

**MINISTRY OF EDUCATION
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**MINISTRY OF HEALTH
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TRANG PHAM THI HA

**A STUDY ON THE PREVALENCE OF SMALL LIVER
FLUKE INFECTION IN TWO KEY COMMUNES IN NINH
BINH AND PHU YEN PROVINCE, AND THE
DEVELOPMENT OF LAMP KITS FOR COMMUNITY
DIAGNOSIS**

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For more information, please visit:

1. National library of Vietnam
2. Institute of Malariology - Parasitology and Entomology's library.

LIST OF THESIS-RELATED PUBLICATIONS OF THE AUTHOR

1. Trang Pham Thi Ha, Hanh Truong Van, Canh Hoang Dinh, Duong Tran Thanh, (2024), Prevalence and some factors relates to *clonorchis sinensis* in human in Yen Loc commune, Kim Son district, Ninh Binh province (2018-2020). *Vietnam medical journal*. Vol 538, May 2024 (N₀2) Article is accepted.
2. Trang Pham Thi Ha, Hanh Truong Van, Canh Hoang Dinh, Duong Tran Thanh, (2024), Prevalence and some factors relates to *Opisthorchis viverrini* in human at An My commune, Tuy An district, Phu Yen province (20218-2019). *Vietnam medical journal*. Vol 538, May 2024 (N₀2), page.... Article is accepted.
3. Trang Pham Thi Ha, Hanh Truong Van, Canh Hoang Dinh, Duong Tran Thanh, (2024), Research on manufacturing LAMP kit to diagnose *Clonorchis sinensis* infection in humans. *Vietnam Journal of Science and Technology*, Vol X(N₀ Y) page. page.... Article is accepted.

INTRODUCTION

Small liver fluke infection is a food-borne infectious disease that has a major impact on public health. Vietnam, being a tropical zone, has natural and social conditions, as well as dietary, agricultural, lifestyle, and living habits, which are the main factors contributing to the high prevalence of the disease. This significantly affects the overall health of the community, particularly with regard to *C. sinensis* and *O. viverrini*.

Vietnam has recorded two species of small liver flukes, *C. sinensis* and *O. viverrini*, distributed in at least 32 provinces and cities. The provinces of Ninh Binh and Phu Yen are considered hotspots with relatively high rates of small liver fluke infection, attributed to various factors, among which the primary cause is the long-standing habit of consuming raw fish salad by the local population.

There are many methods to detect the presence of small liver flukes. Recently, many studies have tended to switch to isothermal DNA amplification methods, especially Loop-mediated Isothermal Amplification (LAMP), due to their numerous advantages. However, in Vietnam, a commercialized LAMP kit for diagnosing small liver flukes has not yet been available. Given the scientific and practical requirements mentioned above, an in-depth study is necessary. Therefore, we conducted research on the topic: **“A study on the prevalence of Clonorchis sinensis infections in two main priority communes in Ninh Binh, Phu Yen province, and the application of LAMP Kit for diagnosis in the community”**, with the following objectives:

1. *Description of the current situation and some associated factors of small liver fluke infection in humans in two key communes in Ninh Binh and Phu Yen provinces (2018-2020).*
2. *Development of a LAMP kit for testing Clonorchis sinensis and Opisthorchis viverrini infections in humans at a laboratory scale.*

SCIENTIFICNESS AND NOVELTY OF TOPIC

1. Description and evaluation of the prevalence of *C. sinensis* và *O. viverrini* infection in humans and factors related to disease in Yen Loc and An My communes of Ninh Binh and Phu Yen provinces. This contributes to the prognosis and effective development of prevention strategies for *C. sinensis* and *O. viverrini*, especially considering the existing habit of consuming fish salad.

2. This is the first study to complete the process and successfully manufacture a LAMP kit for diagnosing *C. sinensis* and *O. viverrini* infections in humans in Vietnam, marking a breakthrough in technical solutions for diagnosing these infections. It contributes to early diagnosis and timely treatment, meeting the practical requirements for preventing *Clonorchis sinensis* infections specifically and intestinal parasitic diseases in our country in general.

STRUCTURE OF THESIS

The thesis covers 140 pages, including: Introduction with 2 pages; Overview with 3. pages; Researching object and methods with 23 pages; Researching findings with 41 pages; Discussion with 39 pages; Conclusion with 2 pages; and Petition with 1 page. The thesis includes 39 figures and 45 tables. There are 127 references, of which more than 40% of the documents have been published within the last 5 years.

Chapter 1: Overview

1.1. Some epidemiological characteristics of liver fluke infection

1.1.1. Pathogens

There are three small liver fluke species belonging to the Opisthorchiidae family worldwide, which cause diseases in humans: *Clonorchis sinensis*, *Opisthorchis viverrini*, and *Opisthorchis felineus*. These species share relatively similar biological, life cycle, and clinical characteristics. In Vietnam, only two small liver fluke species are currently recorded: *C. sinensis* and *O. viverrini*.

1.1.2. Host

- Main hosts include humans and some fish-eating animals.
- The first intermediate host includes many different species of snails, depending on the research sites.
- The second intermediate hosts of small liver flukes include many freshwater fish species.

1.1.3. Transmission and mechanism of disease

1.1.3.1. Transmission route of disease:

Eating fish salad or undercooked fish infected with cysts is the primary risk factor for small liver fluke infection in humans. The infection rate is high in communities where fish salad is commonly consumed.

1.1.3.2. Mechanism of disease transmission:

Small liver fluke are parasites with complex life cycles and involves many intermediate hosts as well as several free-living stages.

1.1.4. Sensitivity and immunity

Everyone can become infected when exposed to the small liver fluke. Those without natural immunity to the small liver fluke, after contracting the parasite, may easily become reinfected.

1.1.5. Factors associated with small fluke infection.

1.1.5.1. Eating fish salad habit:

Eating fish salad is one of the leading risk factors for patients infected with small liver flukes, a fact supported by numerous studies conducted in Vietnam and several other Asian countries

1.1.5.2. Age, gender, occupation

Age: In general, liver fluke infection is observed in all age groups; however, