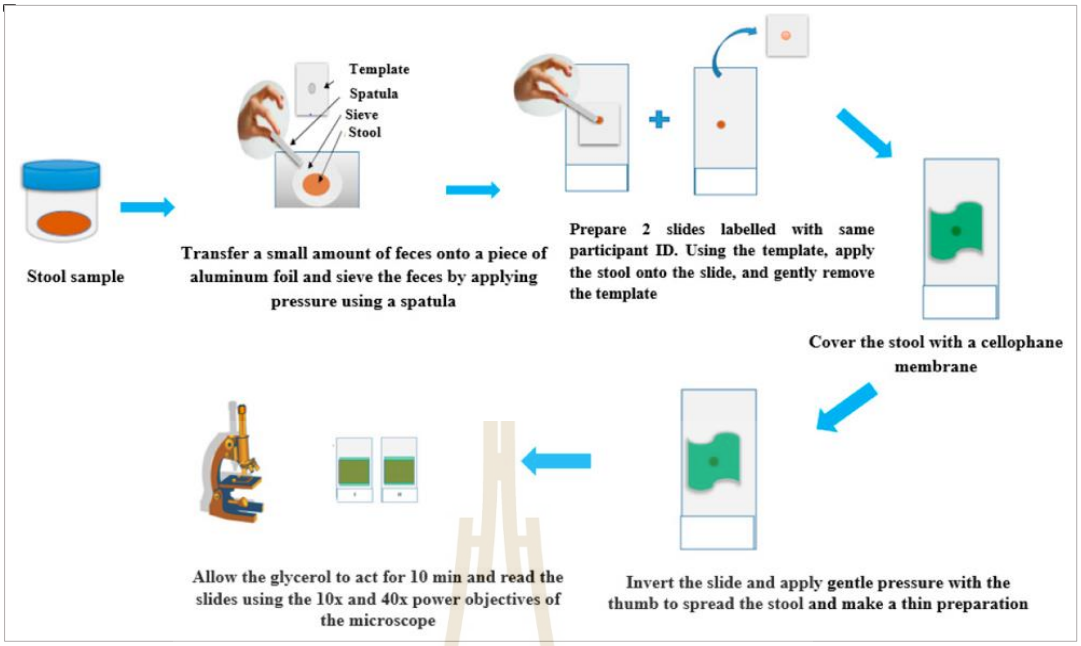

Figure 2.10 Operating steps of the Kato-Katz technique (Mbong Ngwese et al., 2020).

2.10.1.4 Keto's Thick Smear

Keto's thick smear technique is thick glass coverslips are used to detect *Schistosoma mansoni* (*S. mansoni*) infection compares with Kato technique that substituted by cellulose acetate film soaked in glycerin for field use to estimate the sensitivity infection. This technique is widely used to diagnose the helminthic disease that improved various workers (Katz, 1970). This technique was evaluated of modifying comparing with the Bell and digestion technique that specified of both sensitive and suitable for field uses. There were reported of the accuracy and capable comparison with the orthodox Kato & Miura method of thick glass coverslip. Firstly, 1 gram (g) of feces is set on filter paper (5 cm. square) and pressed through a 100 mesh (150 µm pore size) sieve. The schistosome and other helminth eggs are contained in the sieve stool sample, but the no particles larger than sieve mesh size. The wire surface of the sieve with the edge of a microscope slides and packed into the head of disposable syringe, in previous calibrated to deliver 30/g. Next step is place on a slide with fecal smear diameter about 25 mm and then inverted by pressing on the thick glass coverslip firmly. Finally, slide should be examined within 2 hours before it's dried up. The efficacy of modifying technique is estimated the numbers of *S. mansoni* eggs that added into stool of uninfected subject. The observation number of parasite's eggs as

a drop of suspension on slide by using microscopy (40X) of magnification existed by heavy infected stool sieving, sedimentation, washing, and centrifuge of the emulsified stool. Moreover, this procedure has compared with cellulose acetate film technique of Martin & Beaver. Therefore, this method requires expertise and commonly used cellophane sheet instead of cover glass for convenience with feces amount 30-60 milligram (mg) that gave accurate results, but helminth eggs may changes their shape somewhat (Teesdale & Amin, 1976).

2.10.1.5 Formalin-Ethyl Acetate Concentration Technique (FECT)

The surveillance and control for screening individuals of opisthorchiasis using conventional parasitological techniques including Kato-thick smear, Kato-Katz, and simple smear (Jongsuksuntigul & Imsomboon, 2003; Petney et al., 2018). The lower sensitivity analysis in the low-intensity infection has concerned and undetected of the light infection with lower egg per gram by these techniques. Kato-Katz has limited specificity to detect *O. viverrini* eggs that undifferentiated with minute intestinal flukes (MIF) such as *Phaneropsolus bonnei* and *Prosthodendrium molenkampi* without experienced microscopist (Chai et al., 2005; Sayasone et al., 2009). However, there was mentioned that FECT is superior to K-K technique for detection of *O. viverrini* egg, but it is complicated. The disadvantage of FECT is required centrifugation and dangerous with toxic solvent in procedure (Charoensuk et al., 2019). Quantitative concentration FECT is performing with stool concentrator kits such as Mini Parasep® Kit, which is ease to keep the sample for further study of numerous people if demands screening after fecal specimens preparation experience and minimized specimens handling and disposable enclosed system (Saez et al., 2011).

2.10.2 Molecular Methods

2.10.2.1 Polymerase Chain Reaction (PCR)

Mullis developed the polymerase chain reaction (PCR), which was patented in 1985. The polymerase has utilized particular DNA amplification in vitro replication. With this technique, a DNA template (a particle fragment from a DNA extracted gene) may be replicated tens of billions of times. PCR is competency based on accurate amount of matrix DNA and purification or cloning. Finding eggs in fecal samples is the traditional diagnostic procedure for human fish-borne trematodes, including *O. viverrini*

Several techniques, including the modified thick Kato smear (Illuminati et al., 1988), the modified formalin ether concentration technique (Elkins et al., 1991), and the Stoll's dilution egg count approach (Viyanant et al., 1983) have been employed effectively in the past. Under a microscope, it is difficult to distinguish between the infection of small intestinal flukes and other flukes and their eggs (Lee et al., 2012; Sithithaworn et al., 1991). An autopsy investigation that revealed several infected persons with low infection intensities and restricted egg output, resulting in a 20% underdiagnosis, was used to demonstrate the trustworthiness of fecal examinations (Sithithaworn et al., 1991). If a microscope examination is negative, a PCR-based approach is capable of identifying DNA genes for diagnosis from 28.6% to 76.6% and identifying opisthorchis-like eggs from other small intestinal flukes' sensitivities up to 93.7% of Opisthorchis genetic from fecal specimens (Lovis et al., 2009; Umesha et al., 2008; Won et al., 2016). The sensitivity of traditional stool tests is also compromised by sporadic egg excretion, bile duct blockage brought on by chronic infection or cholangiocarcinogenesis, and low infection intensities (Johansen et al., 2015). The concomitant transmission of *O. viverrini* with numerous species of fish-borne zoonotic trematodes from the families Heterophyidae and Lecithodendriidae, often known as minute intestinal flukes (MIF), presents another diagnostic challenge (Chai et al., 2005; De & Le, 2011; Lan-Anh et al., 2009). The size and structure of MIF eggs are comparable to those of liver flukes, which may result in false positive diagnoses and worse diagnostic specificity (Chai et al., 2005; Kaewkes, 2003). By using PCR to target a repetitive DNA element, it was possible to diagnose opisthorchiasis molecularly with excellent specificity but variable sensitivity (Duenngai et al., 2008; Qiao et al., 2012; Stensvold et al., 2006; Wongratanacheewin et al., 2003). Therefore, DNA extraction protocol using cetyltrimethylammonium bromide to remove inhibitors have been applied for higher sensitivity diagnosis (Umesha et al., 2008). This method highly sensitive on specimens contained more than 200 eggs per gram of feces according to *O. viverrini* infection intensity (Wongratanacheewin et al., 2002). However, DNA template analysis using PCR-based on ITS2 have been used as markers to differentiate *O. viverrini*, *C. sinensis*, and small intestinal flukes. In addition, mitochondrial genes also have been used *COX*1 and *NAD*1 to discriminate *O. viverrini*, *C. sinensis*, and *H. taichui*

(Kaewkong et al., 2013; Lamaningao et al., 2017; Traub et al., 2009). Thus, the validation potential of ITS2 region is classified of related sequencing species targeting rDNA, whereas *COX*1 and *NAD*1 are identified of opisthorchis species and its complementary in mtDNA (Yao et al., 2010). The following liver fluke species may now be distinguished using species-specific PCRs: *O. viverrini, Opisthorchis felineus*, and *C. sinensis* (Le et al., 2006; Pauly et al., 2003; Wongratanacheewin et al., 2001). The earlier described molecular techniques will substantially aid in making a more precise and successful diagnosis of trematode infections. It is necessary to further simplify the tests and comprehend cost efficiency in diverse socioeconomic settings (Khuntikeo et al., 2018).

2.10.2.2 Internal Transcribed Spacer 2 (ITS2)

In all living organisms have existed protein synthesis and gene expression, which is majority roles in maintained by ribosomes, intracellular, and molecular machines. The phylogenetic information in prokaryotes and eukaryotes have extended by ribosomal RNA (rRNA), whereas ribosomal DNA (rDNA) has related with composes coding regions (18S, 5.8S and 28S) and non-coding region of internal transcribed spacers 2 (ITS2) (Figure 12) (Wei et al., 2006). Molecular markers of ITS regions have extending utilization to analyze for taxonomy and phylogenetic, which preferred over non-coding region including multiple copies of rRNA genes, suitable for PCR amplification by typical universal primers for diversity organisms, average sequence length for sequencing, and high degree of diversity at the generic and species level that suitable for frequent nucleotide polymorphism in sequences (Poczai & Hyvönen et al, 2010). ITS region was emphasized depended on both high and low degree of inter-specific genetic diversity of population in the different hosts and geographical areas of species level. Three mature worms' genomic DNA was extracted using a Genomic DNA micro kit (tissue, Geneaid, Taipei, Taiwan) in accordance with the manufacturer's instructions. *COX*1-Ov-Hap-F&R was used to amplify a part of the cytochrome c oxidase subunit I (*COX*1) gene and the internal transcribed spacer subunit II (ITS2) gene (Thaenkham et al., 2007). To confirm the location of any nucleotide mutation, the *COX*1 sequences of all the examined species were converted to amino acid sequences and aligned. To calculating 1,000 replications using p-distance and the neighbor-joining (NJ) approach in MEGA, cluster diagrams were created from the *COX*1 and ITS2 sequences (Kumar et al., 2008).

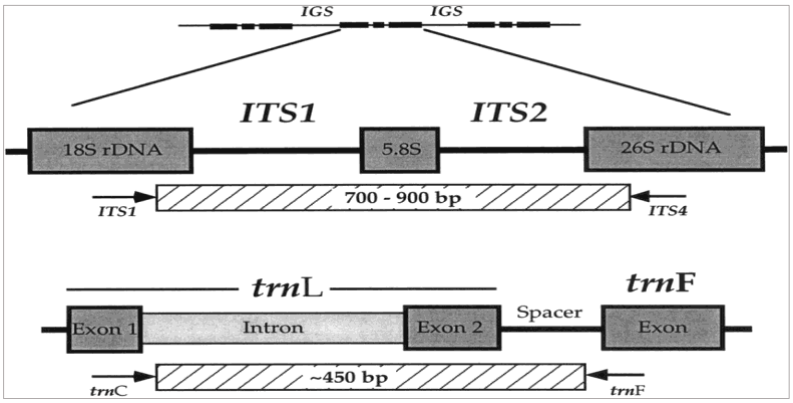

Figure 2.11 The internal transcribed spacer region of ribosomal gene (above) and the *trn*L region of chloroplast DNA (below). Ribosomal DNA diagrams showed the structure of a single array made up of three genes and spacers, as well as the link between individual arrays and IGS sections (Shaw & Stuart, 2002).

2.10.2.3 Mitochondrial DNA markers

The diversity of phylogenetic and genetic were analyzed in the mitochondrial DNA polymorphisms. A maternal mode of inheritance has played the principal role of inherit haploid mtDNA to the next generation and high mutation, which transported by mitochondrial cell of cytoplasm. The interracial evolutionary relationship revealed more features of biologist to evaluate mtDNA mutation patterns that detected hybridization between farmed species and subspecies (Nijman et al., 2003). The nad1 gene and other mitochondrial DNA (mtDNA) sequences have been used to examine animal population structures (Moritz & Brown, 1987). The different maternal lineage may be identified since mtDNA is transmitted from the mother and does not undergo any recombination. In particular, bottlenecks and gene flow may be studied in the evolution of species using the mtDNA sequence (Harrison, 1989; Shaw & Stuart, 2002).

2.10.2.4 Mitochondrial cytochrome c oxidase 1 (*COX*1)

Cytochrome c oxidase subunit 1 (*COX*1) is a mitochondrial DNA mainly used to discriminate animal species downward to mutate and it possible to differentiate of close relationship species. The liver fluke, *O. viverrini*, mtDNA has a circular shape 510 base pairs (bp) existed of 12 protein-producing gene, 2 rRNA-producing-gene, and 20 tRNA-producing gene. The polymorphism including *COX*3, Cyt b, *NAD*4L, *NAD*4, ATP6,

*NAD*2, *NAD*1, *NAD*3, *COX*1, *COX*2, *NAD*6, and *NAD*5 has used in the most common 12 genes. Interestingly, *O. viverrini COX*1 gene are 1551 bp at 6,934-8,484 and in *C. sinensis* 1560 bp at 6,972-8,531 that found in China and 6971-8,530 in Korea (Supaporn Pumpa, 2019). The partial sequence of cytochrome c oxidase subunit 1 (*COX*1) of three metacercariae was amplified by PCR under the amplification conditions supplied by *COX*1-Ov-Hap (F) and (R) primers (Thaenkham et al., 2007). A genetic analyzer named the ABI PRISM 3100, the PCR results were sequenced. Three metacercariae provided partial *COX*1 sequences of 369 base pairs, which CLUSTAL W was used to match with isolates of *O. viverrini* from Thailand and Laos (accession numbers EU022351 and EU022357) with the default setting (Thompson et al., 1994). The MEGA tool, the genetic distances were estimated using Kimura's two-parameter model (Tamura et al., 2007).

The development of innovative molecular approaches for species identification and delimitation, community composition evaluation, species richness in biodiversity investigations, and ecological research has significantly accelerated in recent years (Amaral-Zettler et al., 2009; Hebert et al., 2003; Pawlowski et al., 2016; van der Loos & Nijland, 2021; Wangensteen & Xavier, 2017). The identification of a variety of species is made possible by metabarcoding techniques based on high-throughput sequencing, particularly when mitochondrial markers are used (Wangensteen & Xavier, 2017). The mitochondrial cytochrome oxidase subunit 1 (*COX*1), which is present in most phyla in many Kingdoms, has just recently been discovered in foraminifera (Macher et al., 2021a). Unicellular protists known as foraminifera often inhabit watery habitats and are found in marine ecosystems. These tiny organisms are significant carbonate producers in addition to serving as reliable proxies for biochronology, coral reef ecosystem health, and (paleo) climatic changes (Billups, 2003; Pawlowska et al., 2020). However, little is known about the foraminifera genome as a whole. Using molecular markers from the internal transcribed spacer (ITS) and ribosomal small-subunit (18S SSU rRNA) regions of the genome, known foraminiferal morphospecies and cryptic species have been found (Borrelli et al., 2018; Macher et al., 2021a; Morard et al., 2015). It is recognized that a number of Foraminifera display notable degrees of chromosomal polymorphism (Morard et al., 2015), which may be related to the fact that these organisms have several nuclei per cell (Weber &

Pawlowski, 2014). Some taxa show insufficient diversity across species; for example, the ITS gene identified just one single nucleotide polymorphism (SNP) between two species of Amphisorus (Macher et al., 2021b). As a result, identifying species with 18S rRNA and ITS markers might be challenging (Morard et al., 2015; Macher et al., 2021a). Additionally, misclassified specimens that were difficult to identify visually down to the species level were identified with the use of the *COX*1 marker. An accurate representation of the diversity of foraminiferal taxa is provided by integrative taxonomy, which integrates molecular and morphological characteristics. Several studies also emphasize the need of combining several markers to get a trustworthy species identification and to gain a better understanding of evolutionary traits and interactions within and between species (Zhao et al., 2016). Foraminifera's first mitochondrial *COX*1 gene was discovered recently by (Macher et al., 2021b). The molecular marker *COX*1 is often utilized in molecular studies on animals (Hebert et al., 2003).

2.10.2.5 Nicotinamide adenine dinucleotide hydrogen dehydrogenase (NADHD)

Nicotinamide adenine dinucleotide (*NAD*) is an enzyme that is encoded in the mitochondrial genome. Its function is to convert NADH from its reduced form of NADH to its oxidized form of *NAD*+ (Xue et al., 2019). The mitochondria's inner membrane contains the NADH dehydrogenase complex. By moving protons from the mitochondrial matrix into the intermembrane gap and oxidizing NADH to *NAD*+, the complex produces energy. The complex also carries out a number of metabolic functions, including fatty acid oxidation, the TCA cycle, amino acid catabolism, and cytosolic activities, due to the vital role that metabolic pathways play in the breakdown of carbohydrates, fatty acids, and amino acids (Kerscher, 2000). Since *NAD* is abundantly expressed in all cells and is involved in many vital cell functions, it is a prime candidate for molecular detection. In addition to *NAD*1, which has already been studied, *NAD* subunits for development can be found as a diagnostic target since *O. viverrini*'s mitochondrial genome is well-established (Buathong et al., 2017).

The parasitologists diagnosed *C. sinensis* from DNA analysis of ITS, *COX*, and *NAD.* The molecular analysis has claimed the different between *C. sinensis* genetic and

other trematodes (Park, 2007). The researcher demonstrated the genetic characteristics of *C. sinensis* attempted DNA analysis and paleoparsitologists proofed genetic characteristic of ancient *C. sinensis* among their study on samples at archeological sites in Korea. Thus, they have succeeded analyzed ancient DNA (aDNA) sequencing of *C. sinensis* egg using amplifies sequences of ITS1, ITS2, and *COX*1 analysis in Korean mummy feces since 17th century (Shin DH et al., 2013). RAPD revealed genetic variation in the *O. viverrini* isolates from the wetlands of Thailand and the Lao People's Democratic Republic (Sithithaworn et al., 2007) and multilocus enzyme electrophoresis (Saijuntha et al., 2006; Saijuntha et al., 2007). Additionally, *O. viverrini* isolates from northern and northeastern Thailand, as well as two provinces in Lao PDR, were found to contain 4 different nad1 haplotypes and 2 different *COX*1 haplotypes, respectively, using data from 2 mitochondrial genes, nad1 and *COX*1 (Saijuntha et al., 2008). The study examined the possible advantages of utilizing the *NAD*1 gene as a genetic marker for *O. viverrini*, given that genetic diversity within the species may be linked to regional variations in the occurrence of CCA (Andrews et al., 2008).

2.10.2.6 DNA Barcoding

A short DNA sequence known as a barcode from the mitochondrial DNA (mtDNA) of the cytochrome c oxidase subunit 1 (*COX*1) is used in genetic studies and is a crucial identifier for classifying animal species (Figure 13) (Hebert et al., 2003). However, a sequence of the nucleotide based on adenine (A), thymine (T), cytosine (C), and guanine (G), which is single barcode contained form C C G G C A T A G T A G G C A C T G and different ranges in length from 255 to 700 nucleotide based. Moreover, barcodes of same species will be classified not more than 2% divergences that found of related species sequence divergence between 6.8% and 99.98%, which were sequenced greater than 3% (Johns & Avice, 1998). The species not closely related was highly found by sequence divergence, so this barcode has discriminated the species challenged with taxonomy validation effectively. More significantly, barcodes may be used to find and compare new barcodes in order to identify species or distinguish them from those that do not belong to a species in the database, particularly when expressing new species with competence. Furthermore, it is demonstrated that a very little volume of an organism's tissue (1-3 mm3) may be retrieved at any stage of life.

Therefore, regardless of how an organism develops or changes morphologically over time, DNA barcoding has the ability to represent major portions of that organism (Ivanova et al., 2007).

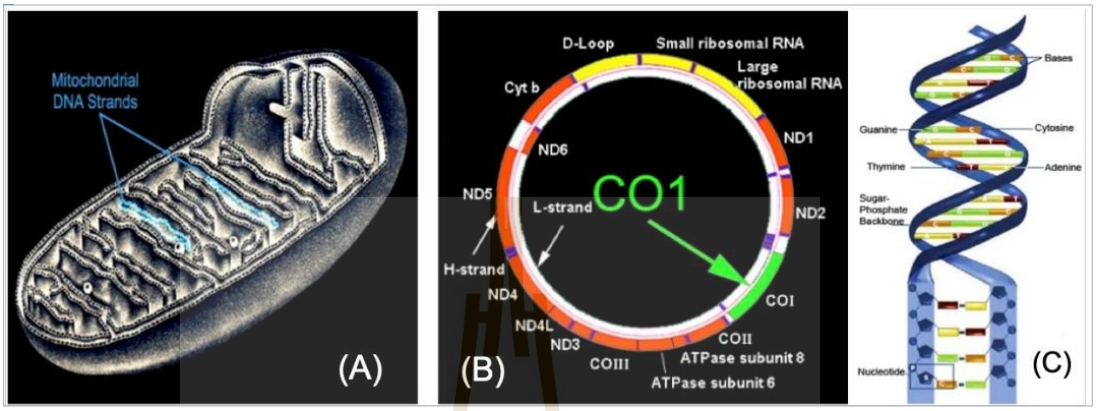

Figure 2.12 DNA barcoding identifies the following: (a) mitochondria; (b) where on an mtDNA strand the cytochrome c oxidase subunit 1 (*COX*1) is located; and (c) the nucleotide bases in an mtDNA strand (Hebert et al., 2003).

2.11 Treatment

Praziquantel is the current drug of choice for treatment opisthorchiasis patients that recommended as follows. Praziquantel 25 mg/kg three times daily for 2-3 consecutive days (Chai et al., 2005; Sithithaworn et al., 2007). The WHO advised another chemotherapy in 1995 that included praziquantel 40 mg/kg in a single dosage, however it was unclear how this method would regulate sustainability (Chai et al., 2005; Clausen et al., 2015). Treatment for human *O. felineus* infections also involves praziquantel (25 mg/kg orally three times a day for one to two days). The usual dosage of praziquantel is 75 mg/kg taken three times a day for one day, which causes an egg's risk of infection to drop from 98% to 99% in both *O. viverrini* and *C. sinensis* cases (Keiser J & Utzinger, 2010). As a result, side symptoms like headache, nausea, and dizziness are rare from praziquantel therapy. Furthermore, according to many books, trimedimidine was previously one of the drugs used to cure liver flukes. Trindimidine, in fact, was a derivative of amidantel that acted against intestinal roundworms such as hookworms, ascariasis, and enterobiasis, in addition to liver flukes (Xu LL et al., 2014). In comparison to praziquantel, a 400 mg single dosage of tribendimidine had a greater

effect (98%–99%) in reducing the egg rate of *O. viverrini* and *C. sinensis*. It also had less adverse effects. Trindimidine's most typical adverse effects are headache, nausea, lethargy, dizziness, and vertigo (Sayasone et al., 2016). Even though the efficiency of liver fluke therapy should be determined by looking at the eggs in stool specimens, relatively few eggs are generated when treatment is ineffective.

2.12 Prevention

Opisthorchiasis, foodborne zoonotic disorder is neglected by the government health authorities in the Greater Mekong Subregion (GMS), except Thailand (WHO, 2010a). Liver fluke infection remains majority concerning in public health which is difficulty to control by traditional methods (Upatham et al., 1984). The first-choice drug treatment of *Opisthorchis* parasite infections is praziquantel that available in the market, which is effective chemotherapy among opisthorchiasis patient (WHO, 1995b). However, the recurrent infection and treatment *O. viverrini*, praziquantel was often used, which is high risk to increase CCA condition. The main playing role of reduce infection is basically prevention the consumption of infected and changing attitude traditional of consuming freshwater fish better prolong the effective to lower the prevalence in the future (Pinlaor et al., 2008; Pinlaor et al., 2004). Moreover, there was control program for *O. viverrini* infection throughout primary care system and arrangement selective treatment to empowerment targeting in communities with high-risk intensities and areas with high-risk of CCA (Jongsuksuntigul & Imsomboon, 2003; Sriamporn et al., 2004; Sripa & Pairojkul, 2008). In other hand, the principal of reduction the liver fluke infection among schoolchildren should be issue in campaign and education program in the communities including knowledge of parasitic disease and biomarkers technology detection of CCA (Dechakhamphu et al., 2008; Thanan et al., 2008). Success control programs for *O. viverrini* are based on epidemiological factors like human hosts, population genetic data, and human movement restrictions. To minimize prevalence, an adequate health system is needed, especially in Cambodia, Vietnam, and Lao PDR, and Thailand, to improve surveillance efforts effectively.

2.13 Conceptual framework

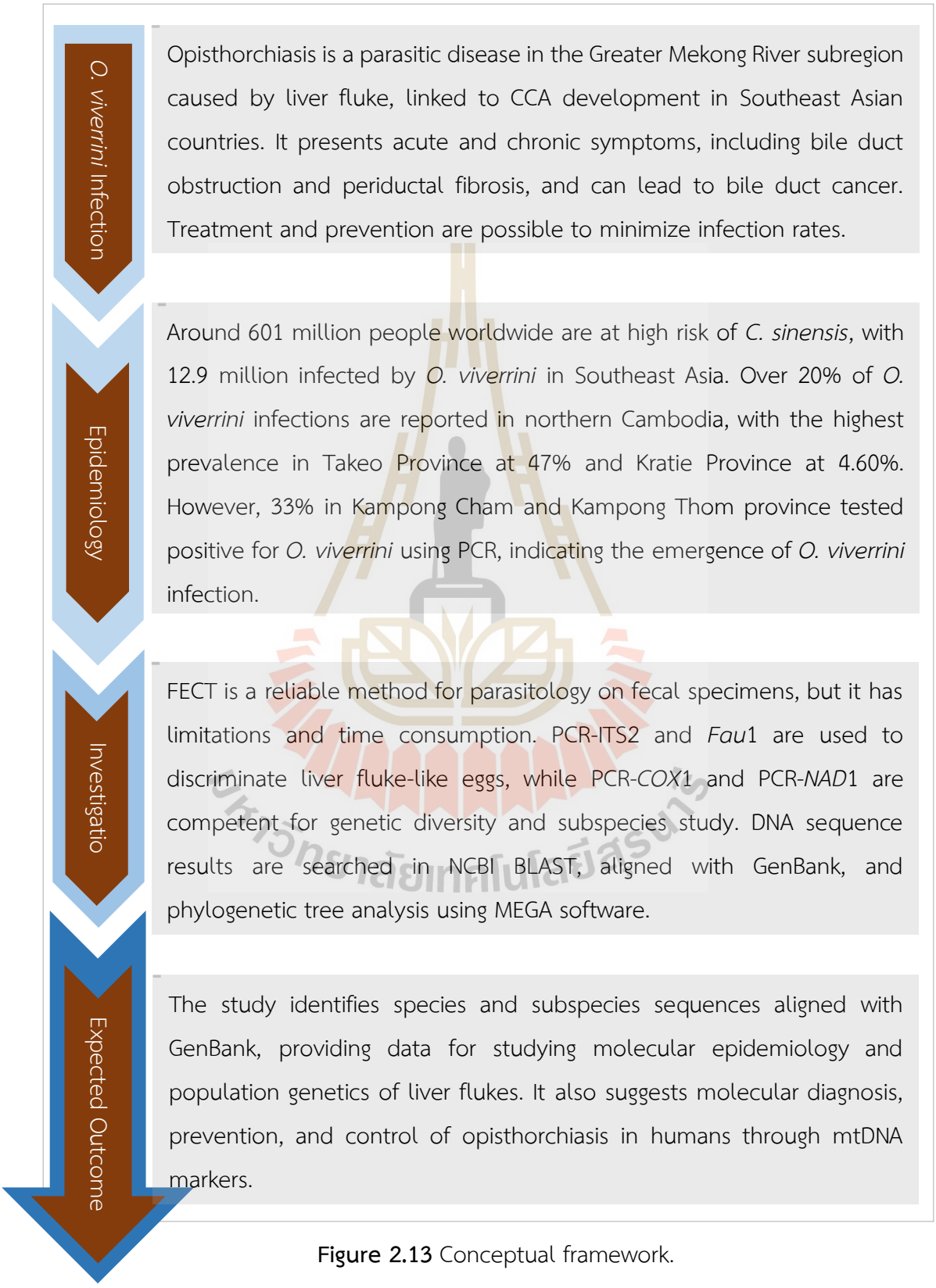

Figure 2.13 Conceptual framework.

CHAPTER III

RESEARCH METHODOLOGY

3.1 Study design

A cross-sectional study design to observe and analyze data from a particular population in the Snuol district, Kratie Province, Cambodia to determine the prevalence of liver fluke infections using parasitological and molecular methods. The data analysis was descriptive analysis of risk factor to determine the liver fluke infection within the study areas in Kratie Province. The population and sample sizes used in this study were calculated, and sampling methods such simple random sampling was used to determine data analysis for quality control, which was claimed under ethical concerns for interpretation and conclusion.

The analytical evaluation for this investigation was conducted utilizing a parasitological approach that required quantitative concentration FECT under light microscopic and then differentiation of the Opisthorchis-like eggs from fecal materials. A total of 377 from fecal specimens were set and accessible at the PDRC, Institute of Medicine, SUT were used to establish this study. The specimens were observed for liver flukes and other parasites lava and eggs read under light microscopic. Whereas the results of parasitological technique were discriminated by PCR-ITS2 gene targeting in ribosomal DNA the molecular method. Moreover, the discrimination of liver flukews between *O. viverrini*, *C. senensis*, and small intestinal flukes were used PCR-RFLP using restriction enzyme *Fau*1. Additionally, PCR-ITS2 results for mitochondrial PCR-*COX*1 and PCR-*NAD*1 genes were sequenced for genetic diversity analysis based on the references sequence in NCBI GenBank database. Following alignment and construction of phylogenetic trees using the reference sequencing of *COX*1 and *NAD*1, comparable subspecies and isolated of *O. viverrini* DNA sub-type of Kratie Province with others region (Figure 14). This study was performed within 12 months from 08/4/ 2022 to

07/4/ 2023, accordingly under assertion BioEthic committee by research institute and development, SUT.

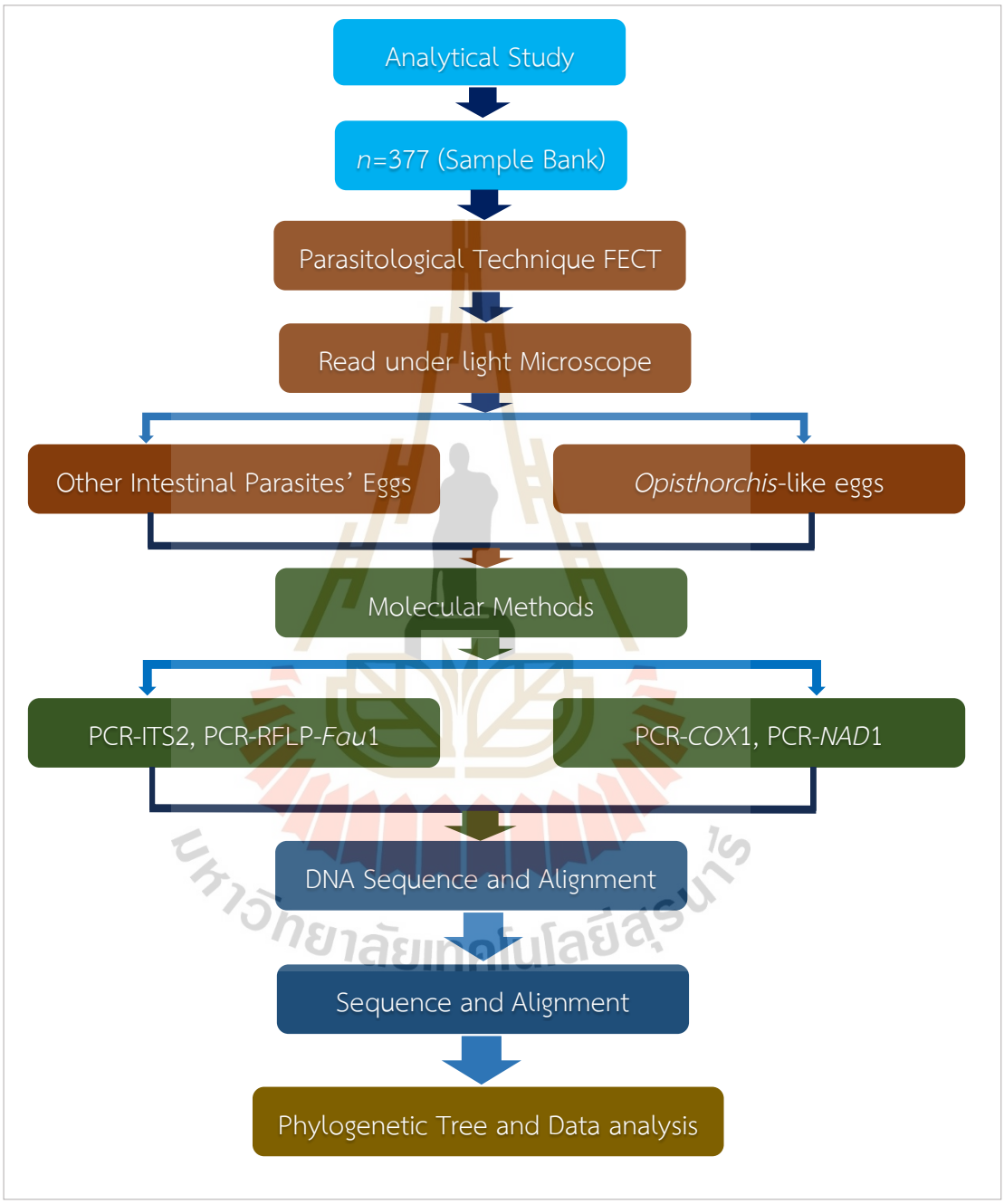

Figure 3.14 Flowchart of study design.

3.2 Population and sample size

This study was used the secondary data and fecal specimens collected within Kratie Province, Cambodia that stored in PDRC for purposive study if it is required. However, the primary data that provided from the PDRC based on the original study areas was clearly defined the population of residents in a particular region, age group, genders, educational levels, socioeconomic, and location, respectively. Moreover, the fecal specimens were organized and sorted in order to preserve in the appropriate temperature in the refrigerator at 4 °C until examination according to the protocols of sample collection. They looked examined the derived microbial community's response to storage at 80 °C for up to 2 years (Shaw at al., 2016).

Fecal samples were used to investigate in the present study to distinguish between Opisthorchis-like eggs by divided into two parts for the parasitological and molecular study. However, the following sample size was calculated by the equation n=N/[1+(N*e^2)] (Ngrenngarmlert et al., 2012), which is represented by n=sample size, N=population of Kratie Province 374,755 by Cambodia census in 2019 (NIS, 2020) and e^2= precision margin of error (0.0025). Therefore, the sample size was set at 399 individuals, however based on available sample kept in PDRC was 377 of fecal specimens.

3.3 Sample and sampling

The study design of accept the fecal samples was a cross-sectional design for collecting fecal samples as a simple random sampling technique. The fecal specimens were heap up from Kratie Province and perform for purposive simple random sampling that available in PDRC. The analytical study was applied as descriptive analysis to describe on *Opisthorchis*-like eggs and other intestinal fluke infections (Figure 15).

Figure 3.15 Sorting of 377 fecal sample for laboratory performance.

3.4 Materials

3.4.1 Protocol

The fecal specimens were approved by the human ethic committee with number EC: NRPH013 from Health Office in Nakhon Ratchasima province. Regarding this study, the researcher has received training in biosafety and biosecurity, which has been approved by the BioEthic Committee, Research Institute and Development (RID), SUT. This training supports the researcher's professional conduction and performance in the laboratory when practical experiments for the thesis project (Figure 16).

Figure 3.16 Observing and identifying the parasite through fecal samples.

3.4.2 Specimen

This study used fecal samples from the Cambodian population that were accessible at the PDRC, Institute of Medicine, SUT. The fecal specimens were freeze in refrigerator at 4 °C for purposive study if required by the head of PDRC (Figure 17).

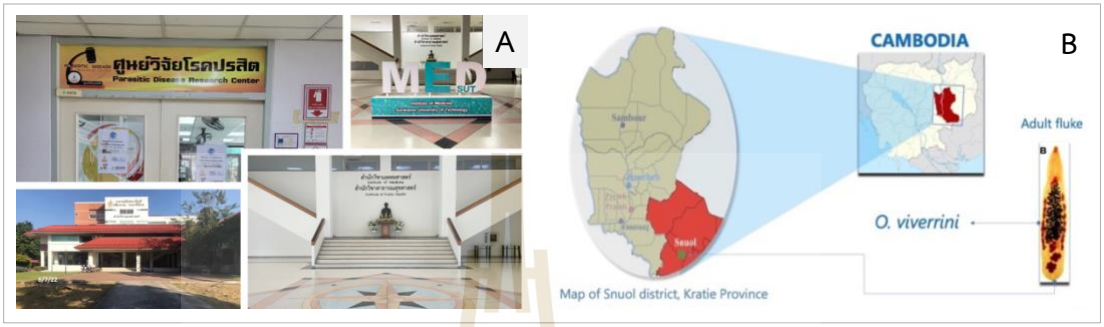

Figure 3.17 Institution (A) and (B) the map of study area. (NIS, 2020).

3.5 Methods

3.5.1 Parasitological Technique

3.5.1.1 Formalin-Ethyl Acetate Concentration Technique

Clinical laboratories frequently utilize the formalin-ethyl acetate concentration technique, also known as the formalin-ethyl acetate sedimentation technique, to evaluate parasite eggs, larvae, cysts, and oocysts in fecal specimens (Figure 18). This technique concentrates the parasitic stages that made them easier to observe under a light microscope. Three grams of feces weight was dissolved in 10 milliliters (mL) of 0.85% normal saline solution. The initial step, the debris specimen was strained by veil into a centrifuge tube of 15 mL then placed into centrifuge at 2,500 round per minute (rpm) for 5 minutes (min). The second was completely mixed with the sediment with 7 mL of 10% formalin and 3 mL of ethyl acetate. After five minutes of centrifuging the sample, the top layer of supernatant was removed. An applicator with a cotton tip was used to remove the material from the centrifuge tube's sidewalls. Finally, to resuspend the real specimen was added 3 drops of 0.85% saline and then the *O. viverrini*-like eggs were seen and reported counting under the light microscopic that utilized of 10x and 40x magnification objective lens. Although, quantitative concentration FECT is the capable diagnosis testing technique but the

lower of flukes and sensitivity of stool quantitative concentration technique is considering burden of illness does affect the accuracy of detection of parasites' egg existing.

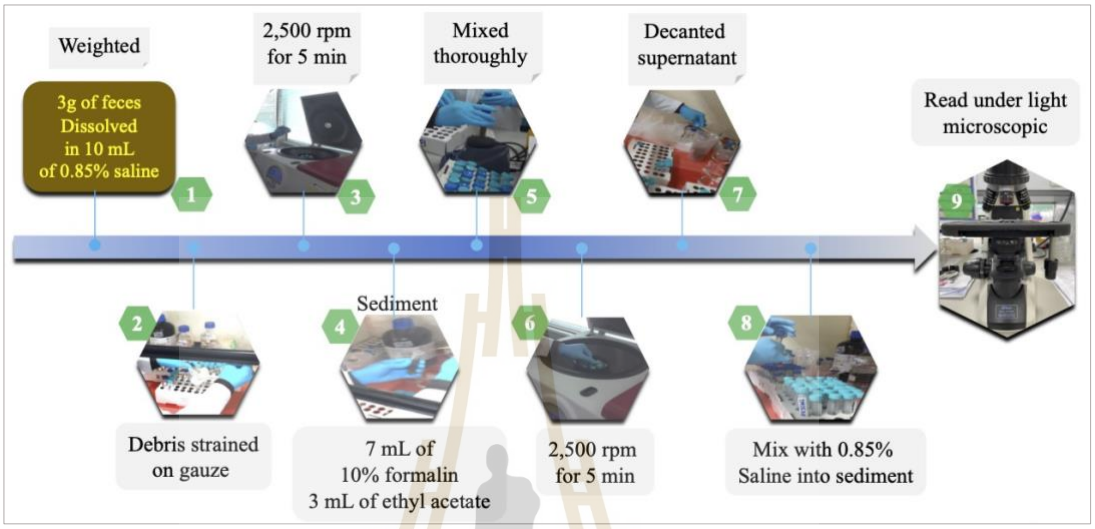

Figure 3.18 The formalin-ethyl acetate concentration technique.

3.5.1.2 Light microscopic examination

Traditional technique, light microscopic examination is known as a gold standard to examine the liver fluke-like eggs through fecal sample based on FECT (Bogoch et al., 2016) but it is difficulties to differentiate between human liver flukes and small intestinal flukes (Sripa et al., 2011). The saline preparation has the benefit of being a rapid, easy process that yields a prompt result when positive, which aided in the identification of the distinctive motility of trophozoites (Holgate & Webb, 2003). The light microscopic was performed to observe the parasite eggs and larvae that maintained by using 4 objective lenses, which functioned as 4x, 10x, 40x, and 100x objective lenses (Figure 19). However, 10x objective lens was use for screening and then moving to 40x for confirm more characteristic morphology of existed egg. In contrast, the discrimination the true prevalence of the liver flukes were diagnosed by using molecular methods PCR-ITS2 gene targeting in rDNA (Sato et al., 2009).

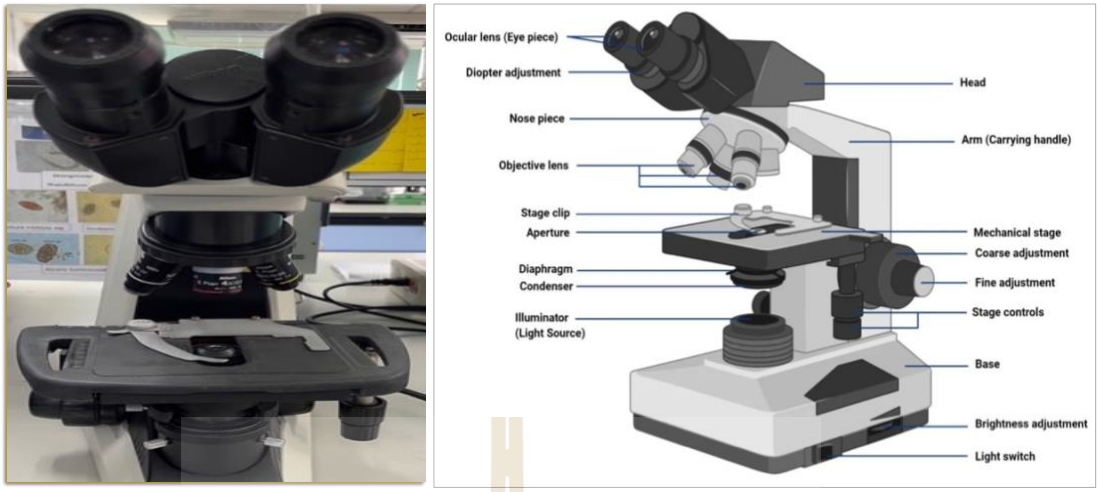

Figure 3.19 Light microscopic in PDRC and graphic (Prescott & Mokobi, 2022).

3.5.2 Molecular methods

3.5.2.1 Genomic DNA extraction

Using a feces QIAamp1 DNA mini kit (Qiagen, Hilden, Germany), a standard DNA extraction kit intended for fecal samples, and adhering to the manufacturer's recommendations, a genomic DNA mini kit was used to extract via fecal specimens (Figure 20). In concise, each specimen received 180–220 mg of feces in 2 mL along with 1 mL of inhibit EX buffer in a microcentrifuge tube. Initiative step vortex 1 min or until stool sample has homogenized, heat suspension temperature at 95 ºC for 5min. And then centrifuge with 14,000 rpm for 1 min to pellet stool particles. Add 200 µL of supernatant into 15 µL of proteinase K in the tube and add 200 µL buffer AL and vortex with 15 second (sec). After adding 200 µL of 95% ethanol to 415 µL of the previously combined solution, it was incubated for 10 minutes at 70 ºC and vortexed for 15 seconds. After transferring 600 µL of the supernatant to a fresh spin column and centrifuging it for a minute, the QIAamp spin column was placed into a 2 mL collecting tube. After centrifuging for one minute with 500 µL of AW1 buffer, the tube holding the filtrate was disposed away. After adding 500 µL of AW2 buffer to the spin column and centrifuging for three minutes, the collection tube holding the filtrate was disposed away. After being put into a fresh collecting tube, the spin column was centrifuged for three minutes. After moving the QIAamp into a fresh, labeled 1.5 mL microcentrifuge tube, 50 µL of ATE buffer was added to the spin column. It was then allowed to

incubate for one minute at room temperature before being centrifuged for one minute to extract the DNA gene. Finally, extracted DNA stored at –20 °C until use.

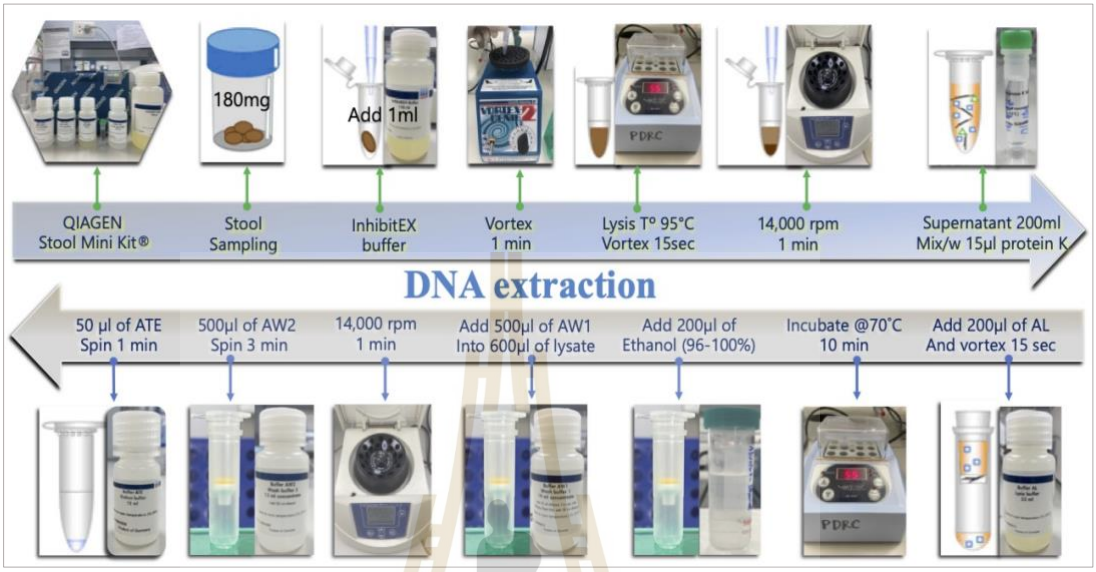

Figure 3.20 The graphic of DNA gene extraction from fecal specimens.

3.5.2.2 Primer design

Design specific primers that face the ITS2, *COX*1, and *NAD*1 genes, which would be species or genus-specific based on existing databases. The DNA template was used to bind a conserved portion of the mitochondrial *COX*1 and *NAD*1 gene of *O. viverrini.* PCR are most important in molecular biology with specific primer based on rDNA and mtDNA from available search of the nucleotide database in NCBI GenBank. Nevertheless, the primers for amplifying or identifying and sequencing of mitochondrial protein-coding and ribosomal nuclear genes were designed for this study. The initially, PCR-ITS2 sequence-Ov-6F 5′-CTG AAT CTC TCG TTT GTT CA-3′ as a forward primer and Ov-6R 5′-GTT CCA GGT GAG TCT CTC TA-3′ (GenBank accession number: S80278) as a reverse primer that generated an amplicon fragment 330 bp from nucleus ribosome genes (Wongratanacheewin et al., 2001). However, PCR-ITS2 were amplified and then PCR-product was use for PCR-RFLP as RTFlukeFau1-F 5'- CTT GAA CGC ACA TTG CGG CC-3' And RTFlukeFau1-R 5'-CAC GTT TGA GCC GAG GTC AG-3'was designed of these *O. viverini*, *C. senensis*, and *H. Taichui* gene amplifying, which generated fragment 377, 381, and 321 bp (accession No.: MK886663.2 and MK415601.1). PCR-RFLP was

performed to discrimination of *O. viverrini*, *C. sinensis*, and *H. Taichui* egg by restriction enzyme *Fau*1 (Traub et al., 2009). The PCR-*COX*1 sequences *COX*1-OvF; 5'-TGA TCC GTT GTT GTT TCA-3' and *COX*1-OvR; 5'-ACG GAT ATA ACC ACC GTT CT-3') (GenBank accession No.: MF287782) (Buathong et al., 2017) that generated fragment 504 bp were used. The PCR-*NAD*1 primers sequence *NAD*1-OvF; 5'-TAC GCA GGT GGT TTG GTT GG-3' and *NAD*1-OvR; 5'- CCC AAA GCT CAC ATC CTT GT -3' (GenBank accession No.: DQ119551) generated fragment 668 bp were applied, respectively (Saijuntha et al., 2008).

3.5.2.3 Internal transcribed spacer 2 (ITS2) based on PCR

Polymerase chain reaction (PCR) is well known used to manipulate DNA with small amounts of genetic material amplifying to identify or detect of the specific infectious organisms such as viruses, bacteria, and parasites including genetic variations, mutation in human genes and other numerous tasks (Figure 21). A PCR mixture solution including DNA template, primers, nucleotides, Taq polymerase, and other necessary buffer components. However, amplify the DNA using thermal cycler requiring a standard PCR following by denaturation, annealing, and extension. This was included multiple cycles to ensure ample DNA amplification. Finally, gel electrophoresis was used to visualize the amplified products consisted by DNA ladder might be run alongside the samples to identify the size of the amplified fragments. If the size matched the expected size for the *O. viverrini* target region, it indicates a positive result.

The PCR-ITS2 was amplified by using Ov-6F 5´-GTT CCA GGT GAG TCT CTC TA-3´ as a forward primer and Ov-6R 5´-CTG AAT CTC TCG TTT GTT CA-3´ as revers by product size 330 bp. Although, PCR-ITS2 reaction 25 µL of total volume and containing PCR buffer 16.8 µL of distill water (DW), 2.5 µL of 10x taq buffer, 1 µL of dNTP, 1 µL of Ov-IST2-F, 1 µL of Ov-ITS2-R, 0.2 µL of Taq DNA polymerase, 1.5 µL of $MgCl_2$, and DNA template 1µL (Wongratanacheewin et al., 2002). The PCR products was amplified in the G-STORM™ GS482 thermal cycler. There were three steps of PCR condition including denaturation, annealing, and extension, which were initial denaturation temperature at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 45 sec, which was accompanied by 30 cycles and final extension 72°C for 5 min. Finally holding temperature at 4 °C infinite (Duenngai et

al., 2008). Because of the greater specificities of PCR, if a PCR inhibitor is present in the feces, the test is not negative. For improved diagnostic sensitivity, a DNA extraction procedure employing cetyltrimethylammonium bromide to eliminate inhibitors has been used. Depending on the degree of *O. viverrini* infection, a PCR-based technique test is very sensitive if the specimens had more than 200 eggs per gram of feces (Wongratanacheewin et al., 2002).

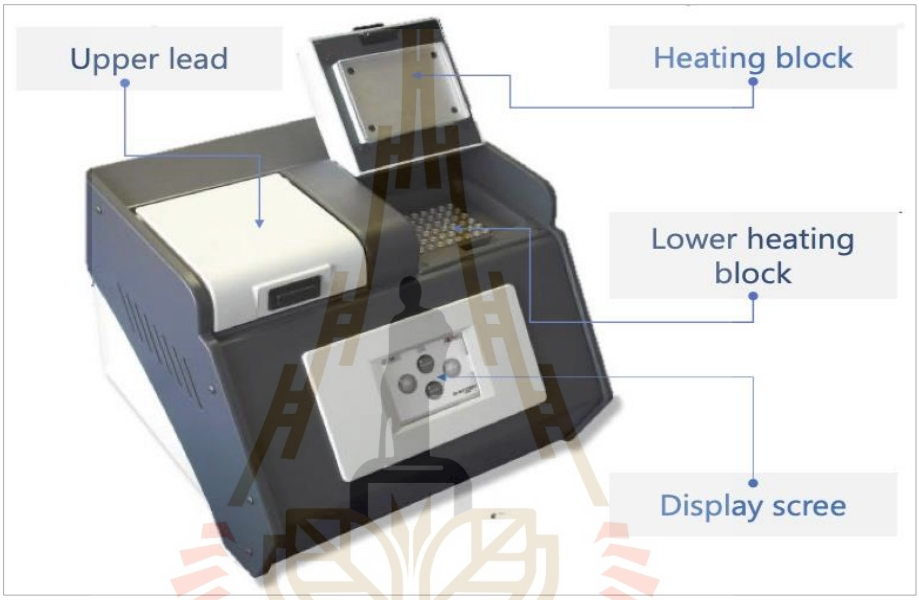

Figure 3.21 G-STORM thermocycler in PDRC, Institute of Medicine, SUT.

3.5.2.4 PCR-restriction fragment length polymorphism (PCR-RFLP)

Opisthorchis-like eggs of *O. viverrini*, *C. senensis*, and MIFs were discriminated from the positive PCR-ITS2 products as the template for PCR-RFLP amplifying using RTFluke, whereas restriction enzyme *Fau*1 for PCR assay to confirm of the true-negative results by PCR amplification (Buathong et al., 2017; Sato et al., 2009). PCR-RFLP was developed to identify the species *O. viverrini* and *C. senensis* by size, which is competency to differentiate artificially mixed infection. These amplifying ITS2-PCR products was performed as the template to RTFlukeFaF 5'- CTT GAA CGC ACA TTG CGG CC-3' And RTFlukeFaR 5'-CAC GTT TGA GCC GAG GTC AG-3' of both species were generated. The total volume 25 µL of PCR-RFLP amplifying was designed as consist of DNA template. The components of this procedure including buffer 16.8 µL of DW, 2.5

µL of 10x Taq buffer, 1 µL of dNTP, 1 µL of Fluke-F, 1 µL of Fluke-R, 1.5 µL of $MgCl_2$, 0.2 µL of Taq DNA pol., and 1 µL ITS2-PCR product as the template. The PCR-RFLP assay was existed of initial denaturation 94 ˚C for 15 min, annealing temperature at 60 ˚C for 1 min, extension step at 72 ˚C for 2 min that accompanied by 35 cycles. The denaturation at 94 ˚C for 30 sec, 60 ˚C of annealing for 30 sec, extension temperature at 72 ˚C for 30 sec, a final extension temperature at 72 ˚C for 7 min and holding temperature at 4 ˚C infinite to complete amplification (Traub et al., 2009). Therefore, the discriminate of *O. viverrini*, *C. sinensis* and MIF by PCR-RFLP was performed of total volume 25 µL that mixture reaction included molecular water based on PCR-RFLP product concentration converted from nanogram (ng) to µL, 5 µL of NEBbuffer, restriction enzyme, 0.5 µL of *Fau*1. The PCR-RFLP products that digested with 2 units of *Fau*1-F-5'...CCCGC $(N)_4$ 3' and *Fau*1-R-3'...GGGCG $(N)_6$ 5' (New England Biolabes) of total volume 25 µL and incubated at 55 ˚C for 5-15 min and heat inactivated at 65 ˚C for 20 min (Buathong et al., 2017).

3.5.2.5 Genetic diversity and genetic structure using the PCR-*COX*1

PCR tests were used to determine the genetic diversity of *O. viverrini* eggs in accordance with Bauthong, 2015 (Buathong et al., 2015). The primers were used in this study for the genetic diversity of the positive results from mitochondrial DNA (mtDNA) sequencing and then subject to align in NCBI BLAST GenBank search to classify the nucleotide and species (Hall, 1999) includes *COX*1-OvF; 5'-TGA TCC GTT GTT GTT TCA-3' and *COX*1-OvR; 5'-ACG GAT ATA ACC ACC GTT CT-3') (Buathong et al., 2017). The amplification of mitochondrial and ribosome genes of PCR reaction was performed *COX*1-*O. viverrini* in total volume 25 µL, which was composed 1 µL of each primer *COX*1-OvF and *COX*1-OvR, 12.5 µL Thermo-Scientific Phusion, 1 µL DMSO (dimethyl sulfoxide), 8.5 µL H_2O, and DNA template 1 µL. The Master cycler G-STORM^{TM} GS482 thermal cycler was amplified to generate the PCR products. Pre-denaturation at 95 ˚C for 5 minutes was the starting point for the *COX*1-*O. viverrini* primer PCR assay. Thereafter, there were 30 cycles: denaturation at 95 ˚C for 30 seconds, annealing at 50 ˚C for 30 seconds, extension at 72 ˚C for 30 seconds, final extension at 72 ˚C for 7 minutes, and holding temperature at 4˚C infinite to finish amplification.

3.5.2.6 Genetic diversity and genetic structure using the PCR-*NAD*1

The PCR-*NAD*1 primers *NAD*1-OvF; 5'-TAC GCA GGT GGT TTG GTT GG-3' and *NAD*1-OvR; 5'-CCC AAA GCT CAC ATC CTT GT-3', which generated amplicon fragment 668 bp, respectively (Saijuntha et al., 2008). The amplification of mitochondrial and ribosomal genes on PCR reaction was performed for *NAD*1-*O. viverrini* of total volume 25 µL, which were composed 1 µL of each primer *NAD*1-OvF and *NAD*1-OvR, 12.5 µL Thermo-Scientific molecular water 16.8 µL, 10x taq buffer 2.5 µL, dNTP 1 µL, Ov-NAD1-F 1 µL, Ov-NAD1-R 1 µL, Tag DNA Polymerase 0.2 µL, $MgCl_2$ 1.5 µL, and DNA template 1 µL. The Master cycler G-STORM^{TM} GS482 thermal cycler will be used for amplifying to generate the PCR products. Pre-denaturation at 94 ˚C for 5 minutes was the starting point for the *NAD*1-*O. viverrini* primer PCR assay. This was followed by 30 cycles of denaturation at 94 ˚C for 1 minute, annealing at 52 ˚C for 1 minute, extension at 72 ˚C for 2 minutes, final extension at 72 ˚C for 7 minutes, and holding temperature at 4˚C infinite to complete amplification (Saijuntha et al., 2008).

3.5.2.7 Agarose gel electrophoresis

The ITS2-PCR, PCR-*COX*1, PCR-*NAD*1 products and PCR-RFLP with *Fau*1 were observed under 1.5% agarose gel electrophoresis weight 0.45g was mixed with buffer 30 mL of TAE 1x solution (Figure 22). This diluted solution was solid after melted about 3 min by the microwave and incubated in room temperature within the gel tray to make gel solid approximately 4mm thickness. The gel was solidified in 10-15 min then placed the gel with gel bed into the migration tank that flood by approximately 200-250 mL of TAE buffer, which was appropriated for electrode migration from the negative to positive pole.

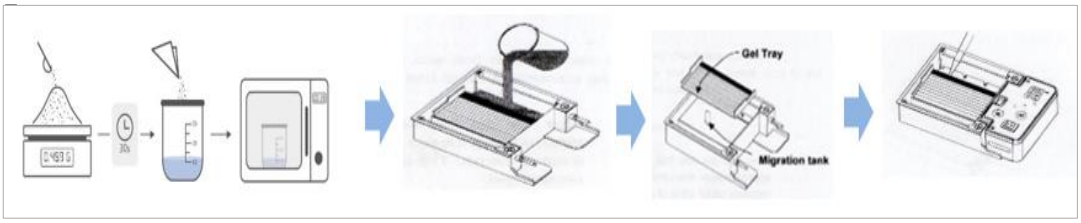

Figure 3.22 PCR products under 1.5% agarose gel electrophoresis.

3.5.2.8 PCR products analysis

The PCR-product was confirmed the positive DNA band by electrophoresis under 1.5% of agarose powder to detect ITS2-PCR, PCR-*COX*1 and PCR-*NAD*1 products by using TAE buffer, 6x DNA loading, DNA ladder, Maestrosafe nucleic acid stain (MNAS), and PCR-product (Figure 23). Before the gel electrophoresis run the solution was diluted comprising of 1µL MNAS, 1 µL of 6x DNA loading dye, and 3-5 µL of DNA template depending on the size of gel well, respectively. However, 1 µL DNA ladder dye approximately 100 bp mixed with MNAS only for ladder band. The PCR-product was set on 100 V at room temperature approximately 30 min. The final step, the agarose gel was transferred to visualize under molecular image® Gel Doc TMXR + Imaging System (Bio-Rad). The positive PCR-product result was sent to sequence at the QIAquick Gel Extraction Kit (Qiagen) Co., Ltd with randomly selected PCR-products of *Opisthorchis*-like eggs.

Figure 3.23 The agarose gel electrophoresis and imaging documentary.

3.5.2.9 Sanger Sequencing of *O. viverrini*'s DNA

Sequences on DNA, RNA, or proteins were categorized using bioinformatics sequencing alignment to find regions of similarity that could result from functional, structural, or evolutionary relationships between the sequences. The PCR-ITS2 product that generated of 330 bp was analyzed and subjected to NCBI BLAST GenBank that genetically identified *O. viverrini* and obtained as accession number after DNA sequent. *O. viverrini*'s DNA were purified after fecal specimens were extracted and positive PCR-

products were sent for sequencing to QIAGENE company. The PCR-product contained within 10 µL of PCR tube that labeled by ID code as the accession numbers and followed by printed paper sheet as a note for each product identities. The sequencing results were sent Fasta electronic files as accession numbers of PCR-ITS2 sequence that available searching in NCBI BLAST GenBank database. There were 26 results of *O. viverrini*'s DNA sequencing, which were indicated matched with *O. viverini* genomic mRNA 333 bp in the GenBank by the previous publication in 1997 throughout the accession number S80278.1 with varieties per identities between (93.74% to 99.20%). The PCR-*COX*1, and PCR-*NAD*1 with generated of fragment 504 and 668 bp, respectively, was sent for DNA sequencing, which are PCR-product or cuts off the positive results band from agarose gel to purifying. The DNA sequence was analyzed via Program BLAST search at NCBI verifying and confirm with positive for alignment with another deposited DNA database in the GenBank (Buathong et al., 2020; Saijuntha et al., 2008). The references sequences of *COX*1 and *NAD*1 will be constructed as genetic diversity. Additionally, the PCR result was purified by DNA sequencing using a Qiagen QIAquick Gel extraction kit in accordance with the manufacturer's instructions. In order to categorize species and sequence DNA, PCR results were submitted to First BASE laboratory for sequencing. After that, the sequence was subjected to NCBI BLAST nucleotide sequence analysis.

3.5.2.10 Phylogenetic analysis

The ITS2-PCR positive samples were used for PCR-based mitochondrial *COX*1 and *NAD*1 to amplifies, bidirectionally sequence, and population genetics analysis. However, *COX*1 and *NAD*1 sequence of *O. viverrini* was isolated. The reference *COX*1 and *NAD*1 sequence of *O. viverrini* was aligned by separately to generates phylogenetic tree diversity (Buathong et al., 2017). PCR products of mitochondrial sequences was identified for analysis by the BioEdit version 7.0.9 program and the MEGA5 program, version 5.05 (http://www.megasoftware.net/) was aligned for DNA sequencing to distinguish the similarity by phylogenetic relationship of Opisthorchiidae and Heterophyidae families and others intestinal flukes. The taxonomic location was examined by phylogenetic analysis employing two mitochondrial protein-coding genes, *COX*1 and *NAD*1. Sequences of trematode species belonging to the Opisthorchiidae,

Heterophyidae, Fasciolidae, and Schistosomatidae families of *Opisthorchis* were employed (Buathong et al., 2020). Therefore, DNA sequence was aligned using MEGA software to observe of the similarities.

3.6 Statistical analysis

Descriptive analysis was computed the overall prevalence and stratified prevalence base on demographics or regions within Snuol district, Kratie Province. This study was analyzed by using IBM SPSS® statistic for Window version 23 software program for data management and analysis. Bivariate binary logistic regression was used to examine the associated prevalence of *O. viverrini* infections, while crosstabs and chi-square tests were used to define the frequencies of the research data (Sangwalee et al., 2021). Regression analysis was therefore used in risk factor analysis to identify factors that were significantly associated with liver fluke infection by selecting the variable from the binary logistic regression test as a significance, with the results being expressed as a percentage, 95% confidence interval (95% CI), and p-value<0.05.

3.7 Ethical statement

This study was certified by BioEthic committee number (IBC-65-05), Research Institute and Development, SUT for validation on the thesis defense, but thesis proposal project sample was used fecal specimens of Cambodian population that available in PDRC, Institute of Medicine, SUT, which was approved by the Human Ethic Committee, Health Office of Nakhon Ratchasima Province number (EC: NRPH013).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Parasitological detection

4.1.1 Morphology of *O. viverrini* using FECT read under light microscopic

For viewing the existing parasite eggs and larva, the parasitological concentration techniques that were based on microscopic observations were advantageous. After performing a quantitative concentration FECT from fecal specimens was used to examine the existence of *O. viverrini's* egg by objective lens 10x, 40x, and 100x, the morphology of *O. viverrini* was analyzed under a light microscope. The clarity and egg's size were observed by eyes via objectives lens 10x (Fig. 24A) for screening the existing egg on the microscopic slide, 40x (Fig. 24B) for conforming the *O. viverrini*-like egg, which was more extending size clearer, and 100x was extended the transparency of the egg (Fig. 24C).

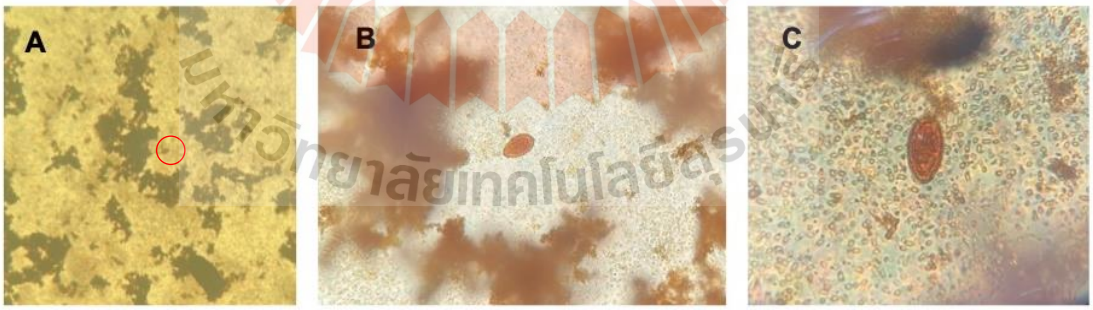

Figure 4.24 Morphological of *O. viverrini* observing under light microscopic. (A) *O. viverrini*'s egg using 10x objective lens to screen, (B) *O. viverrini*'s egg using 40x lens to confirm, and (C) *O. viverrini*'s egg using objective lens 100x to characteristic.

4.1.2 Morphology of intestinal parasites infections

Measured concentration FECT The gold standard was utilizing fecal specimens to observe the intestinal parasites' eggs and larva under light microscopy with a scale bar of 50 µm. In relation to this investigation, a total of 64 helminth eggs were discovered in fecal collections. However, evidence of IPIs' egg included *O. viverrini*'s (Fig. 25B), *Taenia* (Fig. 25A), Hookworm (Fig. 25C), *Trichuris Trichiura* (*T. trichiura*) (Fig. 25E), *Ascaris lumbricoides* (*A. lumbricoides*) (Fig. 25D), *Hyminolepis nana* (*H. nana*) (Fig. 25G), and *Entamoeba coli* (*E. coli*) (Fig. 25F).

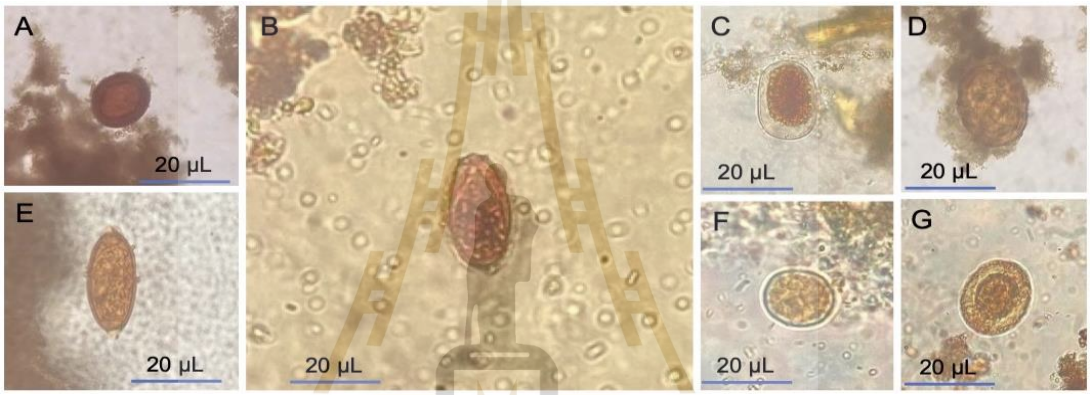

Figure 4.25 Morphological of intestinal parasites' egg under light microscopic. (A) *Tenia*, (B) *O. viverrini*, (C) Hookworm, (D) *A. lumbricoides*, (E) *T. trichiura*, (F) *E. coli*, and (G) *H. nana*.

4.1.3 Intensity of intestinal parasite infection

Each individual provided a single feces sample, which was collected. The formalin-ethyl acetate concentration (FECT) technique and the basic wet smear were used to evaluate the fecal specimens. If one or more *O. viverrini*-like eggs were found using of this procedure, a fecal sample was deemed positive for the presence of such eggs. Under a microscope, the number of *O. viverrini*-like eggs was counted, and the FECT was used to quantify the number of eggs per gram (EPG) of feces. Light infection (1,000 EPG), moderate infection (1,000–19,000 EPG), and high infection (> 29,000 EPG) were used to categorize the severity of Opisthorchis-like egg (Maleewong et al., 1992).

According to the formula, the number of eggs per gram was determined to

distinguish the intensity range of *O. viverrini*'s egg (Albonico et al., 2003; Boonjaraspinyo et al., 2011). The egg presentation using microscope revealed on average, 61 eggs per slide and 671 eggs per gram. Additionally, the EPG of *O. viverrini* infection was classified as ranging from mild infection to highly intensity, followed by hookworm, *E. coli*, *A. lumbricoides*, and *T. trichiura*, which were classified as low intensity of infection. Because the threshold for light infection was 1,000 EPG, the *O. viverrini* infection in the Sunuol district should be categorized as light infection given that 5.57% was below that threshold (Table 4.1). Contrarily, the *H. nana* and *Taenia* are rodent cestodes after the egg was expelled from proglottids via the vaginal atrium and subsequently dissolved in the small intestine after passing through the stool (Flisser, 1994).

Table 4.1 The intensity egg per gram (EPG) of intestinal parasite.

Parasites	Egg positive (*n*)	Egg examined (%)	Egg per gram	Intensity of infection (normal range)		
				Light	Moderate	High
O. viverrini	21	5,57	671.00	1-1000	1000-9999	≥29,999
Hookworm	16	4,24	256.67	1-1999	2000-3999	≥4000
E. coli	15	3,97	666.67	1-1000	1000-9999	≥29,999
A. lumbricoides	4	1,06	180.00	1-499	5000-9999	≥10,000
T. trichiura	2	0,53	7.00	1-999	1000-9999	≥10,000
Taenia	2	0,53	N/A	-	-	-
H. nana	4	1,06	N/A	-	-	-

4.1.4 Prevalence of Intestinal parasites infections (IPIs)

In Snuol district, Kratie Province, northeastern Cambodia, the frequency of IPIs was examined of a total 377 fecal samples, which was more likely in women (*n*=220, 58.36%) than men (*n*=157, 41.64%). The IPIs were found (*n*=60, 15.91%), it was found in men to be higher infection rates (*n*=32, 20.38%) than women (*n*=28, 12.72%) (Table 4.2). The binary logistic regression test was used to examine the prevalence of the association between gender and the results were significant for crude odds ratio (OR_{cru})=0.570, 95% confidence interval (CI)=0.327-0.992, *p*=0.047*, and adjusted odds

ratio (OR_{adj})=0.582, CI=0.325-1.041, *p*=0.068. Age groups under 20 (*n*=13, 13.40%), those between 21-30 (*n*=17, 16.34%), 31-40 (*n*=7, 10.0%), and 41-50 (*n*=11, 18.96%) was lower rates of infection. The highest rates were found in age groups above 51 years old (*n*=12, 25.0%). Primary school had the highest infection prevalence (*n*=26, 19.54%), followed by illiteracy (*n*=16, 15.53%), and secondary & higher education (*n*=18, 12.76%) were not very noteworthy. Farmers (*n*=30, 20.83%) and employed (*n*=4, 9.75%), shopkeepers (*n*=9, 13.23%), housewives (*n*=10, 16.12%), government officers (*n*=3, 15.78%), and others (*n*=4, 9.30%) were found most frequent in occupational. Trapaing Srae village was the highest prevalence of infection (*n*=31, 19.25%), followed by Chrab (*n*=10, 15.87%), Cheung Khei (*n*=3, 5.76%), Pravanh (*n*=7, 16.66%), and Cheung Khlue (*n*=9, 15.25%), with no statistically significant difference.

A total of 377 fecal sample were observed by using FECT based on light microscopic in this study was found 15.91% infected by IPIs among the population in the Snuol district, Kratie Province. In-depth analysis highlights once more the significant multiparasitism, variety, and intestinal helminthic infection in a semi-rural Cambodian context. The research subjects were found (70.20%) of helminth infections in Kandal Province, Cambodia (Bless et al., 2015). The subjects of (33.8%) had multiparasitism in rural Laos, even higher rates were seen (Sayasone et al., 2011; Sayasone et al., 2009). Therefore, in this study was found of IPIs lower rates than the previously study as well as comparing to Loa PDR's IPIs prevalence. This finding is reflexing due to the National Helminth Control Program (NHCP) was provided and an effectiveness of inti-helminthic prophylaxis treatment in the countrywide since their launched to conduct of field survey on parasites endemic area in 2006 (Khieu et al., 2019).

Table 4.2 Detection of IPIs infection using quantitative concentration FECT (*n*=377).

Variable	Examined (n (%))	Crude and Adjusted OR (95% CI) and *P*-value				
		IPIs (n (%))	OR_{cru} (95% CI)	*P*-value	OR_{adj} (95% CI)	*P*-value
Sex			0.570		0.582	
Male	157 (41.64)	32 (20.38)	(0.327-	0.047*	(0.325-	0.068
Female	220 (58.36)	28 (12.72)	0.992)		1.041)	
Age groups						
< 20 years	97 (25.72)	13 (13.40)				
21-30 years	104 (27.58)	17 (16.34)	1.172		1.213	
31-40 years	70 (18.56)	7 (10.00)	(0.959-	0.121	(0.989-	0.064
41-50 years	58 (15.38)	11 (18.96)	1.433)		1.487)	
>51 years	48 (12.73)	12 (25.00)				
Educational level						
Illiteracy	103 (27.32)	16 (15.53)			0.815	
Primary	133 (35.27)	26 (19.54)	1.179	0.106	(0.568-	0.265
Secondary & higher	141 (37.40)	18 (12.76)	(0.965- 1.441)		1.168)	
Occupation						
Employees	41 (10.87)	4 (9.75)				
Farmers	144 (38.19)	30 (20.83)				
Shopkeepers	68 (18.03)	9 (13.23)	1.167		0.924	
Housewives	62 (16.44)	10 (16.12)	(0.954-	0.133	(0.751-	0.460
Government officer	19 (5.03)	3 (15.78)	1.428)		1.138)	
Others	43 (11.40)	4 (9.30)				

Table 4.2 (Continued).

Variable	Examined (n (%))	Crude and Adjusted OR (95% CI) and *P*-value				
		IPIs (n (%))	OR_{cru} (95% CI)	*P*-value	OR_{adj} (95% CI)	*P*-value
Location						
Trapaing Srae	161 (42.70)	31 (19.25)	1.188 (0.970-1.455)	0.096	0.880 (0.724-1.070)	0.200
Chrab	63 (16.71)	10 (15.87)				
Cheung Khie	52 (13.79)	3 (5.76)				
Pravanh	42 (11.14)	7 (16.66)				
Cheung Khlue	59 (15.64)	9 (15.25)				

*Binary logistic regression test. Significant different $P<0.05$.

4.1.5 Prevalence of helminths and protozoa infection

The examination intestinal parasite was classified into helminths such as *O. viverrini*, Hookworm, *Taenia*, *T. trichiuara*, *A. lumbricoides*, and *H. nana*, and protozoa was *E. coli*, respectively, which were associated in each variable distributed. The prevalent of helminths was more frequent than protozoa infections (*n*=45, 11.93%) and (*n*=15, 3.97%) from 377 fecal samples, respectively. The helminths were found in two groups that higher in men (*n*=27, 17.19%) than in women (*n*=18, 8.18%) were analyzed by binary logistic regression test OR_{cru}=0.429, 95% CI=0.227-0.810, *P*=0.008 and OR_{adj}=0.420, 95% CI=0.217-0.811, *P*=0.010, which was significantly (Table 4.3). Whereas prevalent of protozoa infection with a total (*n*=15, 3.97%) was found in both gender women (*n*=10, 4.54%) higher than men (*n*=5, 3.18%), however there was not significantly. The prevalent of helminth was related with age groups of over 51 years old (*n*=9, 18.75%) higher than other age groups and followed by lower than 20 years old (*n*=6, 6.18%), 21-30 years old (*n*=15, 14.42%), 31-40 years (*n*=6, 8.57%), and 41-50 years (*n*=9, 15.51%) by binary logistic regression that associated with dependent and co-variate OR_{cru}=1.260, 95% CI=1.005-1.580, *P*=0.45 and OR_{adj}=1.280, 95% CI=1.016-1.614, *P*=0.036 was significantly. The following age groups including lower than 20 years old to 41-50 years old were no significant. The infection associated with education level was common in primary schoolchild (*n*=21, 15.78%), which followed by

secondary & higher (*n*=16, 11.34%), and (*n*=8, 7.76%) of illiteracy. The prevalent of occupational was included that classified into multiple occupation was found highest in government officers (*n*=3, 15.78%) and followed by farmers (*n*=20, 13.88%), shopkeepers (*n*=9, 13.23%), housewives (*n*=8, 12.90%), employees (*n*=4, 9.75%), and others (*n*=1, 2.32%). The location was included such as Cheung Khlue (*n*=9, 15.25%) was common frequency than Trapaing Srae village (*n*=19, 11.80%) which followed by Chrab (*n*=9, 14.28%), Pravanh (*n*=6, 14.28%), and Cheung Khie village (*n*=2, 3.84%). Therefore, the helminths infection was associated within age groups with significantly, however, the education, occupation, and location were not significant.

Table 4.3 The prevalence of helminths infection (*n*=377).

Variable	Examined (n (%))	Number of parasite infections n (%)				
		Helminths (%)	OR_{cru} (95% CI)	*P*-value	OR_{adj} (95% CI)	*P*-value
Sex			0.429		0.420	
Male	157 (41.64)	27 (17.19)	(0.227-	0.008*	(0.217-	0.010*
Female	220 (58.36)	18 (8.18)	0.810)		0.811)	
Age groups						
<20 years	97 (25.72)	6 (6.18)				
21-30 years	104 (27.58)	15 (14.42)	1.260		1.280	
31-40 years	70 (18.56)	6 (8.57)	(1.005-	0.045*	(1.016-	0.036*
41-50 years	58 (15.38)	9 (15.51)	1.580)		1.614)	
>51 years	48 (12.73)	9 (18.75)				
Educational						
Illiteracy	103 (27.32)	8 (7.76)	1.149		1.095	
Primary	133 (35.27)	21 (15.78)	(0.774-	0.491	(0.723-	0.669
Secondary & higher	141 (37.40)	16 (11.34)	1.704)		1.658)	

Table 4.3 (Continued).

Variable	Examined (n (%))	Number of parasite infections n (%)				
		Helminths (%)	OR_{cru} (95% CI)	*P*-value	OR_{adj} (95% CI)	*P*-value
Occupation						
Employees	41 (10.87)	4 (9.75)				
Farmers	144 (38.19)	20 (13.88)	0.871		0.953	
Shopkeepers	68 (18.03)	9 (13.23)	(0.696-	0.227	(0.752-	0.690
Housewives	62 (16.44)	8 (12.90)	1.090)		1.207)	
Government officers	19 (5.03)	3 (15.78)				
Others	43 (11.40)	1 (2.32)				
Location						
Trapaing Srae	161 (42.70)	19 (11.80)				
Chrab	63 (16.71)	9 (14.28)	1.043		1.025	
Cheung Khie	52 (13.79)	2 (3.84)	(0.850-	0.684	(0.832-	0.819
Pravanh	42 (11.14)	6 (14.28)	1.280)		1.262)	
Cheung Khlue	59 (15.64)	9 (15.25)				

*Binary logistic regression test. Significant different $P<0.05$.

The prevalence of protozoa infection was found within men and women with a total (*n*=15, 3.97%) from 377 fecal samples whereas infections were found higher in women (*n*=10, 4.54%) than men (*n*=5, 3.18%). The prevalence was included in age groups was classified of age groups less than 20 years old (*n*=7, 7.21%) highest than the following of age group 21-30 years (*n*=2, 1.92%), 31-40 (*n*=1, 1.42%), 41-50 (*n*=2, 3.44%), and over 51 years old (*n*=3, 6.25%). However, education levels were prevalent among illiteracy (*n*=8, 7.76%) higher than the following of primary school (*n*=5, 3.75%) and secondary & higher (*n*=2, 1.41%) analyzed by binary logistic regression with OR_{cru}=0.431, 95% CI=0.212-0.873, *P*=0.020, OR_{adj}=0.359, 95% CI=0.175-0.739, *P*=0.005 was remarked as significantly (Table4.4). For occupational prevalence was most

frequency in other careers (*n*=3, 6.97%) and followed by farmers (*n*=10, 6.94%) and housewives (*n*=2, 3.22%). The location was detected of IPIs in Trapaing Srae village (*n*=12, 7.45%) analyzed by binary logistic regression OR_{Cru}=0.483, 95% CI=0.260-0.894, *P*=0.021, OR_{Adj}=0.415, 95% CI=0.209-0.822, *P*=0.012 was significantly. Therefore, the prevalence of protozoa was associated with education level and location.

In discussion on this study that classified into two group of parasites infection by helminths and protozoa, which were found (11.93% and 3.97%) among population in the Snuol district, Kratia Province. The co-relationship predominance between IPIs was discovered in this study with a dependent variable. Helminths were associated with gender as a risk factor that both men and women were susceptible to.

It has long been known by epidemiologists that males of vertebrate species, including humans, typically exhibit higher rates of illness and parasitism than females (Bundy, 1988; Zuk, 1990). A prior research on the prevalence of helminth infections in Cambodia and the Lao People's Democratic Republic indicated that males (51.5%) were more likely than women (45.5%) to be infected, in contrast to Laos, where women (56.4%) were more likely to be infected than men (43.7%), respectively (Vonghachack et al., 2017).

Numerous empirical studies have demonstrated age-intensity, demonstrating either a steadily increasing parasite load or a gradually increasing parasite burden with age (Hudson, 1995). However, the diagnosis tool to detect the parasite infection using serum and urine specimens were found the relationship of helminths infection with age group. Moreover, the peak in lower age group 10-16 years was observed higher than in older age group by using circulating anodic antigen (CAA) (Vonghachack et al., 2017). However, a recent assessment of *O. viverrini* infection in Preah Vihear and Stung Treng Province found that the age range of 30-39 years had the highest frequency (Jung et al., 2023). In contrast, this was found the highest prevalence related with age group of over 51 years (18.75%). The parasites really multiply after being transferred within the host, adapting to the environment over time and progressively getting bigger as the host ages (Hudson & Dobson, 1995).

Regarding to the protozoa infection was related with educational level and location in this study. The highest frequency infection of this parasite was among

population no educational, illiteracy (7.76%) and followed by primary school kids (3.75%). Poor literacy is strongly correlated with intestinal parasite infection, according to certain research. *O. viverrini* infection prevalence among the age groups was associated with illiteracy (8.74%) levels of education. The risk factor for the illiteracy rate was present (Nematian et al., 2004). According to several research, parasitoses are correlated with illiteracy, a lack of latrines causing diarrhea, a lower socioeconomic standing, improper disposal of human waste, and a lack of cleanliness in homes.

The area linked to protozoa infection as notable at high-risk factor identified in this study was Trapaing Srae village, Snuol district, Kratie, which had the greatest prevalence (7.45%). The demographic risk variables associated with the importance of the covariate location were the main focus of the study at endemic areas. Such a sign can be used to inform the public and the authorities about untreated foodborne trematode infections, in an effort to increase awareness of parasite infection and take action to control (Zhou et al., 2009).

Table 4.4 The prevalence of protozoa infection (*n*=377).

Variable	Examined (n (%))	Number of parasite infections n (%)				
		Protozoa (%)	OR_{cru} (95% CI)	*P*-value	OR_{adj} (95% CI)	*P*-value
Sex			1.448		2.146	
Male	157 (41.64)	5 (3.18)	(0.485-	0.507	(0.638-	0.217
Female	220 (58.36)	10 (4.54)	4.321)		7.217)	
Age groups						
<20 years	97 (25.72)	7 (7.21)				
21-30 years	104 (27.58)	2 (1.92)	0.915		1.024	
31-40 years	70 (18.56)	1 (1.42)	(0.618-	0.658	(0.712-	0.900
41-50 years	58 (15.38)	2 (3.44)	1.355)		1.471)	
>51 years	48 (12.73)	3 (6.25)				

Table 4.4 (Continued).

Variable	Examined (n (%))	Number of parasite infections n (%)				
		Protozoa (%)	OR_{cru} (95% CI)	*P*-value	OR_{adj} (95% CI)	*P*-value
Educational						
Illiteracy	103 (27.32)	8 (7.76)	0.431		0.359	
Primary	133 (35.27)	5 (3.75)	(0.212-	0.020*	(0.175-	0.005*
Secondary & higher	141 (37.40)	2 (1.41)	0.873)		0.739)	
Occupation						
Employees	41 (10.87)	00				
Farmers	144 (38.19)	10 (6.94)				
Shopkeepers	68 (18.03)	00	1.023		0.802	
Housewives	62 (16.44)	2 (3.22)	(0.727-	0.897	(0.542-	0.271
Government officers	19 (5.03)	00	1.440)		1.188)	
Others	43 (11.40)	3 (6.97)				
Location						
Trapaing Srae	161 (42.70)	12 (7.45)				
Chrab	63 (16.71)	1 (1.58)	0.483		0.415	
Cheung Khie	52 (13.79)	1 (1.92)	(0.260-	0.021*	(0.209-	0.012*
Pravanh	42 (11.14)	1 (2.38)	0.894)		0.822)	
Cheung Khlue	59 (15.64)	00				

*Binary logistic regression test. Significant different $P<0.05$.

4.1.6 Prevalence and risk factors of *Opisthorchis*-like infection

A total of 377 fecal specimens were examined for intestinal parasite eggs using concentration FECT under light microscopy, and stool examination, prevalence of *Opisthorchis*-like infection was found (*n*=21, 5.57%), followed by hookworm (*n*=16, 4.24%), *E. coli* (*n*=15, 3.97%), and other *A. lumbricoides* (*n*=4, 1.06%), *H. nana* (*n*=4, 1.06%) (Figure 26). The prevalence of the other top three intestinal parasites, which are listed (Table 4.5) was 5.57% for *O. viverrini*, 4.24% for Hookworm, and 3.97% for E. coli. Prevalence in each age-group was 4.12% (< 20 years), 8.65% (21–30), 1.42% (31–40), 6.89% (41-50), and 6.25% (> 51 years), respectively. Each age group's prevalence did not differ appreciably. Of the 21 positive *Opisthorchis*-like egg samples analyzed, the EPG of the feces is as follows, as indicated in Table 1: One sample each of <1,000 EPG, <50 EPG, and <20 EPG, out of which all positive samples were regarded as light intensity. The frequency of infection in age group 21-30 years (*n*=9, 10.41%) than the age group behind them, 41-50 years (*n*=4, 6.89%), <20 year (*n*=4, 4.12%), 30-40 years (*n*=1, 1.42%), and >51 years (*n*=3, 6.25%), OR=1.065, 95% CI=0.766-1.483, *P*=0.707 were no significant. Therefore *O. viverrini* was found higher than the other intestinal flukes such as Hookworm, *Taenia*, *T. trichiura*, *A. lumbricoides*, *H. nana*, and *E. coli* infection.

However, a binary logistic regression analysis revealed that, overall, there was a significant correlation between the presence of hookworm and gender (n=16, 4.24%), with males being more likely to be infected (n=14, 8.91%) than women (n=2, 0.90%); OR=0.096, 95% CI=0.021-0.443, P=0.003 (Table 4). Over 51 years old age groups are more common (n=5, 10.41%) than the age group behind them, which is less than 20 (n=2, 2.06%), 21-30 years (n=3, 2.88%), 31-40 years (n=2, 2.85%), and 41- 50 years old (n=4, 6.89%), OR=1602, 95% CI=1.085-2.365, *P*=0.014 were significant. The prevalence of Hookworm infection, however, did not significantly among occupation and location. According to binary logistic regression analysis, *E. coli* was associated with educational level and location were common among illiteracy in various regions of the Snuol district with OR=0.356, 95% CI=0.171-0.740, P=0.006 and OR=0.419, 95% CI=0.212-0.828, *P*=0.012, which were remarked significantly. This study was found the prevalence of IPIs associated with genders, age group, educational level, and location areas, especially hookworm and *E. coli* infection, which was significant, respectively. Males

were substantially more likely to get hookworm infection (21.8%) than females (16.0%) were (P<0.01). Hookworm (81.4%) was found in Khoua District, Phongsaly Province, and Namor District, Oudomxay Province (Rim et al., 2003). In Lao PDR, 161 of the 451 students (35.7%) in Khammuane, Vientiane and Champassak Province, as well as the Vientiane Municipality, tested positive for *E. vermicularis* eggs. The rate of *E. vermicularis* infection in men (35.2%) was different as that in women (36.1%). Statistically (Rim et al., 2003). There was known that the IPIs found to be higher in age group 8-10 and 11-13 years old among the school age (Bless et al., 2015). In fact, the parasites are reproduced after transmitted within the host and they are adapted from environment over time and gradually increasing with the host age. Numerous empirical research has documented age-intensity, showing either a persistent rise in parasite load or a gradual elevated of parasite burden with age (Hudson & Dobson, 1995). A 5-year Laos-Korea collaboration on the prevention of intestinal parasite illnesses in Lao primary students was started in 2000 in light of the aforementioned condition. With moderate and heavy infections, Luang Namtha Province had the highest hookworm and whipworm burden, measuring 37.4% and 35.3% for hookworm and 87.1% and 2.0% for whipworm, respectively (Rim et al., 2003). Among patients with hookworm infection, light infections were more common than severe or strong infections. The prevalence of soil-transmitted intestinal parasites showed the relationship between such prevalence and socioeconomic factors like agricultural practices, sanitary conditions, and degree of environmental contamination by human excreta, while the distribution of food-borne intestinal parasites like *O. viverrini* is closely related to water courses and dietary habits (Rim et al., 2003). It has been stated that 40% of youngsters in the Bolikhamxay province consume raw beef, utilize animal and/or human feces as fertilizer, 95.5% of dwellings lack latrines, and two-thirds consume raw fish (Panzer et al., 1998). Although it was unable to pinpoint the precise cause, environmental variables and sanitary conditions may both contribute to these variations. In this investigation, it was shown that male pupils had a greater risk of hookworm infection. One of the potential explanations for this intriguing discovery is that men engage in more outdoor exercise than women.

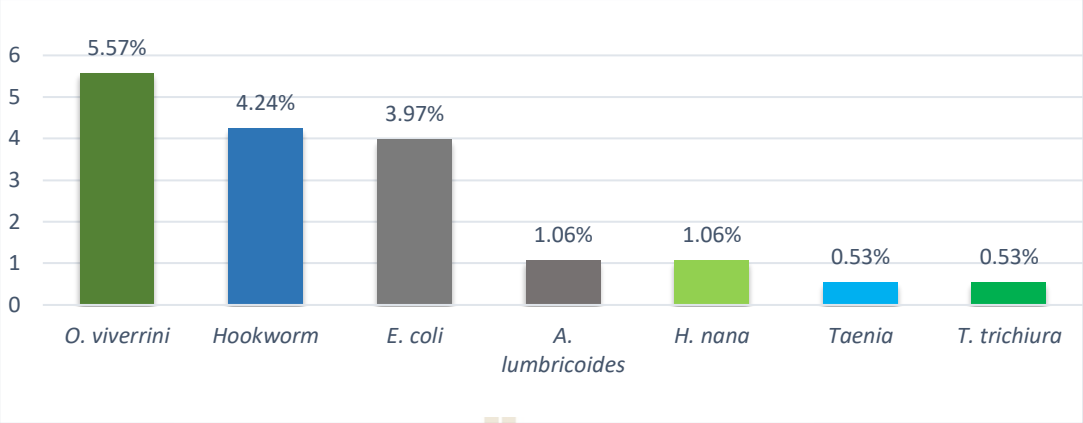

Figure 4.26 Diagram of IPIs eggs positive prevalence using FECT read under light microscopic.

Table 4.5 The prevalence of intestinal parasite infections associated with multivariable (*n*=377).

Variable	*n* (%), OR (95% CI), and *P*-value					
	O. viverrini	OR (95% CI)	*P*- value	Hookworm	OR (95% CI)	*P*-value
Sex		0.468			0.096	
Male	13 (8.28)	(0.182-	0.116	14 (8.91)	(0.021-	0.003*
Female	8 (3.63)	1.205)		2 (0.90)	0.443)	
Age groups						
<20 years	4 (4.12)			2 (2.06)		
21-30 years	9 (8.65)	1.065		3 (2.88)	1.602	
31-40 years	1 (1.42)	(0.766-	0.707	2 (2.85)	(1.085-	0.018*
41-50 years	4 (6.89)	1.483)		4 (6.89)	2.365)	
>51 years	3 (6.25)			5 (10.41)		
Educational						
Illiteracy	3 (2.91)			3 (2.91)	1.098	
Primary	11 (8.27)	1.055		6 (4.51)	(0.544-	
Secondary & higher	7 (4.96)	(0.596-1.866)	0.854	7 (4.96)	2.218)	0.794

Table 4.5 (Continued).

Variable	*n* (%), OR (95% CI), and *P*-value					
	O. viverrini	OR (95% CI)	*P*- value	Hookworm	OR (95% CI)	*P*-value
Occupation						
Employed	1 (2.43)			3 (7.31)		
Farmer	11 (7.63)	0.844		8 (5.55)	0.751	
Shopkeeper	6 (8.82)	(0.589-1.210)	0.357	3 (4.41)	(0.475-	0.219
Housewife	2 (3.22)			2 (3.22)	1.186)	
Gov.-officer	1 (5.26)			00		
Others	00			00		
Location						
Trapaing Srae	11 (6.83)			4 (2.48)	1.290	
Chrab	5 (7.93)	0.806	0.207	4 (6.34)	(0.925-	0.134
Cheung Khie	1 (1.92)	(0.576-1.127)		1 (1.92)	1.800)	
Pravanh	2 (4.76)			1 (2.38)		
Cheung Khlue	2 (3.38)			6 (10.16)		

*Binary logistic regression test. Significant different *P*<0.05.

Table 4.5 (Continued).

Variable	*n* (%), OR (95% CI), and *P*-value					
	Taenia spp	OR (95% CI)	*P*-value	*Trichuris trichiura*	OR (95% CI)	*P*-value
Sex		0.377				
Male	1 (0.63)	(0.008-	0.618	00	N/A	0.995
Female	1 (0.45)	17.514)		2 (0.90)		
Age groups						
<20 years	00			00		
21-30 years	00	26.099		1 (0.96)	1.647	
31-40 years	00	(0.428-	0.120	00	(0.614-	0.321
41-50 years	1 (1.72)	1591.237)		00	4.418)	
>51 years	1 (2.08)			1 (2.08)		
Educational						
Illiteracy	00			2 (1.94)		
Primary	00	N/A	0.989	00	N/A	0.992
Secondary & higher	2 (1.41)			00		
Occupation						
Employed	00			00		
Farmer	00	6.426		00	1.855	
Shopkeeper	1 (1.47)	(0.552-	0.138	00	(0.477-	0.372
Housewife	00	74.868)		1 (1.61)	7.206)	
Gov.-officer	1 (5.26)			00		
Others	00			1 (2.32)		
Location						
Trapaing Srae	1 (0.62)			1 (0.62)		
Chrab	1 (1.58)	0.601		00	1.293	
Cheung Khie	00	(0.079-	0.623	00	(0.492-	0.602
Pravanh	00	4.572)		00	3.397)	
Cheung Khlue	00			1 (1.69)		

Table 4.5 (Continued).

Variable	*n* (%), OR (95% CI), and *P*-value					
	A. lumbricoides	OR (95% CI)	*P*-value	*Hymenolepis nana*	OR (95% CI)	*P*-value
Sex		0.654			1.766	
Male	2 (1.27)	(0.088-	0.679	1 (0.63)	(0.170-	0.634
Female	2 (0.90)	4.885)		3 (1.36)	18.370)	
Age groups						
<20 years	1 (1.03)			00		
21-30 years	1 (0.96)	0.899		1 (0.96)	1.259	
31-40 years	1 (1.42)	(0.428-	0.780	2 (2.85)	(0.601-	0.542
41-50 years	1 (1.72)	1.892)		1 (1.72)	2.635)	
>51 years	00			00		
Educational						
Illiteracy	1 (0.97)	0.592		00	1.309	
Primary	3 (2.25)	(0.143-	0.470	3 (2.25)	(0.343-	0.694
Secondary & higher	00	2.454)		1 (0.70)	4.985)	
Occupation						
Employed	00			00		
Farmer	1 (0.69)	1.1016		1 (1.69)	1.242	
Shopkeeper	1 (1.47)	(0.519-	0.963	1 (1.47)	(0.610-	0.551
Housewife	2 (3.22)	1.991)		1 (1.61)	2.530)	
Gov.-officer	00			1 (5.26)		
Others	00			00		
Location						
Trapaing Srae	00			3 (1.86)		
Chrab	1 (1.58)	1.771		00	0.714	
Cheung Khie	00	(0.887-	0.105	00	(0.309-	0.430
Pravanh	2 (4.76)	3.536)		1 (2.38)	1.650)	
Cheung Khlue	1 (1.69)			00		

Table 4.5 (Continued).

Variable	*n* (%), OR (95% CI), and *P*-value		
	Entamoeba coli	OR (95% CI)	*P*- value
Sex			
Male	5 (3.18)	2.123	0.223
Female	10 (4.54)	(0.633-7.123)	
Age groups			
<20 years	7 (7.21)		
21-30 years	2 (1.92)	1.009	0.963
31-40 years	1 (1.42)	(0.685-1.485)	
41-50 years	2 (3.44)		
>51 years	3 (6.25)		
Educational			
Illiteracy	8 (7.76)	0.356	0.006*
Primary	5 (3.75)	(0.171-0.740)	
Secondary & higher	2 (1.41)		
Occupation			
Employed	00		
Farmer	10 (6.94)		
Shopkeeper	00	1.806	0.277
Housewife	2 (3.22)	(0.545-1.190)	
Gov.-officer	00		
Others	3 (6.97)		
Location			
Trapaing Srae	12 (7.45)		
Chrab	1 (1.58)	0.419	0.012*
Cheung Khie	1 (1.92)	(0.212-0.828)	
Pravanh	1 (2.38)		
Cheung Khlue	00		

*Binary logistic regression test. Significant different *P*<0.05.

4.2 Molecular Identifying

4.2.1 Prevalence of *O. viverrini* infection by using PCR-ITS2

In order to discover *O. viverrini*'s ITS2 gene-based PCR technique, a total of 377 fecal specimens were available for DNA extraction. This approach produced a distinct amplicon of about 330 bp for each forward and reverse primer individual diagnostic. The following was the determination of the amplified bands (Figure 27). Thus, the PCR products of about 330 pb that revealed with *O. viverrini*'s ITS2 gene were confirmed with DNA sequencing. There were 26 results of *O. viverrini*'s ITS2 sequence, which were indicated matching with *O. viverini* genomic mRNA 333 bp in the GenBank with the previous publication in 1991 (Sermswan et al., 1991) by accession number S80278 that represented the varieties per identities between 93.74%-99.20% templates individually. The *O. viverrini*'s ITS2 gene was identified (*n*=26/377, 6.89%) of positive cases. The prevalent was common in male (*n*=14, 8.91%) higher than in female (*n*=12, 5.45%). However, the age groups variable was related with *O. viverrini* infection that was demonstrated of prevalent highest in age under 20 years old (*n*=13, 13.40%), which was followed by 21-30 years old (*n*=8, 7.69%), 31-40 years old (*n*=2, 2.85%), 41-50 years old (*n*=2, 3.44%), and over 51 years old (*n*=1, 2.08%) by binary logistic regression that associated with dependent and co-variate of OR_{cru}=0.569, 95% CI=0.388-0.836, *P*=0.004 and OR_{adj}=0.601, 95% CI=0.410-0.882, *P*=0.009, which was significant (Table 4.6). The other variables were included such as education level, occupation, and location. For education level was showed that higher in illiteracy (*n*=9, 8.73%), primary school (*n*= 11, 8.27%), and secondary & higher (*n*=6. 4.25%). The occupation was demonstrated higher in other career (*n*=5, 11.62%) which followed by farmers (*n*=12, 8.33%), employed (*n*=2, 4.87%), shopkeeper (*n*=4, 5.88%), housewives (*n*=2, 3.22%), and government officers (*n*=1, 5.26%). In the location was higher at Trapaing Srae village (*n*=16, 9.93%), Pravanh (*n*=4, 9.52%), Cheung Khlue (*n*=4, 6.77%), Cheung Khie (*n*=1, 1.92%), and Chrab village (*n*=1, 1.58%), but these variables were not remarkable.

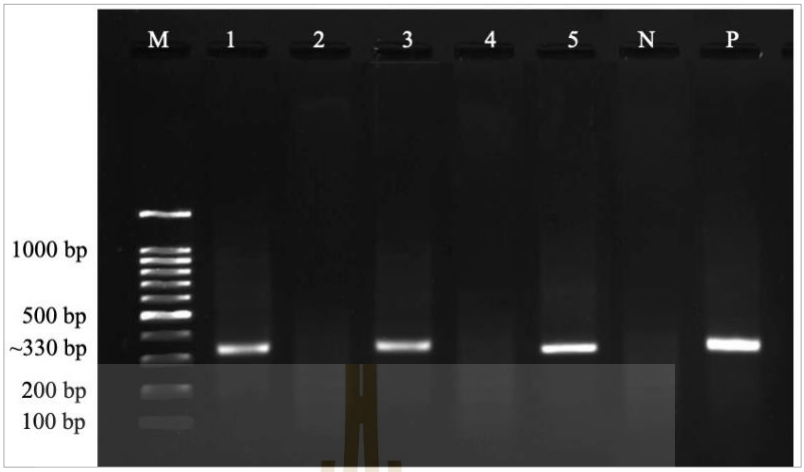

Figure 4.27 PCR products of *O. viverrini* amplifying based on internal transcribed spacer 2 (ITS2). M-marker was100 bp DNA ladder; N-negative control; P-positive control; the primer ITS2 was set at 330 bp. The lenes 1, 3, and 5 are DNA templates of PCR products showed of positive bands, however lenes 2 and 4 were DNA templates of PCR products showed of negative bands. Each PCR templates (5µl/well) were stained with MNAS and 6x DNA loading. Read under 1.5% agarose gel diluted with 30 ml of TAE 1x solution and then was took in gel documentary imaging.

The creation of a successful eradication campaign is hampered by the lack of an accurate, swift, sensitive, and specific diagnostic approach suited for epidemiological investigation and for monitoring the outcome of anthelmintic therapy. The large copy number of the target sequence may be responsible for this method's exceptional sensitivity (Sermswan et al., 1991). *O. viverrini* is frequently discovered in mixed infections with tiny intestinal flukes, which produce eggs that are morphologically identical (Radomyos et al., 1998). To differentiate *O. viverrini* eggs from those of the other minute flukes, this particular PCR will be extremely helpful. The approach has a number of benefits over the traditional parasitological method. First, it is very sensitive, making it appropriate for the identification of mild infections that a microscopical approach may otherwise produce false negatives. Second, findings can be received in a day when employed with a high number of samples at once. Third, it eliminates misunderstanding with tiny flukes and is independent of the subjective bias that may

occasionally be overcome in microscopical inspections. Last but not least, it might be further altered to allow for the parasite's identification in intermediate hosts (Sermswan et al., 1991). The technique offers a possible way to keep track of how well pharmacological therapy is working.

The National Center for Malaria Control, Parasitology and Entomology (CNM) departments and the Ministry of Health (MOH) of Cambodia was interested researching on epidemiological survey of parasitology in the endemic province after the first publishing of *O. viverrini* infection in Cambodia in PubMed database by Korean researcher team in 2002 (Lee et al., 2002). The initial investigation into *O. viverrini* infection has been started since 2006 in two villages in the Prey Kabas district, Takeo Province (Miyamoto et al., 2014). The National Helminth Control Program (NHCP) launched to conduct of field survey of *O. viverrini* infection that covered almost countrywide by collaboration with oversea researcher team especially Korean researchers. According to Vireak Khieu in 2019, the geographic distribution of *O. viverrini* infection covers more than 20% in the north across the center to southern province. The prevalence of egg positive rate among the residence was found in central and southeastern province, began from Kampong Thom (34.8%), Kampong Cham (24.0%), Kandal (20.2%), and especially in three villages of Takeo Province range from 46.4 to 50.6%, respectively (Khieu et al., 2019; Yong et al., 2014; Yong et al., 2012). However, according to Korean researcher team was reported about a nationwide survey by highest prevalence of *O. viverrini* infection in Kampong Cham (24.0%), Takeo (23.8%), and lower prevalence followed by Preah Vihear (2.7%), Stung Treng (2.5%), Kratie (3.4%), and (2.8%) Ratanakiri Province, respectively. Another study of riparian population was infected by the *O. viverrini* in the Prey Kabas district, Takeo Province with higher prevalence (47.5%) was reported (Yong et al., 2012). A couple years latter there was clarified of *O. viverrini* infection distribution through the Provinces along the Mekong River basin in Cambodia was found higher prevalence in Kampong Thom (34.8%), which followed by Kampong Cham (33.1%), Takeo (21.4%), and (18.7%) Kandal Province (Miyamoto et al., 2014). The prevalence (4.6%) of *O. viverrini* egg-positive cases was reported in Kratie Province (Sohn et al., 2012). The highest prevalence was (59.8%) of egg-positive cases among population in theses provinces that found to be

highest increasing ever reported in Cambodia (Jung et al., 2023). The estimation prevalence among the population of *O. viverrini* infection in Southeast Asia based on various geographical region in some countries including Thailand (6.71 million), Lao PDR (2.45 million), Vietnam (2.07 million), and (1 million) Cambodia (Zhao et al., 2021a). The classification of risk factors for liver fluke infection based on parasitological and molecular testing is another frequent claim made in studies of demographics. Evidently, northeast Thailand has the highest prevalence (70%) of *O. viverrini* (Sripa & Pairojkul, 2008). In the southern Saravane district of Lao PDR, 58.5% of people had *O. viverrini* infection, which was the greatest incidence (Sayasone et al., 2007). However, prevalence rates in Vietnam's southern regions have been reported to range from 15.2% to 36.9% (De et al., 2003). The revealed 24% of respondents (49.2%) said they have eaten raw fish in the Phon Sawan District of the Nakhon Phanom Province, Thailand, where *O. viverrini* was prevalent (Srithai et al., 2021). According to the latest findings, *O. viverrini* risk factors and prevalence rates both grew between 2017 and 2020. In 2017, 2018, 2019, and 2020, respectively, the prevalence rates of *O. viverrini* infection in the participants were 2.47%, 3.60%, 5.21%, and 7.01% (Perakanya et al., 2022). In previous study was found a highest prevalence (4.6%) of *O. viverrini* infection in Kratie Province (Sohn et al., 2012). However, there was revealed of *O. viverrini* infection with higher prevalence (5.36%) among population in the Snuol district, Kratie Province (La et al., 2022). Therefore, in this study the prevalence highest was (6.89%) of *O. viverrini* infection which was identified among the population in the Snuol district, is highest at any point in Kratie Province by using PCR-ITS2.

Ecological and physiological mechanisms are frequently used to categorize the origins of sex biases in parasitism rates. Ecological mechanisms include sex variations in behavior, nutrition composition, and body size (Zuk et al., 1996). It has long been known by epidemiologists that males of vertebrate species, including humans, typically exhibit higher rates of illness and parasitism than females (Bundy, 1988; Zuk, 1990). Due to various male social traditions, such as the frequent ingestion of raw or undercooked freshwater fish with alcohol in social settings, males in South Korea showed a higher incidence of *C. sinensis* infection than females, whereas in China an indigenous species the comparable trends were seen (Rim et al., 1982). On the

other hand, housewives, or females, were shown to have the highest incidence of *O. felineus* infection in Russia (Rim et al., 1982). However, it has been noted that there is a male preponderance or no discernible sex difference in Thailand, where *O. viverrini* is common (Kobayashi et al., 2000; Rim et al., 1982; Wattanawong et al., 2021). In Takeo province, Cambodia, there was a reported male predominance (Yong et al., 2012). There was no discernible sex difference in the current study. One explanation could be that traditional fish meals, which are widely consumed by individuals of all genders in the investigated locations, are the main source of infection such as 'plea trey (fish salad), 'plea trey chou' (sour fish salad), and 'ma'am' (fermented fish, kept for 2–5 days) (Jung et al., 2023). Therefore, this study was found more frequently in men (8.91%) than in women (5.45%) of *O. viverrini* infection.

In a rural village in Lao PDR, a child 6 months old was found to have contracted *O. viverrini*; nevertheless, despite the fact that all age groups are at considerable risk, infection rates among younger people were lower than those among older people (Kobayashi et al., 2000), and similar to the age-prevalence observation in Thailand (Kaewpitoon et al., 2008; Sithithaworn et al., 2003). In 2013, there was countrywide research of the students that funded by USAID in Cambodia to determine the prevalence infection caused by *O. viverrini* in children was found (4.10%) (Khieu et al., 2019; Lee et al., 2002). However, one of recently investigation of *O. viverrini* infection in Preah Vihear and Stung Treng Province was prevalence higher by the age group 30-39 years and followed by age group 40-49, 50-59, and over 60 years, whereas the prevalence was lower among children under 9 years, age group 10-19, and 20-29 years (Jung et al., 2023). Their finding was similar trended of liver fluke infections that have been seen in other nations, including *O. viverrini* in Lao PDR and Thailand (Forrer et al., 2012; Suwannahitatorn et al., 2019; Pengput & Schwartz, 2020), *O. felineus* in Russia (Rim et al., 1982), and *C. sinensis* in South Korea (Rim et al., 1982). Additionally, the riverside population in Cambodia's Takeo province showed a nearly same age-prevalence trend for *O. viverrini* infection (Khieu et al., 2019; Yong et al., 2012). Thus, in this present study was more frequently higher prevalence in young age groups less than 20 years was significantly and the lower prevalence followed by age group 21-30 years.

Moreover, in this study, the illiteracy rate was greater among people with higher levels of education. Poor literacy is strongly correlated with intestinal parasite infection, according to certain research. *O. viverrini* infection prevalence among the age groups was associated with illiteracy (8.74%) levels of education. The risk factor for the illiteracy rate was present (Nematian et al., 2004). According to several research, parasitoses are correlated with illiteracy, a lack of latrines causing diarrhea, a lower socioeconomic standing, improper disposal of human waste, and a lack of cleanliness in homes (Holland et al., 1996; Tshikuka et al., 1995; Gambao et al., 1998, 2003). The findings indicate that low levels of education and the ensuring poor socioeconomic and sanitary conditions of families are significant risk factors for infection (Nematian et al., 2004). Another study showed that the heads of home had a relatively high average illiteracy rate. This might be a major contributor to parasite infections. Numerous studies have shown a strong correlation between low educational attainment and intestinal parasite infection (Sayasone et al., 2007). General speaking, poor cleanliness is a major factor in helminth infections. There is ample evidence of its association (Esrey et al., 1991; Fewtrell et al., 2005).

The *O. viverrini* infection rates in this region have been observed to range from 4.6% to 60%, while the topography of the region is significantly variable between provinces (Sithithaworn et al., 2012). The epidemiology of liver fluke infection varies by endemic area, where foodborne trematode infection is widespread, especially in connection to the attitude of consumer culture and lifestyle (Jung et al., 2023). The classification of risk factors for liver fluke infection based on parasitological and molecular testing is another frequent claim made in studies of demographics. Evidently, northeast Thailand has the highest prevalence (70%) of *O. viverrini* (Sripa & Pairojkul, 2008). In the southern Saravane district of Lao PDR, 58.5% of people had *O. viverrini* infection, which was the greatest incidence (Sayasone et al., 2007). However, prevalence rates in Vietnam's southern regions have been reported to range from 15.2% to 36.9% (De et al., 2003). The very recently publication was reported the emerging of *O. vierrini* infection in Preah Vihear and Stung Treng Province. There appear to be certain elements that might make the investigated locations turn out to be opisthorchiasis hotspots. One is the vicinity of the communities that were surveyed.

These provinces are found along the Mekong River's main stem or major tributaries (Jung et al., 2023). The 5 villages that make up Preah Vihear province are located right at the Cambodia-Lao PDR border. The *O. viverrini* infection has been reported to be very prevalent in Lao PDR, particularly in the Vientiane Municipality, Khammouane, Savannakhet, and Champasak Province (Chai et al., 1998, 2005, 2007, 2009; Kobayahi et al., 2000). The five villages of Stung Treng province are close to the border and are situated along rivers and streams. The majority of villagers work in the fishing industry and have historically consumed freshwater fish uncooked or undercooked. These are additional risk factors (Jung et al., 2023). Trapaing Srae village, Snuol district, Kratie Province, which borders Stung Treng Province, had the greatest frequency (9.94%), while the high-risk factor identified in this study was not noteworthy. Since the prevalence of metacercariae in fish remained constant in the flood plain during the rainy season and consuming raw fish meal (Trey Bok), the risk of human infections appeared to be much higher during the rainy season than during the dry season by locals was widespread during this time. For the seasonal variations in fish illness and local residents' eating habits, a more thorough survey is needed (Touch et al., 2009). In summary, the demographic risk variables associated with the importance of the covariates age group, education level, employment, location was the main focus of the study at endemic areas. Such a sign can be used to inform the public and the authorities about untreated foodborne trematode infections, in an effort to increase awareness of opisthorchiasis and take action to control *O. viverrini* infection (Sripa & Pairojkul, 2008; Zhou et al., 2009). School-aged children have the greatest burden of illness brought on by intestinal nematodes when compared to other age groups. According to a mathematical model, treating schoolchildren alone can reduce the burden of disease on the entire population in high incidence neighborhoods by almost 70% (Chan, 1997).

When the hepatobiliary cell stimulated by *O. viverrini* soluble ES products from liver flukes, human biliary cell lines proliferated excessively (Sripa, 2003). They have been discovered a human granulin homologue in the ES of *O. viverrini*, a powerful growth factor involved in cell proliferation and wound repair. The *O. viverrini* granulin, also known as Ov-GRN-1, was found in the biliary epithelial cells of experimentally

infected hamsters in addition to being expressed in the majority of parasite organs, including the gut and tegument. Murine fibroblasts' proliferation was enhanced by recombinant Ov-GRN-1, and the afflicted cells underwent morphological alterations as a result. Antibody against Ov-GRN-1 inhibited Human CCA cell lines proliferated when exposed to *O. viverrini* ES, suggesting that Ov-GRN-1 is an important growth factor. According to their findings, liver fluke granulin is important in creating a tumor-genic environment infected livers that may eventually result in CCA (Smout et al., 2009). Nevertheless, data on the morbidity and mortality linked to liver fluke infection are still unclear. Nevertheless, it has been discovered that over 20% of the general population in several parts of the nation have an *O. viverrini* infection prevalence (Khieu et al., 2019). To evaluate the risk factors for *O. viverrini* infection that are linked to an increase in the incidence of opisthorchiasis that is related with the development of CCA, more study on liver fluke infection is generally necessary (Keiser & Utzinger, 2009; Sripa et al., 2010). On the other hand, *O. viverrini* infection is linked to CCA and was described as a family issue that affects mostly men since they are the primary breadwinners in their families (Andrews et al., 2008).

Cholangiocarcinoma (CCA), a primary liver cancer in this location, and hepatobiliary disorders are linked to chronic infection with *O. viverrini* (Sripa et al., 2011). In Thailand, four main regions, a strong correlation was discovered by another investigation between the prevalence of CCA and the frequency of *O. viverrini* infection (Sakhon Nakhon, Prae, Roi-Et, and Nongbua Lampoo) (Sripa et al., 2007). A severe health problem has been created in the area due of the 10% CCA that has been developed in individuals who contract *O. viverrini* (Mairiang et al., 1993). Infection with *O. viverrini* can cause inflammation of the connective tissues in the portal vein and hepatic bile ducts, which can lead to the development of CCA (Hughes et al., 2017; Mantovani et al., 2008). Patients with intrahepatic, distal extrahepatic, and hilar CCA who have surgery had five-year survival rates of 22-44%, 27-37%, and 11-41%, respectively (Hasegawa et al., 2007). According to the latest findings, *O. viverrini* risk factors and prevalence rates both grew between 2017 and 2020. In 2017, 2018, 2019, and 2020, the prevalence rates of *O. viverrini* infection in the participants were 2.47%, 3.60%, 5.21%, and 7.01%, respectively. According to a recent study by Srithai et al.,

(Srithai et al., 2021), *O. viverrini* was prevalent in the Phon Sawan District of Thailand's Nakhon Phanom Province at a rate of 24%, and nearly half of respondents (49.2%) said they had eaten raw fish there. Praziquantel, given as a single dosage of 40 mg/kg, is an effective therapy for *O. viverrini* infection. Praziquantel has a cure rate of up to 95.5%. Some people disregard this information since, if they were never sick, there was no need to be aware of the treatment (Pengput & Schwartz, 2020). Despite the fact that praziquantel is a medication that can eliminate the parasite that causes *O. viverrini* reinfection and its possible long-term hazards, people are still eating raw or undercooked freshwater fish (Kim et al., 2017). Koi-pla and lab-pla were often the types of raw fish consumed by the *O. viverrini* infection patients in this investigation. Instead of getting hooked to the flavor, habit drove people to consume uncooked food.

4.2.2 PCR-Restriction Fragment Length Polymorphism Restriction *Fau*1

In order to distinguish between liver fluke and MIF, including *O. viverrini*, *C. sinensis*, and *H. Taichui*, the extracted DNA was amplified using the ITS2 gene to identify the *O. viverrini* DNA. 26 of the PCR-products positive bands were then chosen for PCR-RFLP using the RTFluke primer to confirm *O. viverrini* species. Fragments 377 bp were generated, and after the PCR-RFLP (Traub et al., 2009). However, the RFLP results based on PCR positive bands were markedly related to the produced fragment of roughly 377 bp, meaning the band present was notably indicative of *O. viverrini* discriminated (Figure 28). Meanwhile, the enzyme *Fau*1 was used to confirm that the *O. vivierrini* fragment length discrimination from opisthorchis-like eggs were differentiated. Additionally, *Fau*1 was used to digest the PCR-RFLP product, which produced the 129 bp and 247 bp fragments after being incubated. The digestion of the enzyme produced patterns in the positive samples in lane T1, S1, R1 from Cambodia produced 129 and 147 bp the positive product and P1 from Thailand (Figure 29). For DNA extraction and amplifying identifying from 26 *Opisthorchis*-like egg of positive fecal samples were confirmed. The ITS2-PCR-RFLP experiment produced unique amplicons of 377 bp for *O. viverrini*, allowing species of *Opisthorchis*-like eggs to be identified. The incidence of *Opisthorchis*-like egg infection and the PCR-RFLP sensitivity in Kratie Province. The PCR-RFLP-*Fau*1 results for *O. viverrini* generated fragments of 129 and 247 bp. Therefore,

Neutrality testing for *O. viverrini* revealed that the adult worms from Thailand and Kratie Province had similarly patterns when they were digested by *Fau*1.

The molecular investigation revealed that the positive samples of *Opisthorchis*-like eggs were *O. viverrini* eggs. This was done using PCR-RFLP of the ITS2 region to differentiate the eggs of *O. viverrini*, *C. sinensis*, and *H. taichui*. In the investigation, no particular amplicons of 381 bp for *C. sinensis* and 321 bp *for H. taichui* were discovered. When EPG in feces was less than 1,000, the sensitivity of ITS2-PCR was 6.89%, but EPG in feces 671 exhibited sensitivity (Table 1). According to a similar investigation, the ITS2-PCR test has a sensitivity limit of detecting Opisthorchis-like eggs of 6.89% (Traub et al., 2009). In this investigation, *O. viverrini* eggs were distinguished from those of *C. sinensis*, a human liver fluke described in 200913, and *H. taichui*, a common small intestine fluke coinfection with *O. viverrini*, using the ITS2-PCR-RFLP assay (Radomyos et al., 1998).

Figure 4.28 PCR-Restriction fragment length polymorphism products of *O. viverrini*. M-marker was 100 bp DNA ladder, N-negative control, P-positive control. T1, T2, and T3 are DNA templates, which was indicated of positive bands.

Table 4.6 The prevalence of *O. viverrini* infection by using PCR-ITS2 (n=377).

Variable	*n* (%), OR (95% CI), and *P*-value				
	O. viverrini	ORcru (95% CI)	*P*-value	ORadj (95% CI)	*P*-value
Sex					
Male	14 (8.91)	0.589 (0.265-1.311)	0.195	0.626 (0.269-1.457)	0.277
Female	12 (5.45)				
Age groups					
<20 years	13 (13.40)				
21-30 years	8 (7.69)	0.569 (0.388-0.836)	0.004*	0.601 (0.410-0.882)	0.009*
31-40 years	2 (2.85)				
41-50 years	2 (3.44)				
>51 years	1 (2.08)				
Educational					
Illiteracy	9 (8.73)	0.696 (0.422-1.148)	0.156	0.765 (0.451-1.299)	0.322
Primary	11 (8.27)				
Secondary & higher	6 (4.25)				
Occupation					
Employed	2 (4.87)	1.052 (0.810-1.368)	0.703	1.022 (0.776-1.347)	0.875
Farmer	12 (8.33)				
Shopkeeper	4 (5.88)				
Housewife	2 (3.22)				
Gov.-officer	1 (5.26)				
Others	5 (11.62)				
Location					
Trapaing Srae	16 (9.93)	0.900 (0.681-1.189)	0.460	0.934 (0.701-1.243)	0.638
Chrab	1 (1.58)				
Cheung Khie	1 (1.92)				
Pravanh	4 (9.52)				
Cheung Khlue	4 (6.77)				

*Binary logistic regression test. Significant different *P*<0.05.

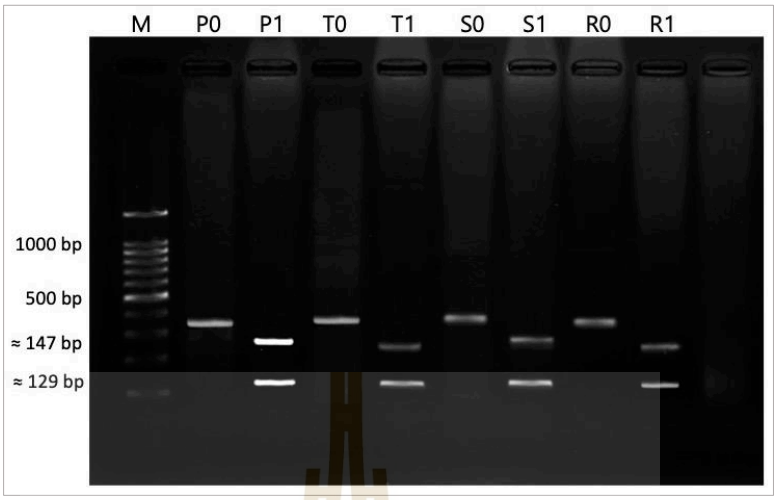

Figure 4.29 PCR-RFLP of *O. viverrini* digested by enzyme *Fau*1. M-marker, P0 was undigested product and P1 was digested product of positive samples from *O. vivierrini* adult worm of Thailand. T0, S0, and R0 were undigested products and T1, S1, and R1 were digested with enzyme *Fau*1 of DNA templates generated fragment 129 and 147 bp.

4.2.3 Genetic diversity using *COX*1

In order to detect the *O. viverrini* DNA from DNA templates that generated fragment of 504 bp, the *COX*1 gene was used as a primer design. However, using PCR focusing on the ITS2 gene, all 26 *O. viverrini*-positive fecal samples were verified. These fecal samples that tested positive for *O. viverrini* were examined for *COX*1-Ov primers targeting *COX*1 and did not sensitivity with the ITS2 region was compared. Contrarily, among 26 DNA-templates were negative bands compared with the adult worm *O. viverrini* control band (Figure 30). The development of innovative molecular approaches for species identification and delimitation, community composition evaluation, species richness in biodiversity investigations, and ecological research has significantly accelerated in recent years (Hebert et al., 2003; Amaral-Zettler et al., 2009; Pawlowski et al., 2016; Wangensteen et al., 2017; van der Loos & Nijland, 2021). Additional nuclear markers have been investigated, including the ITS (Macher et al., 2021a), the ribosomal large subunit (Pawlowski et al., 1994), actin (Flakowski et al.,

2005), tubulin (Takishita et al., 2005), and many genes according to data from expressed sequence tags (Sierra et al., 2013). For general protist molecular studies, these markers are widely utilized. When opposed to the *COX*1, the 18S maker has a lower success rate during amplification, which might be attributed to a poor amplification technique, a poor master mix selection, or an insufficient DNA template to reagents ratio. Ribosomal areas have frequently been amplified using nested or semi-nested PCRs, which may lead to superior amplification outcomes (Pawlowski et al., 2003; Holzmann et al., 2017; Morard et al., 2018). The targeted segment for both markers was around the same size (up to 504 bp), and the *COX*1 marker amplified successfully, suggesting that neither the extraction process nor the materials' preservation in ethanol were the issue.

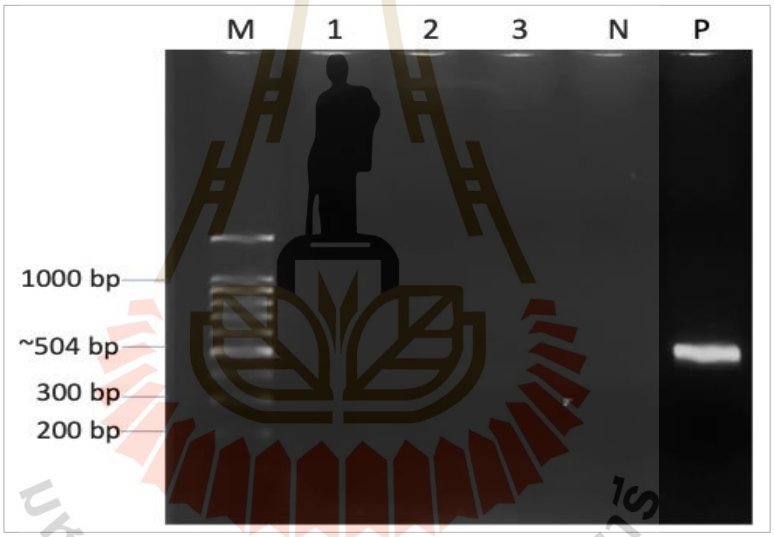

Figure 4.30 PCR-product of *O. viverrini* using *COX*1 gene. M-marker was100 bp DNA ladder; N-negative control; P-positive control; the primer *COX*1 was set at 504 bp. The number 1, 2, and 3 were DNA templates that showed PCR products negative bands. Each PCR templates (5µl/well) were stained with MNAS and 6x DNA loading. Read under 1.5% agarose gel diluted with 30 ml of TAE 1x solution and then was took in gel documentary imaging.

4.2.4 Genetic diversity using *NAD*1

PCR focusing on the ITS2 gene, all 26 *O. viverrini*-positive fecal samples were verified. These fecal samples tested positive for *O. viverrini* were identified for *NAD*1-

Ov primers targeting *NAD*1. *NAD*1 amplification was optimized with (*n*=9) of positive templates, which was less than ITS2 gene sensitivity (Figure 31).

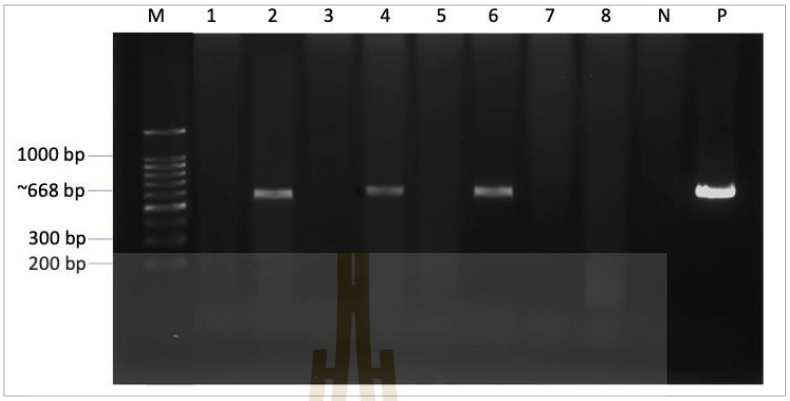

Figure 4.31 PCR-product of *O. viverrini* using *NAD*1 gene. M-marker was100 bp DNA ladder; N-negative control; P-positive control; the primer *NAD*1 was set at 668 bp. The band number 2, 4, and 6 are DNA templates of PCR products showed the positive, and 1, 3, and 5 were negative bands. Each PCR templates (5µl/well) were stained with MNAS and 6x DNA loading. Read under 1.5% agarose gel diluted with 30 ml of TAE 1x solution and then was took in gel documentary imaging.

Many researchers have developed a number of diagnostic techniques for identifying any diseases, particularly infectious diseases with dangerous complexity. Although several recognized molecular diagnostic methods may used to diagnose *O. viverrini* infection, their sensitivity has been found to be insufficient. The ITS-based PCR is the earliest and most well-known target for molecular identification; nevertheless, it later shown variable sensitivity compared to recently discovered targets including *COX*1, Cytochrome B (CytB), and *NAD*1 (Buathong et al., 2017). The Ov*NAD* sequences, which related to the *O. viverrini*-eggs with less resemblance to the tiny intestinal fluke, were validated by DNA sequencing of the PCR products produced by the inserted primers.

In previous study was reported of molecular characteristics of recovery *O. viverrini* in the patient using the NADH dehydrogenase subunit 1 (*nad*1) among the population in Kratie Province (Sohn *et al*., 2012). There was reported of the MND1-A

and MND1-B primers were used successfully sequence PCR products (760 bp of *nad*1) for molecular characteristics of adult flukes. The *nad*1 of *O. viverrini* from Thailand, GenBank (DQ119551.1; Le et al., 2006), Laos (GQ401025.1 and GQ401077.1; Thaenkham et al., 2010), Cambodia (Kandal) (GQ401085.1; Thaenkham et al., 2010), and China (JN936242.1) were also examined. The current *O. viverrini* isolate (Kratie) shared 97–99% nucleotide similarity with the *O. viverrini* isolates from Thailand, Laos, and Cambodia (Kandal). However, there was very little sequence similarity between the Kratie isolate of *O. viverrini* and the Chinese *C. sinensis*. However, the genetic diversity structure was used in this study according to (Saijuntha et al. 2008), the PCR-*NAD*1 primers *NAD*1-Ov-F and *NAD*1-Ov-R produced amplicons of 668 bp and 5'-TAC GCA GGT GGT TTG GTT GG-3', respectively. Since the *NAD*1 gene was identified from fecal samples with more sensitivity than the *COX*1 gene in this investigation, the findings were determined to be (34.61%) by using *NAD*1 gene. In experimental animals, *O. viverrini*'s infectivity, growth, fecundity, and body size was also demonstrated to differ considerably across 5 distinct wetland environments in Thailand and the Lao PDR (Laprom et al., 2009).

4.2.5 Sequences and alignment

The ITS2 region of *O. viverrini*'s positive amplicons were isolated and delivered to the QIAGENE firm (Bangkok, Thailand) for sequencing. The 10 L PCR tube containing the PCR-amplicons was labeled with an ID number to identify each product. The sequence findings were given as Fasta file accession numbers for the PCR-ITS2 and PCR-*NAD*1 sequence that available searched in the GenBank database NCBI BLAST. However, BioEdit Copyright 1997-2013 Tom Hall version 7.2.5 (12/11/2013) entered the sequence alignment findings. In contrast to the original publishing product primers sequenced of ITS2, the forward sequence of the primer products was switched to the reverse primer of alignment, and the reverse primer products were utilized as forward sequence in this alignment and graphic view. The primer product sequence is shown as blue lines, while the forward (top) and reverse (bottom) of the template sequences are shown as red lines. The *O. viverrini* ITS2 gene's nucleotide sequence was aligned in the graphic view alignment to show that the product primer and sample sequence, which was discovered in the human host, were compatible (Figure 32).

Due to the particular primer's alignment with the ITS2 sequence of *O. viverrini*, the first reference sequence was chosen from the positive PCR-template. These DNA gene sequences were suggested for alignment in order to distinguish between molecular diagnoses of the regional variations that *O. viverrin* was impacted by. The visual alignment result demonstrated that the *O. viverrini* sequence that the product with the sample and references sequence of GenBank most closely resembled and was most likely paired (Figure 32). The Mekong River basin is home to the O. viverrini specie genus. On the other hand, homologous nucleotide sequences (97–99%) were revealed using PCR-nad1. Between *O. viverrini* and *O. lobatus*, ITS2 sequencing demonstrated more specificity than cytochrome c oxidase subunit 1 (*cox*1), with nucleotide differences of 0.86% and 3.03%, respectively (Thaenkham et al., 2011). The molecular methods were used to study in the form of identifying the genus population and sub-type of adult flukes, *O. viverrini* spp. by some researchers in Cambodia (Thaenkhm *et al*., 2010; Sohn *et al*., 2012). This study is demonstrated of molecular identification of *O. viverrini* rDNA in northeastern Cambodia using ITS2-PCR and graphic alignment of nucleotide sequences between the product primers sequences and samples sequences by using references sequence in GenBank data base to confirm the molecular alignment. The nucleotide sequence for the *O. viverrini* ITS2 gene was assembled into a visual representation of alignment, which indicated the matches between the product and sample sequences discovered in the human host. It was advised to use these DNA sequences for designated alignment in order to distinguish between molecular diagnoses of gene mutations. The most comparable *O. viverrini* sequence between the sample and product sequences was most likely matched, according to the visual alignment result.

In the molecular marker including the ITS2, cytochrome *c* oxidase subunit 1 (*cox*1), and *nad*1 sequence were most used to compare of the nucleotide difference between Opisthorchis sp. and *C. sinensis* (Thaenkhm *et al*., 2011). On other hand, the mitochondrial DNA (mtDNA) genes marker is extending to identify genetic diversity of liver flukes' sub-type in different regions along the Mekong River including Thailand, Lao PDR, Cambodia, and Vietnam (Thaenkhm *et al*., 2010). Additionally, plausible

connections between various *O. viverrini* genotypes and variations in cholangiocarcinoma incidence have been proposed (Sithithaworn et al., 2012).

Figure 4.32 The graphic view alignment of *O. viverrini*'s ITS2 gene product with the sample sequence of the difference countries in the region. The graphic view was showed the molecular diagnosis to confirm the *O. viverrini* species including (a) sequence of primer product generated amplicons about 333 bp (accession number: S80278.1), (b) the positive fecal specimen sequence from Cambodia (accession number: CK0096.1) were amplified by PCR-ITS2 with specific amplicons generated about 330 bp,

(c) sequence as reference in GenBank of Thailand (accession number: AY584735), (d) sequence of Vietnam (accession number: KT726408), and (e) sequence of Lao PDR (accession number: HQ328549). A dash (-) is indicates the absence of residues, and *O. viverrini* species existing simulation residues with other references sequences.

4.2.6 Phylogenetic trees based on the sequences of ITS2

MEGA version 11.0.13 (1993-2023) was used to compare the pairwise nucleotide sequences of the ITS2 gene in the GenBank database, including *O. viverrini*, *C. sinensis*, and other MIFs with ITS2 gene (330 bp) of product sequence (Figure 33). The pairwise sequences of *O. viverrini* reference among GenBank data base sequences (accession number: S80278.1) of Thailand (Sermswan et al., 1991) and DNA templates code as (CK = Cambodia, Kratie) were isolated from the other references of bootstrap value (30%-61%) (Figure 33A). however, the pairwise O. viverrini DNA between samples sequence and other references nucleotide were likelihood of bootstrap value from 31%-67% (Figure 33A and C).

The following accession numbers, all data are accessible through the GenBank data source accession number, ITS2. DNA templates sequence in Kratie Province of *O. viverrini* ITS2 sequences results including CK0005.1, CK0018.1, CK0020.1, CK0025.1, CK0029.1, CK0031.1, CK0033.1, CK0065.1, CK0066.1, CK0068.1, CK0073.1, CK0075.1, CK0096.1, CK0098.1, CK00100.1, CK00111.1, CK00112.1, CK00116.1, CK00117.1, CK00138.1, CK00152.1, CK00153.1, CK00161.1, CK00167.1, CK00176.1, and CK00187.1. References sequences from GenBank using ITS2 gene Opisthorchis sp. including Thailand: AY584735 (Parvathi et al., 2008), Vietnam: KT726408 (Dao et al., 2016), Myanmar: MG797538 (Sanpool et al., 2018), Lao PDR: HQ328549 (Thaenkham et al., 2011), Japan: EF688144 (Katokhin et al., 2008), S. Korea: AF217094 (Lee & Huh, 2004), China: AF217097 (Lee & Huh, 2004), Russia: DQ513404, DQ513405, EF688140 (Katokhin et al., 2008). HM004161, HM004158, and HM004167 Thailand (Thaenkham et al., 2010). HM004170, EF612489 Vietnam (Thaenkham et al., 2010; Lotfy et al., 2008), AJ557569 China (Huang et al., 2004), KJ200622 France (Wannasan et al., 2014), and NW017386862.1 United State (Protasio et al., 2016). The evolutionary relationship of

ITS2 region sequences were used as DNA marker for molecular diagnosis has weakly supported between liver fluke, *O. viverrini* and *C. sinensis*.

The phylogenetic connections of *O. viverrini* as determined by MEGA are shown in figure 33 using the ITS2 gene sequences about 330 bp. The reference isolates of *O. viverrini* were typically grouped in a polyphyletic group with a weakly bootstrap value between 57% to 77% (Figure 33B), according to the tree topologies of this gene's ITS2, but *O. viverrini* in this study was shown to be paraphyletic to most of the reference isolates. Low bootstrap value (50%) suggests that there may have been a change in the divergent order, which might have affected the diversity of *O. viverrini* in the ITS2 tree. In addition, 6 separate clades were discovered. Because *O. viverrini* from Kratie Province groups with the widely found of *O. viverrini* in the same phyletic with the product sequence (S80278.1) (figure 33C), it is evident that no different from common *O. viverrini* found in Thailand.

The *O. viverrini* (S80278.1) product reference from Thailand was the only *O. viverrini* nucleotide sequence acquired from this investigation that was grouped with this study isolates, suggesting that it was most likely the first isolate of *O. viverrini* in this population. However, no discernible variations in genetic diversity were found across and within populations of *O. viverrini*. So, the ITS2-PCR test has been successful in identifying and detecting eggs that resemble Opisthorchis. According to this research, the rural Snuol district in Kratie Province continues to be an opisthorchiasis endemic region. *O. viverrini* in this research region has a genetic structure that is more similar to *O. viverrini* from Thailand.

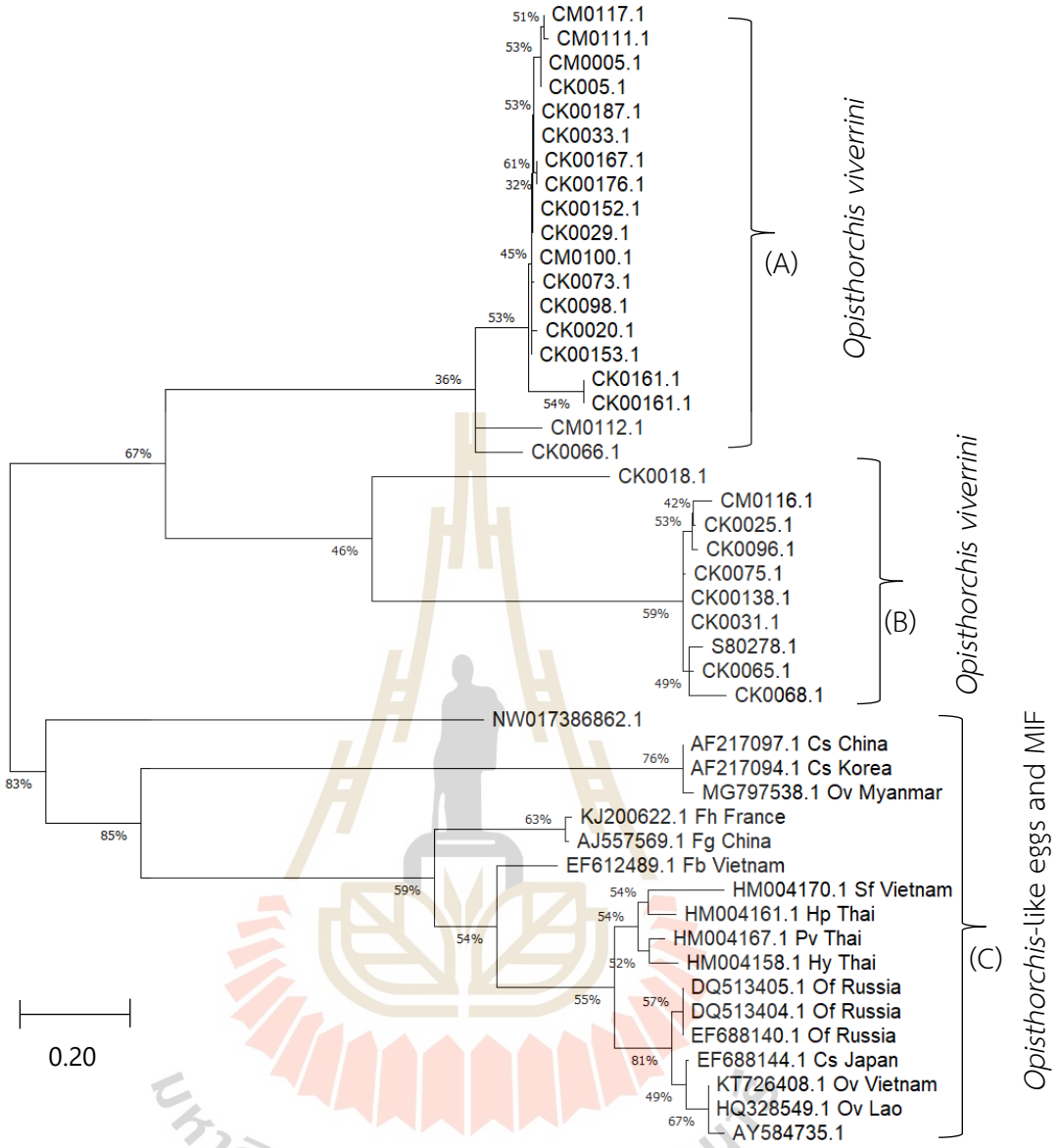

Figure 4.33 Cluster diagram resulting from a neighbor-joining analysis of ITS2 sequences of opisthorchiid liver flukes and MIF after excluding all sequence gap positions. (A) The monophyletic of pairwise relationship with different sequences between liver flukes, (B) the paraphyletic of pairwise fixed between primer products and DNA templates sequences, and (C) the polyphyletic of pairwise relationship between liver flukes and MIFs of GenBank. Phylogenetic tree for *Opisthorchis* spp. and other representative species using ITS2, including the Opisthorchiidae, Heterophyidae, Fasciolidae, and Schistosomatidae. The number of substitutions per site is shown by the scale-bar (Tamura et al., 2013). At the begin of each species name are accession numbers (available). Geographical locales and isolating regions are listed in parenthesis (available). The scale bar shows the number of nucleotide changes made per location.

4.2.7 Phylogenetic trees based on the sequences of *NAD*1

MEGA version 11.0.13 (1993–2023) was used to compare the pairwise nucleotide sequences of the *NAD*1 gene in the GenBank database, including *O. viverrini*, *C. sinensis*, and *Tenia solium* with DNA template sequence of *NAD*1 by generated nucleotides (668 bp) (Figure 34). The following accession numbers of DNA templates sequence *O. viverrini* using *NAD*1 gene including CK0033.1, CK0065.1, CK0066.1, CK0075.1, CK0096.1, CK00100.1, CK00112.1, CK00138.1, CK00152.1. All data are accessible through the NCBI GenBank data source accession number *NAD*1 gene of *O. viverrini*: GQ401110.1, EU022348.1, GQ401040.1, EU022348.1, EU022344.1, EU022343.1, EU022345.1, EU022337.1 Thailand (Thaenkham et al., 2010; Saijuntha et al., 2008), DQ882173.1, DQ119551.1, DQ882172.1, EU443832.1, DQ882174.1, EU443831.1, DQ882175.1, DQ882173.1, EU443833.1 Vietnam (Ngo et al., 2008; Ngo et al., 2006; Le et al., 2006), GQ401025.1, GQ401077.1 Lao PDR (Thaenkham et al., 2010); GQ401085.1 Cambodia (Thaenkham et al., 2010) *C. sinensis*: JN936242.1, JF729304.1, JF739555.1, JN936242.1, China (Cai et al., 2012; Liu et al., 2012; Li et al., 2001).

The pairwise sequences of *O. viverrini* reference from GenBank data base and DNA templates sequence code (CK=Cambodia, Kratie) were isolated from the GenBank references with bootstrap value lower than the standard (50%) (Figure 34B). However, the pairwise *O. viverrini* DNA between samples sequence and other references nucleotide were likelihood of bootstrap value 50% that is weakly supported (Figure 34A, B). Using pairwise comparison of the nucleotide sequences between sample collection and *O. viverrini* and *C. sinensis* sequence in GenBank. The percentages of fixed differences in the nucleotide variants of the partial *NAD*1 gene (668 bp) were found from 2% to 6% and references sequence between 3% to 31% respectively. When the *NAD*1 region were utilized as DNA markers, the cluster diagram revealed that *O. viverrini* has the lower degree of sequence similarity of *O. viverrini* samples and references sequences were weakly supported by bootstrap value of sequence similarity, and both are differed in sequence according to the cluster diagram built from the amino acids of the *NAD*1 genes sequences (Figure 34A, B). The connection between *O. viverrini* in the Kratie Provinces is still another issue that has to be clarified Opisthorchis species and other references in GenBank. Both were discovered in

humans; however some morphological variations appear to be present. The author would like to keep the subject open for now, awaiting more morphological and molecular research.

The *O. viverrini* product reference from Thailand, Lao PDR, and Vietnam *O. viverrini* nucleotide sequence was acquired from this investigation that was not grouped with reference isolates, suggesting that it was most likely isolated of *O. viverrini* in this population. However, no discernible variations in genetic diversity were found across and within populations of *O. viverrini*. While the causes of genetic variety are unknown, this work has revealed the diversity of *O. viverrini* in the Snuol area of Kratie Province, which may or may not be an isolated population. As a result, more research on the genetic diversity of *O. viverrini* in this endemic region should be done utilizing other potent genetic markers as well as samples acquired from many other populations in surrounding districts or provinces.

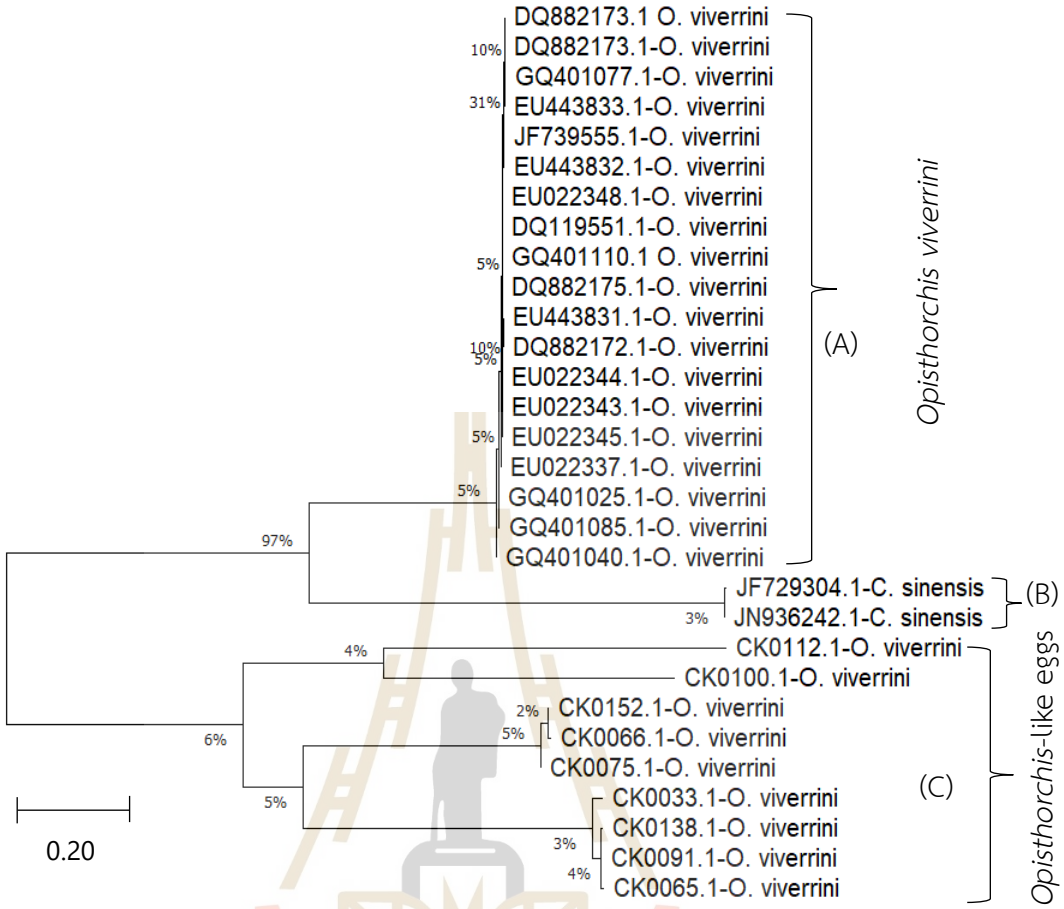

Figure 4.34 Cluster diagram resulting from a neighbor-joining analysis of *NAD*1 sequences of opisthorchiid liver flukes and MIF after excluding all sequence gap positions. (A) The monophyletic of pairwise relationship with different sequences of *O. viverrini* spp., (B) the paraphyletic of pairwise fixed between *O. viverrini* and *C. sinensis* GenBank sequences, and (C) the polyphyletic out group from GenBank and pairwise relationship between liver flukes of DNA templates. Phylogenetic tree for *Opisthorchis* spp. and other representative species using *NAD*1, including the Opisthorchiidae. The MEGA software program was used to do phylogenetic reconstruction using maximum likelihood analysis using the general time-reversible model, a gamma distributed rate heterogeneity, and a fraction of invariant sites. The number of substitutions per site is shown by the scale-length (Tamura et al., 2013). At the begin of each species name are accession numbers (available). Geographical locales and isolating regions are listed in parenthesis (available). The scale bar shows the number of nucleotide changes made per location.

CHAPTER V

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The IPIs is very prevalent (15.91%) based on this study in Snuol district, Kratie Province compared to the countrywide endemic area that the risk factors like sex, age, and region were associated with higher IPI rates in Cambodia. The frequency of *Opisthorchis*-like egg infection was 5.57%, and the ITS2-PCR test was used to detect 6.89% of *O. viverrini* eggs. All infected people had low-intensity infections (EPG 1,000), according to faecal EPG.

This study clarifies the nature of *O. viverrini* infection and identifies age group as a predictor of prevalence. According to this research, the rural Snuol district in Kratie Province continues to be an opisthorchiasis endemic region. *O. viverrini* in this research region has a genetic structure that is more similar to *O. viverrini* from Thailand. *O. viverrini*, despite its little size, has the capacity to seriously harm the hepatobiliary system, ranging from cancer to major infection. With the right safeguards in place, this might all be avoided, even if it has serious socioeconomic effects in addition to harmful effects on people's health. It is necessary to address the incidence of *O. viverrini* infection in Kratie Province and implement community-based health interventions that encourage the eating of properly cooked freshwater fish. Primary prevention should be used to lower infection rates in low-prevalence populations, such as those under the age of 20, and to keep them from contracting the infection by eating raw fish. Infected cases should be addressed as secondary prevention in higher risk categories, such as men and older age groups, and long-term strategies should be advised to establish preventative programs to decrease reinfection.

However, no discernible variations in genetic diversity were found across and within populations of *O. viverrini*. While the causes of genetic variety are unknown, this work has revealed the diversity of *O. viverrini* in the Snuol area of Kratie Province which may or may not be an isolated population. As a result, more research on the genetic diversity of *O. viverrini* in this endemic region should be done utilizing other potent genetic markers as well as samples acquired from many other populations in surrounding districts or provinces.

5.2 Recommendation

Enhance and encouragement public health campaigns focusing on hygiene practices, invest in sanitation infrastructure, prioritizing rural regions, introduce school-based deworming program, and promote community-based health initiatives for early detection and treatment more widely.

The plausible connections between various *O. viverrini* genotypes and variations in cholangiocarcinoma incidence have been proposed. Therefore, health education and suggestions for hygienic measures to avoid infections in the targeted locations should alleviate these major risk factors for *O. viverrini* infection.

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APPENDICES

APPENDICES A

DNA EXTRACTION INSTRUCTION

Notes before starting PCR amplifying.

- Prepare a thermomixer with 2 ml inlays or a water bath at 70, 95 °C for use in steps 3 and 8.
- Perform all centrifugation steps at room temperature (15-25°C) at 20,000 x g (~14,000 rpm).
- Redissolve any precipitates in Buffer AL and InhibitEX® Buffer by heating and mixing.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Mix all buffers before use.
- Symbols: pathogen detection; Δ human DNA analysis

1. Weight 180-220 mg stool in a 2 ml microcentrifuge tube (not provided) and place tube on ice.
2. Add 1 ml InhibitEX Buffer to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized.
3. Δ Skip this step and continue with step 4. Heat the suspension for 5 min at 95 °C. The lysis temperature can be increased to 95 °C for cells that are difficult to lyse. Vortex for 15 s.
4. Centrifuge sample for 1 min to pellet stool particles.
5. Pipet 15 µl or Δ 25 µl Proteinase K into a new 1.5 ml or Δ 2 ml microcentrifuge tube (not provided).
6. Pipet 200 µl or Δ 600 µl supernatant from step 4 into the 1.5 ml or Δ 2 ml microcentrifuge tube containing Proteinase K.
7. Add 200 µl or Δ 600 µl Buffer AL and vortex for 15s. Note: Do not add Proteinase K directly to Buffer AL. it is essential that the sample and Buffer AL are thoroughly mixed to form a homogeneous solution.
8. Incubate at 70 °C for 10 min.
9. Add 200 µl or Δ 600 µl of ethanol (96-100%) to the lysate and mix by vortexing.
10. Carefully apply 600 µl lysate from step 9 to the QIAamp spin column. Close the cap and centrifuge for 1 min. place the QIAamp spin column in a new 2 ml collection tube and discard the tube containing the filtrate. Δ Repeat step 10 until all lysate is loaded.

11. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Centrifuge for 1 min. place the QIAamp spin column in a new 2 ml collection tube and discard the collection tube containing the filtrate.
12. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Centrifuge for 3 min. discard the collection tube containing the filtrate.
13. Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge for 3 min.
14. Transfer the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube (not provided) and pipet 25 µl Buffer ATE directly onto the QIAamp membrane. Incubate for 1 min at room temperature, then centrifuge for 1 min to elute DNA. If yield will be quantified by UV absorbance, blank the measuring device using Buffer ATE to avoid false results.

APPENDICES B

PCR PROCEDURES

PCR-Reaction Components

- DNA template
- Primers
- Enzyme (Thermostable DNA polymerase)
- Nucleotides (dNTPs)
- Mg^{2+} (co factor for DNA polymerase)
- Buffers

Method of setting PCR running Procedure

Sample preparation PCR Reactions Volume (number of Extract DNA sample + N- + P+ + 2 extra)

1. DW 16.8 x 13 = 218.4 (DW- Distilled water)
2. 10x Taq Buffer 2.5 x 13 = 32.5
3. dNTP 1 x 13 = 13
4. Ov-6F 1 x 13 = 13 (F-forward) Primers
5. Ov-6R 1 x 13 = 13 (R-reverse)
6. Tag DNA Pol. 0.2 x 13 = 2.6
7. MaCl2 1.5 x 13 = 19.5
8. DNA template 1 x 13 = 13 (Extracted DNA sample)

Preparation for PCR master mix:

1. Draw DW = 218.4 µL into master mix tube and (total volume 325 µL) add all of reagents into master mixtube => (Exception Extracted DNA sample)
2. Voltage shaking of Master mix solution tube.
3. Negative control (add DW in new tube) (24µL of M.mix + 1µL of DW)
4. Positive control (add OV P.C in new tube) (24µL of M.mix + 1µL of OV P.C)
5. Draw 24µL of reaction mixed from master mix tube into new tube (the code number of new tube must write the same number of extract DNA sample tube).
6. Draw extract DNA sample = 1µL drop into new master mixed tub (Voltage shaking)
7. Repeat the same number (6) until completed all of your extract DNA into each tube preparation.

8. Total volume in new set tube were (24µL + 1µL = 25µL) => (notice for PCR machine set volume) Ready for PCR running => (Method of setting PCR running)

Gel and Buffer preparation:

1. Gel: Agarose = 1.5%
2. Buffer: TAE 1x = 30 mL => Agarose = 1.5 x 30/ 100 = 0.45 g (Boil mixture)
3. Pure agarose powder 0.45g add into 30mL of buffer in the boil mixture.
4. Place the solution in the microwave to melt approximately ~4 min until it diluted. Agarose
5. Pure the agarose solution into the gel tray to make a gel approximately 4mm thick = 27 mL of agarose solution.
6. Waiting until the gel solidified = 20 min.
7. Pure some of 1x TAE buffer to flood onto the gel tray.
8. Hold the comb gently lift it out of the gel to prevent the wells straight and undamaged.
9. Remove the gel tray with the gel from the stand and place it into the migration tank.

How to make 1000ml of 1x TAE solution from 50x TAE buffer:

- $C_1 V_1 = C_2 V_2$ (C_1 = 50x TAE, V_1 =?; C_2 = 1x TAE, V_2 = 1,000ml
- 50 x V_1 = 1 x 1,000 => V_1 = 1 x 1,000/ 50 = 980mL => V_1 = 980 mL

  980mL of DW or DI + 20mL of 50x TAE buffer => 1x TAE buffer = 1,000mL (Note: DI = Distilled Ionized water)

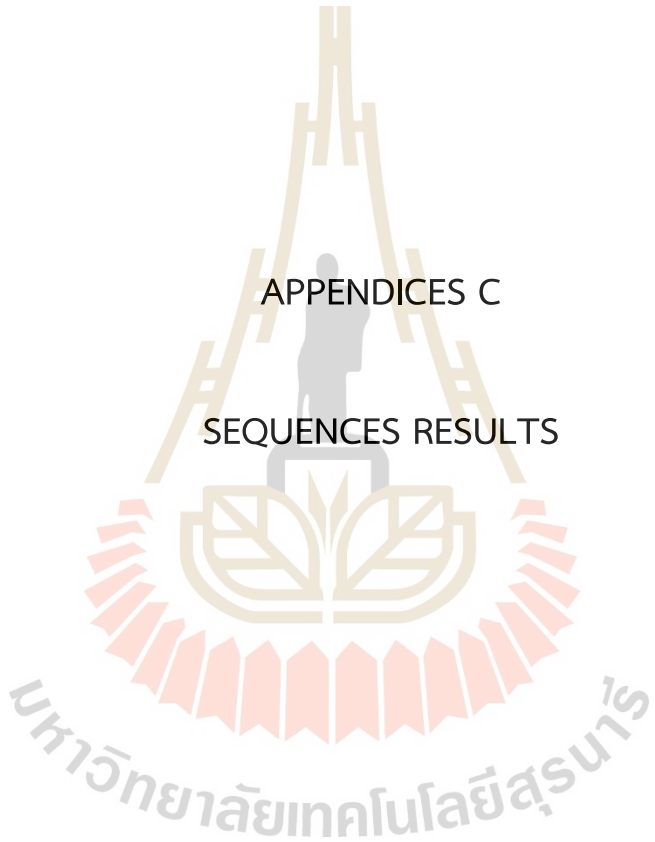
  Electrophoresis - make sure that MiniGel Electrophoresis system is installed on a stable level surface to ensure even sample migration. Pure 1x TAE ≈ 250-300mL of buffer into the migration tank. Place gel with gel tray onto the gel bed inside the gel migration tank.

1. Draw = 1µL of MaestroSafe Nucleic acid stain (MNAS) then drop onto the mixture plate for 12 drips.
2. Draw 6x DNA loading= 1µL to add with MNAS for 11 drips, start from the 2nd drip to the last drip. Draw DNA ladder = 1µL mix with MNAS start from the 1st drip then drop into the gel wells in the migration tank.

3. PCR products = 4μL mix with the reagents start from 2nd drip then drop into the gel wells in the migration tank
4. Use an appropriate pipette to dispense samples into the wells of gel
5. Attach the lid. The display screen will on when the lid is properly seated, and the power switch is turned on.
6. Press set selection button to run for 40min.
7. Press voltage selector button to select output voltage.
8. Press start/stop button briefly to start a run.
9. At the end of an electrophoresis run, turn off the power and open the lid to remove the gel tray.

APPENDICES C

SEQUENCES RESULTS



Sequences results of ITS2-PCR-product by next generation sequencing as Fasta files.

Accession No.	Sequences results
CK0018.1	ATTACAATAGAAATATATACATGTATCGCGATATTTGAAATCTGCTGGCGACTGAAACATGAGGCGGCCTGGTGCATGCAGCACA CTCACTTGCCTGAGAACATCATGAACAAACGAGAGATTCAGATGTGTTCCAGGTGAGTCTCTCTAGAGAACCAAATTAGTTTGCAA ATCAGTGTCTATTGGCTAATTTGGTGCAATTTGGTTTGAAGTAGAACATAAGGTTGACGGAAACCCGGGGACTGCGCCTACCTGAT GAGCCCGAA
CK0020.1	CAGCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTCAGTCGCCAGC AGATTTCAAATATCGCCATACATGTATATATTTCTATTGTAATGTACTACTCATAAGGTTGCTGAAAATCCATCCACAGCCCACGAC CGGTTTCGCCCTTGTTCGGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAACAAAAATATTG CACCAAATTAGCCAATAGACACTCACTCATTTGCAAACTAATTTGGTTCTCTAGAGAGACTCACCTGGAACACATCTGAATCTCTC
CK0025.1	GAGATTCAGATGTGTTCCAGGTGAGTCTCTCTAAAGAACCAAATTAGTTTGCAAATGAGTCAGTGTCTATTGGCTAATTTGGTGCA ATTTGGTTTGAAGTAGAACATAAGGTTGACGGAAACCCGGGGACTGCGCCTACCTGATGAGCCCGAACAAGGGCGAAACCGGTCGT GGGCTGTGGATGGATTTTCAGCAACCTTATGAGTAGTACATTACAATAGAAATATATACATGTATGGCGATATTTGAAATCTGCTGG CGACTGAAACATGAGGCGGCCTGGTGCATGCAG
CK0029.1	ACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTC AGTCGCCAGCAGATTTCAAATATCGCCATACATGTATATATTTCTATTGTAATCTACTACTCATAAGGTTGCTGAAAATCCATCCAC AGCCCACGACCGGTTTCGCCCTTGTTCGGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAAC AAACAAATTGCACCAAATTAGCCAATAGACACTCACTGATTTGCAAACTAATTTGGTTCTCTAGAGAGACTCACCTGGAACACATCT GAATCT
CK0031.1	GTGAGTCTCTCTAGAGAACCAAATTAGTTTGCAAATCAGTGAGTGTCTATTGGCTAATTTGGTGCAATTTGTTTGTTTGAAGTAGAA CATAAGGTTGACGGAAACCCGGGGACTGCGCCTACCTGATGAGCCCGAACAAGGGCGAAACCGGTCGTGGGCTGTGGATGGATTTT CAGCAACCTTATGAGTAGTAGATTACAATAGAAATATATACATGTATGGCGATATTTGAAATCTGCTGGCGACTGAAACATGAGGCG GCCTGGTGCATGCAG
CK0033.1	GGGTTTCCGTCAACCTTATGTTCTACTTCAAACAAAAAAATTGCACCAAATTAGCCAATAGACACTCACTCATTTGCAAACTAATTT GGTTCTCTAGAGAGACTCACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACC AGGCCGCCTCATGTTTCAGTCGCCAGCAGATTTCAAATATCGCGATACATGTATATATTTCTATTGTAATCTACTACTCATAAGGTT GCTGAAAATCCATCCACAGCCCACGACCGGTTTCGCCCTTGTTCGGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTC
CK0065.1	AGTGAGTGTCTATTGGCTAATTTGGTGCAATTTTTTTGTTTGAAGTAGAACATAAGGTTGACGGAAACCCGGGGACTGCGCCTACCT GATGAGCCCGAACAAGGGCGAAACCGGTCGTGGGCTGTGGATGGATTTTCAGCAACCGCATGAGTAGTAGATTACAATAGAAATAT ATACATGTATGGCGATATTTGAAATCTGCTGGCGACTGAAACAT
CK0066.1	CTGAATCTCTCGTTTGTTCACTGAATCTCTCGTTTGTTTCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGC ATGCACCAGGCCGCCTCATGTTTCAGTCGCCAGCAGATTTCAAATATCGCGATACATGTATATATTTCTATTGTAATCTACTACTCA TAAGGTTGCTGAAAATCCATCCACAGCCCAC
CK0068.1	GTTTGAAGTAGAACATAAGGTTGACGGAAACCCGGGGACTGCGCCTACCTGATGAGCCCGAACAAGGGCGAAACCGGTCGTGGGCT GTGGATGGATTTTCAGCAACCTTATGAGTAGTAGATTACAATAGAAATATATACATGTATCGCGATATTTGAAATCTGCTGGCGACT GAAACATGAGGCGGCCTGGTGCATGCAGCACACTCACTTGCCTGAGAACATCATGAACAAACGAGAGATTCAGGCGAGAGATTCAG CTCTCTGTTCCAGGTGAGTCTC
CK0073.1	TCACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTT CAGTCGCCAGCAGATTTCAAATATCGCCATACATGTATATATTTCTATTGTAATGTACTACTCATAAGGTTGCTGAAAATCCATCCA CAGCCCACGACCGGTTTCGCCCTTGTTCGGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAAC CAAATTGCACCAAATTACCCAATAGACACTCACTGATTTGCAAACTAATTTGGTTCTTTAGAGAGACTCACCTGGAACACATCTGAA TCTCTCGTTTGTTCA
CK0075.1	AGTGAGTGTCTATTGGCTAATTTGGTGCAATTTGGTTTGAAGTAGAACATAAGGTTGACGGAAACCCGGGGACTGCGCCTACCTGAT GAGCCCGAACAAGGGCGAAACCGGTCGTGGGCTGTGGATGGATTTTCAGCAACCTTATGAGTAGTAGATTACAATAGAAATATATA CATGTATCGCGATATTTGAAATCTGCTGGCGACTGAAACATGAGGCGGCCTGGTGCATGCAGCACACTCACTTGCCTGAGAACATC ATGAACAAACGAGAGATTCAGATGTGTTCCAGGTGAGTCTCTCTAATGAACAAACGAGA

Accession No.	Sequences results
CK0096.1	AGATTCAGATGTGTTCCAGGTGAGTCTCTCTAGAGAACCAAATTAGTTTGCAAATTAGTGAGTGTCTATTGGCTAATTTGGTGCAAT TTTTTTGTTTGAAGTAGAACATAAGGTTGACGGAAACCCGGGGACTGCGCCTACCTGATGAGCCCGAACAAGGGCGAAACCGGTCG TGGGCTGTGGATGGATTTTCAGCAACCTTATGAGTAGTACATTACAATAGAAATATATACATGTATGGCGATATTTGAAATCTGCTG GCGACTGAAACATGAGGCGGCCTGGTGCATGCAGCACACTCACTTGCCTGAGAACATCATGAACAAACGAGAGATTCAGATGTGTT CCAGGT
CK0098.1	TCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTCAGTCGCCAGCAGA TTTCAAATATCGCCATACATGTATATATTTCTATTGTAATGTACTACTCATAAGGTTGCTGAAAATCCATCCACAGCCCACGACCGG TTTCGCCCTTGTTCGGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAACAAACAAATTGCACC AAATTAGCCAATAGACACTCACTGATTTGCAAACTAATTTGGTTCTCTAGAGAGACTCACCTGGAACACATCTGAATCTCTCG
CK00138.1	GTTTGAAGTAGAACATAAGGTTGACGGAAACCCGGGGACTGCGCCTACCTGATGAGCCCGAACAAGGGCGAAACCGGTCGTGGGCT GTGGATGGATTTTCAGCAACCTTATGAGTAGTAGATTACAATAGAAATATATACATGTATGGCGATATTTGAAATCTGCTGGCGACT GAAACATGAGGCGGCCTGGTGCATGCAGCACACTCACTTGCCTGAGAACATCATGAACAAACGAGAGATTCAGATGTGTTCCAGGT
CK00152.1	TTCGGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAACAAAAAAATTGCACCAAATTAGCCAA TAGACACTCACTCATTTGCAAACTAATTTGGTTCTCTAGAGAGACTCACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTT CTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTCAGTCGCCAGCAGATTTCAAATATCGCCATACATGTATATAT TTCTATTGTAATCTACTACTCATAAGGTTGCTGAAAATCCATCCACAGCCCAC
CK00153.1	CTCACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTT TCAGTCGCCAGCAGATTTCAAATATCGCCATACATGTATATATTTCTATTGTAATGTACTACTCATAAGGTTGCTGAAAATCCATCCA CAGCCCACGACCGGTTTCGCCCTTGTTCGGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAAC
CK00161.1	GTTTGTTCAGTTCTAGAGAGACTCACCTGGAACAGAGAGCCGAATCTCTCGTCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCA AGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTCAGTCGCCAGCAGATTTCAAATATCGCCATACATGTATATATTTCTATTG TAATGTACTACTCATAAGGTTGCTGAAAATCCATCCACAGCCCACGACCGGTTTCGCCCTTGTTCGGGCTCATCAGGTAGGCGCAGT CCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAACCAAATTGCACCA
CK00167.1	CTCACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTT TCAGTCGCCAGAACATTTCAAATATCGCGATACATGTATATATTTCTATTGTAATCTACTACTCATAAGGTTGCTGAAAATCCATCCA CAGCCCACGACCGGTTTCGCCCTTGTTCGGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAAC AAACAAATTGCACCAAATTAGCCAATAGACACTCACTGATTTGCAAACTAATTTGGTTCTCTAGAGAGACTCACCTGGAACACATCT GAATCT
CK00176.1	ATTGCACCAAATTAGCCAATAGACACTCACTCATTTGCAAACTAATTTGGTTCTCTAGAGAGACTCACCTGGAACACATCTGAATCT CTCGTTTGTTCTCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTCAG TCGCCAGAACATTTCAAATATCGCGATACATGTATATATTTCTATTGTAATCTACTACTCATAAGGTTGCTGAAAATCCATCCACAGC CCAC
CK00187.1	TCCGTCAACCTTATGTTCTACTTCAAACAAAAAAATTGCACCAAATTAGCCAATAGACACTCACTCATTTGCAAACTAATTTGGTTCT CTAGAGAGACTCACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCG CCTCATGTTTCAGTCGCCAGCAGATTTCAAATATCGCGATACATGTATATATTTCTATTGTAATCTACTACTCATAAGGTTGCTGAAA ATCCATCCACAGCCCAC
CK005.1	GGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAACCAAATTGCACCAAATTAGCCAATAGACA CTCACTGATTTGCAAACTAATTTGGTTCTTTCGAGAGACTCACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGG CAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTCAGTCGCCAGCAGATTTCAAATATCGCGATACATGTATATATTTCTAT TGTAATCTACTACTCATAAGGTTGCTGAAAATCCATCCACAGCCCAC
CK00100.1	TCCGTCAACCTTATGTTCTACTTCAAACAAAAAAATTGCACCAAATTAGCCAATAGACACTCACTCATTTGCAAACTAATTTGGTTCT CTAGAGAGACTCACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCG CCTCATGTTTCAGTCGCCAGCAGATTTCAAATATCGCCATACATGTATATATTTCTATTGTAATGTACTACTCATAAGGTTGCTGAAA ATCCATCCACAGCCCACGACCGGTTTCGCCCTT
CK00111.1	CTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAACCAAATTGCACCAAATTAGCCAATAGACACTC ACTGATTTGCAAACTAATTTGGTTCTCTAGAGAGACTCACCTGGAACCCAGCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAA GTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTCAGTCGCCAGAAGATTTCAAATATCGCGATACATGTATATATTTCTATTGT AATCTACTACTCATAAGGTTGCTGAAAATCCAT

CK00112.1	GGAACACATCTGAATCTCTCGTTTGTTCATAGAGAGACTCACCTGGAACACATCTGAATCTCTCGTTTGTTCAGTTCTAGAGAGACT CACCTGGAACACATCTGAATCTCTCGTTTGTTCATGATGTTCTCAGGCAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTC AGTCGCCAGCAGATTTCAAATATCGCGATACATGTATATATTTCTATTGTAATCTACTACTCATA
CK00116.1	TTTGCAAATGAGTGAGTGTCTATTGGCTAATTTGATGGAATTGGATATGAAGTTAAACATAAGGTTGACGGAAACCCGGGGACTGCG CCTACCTGATGAGCCCGGAGAAGGGCGAAACCGGTCGTGGGCTGTGGATGGATTTTCAGCAACCTTATGAGTAGTACATTACAATA GAAATATATACATGTATGGCGATATTTGAAATCTGCTGGCGACTGAAACATGAGGCGGCCTGGTGCATGCAGCACACTCACTTG
CK00117.1	GGGCTCATCAGGTAGGCGCAGTCCCCGGGTTTCCGTCAACCTTATGTTCTACTTCAAACCAAATTGCACCAAATTAGCCAATAGACA CTCACTGATTTGCAAACTAATTTGGTTCTTTCGAGAGACTCACCTGGAACCCAGCTGAATCTCTCGTTTGTTCATGATGTTCTCAGG CAAGTGAGTGTGCTGCATGCACCAGGCCGCCTCATGTTTCAGTCGCCAGCAGATTTCAAATATCGCGATACATGTATATATTTCTAT TGTAATCTACTACTCATAAGGTTGCTGAAAATCCATCCACAGCCCAC

Sequences results of *NAD*1-PCR-product by next generation sequencing as fasta files

Accession No.	**Sequences results**
CK0033.1	TCACCCTTTTCGGCTCTCACCTTGATGGCTTCAGCCTTTGGCTTCACGTAATGGTCGCGACAGATTCTCGGTGTCGGCTGGATGTTAG CCATCAGAGTGGTATCCTGATCCATCAGGTTGCCGCATCCGCAGAGGTCTTCCGTCATGGAGAGGTTGAAAGCCTTCATCCACTTCTT GTAGGGAAGGGTCTGTGAGAAGAACACCTGCTGCTCGGTATCATTCTTGCGTCTCACTACCACTGCCTGGCTGTTTTTCAGTTTGCTG CTTTTGCGCAGGTATCTGATGTTCTGGTTTTTGCCATTGATGATAATGGGATTCAGGGTGATGGTATCTTCTCCGGCAATAAGCGGAG TGAAAACCAGCTGTTGCTCTGATTTGAGCTGCAGACTATCGAGATGGAGGCGGAAAGCAACCTTGAGACTGTCGCTCTTCTTCTGTGTC AAGACGCTGTCTATCAGAATCTTGCCTCCAACCAAACCACC
CK0065.1	GCTCACATCCTTGTCGTTCTTCACGAGGTCGATGGTGGATGTAATCTTTCTCAGCTCTGTGGCATTCTCACGGAAGTCGGCGAGGATAT CGCTCTTGTTGAGCTTGAAGCTCAGGTAAGCTTCACCCTTTTCGGCTCTCACCTTGATGGCTTCAGCCTTTGGCTTCACGTAATGGTCG CGACCAATCTTGGGTATTGGGTTGATGACCGCCATCTGGGTGGTATCCTGATCCATCAGGTTGCCGCATCCGCAGAGGTCTTCCGTCAT GGAGAGGTTGAATGCCTTCATCCACTTCCTGTAGGGAAGGGTCTGTGAGAAGAGCACCTGCTGCTCGGTATCATTCCTGCGTCTCACTA CCACTGCCTGGCTGTTTTTCAGTTTGCTGCTTTTGCGCAGGTATCTGATGTTCTGGTTTTTGCCATTGATGATAATGGGATTCAGGGCAA TGGTATCTTCTCCGGCAATAAGCGGAGTGAAAACCAGCTGTTGCTCTGATTTGAGCTGCAGACTATCGAGATGGAGGCGGAAAGCAACC TTCAGACTGTCGTTTTTCTTCTGTGTCAAGACGCTGTCTATCAGAATCTTGCCTCCAACCAAACCTAGCTGCGAATCATCTATCTTCTGG GCTTGGACAGTCAGTGCGCTCTGTGCAAACAGAATGCAAAGGGGCGAAATGACCGCATAAAGCTTCATTTTCTTCATATTCTTCATCTT GTAAAAGAGGGT
CK0066.1	GCAGAAGAAGAGCGACAGTCTGAAGGTTGCTTTCCGACTCCATCTCGATAGTCTGCAGCTCAAATCAGAGCAACAGCTGGTTTTCACTC CGCTTATTGCCGGAGAAGATACCATCACCCTGAATCCCATTATCATCAATGGCAAAAACCAGAACATCAGATACCTGCGCAAAAGCAGC AAACTGAAGAACAGCCAGGCAGTGGTAGTGAGACGCAGGAATGATACCGAGCAGCAGGTGCTCTTCTCCCAGACCCTTCCCTACAGGA AGTGGATGAAGGCTTTCAACCTCTCCATGACGGAAGACCTCTGCGGATGCGGCAACCTGATGGATCAGGATACCACCCAGATGGCGGT CATCAACCCAATACCCAAGATTGGTCGCGACCATTACGTGAAGCCAAAGGCTGAAGCCATCAAGGTGAGAGCCGAAAAGGGTGAAGCT TACCTGAGCTTCAAGCTCAACAAGAGCGATATCCTTGCCGACTTCCG
CK0075.1	ATACCAATGGCTGCTGTCGGAGCATCTCAATCTCGAAGGATCCATCGGATTAGGATACATCTACAGCCCATACAAGCTCTACGGAAGA TGTGACAAATGTCTTAACGAGGACCATCGCAACTATGTAGGTCCTACTAAAGCTGCCCTCTCCCTGATATACGCATTCTAGAAACAAT ATCACCGAGATAGCGAAAACCAAAATCAAACCCTCTTTTACAAGATGAAGAATATGAAGAAAATGAAGCTTTATGCGGTCATTTCGCC CCTTTGCATTCTGTTTGCACAGAGCGCACTGACTGTCCAAGCCCAGAAGATAGATGATTCGCAGCTACGGTTGGCTGGAGGCAAGAT TCTGATAGACAGCGTCTTGACACAGAAGAAAAGCGACAGTCTGAAGGTTGCTTTCCGCCTCCATCTCGATAGTCTGCAGCTCAAATCA GAGCAACAGCTGGTTTTCACTCCGCTTATTGCCGGAGAAGATACCATTGCCCTGAATCCCATTATCATCAATGGCAAAAACCAGAACA TCAGATACCTGCGCAAAAGCAGCAAACTGAAAAACAGCCAGGCAGTGGTAGTGAGACGCAGGAATGATACCGAGCAGCAGGTGCTCT TCTCACAGACCCTTCCCTACAGGAAGTGGATGAAGGCATTCAACCTCTCCATGACGGAAGACCTCTGCGGATGCGGCAACCTGATGG ATCAGGATACCAC
CK0091.1	CATCCTTGTCGTTCTTCACGAGGTCGATGGTGGATGTAATCTTTCTCAGCTCTGTGGCATTCTCACGGAAGTCGGCGAGGATATCGCT CTTGTTGAGCTTGAAGCTCAGGTAAGCTTCACCCTTTTCGGCTCTTACCTTGATGGCTTCAGCCTTTGGCTTCACGTAATGGTCGCGA CCAATCTTGGGTATTGGGTTGATGACCGCCATCTGGGTGGTATCCTGATCCATCAGGTTGCCGCATCCGCAGAGGTCTTCCGTCATG GAGAGGTTGAAAGCCTTCATCCACTTCCTGTAGGGAAGGGTCTGTGAGAAGAGCACCTGCTGCTCGGTATCATTCCTGCGTCTCACT

	ACCACTGCCTGGCTGTTCTTCAGTTTGCTGCTTTTGCGCAGGTATCTGATGTTCTGGTTTTTGCCATTGATGATAATGGGATTCAGGG CGATGGTATCTTCTCCGGCAATAAGCGGAGTGAAAACCAGCTGTTGCTCTGATTTGAGCTGCAGACTATCGAGATGGAGGCGGAAAG CAACCTTGAGACTGTCGCTCTTCTTCTG
CK0138.1	GCTCACATCCTTGTCGTTCTTCACGAGGTCGATGGTGGATGTAATCTTTCTCAGCTCTGTGGCATTCTCACGGAAGTCGGCGAGGATA TCGCTCTTGTTGAGTTTGAAGCTCAGGTAAGCTTCACCCTTTTCGGCTCTCACCTTGATGGCTTCAGCCTTTGGCTTCACGTAATGGTC GCGACCAATCTTGGGTATTGGGTTGATGACCGCCATCTGGGTGGTATCCTGATCCATCAGGTTGCCGCATCCGCAGAGGTCTTCCGTC ATGGAGAGGTTGAAAGCCTTCATCCACTTCCTGTAGGGAAGGGTCTGGGAGAAGATCACCTGCTGCTCGGTATCATTCCTGCGTCTCA CTACCACTGCCTGGCTGTTTTTCAGTTTGCTGCTTTTGCGCAGGTATCTGATGTTCTGGTTTTTGCCATTGATGATAATGGGATTCAGG GCGATGGTATCTTCTCCGGCAATAAGCGGAGTGAAAACCAGCTGTTGCTCTGATTTGAGCTGCAGACTATCGAGATGAAGGCGGAAAG CAACCTTGAGACTGTCGCTCTTCTTCTGTGTCAAGACGCTGTCTATCAGAATCTTGCCTCCAACC
CK0152.1	GGTTTGGTTGGTGGCCTCACCTACGGATACCAATGGCTGCTGTCTGAGCATCTCAATCTCGAAGGATCCATCGGATTAGGATACATCT ACAGCCCATACAAGCTCTACGGAAGATGTGACAAATGTCTTAACGAGGACCACCGCAACTATGTAGGTCCAACTAAAGCCGCCCTTTC CCTGATATACGCATTCTAGAAACAATATCACCGAGATAGCGAAAAACAAAATCAAACCCTCTTTTACAAGATGAAGAATATGAAGAAA ATGAAGCTTTATGCGGTCATTTCGCCCCTTTGCATTCTGTTTGCACAGAGCGCATTGACTGTCCAAGCCCAGAAGATAGATGATTCGC AGCTACGGTTGGCTGGAGGCAAGATTCTGATAGACAGCGTCTTGACACAGAAGAAGAGCGACAGTCTGAAGGTTGCTTTCCGACTCCA TCTCGATAGTCTGCAGCTCAAATCAGAGCAACAGCTGGTTTTCACTCCGCTTATTGCCGGAGAAGATACCATCGCCCTGAATCCCATTA TCATCAATGGCAAAAACCAGAGCATCAGATACCTGCGCAAAAGCAGCAAACTGAAGAACAGCCAGGCAGTGGTAGTGAGACGCAGGAA TGATACCGAGCAGCAGGTGCTCTTCTCGCAGACCCTTCCCTACAGGAAGTGGATGAAGGCTTTCAACCTCTCCATGACGGAAGACCTCT GCGGATGCGGCAACCTGA
CK0100.1	CATCCTTGTAGTTTGGAATAACAACGATACCACCTGGGTGTTGTCCAGTCGTACGTTTAACACCAGCTGCACCTTGTGCCAAGCGTTCC ACTTCTGCTTCTGGATAGAATTTGCCATAATCACGTTCGTAACCTTTAACAAATCCGTAAGCAGTACGATCTGCCACCGTACCTACGGTT CCAGCACGAAAGGCGTATTGTTCACCAAAAATATCACGAACATCCAAGTGAGCTGACGGTTGGTCATCACCAGAGAAGTTCAAATCGAT ATCGGGAACCTTGTCTCCATCAAAACCAAGGAAGGTTTCAAACGGAATATCGTGTCCATCTTTCTTGTAACGTGTGCCACATTCTGGGC AATCCTTGTCAGGCAAATCATAACCAGAACCAACAGAACCATCGGTGATGAATTCTGAATGCTGACAATTTGGACAGACATAATGTGGT GGCATTGGGTTAACTTCGGTAATACCAATCATGGTTGCCACAAAACTTGACCCAACCAAACCACC
CK0112.1	GCTCACATCCTTGTAAGCCTGCTGTAATCTTAACAGAGCTGCCTTGGTAGCATCGGTCTGGAATCCATCGTACCAGGTACCGTTGTCAT CCTGCAGGATTGCGGGATAAGAATCCTGCCAGAACTCAGGCAGATAGTTTACATAAGGAGCTTCGTTACCAACCAAACCACCTGCGTAC CAACCAAACCACCTGCGTACCAACCAAACCA

APPENDICES D

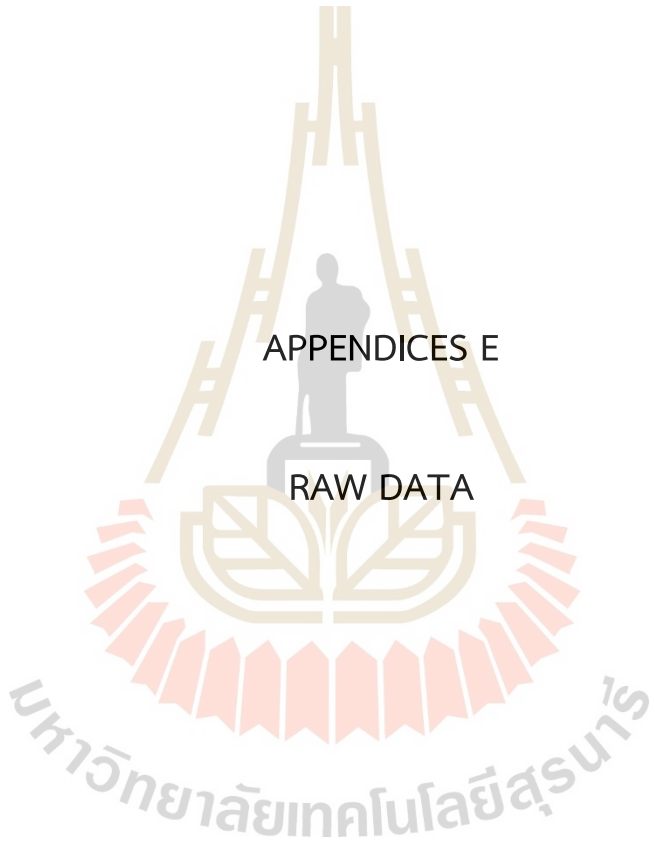
CONFIRM RESULTS WITH GENBANK DATA

The confirming results of sequencing based on ITS2-PCR 330 bp searching in NCBI GenBank

No	Fasta code of samples sequencing	Varieties per identities (%)	Accession number: (NCBI GENBANK)
1	CK0018.1	99.20	S80278.1
2	CK0020.1	96.28	LC230157.1
3	CK0025.1	98.09	LC230157.1
4	CK0029.1	96.28	LC230157.1
5	CK0031.1	96.23	LC230157.1
6	CK0033.1	97.80	LC230157.1
7	CK0065.1	95.65	LC230157.1
8	CK0066.1	99.20	LC230157.1
9	CK0068.1	98.04	LC230157.1
10	CK0073.1	98.11	LC230157.1
11	CK0075.1	98.11	LC230157.1
12	CK0096.1	97.60	LC230157.1
13	CK0098.1	96.74	LC230157.1
14	CK0138.1	97.55	LC230157.1
15	CK0152.1	98.40	LC230157.1
16	CK0153.1	98.04	LC230157.1
17	CK0161.1	98.11	LC230157.1
18	CK0167.1	95.81	LC230157.1
19	CK0176.1	97.60	LC230157.1
20	CK0187.1	99.20	LC230157.1
21	CK005.1	99.20	LC230157.1
22	CK0100.1	99.29	LC230157.1
23	CK0111.1	98.25	LC230157.1
24	CK0112.1	98.54	S80278.1
25	CK0116.1	97.12	LC230157.1
26	CK0117.1	99.20	LC230157.1

APPENDICES E

RAW DATA



Raw data of participants from Snuol district, Kratie Province, Cambodia

No.	PID	Gender	Age	Location (Village,sub-district,district,province)	EPG
1	CK0001	M	48	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
2	CK0002	F	51	Trapaing Srae, Pithnu, Snuol, Kratie	880,00
3	CK0004	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
4	CK0005	F	17	Trapaing Srae, Pithnu, Snuol, Kratie	20,00
5	CK0006	F	17	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
6	CK0007	M	25	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
7	CK0008	M	21	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
8	CK0009	M	29	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
9	CK0010	F	23	Trapaing Srae, Pithnu, Snuol, Kratie	6,67
10	CK0011	F	45	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
11	CK0012	F	40	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
12	CK0014	M	23	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
13	CK0015	F	24	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
14	CK0016	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
15	CK0017	M	25	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
16	CK0018	M	23	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
17	CK0019	M	32	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
18	CK0020	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
19	CK0021	F	32	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
20	CK0022	M	20	Trapaing Srae, Pithnu, Snuol, Kratie	3,00
21	CK0023	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
22	CK0024	F	30	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
23	CK0025	F	21	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
24	CK0026	M	17	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
25	CK0027	M	32	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
26	CK0028	F	21	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
27	CK0029	F	18	Cheung Khlu, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
28	CK0030	F	17	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
29	CK0031	M	23	Trapaing Srae, Pithnu, Snuol, Kratie	11,50
30	CK0032	F	32	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
31	CK0033	F	43	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
32	CK0034	F	17	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
33	CK0035	M	47	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
34	CK0038	F	25	Chrab, Pithnu, Snuol, Kratie	0,00
35	CK0039	F	61	Cheung Khle, Pithnu, Snuol, Kratie	0,00
36	CK0041	F	51	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
37	CK0042	F	53	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
38	CK0043	F	20	Trapaing Srae, Pithnu, Snuol, Kratie	266,67
39	CK0044	F	60	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
40	CK0045	F	56	Cheung Khle, Pithnu, Snuol, Kratie	16,67
41	CK0046	F	46	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
42	CK0048	F	35	Trapaing Srae, Pithnu, Snuol, Kratie	11,67
43	CK0049	F	53	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
44	CK0050	F	57	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
45	CK0051	M	42	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
46	CK0052	F	41	Trapaing Srae, Pithnu, Snuol, Kratie	18,00
47	CK0053	F	34	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
48	CK0054	F	63	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
49	CK0055	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
50	CK0056	M	20	Chrab, Pithnu, Snuol, Kratie	0,00
51	CK0057	F	19	Chrab, Pithnu, Snuol, Kratie	0,00
52	CK0059	F	42	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
53	CK0060	M	21	Pravanh, Pithnu, Snuol, Kratie	0,00
54	CK0061	F	31	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
55	CK0062	F	42	Chrab, Pithnu, Snuol, Kratie	0,00
56	CK0063	F	21	Cheung Khle, Pithnu, Snuol, Kratie	0,00
57	CK0064	F	31	Chrab, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
58	CK0065	M	17	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
59	CK0066	F	21	Pravanh, Pithnu, Snuol, Kratie	165,00
60	CK0067	M	41	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
61	CK0068	F	27	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
62	CK0070	F	71	Cheung Khle, Pithnu, Snuol, Kratie	0,00
63	CK0071	F	19	Cheung Khle, Pithnu, Snuol, Kratie	245,00
64	CK0072	M	35	Cheung Khle, Pithnu, Snuol, Kratie	0,00
65	CK0073	F	18	Pravanh, Pithnu, Snuol, Kratie	0,00
66	CK0074	F	17	Chrab, Pithnu, Snuol, Kratie	0,00
67	CK0075	M	19	Cheung Khle, Pithnu, Snuol, Kratie	0,00
68	CK0076	F	36	Pravanh, Pithnu, Snuol, Kratie	0,00
69	CK0077	F	36	Pravanh, Pithnu, Snuol, Kratie	0,00
70	CK0078	F	47	Pravanh, Pithnu, Snuol, Kratie	0,00
71	CK0079	F	45	Pravanh, Pithnu, Snuol, Kratie	0,00
72	CK0080	F	21	Pravanh, Pithnu, Snuol, Kratie	0,00
73	CK0081	F	19	Pravanh, Pithnu, Snuol, Kratie	0,00
74	CK0082	M	49	Pravanh, Pithnu, Snuol, Kratie	40,33
75	CK0083	F	49	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
76	CK0084	M	44	Trapaing Srae, Pithnu, Snuol, Kratie	48,00
77	CK0085	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
78	CK0086	F	21	Pravanh, Pithnu, Snuol, Kratie	0,00
79	CK0087	F	18	Chrab, Pithnu, Snuol, Kratie	0,00
80	CK0088	M	21	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
81	CK0089	M	21	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
82	CK0090	F	19	Pravanh, Pithnu, Snuol, Kratie	0,00
83	CK0091	F	55	Trapaing Srae, Pithnu, Snuol, Kratie	1,67
84	CK0092	M	19	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
85	CK0093	F	32	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
86	CK0094	M	18	Chrab, Pithnu, Snuol, Kratie	8,00
87	CK0095	F	33	Chrab, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
88	CK0096	M	18	Pravanh, Pithnu, Snuol, Kratie	0,00
89	CK0097	F	19	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
90	CK0098	M	49	Chrab, Pithnu, Snuol, Kratie	0,00
91	CK0099	M	20	Chrab, Pithnu, Snuol, Kratie	6,17
92	CK0100	F	34	Chrab, Pithnu, Snuol, Kratie	0,00
93	CK0101	F	21	Chrab, Pithnu, Snuol, Kratie	0,00
94	CK0102	M	18	Chrab, Pithnu, Snuol, Kratie	0,00
95	CK0103	F	37	Chrab, Pithnu, Snuol, Kratie	0,00
96	CK0104	F	42	Chrab, Pithnu, Snuol, Kratie	0,00
97	CK0105	F	19	Chrab, Pithnu, Snuol, Kratie	0,00
98	CK0106	F	23	Chrab, Pithnu, Snuol, Kratie	0,00
99	CK0107	F	21	Chrab, Pithnu, Snuol, Kratie	0,00
100	CK0108	M	19	Chrab, Pithnu, Snuol, Kratie	0,00
101	CK0109	M	23	Cheung Khle, Pithnu, Snuol, Kratie	0,00
102	CK0110	M	35	Cheung Khle, Pithnu, Snuol, Kratie	0,00
103	CK0111	M	18	Cheung Khle, Pithnu, Snuol, Kratie	0,00
104	CK0112	M	34	Cheung Khle, Pithnu, Snuol, Kratie	0,00
105	CK0113	F	24	Cheung Khle, Pithnu, Snuol, Kratie	0,00
106	CK0114	M	48	Cheung Khle, Pithnu, Snuol, Kratie	0,00
107	CK0115	F	19	Cheung Khle, Pithnu, Snuol, Kratie	0,00
108	CK0116	F	19	Cheung Khle, Pithnu, Snuol, Kratie	0,00
109	CK0117	F	48	Cheung Khle, Pithnu, Snuol, Kratie	0,00
110	CK0118	F	70	Pravanh, Pithnu, Snuol, Kratie	0,00
111	CK0119	F	20	Pravanh, Pithnu, Snuol, Kratie	0,00
112	CK0120	M	17	Pravanh, Pithnu, Snuol, Kratie	0,00
113	CK0121	F	29	Pravanh, Pithnu, Snuol, Kratie	0,00
114	CK0122	F	19	Pravanh, Pithnu, Snuol, Kratie	0,00
115	CK0123	F	28	Pravanh, Pithnu, Snuol, Kratie	0,00
116	CK0124	F	50	Pravanh, Pithnu, Snuol, Kratie	0,00
117	CK0125	M	21	Pravanh, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
118	CK0126	M	15	Pravanh, Pithnu, Snuol, Kratie	0,00
119	CK0127	F	17	Trapaing Srae, Pithnu, Snuol, Kratie	112,67
120	CK0128	M	67	Trapaing Srae, Pithnu, Snuol, Kratie	3,00
121	CK0130	F	33	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
122	CK0131	F	39	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
123	CK0132	M	21	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
124	CK0133	F	32	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
125	CK0134	F	39	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
126	CK0135	F	32	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
127	CK0136	F	23	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
128	CK0137	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
129	CK0138	F	17	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
130	CK0139	F	41	Trapaing Srae, Pithnu, Snuol, Kratie	666,67
131	CK0140	M	5	Trapaing Srae, Pithnu, Snuol, Kratie	8,33
132	CK0141	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
133	CK0142	F	19	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
134	CK0143	F	65	Trapaing Srae, Pithnu, Snuol, Kratie	2,67
135	CK0144	M	65	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
136	CK0145	F	22	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
137	CK0146	M	19	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
138	CK0147	M	44	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
139	CK0148	F	23	Chrab, Pithnu, Snuol, Kratie	0,00
140	CK0149	M	29	Chrab, Pithnu, Snuol, Kratie	0,00
141	CK0150	F	41	Chrab, Pithnu, Snuol, Kratie	0,00
142	CK0151	F	33	Chrab, Pithnu, Snuol, Kratie	0,00
143	CK0152	M	36	Trapaing Srae, Pithnu, Snuol, Kratie	671,00
144	CK0153	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
145	CK0154	M	35	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
146	CK0155	M	53	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
147	CK0156	F	30	Trapaing Srae, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
148	CK0157	M	34	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
149	CK0158	F	26	Trapaing Srae, Pithnu, Snuol, Kratie	30,00
150	CK0159	F	30	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
151	CK0160	F	32	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
152	CK0161	M	17	Trapaing Srae, Pithnu, Snuol, Kratie	630,00
153	CK0163	F	30	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
154	CK0164	M	23	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
155	CK0165	F	19	Trapaing Srae, Pithnu, Snuol, Kratie	16,00
156	CK0166	F	28	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
157	CK0167	F	28	Trapaing Srae, Pithnu, Snuol, Kratie	160,00
158	CK0168	M	8	Trapaing Srae, Pithnu, Snuol, Kratie	133,33
159	CK0169	F	49	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
160	CK0170	M	3	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
161	CK0171	F	43	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
162	CK0172	F	16	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
163	CK0173	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
164	CK0174	F	17	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
165	CK0175	M	19	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
166	CK0176	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
167	CK0177	F	26	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
168	CK0178	F	31	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
169	CK0179	F	32	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
170	CK0181	F	20	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
171	CK0182	F	80	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
172	CK0183	M	36	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
173	CK0184	F	49	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
174	CK0185	F	20	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
175	CK0186	M	31	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
176	CK0187	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
177	CK0188	M	21	Trapaing Srae, Pithnu, Snuol, Kratie	5,00

No.	PID	Gender	Age	Location	EPG
178	CK0189	M	17	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
179	CK0190	M	58	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
180	CK0191	F	19	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
181	CK0192	M	49	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
182	CK0193	F	50	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
183	CK0194	M	24	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
184	CK0195	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
185	CK0196	F	30	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
186	CK0197(n)	F	21	Chrab, Pithnu, Snuol, Kratie	0,00
187	CH0001	F	35	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
188	CH0002	M	49	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
189	CH0003	M	52	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
190	CH0004	M	37	Cheung Khlu, Pithnu, Snuol, Kratie	6,00
191	CH0005	M	36	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
192	CH0006	M	18	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
193	CH0007	F	46	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
194	CH0008	F	22	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
195	CH0009	F	42	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
196	CH0010	M	60	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
197	CH0011	F	52	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
198	CH0012	F	36	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
199	CH0013	F	21	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
200	CH0014	F	23	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
201	CH0015	M	19	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
202	CH0016	M	31	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
203	CH0017	M	52	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
204	CH0018	F	26	Cheung Khle, Pithnu, Snuol, Kratie	0,00
205	CH0019	F	54	Cheung Khle, Pithnu, Snuol, Kratie	0,00
206	CH0020	F	52	Cheung Khle, Pithnu, Snuol, Kratie	0,00
207	CH0021	M	55	Cheung Khle, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
208	CH0022	M	51	Cheung Khle, Pithnu, Snuol, Kratie	0,00
209	CH0023	F	24	Cheung Khle, Pithnu, Snuol, Kratie	0,00
210	CH0024	F	39	Cheung Khle, Pithnu, Snuol, Kratie	0,00
211	CH0025	F	70	Cheung Khle, Pithnu, Snuol, Kratie	0,00
212	CH0026	F	59	Cheung Khle, Pithnu, Snuol, Kratie	16,67
213	CH0027	F	52	Cheung Khle, Pithnu, Snuol, Kratie	0,00
214	CH0028	M	28	Cheung Khle, Pithnu, Snuol, Kratie	0,00
215	CH0029	F	40	Cheung Khle, Pithnu, Snuol, Kratie	0,00
216	CH0030	F	18	Cheung Khle, Pithnu, Snuol, Kratie	0,00
217	CH0031	M	41	Cheung Khle, Pithnu, Snuol, Kratie	0,00
218	CH0032	F	38	Cheung Khle, Pithnu, Snuol, Kratie	0,00
219	CH0033	F	27	Cheung Khle, Pithnu, Snuol, Kratie	0,00
220	CH0034	M	31	Cheung Khle, Pithnu, Snuol, Kratie	0,00
221	CH0035	M	73	Cheung Khle, Pithnu, Snuol, Kratie	0,00
222	CH0036	M	41	Cheung Khle, Pithnu, Snuol, Kratie	0,00
223	CH0037	F	18	Cheung Khle, Pithnu, Snuol, Kratie	0,00
224	CH0038	F	23	Cheung Khle, Pithnu, Snuol, Kratie	0,00
225	CH0039	F	37	Cheung Khle, Pithnu, Snuol, Kratie	0,00
226	CH0040	M	31	Cheung Khle, Pithnu, Snuol, Kratie	0,00
227	CH0041	M	16	Cheung Khle, Pithnu, Snuol, Kratie	0,00
228	CH0042	M	19	Cheung Khle, Pithnu, Snuol, Kratie	0,00
229	CH0043	M	67	Cheung Khle, Pithnu, Snuol, Kratie	0,00
230	CH0044	M	31	Cheung Khle, Pithnu, Snuol, Kratie	0,00
231	CH0045	M	28	Cheung Khle, Pithnu, Snuol, Kratie	0,00
232	CH0046	F	19	Cheung Khle, Pithnu, Snuol, Kratie	0,00
233	CH0047	F	20	Cheung Khle, Pithnu, Snuol, Kratie	0,00
234	CH0048	F	38	Cheung Khle, Pithnu, Snuol, Kratie	0,00
235	CH0049	M	21	Cheung Khle, Pithnu, Snuol, Kratie	0,00
236	CH0050	F	26	Cheung Khle, Pithnu, Snuol, Kratie	0,00
237	CH0051	M	22	Cheung Khle, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
238	CH0052	M	21	Chrab, Pithnu, Snuol, Kratie	0,00
239	CH0053	M	19	Chrab, Pithnu, Snuol, Kratie	0,00
240	CH0054	M	51	Chrab, Pithnu, Snuol, Kratie	0,00
241	CH0055	M	65	Chrab, Pithnu, Snuol, Kratie	0,00
242	CH0056	F	47	Chrab, Pithnu, Snuol, Kratie	0,00
243	CH0057	F	24	Chrab, Pithnu, Snuol, Kratie	0,00
244	CH0058	M	17	Chrab, Pithnu, Snuol, Kratie	0,00
245	CH0059	M	18	Chrab, Pithnu, Snuol, Kratie	0,00
246	CH0060	F	49	Chrab, Pithnu, Snuol, Kratie	0,00
247	CH0061	M	47	Chrab, Pithnu, Snuol, Kratie	0,00
248	CH0062	F	52	Chrab, Pithnu, Snuol, Kratie	23,33
249	CH0063	F	18	Chrab, Pithnu, Snuol, Kratie	0,00
250	CH0064	F	20	Chrab, Pithnu, Snuol, Kratie	0,00
251	CH0065	F	68	Pravanh, Pithnu, Snuol, Kratie	0,00
252	CH0066	F	48	Pravanh, Pithnu, Snuol, Kratie	0,00
253	M0001	F	18	Pravanh, Pithnu, Snuol, Kratie	0,00
254	M0002	M	18	Pravanh, Pithnu, Snuol, Kratie	0,00
255	M0003	F	29	Pravanh, Pithnu, Snuol, Kratie	0,00
256	M0004	F	32	Pravanh, Pithnu, Snuol, Kratie	0,00
257	M0005	M	30	Pravanh, Pithnu, Snuol, Kratie	0,00
258	M0006	M	18	Pravanh, Pithnu, Snuol, Kratie	0,00
259	M0007	F	25	Pravanh, Pithnu, Snuol, Kratie	0,00
260	M0008	F	56	Pravanh, Pithnu, Snuol, Kratie	0,00
261	M0009	M	42	Pravanh, Pithnu, Snuol, Kratie	5,67
262	M0010	F	26	Pravanh, Pithnu, Snuol, Kratie	0,00
263	M0011	F	18	Pravanh, Pithnu, Snuol, Kratie	100,00
264	M0012	F	25	Pravanh, Pithnu, Snuol, Kratie	5,17
265	M0013	M	22	Cheung Khlu, Pithnu, Snuol, Kratie	8,67
266	M0014	F	35	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
267	M0015	F	18	Cheung Khlu, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
268	M0016	F	38	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
269	M0017	M	42	Cheung Khlu, Pithnu, Snuol, Kratie	30,00
270	M0018	M	25	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
271	M0019	F	63	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
272	M0020	M	38	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
273	M0021	F	24	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
274	M0022	M	48	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
275	M0023	M	34	Cheung Khlu, Pithnu, Snuol, Kratie	33,33
276	M0024	M	45	Cheung Khlu, Pithnu, Snuol, Kratie	4,50
277	M0025	M	50	Cheung Khlu, Pithnu, Snuol, Kratie	10,00
278	M0026	F	64	Cheung Khlu, Pithnu, Snuol, Kratie	7,00
279	M0027	F	40	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
280	M0028	F	46	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
281	M0029	F	18	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
282	M0030	M	58	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
283	M0031	F	21	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
284	M0032	F	65	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
285	M0033	M	27	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
286	M0034	M	26	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
287	M0035	F	49	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
288	M0036	F	30	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
289	M0037	M	54	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
290	M0038	M	63	Cheung Khlu, Pithnu, Snuol, Kratie	256,67
291	M0039	F	49	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
292	M0040	M	31	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
293	M0041	F	32	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
294	M0042	F	19	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
295	M0043	M	40	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
296	M0044	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
297	M0045	F	20	Trapaing Srae, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
298	M0046	F	37	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
299	M0047	M	48	Trapaing Srae, Pithnu, Snuol, Kratie	191,67
300	M0048	M	20	Trapaing Srae, Pithnu, Snuol, Kratie	210,00
301	M0049	F	25	Trapaing Srae, Pithnu, Snuol, Kratie	54,00
302	M0050	F	36	Trapaing Srae, Pithnu, Snuol, Kratie	62,50
303	M0051	F	27	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
304	M0052	M	55	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
305	M0053	F	49	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
306	M0054	M	35	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
307	M0055	M	56	Trapaing Srae, Pithnu, Snuol, Kratie	22,00
308	M0056	M	38	Chrab, Pithnu, Snuol, Kratie	0,00
309	M0057	F	39	Chrab, Pithnu, Snuol, Kratie	0,00
310	M0058	F	42	Chrab, Pithnu, Snuol, Kratie	14,00
311	M0059	F	29	Chrab, Pithnu, Snuol, Kratie	0,00
312	M0060	M	55	Chrab, Pithnu, Snuol, Kratie	10,67
313	M0061	F	43	Chrab, Pithnu, Snuol, Kratie	0,00
314	M0062	M	25	Chrab, Pithnu, Snuol, Kratie	0,00
315	M0063	M	27	Chrab, Pithnu, Snuol, Kratie	0,00
316	M0064	M	23	Chrab, Pithnu, Snuol, Kratie	0,00
317	M0065	F	49	Chrab, Pithnu, Snuol, Kratie	0,00
318	M0066	F	38	Chrab, Pithnu, Snuol, Kratie	0,00
319	M0067	F	19	Chrab, Pithnu, Snuol, Kratie	0,00
320	M0068	F	46	Chrab, Pithnu, Snuol, Kratie	0,00
321	M0069	F	47	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
322	M0070	F	48	Trapaing Srae, Pithnu, Snuol, Kratie	3,83
323	M0071	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
324	M0072	F	24	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
325	M0073	F	25	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
326	M0074	F	56	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
327	M0075	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00

No.	PID	Gender	Age	Location	EPG
328	M0076	M	25	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
329	M0077	M	29	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
330	M0078	M	45	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
331	M0079	F	30	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
332	M0081	F	24	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
333	M0082	M	40	Trapaing Srae, Pithnu, Snuol, Kratie	966,67
334	M0083	F	23	Cheung Khle, Pithnu, Snuol, Kratie	0,00
335	M0084	F	30	Chrab, Pithnu, Snuol, Kratie	0,00
336	M0085	F	27	Chrab, Pithnu, Snuol, Kratie	18,00
337	M0086	F	25	Chrab, Pithnu, Snuol, Kratie	10,00
338	M0087	F	28	Pravanh, Pithnu, Snuol, Kratie	0,00
339	M0088	F	33	Pravanh, Pithnu, Snuol, Kratie	0,00
340	M0089	F	33	Pravanh, Pithnu, Snuol, Kratie	0,00
341	M0090	F	35	Pravanh, Pithnu, Snuol, Kratie	0,00
342	M0091	F	40	Pravanh, Pithnu, Snuol, Kratie	3,67
343	M0092	M	22	Pravanh, Pithnu, Snuol, Kratie	7,67
344	M0093	M	27	Chrab, Pithnu, Snuol, Kratie	0,00
345	M0094	M	30	Chrab, Pithnu, Snuol, Kratie	11,00
346	M0095	M	18	Chrab, Pithnu, Snuol, Kratie	0,00
347	M0096	M	24	Chrab, Pithnu, Snuol, Kratie	0,00
348	M0097	M	48	Chrab, Pithnu, Snuol, Kratie	4,50
349	M0098	F	45	Chrab, Pithnu, Snuol, Kratie	180,00
350	M0099	M	18	Chrab, Pithnu, Snuol, Kratie	0,00
351	M0100	F	68	Trapaing Srae, Pithnu, Snuol, Kratie	5,17
352	M0101	F	31	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
353	M0102	F	46	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
354	M0103	M	27	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
355	M0104	M	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
356	M0105	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
357	M0106	M	25	Trapaing Srae, Pithnu, Snuol, Kratie	8,67

No.	PID	Gender	Age	Location	EPG
358	M0107	M	19	Trapaing Srae, Pithnu, Snuol, Kratie	16,00
359	M0108	F	45	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
360	M0109	M	35	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
361	M0110	M	25	Trapaing Srae, Pithnu, Snuol, Kratie	17,00
362	M0111	F	20	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
363	M0112	F	40	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
364	M0113	M	28	Trapaing Srae, Pithnu, Snuol, Kratie	8,33
365	M0114	F	18	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
366	M0115	M	24	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
367	M0116	M	19	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
368	M0117	M	28	Cheung Khlu, Pithnu, Snuol, Kratie	10,33
369	M0118	M	39	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
370	M0119	M	18	Cheung Khlu, Pithnu, Snuol, Kratie	9,00
371	M0120	F	24	Cheung Khlu, Pithnu, Snuol, Kratie	20,00
372	M0121(n)	F	52	Cheung Khlu, Pithnu, Snuol, Kratie	0,00
373	M0122(n)	F	28	Chrab, Pithnu, Snuol, Kratie	0,00
374	M0123(n)	M	33	Chrab, Pithnu, Snuol, Kratie	0,00
375	M0124(n)	M	27	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
376	M0125(n)	F	24	Trapaing Srae, Pithnu, Snuol, Kratie	0,00
377	M01126(n)	M	29	Trapaing Srae, Pithnu, Snuol, Kratie	0,00

APPENDICES F

SUT IBC

เลขที่รับรอง SUT-IBC-006/2022

ใบรับรองการดำเนินงานด้านความปลอดภัยทางชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี

ชื่อโครงการ : Discrimination of Human Carcinogenic Liver Fluke-Like Eggs from Cambodia using Parasitological and Molecular methods.

รหัสโครงการ : IBC-65-05

หัวหน้าโครงการ : Mr.Nav La

อาจารย์ที่ปรึกษา : รองศาสตราจารย์ แพทย์หญิง ชวัลญ์ญา รัตนพิทูลย์

สังกัด : สำนักวิชาแพทยศาสตร์

ประเภทงานวิจัย : งานวิจัยประเภทที่ 1

ระดับห้องปฏิบัติการ : Biosafety Level 1 (BSL 1)

รายงานความก้าวหน้า : ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี

ข้อเสนอโครงการวิจัยและเอกสารประกอบของข้อเสนอโครงการวิจัยนี้ ได้รับการพิจารณาจากคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มหาวิทยาลัยเทคโนโลยีสุรนารีแล้ว คณะกรรมการฯ ลงความเห็นว่า ข้อเสนอโครงการวิจัยที่จะดำเนินการมีความสอดคล้องกับแนวทางปฏิบัติเพื่อความปลอดภัยทางชีวภาพ และพระราชบัญญัติเชื้อโรคและพิษจากสัตว์ พ.ศ. 2558 จึงเห็นสมควรให้ดำเนินการวิจัยตามข้อเสนอการวิจัยนี้ได้

กรณีที่มีการปฏิบัติอย่างหนึ่งอย่างใดนอกเหนือจากที่กรอกไว้ในข้อมูลและที่เสนอไว้ในโครงการ คณะกรรมการฯ จะดำเนินการงดใบรับรองนี้ และแจ้งมายังคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ มทส.หรือหน่วยงานที่เกี่ยวข้องทราบ

ลงชื่อ.....

(รองศาสตราจารย์ ดร.ระพี อูทเคอ)

ประธานคณะกรรมการควบคุมความปลอดภัยทางชีวภาพ

มหาวิทยาลัยเทคโนโลยีสุรนารี

วันที่ออกใบรับรอง 8 เมษายน 2565

วันที่ใบรับรองหมดอายุ 7 เมษายน 2566

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PERSONAL INFORMATION

Date of Birth : 01-January-1987

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Nationality : Cambodian (Khmer)

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Marital Status : Single

EDUCATION HISTORY

2022-2023 : Ph.D. candidate in Translational Medicine (International program), Institute of Medicine, Suranaree University of Technology (SUT), Nakhon Ratchasima 30000, Thailand

2019-2021 : MSc. in Translational Medicine (International program), Institute of Medicine, Suranaree University of Technology (SUT), Nakhon Ratchasima 30000, Thailand

2013-2014 : Resident at ward of Medicine, Khmer-Soviet Hospital (6 months)

: Resident at ward of Surgery, Khmer-Soviet Hospital (6 months)

: Resident at ward of Pediatric, Referral Hospital of Phnom Penh (6 months)

: Resident at Ward of Gynecology & Obstetric, Preah Ketomilea Hospital (6 months)

2006-2012 : Bachelor of Health Science, Faculty of Pediatric and Medicine, International University (IU), Phnom Penh, Cambodia.

SCHOLARSHIP

1. Thailand Science Research and Innovation (TSRI), and National Science, titled "SUT Scholarship for Graduate International Students (Vithedbundit)" and dated October 7, 2016.
2. Suranaree University of Technology, Thailand Science Research and Innovation (TSRI), and National Science, Research, and Innovation Fund (NSRF) (NRIIS number 160366 and NRIIS number 179304).
3. Parasitic Disease Research Center, Institute of Medicine, SUT, Thailand

PhD. THESIS

International Journal Publication

1. LA N., Leng, M., Rattanapitoon, K., Pechdee, P., Boonsuya, A., Arunsan, P., & Rattanapitoon, S. K. (2022). Intestinal parasitic infections and risk factors among the population in Cambodia. *Tropical biomedicine*, *39*(4), 539–546. https://doi.org/10.47665/tb.39.4.009

International Journal Publication Acceptant

1. LA N, Leng M, Rattanapitoon K, Pechdee P, Boonsuya A, Arunsan P, Rattanapitoon SK. Molecular identification of *Opisthorchis viverrini* among the northeastern Cambodian population by internal transcribed spacer 2 based polymerase chain reaction. *Trop Biomed*. JTB2-23-0409. 27th August 2023

International Congress Poster Presentation

1. NAV LA, Monica Leng, Patpitcha Arunsan, Phornphitcha Pechdee, Alisa Boonsuya, Chutharat Thanchonnang, Nathkapach Rattanapitoon, and Schawanya Rattanapitoon. Dection of Opisthorchiasis Among Northeastern Population using Parasitological and Molecular Methods. In the 19th *Asia Pacific Congress of Clinical Microbiology and Infection* on 06-08 July 2023 at COEX, Seoul, Korea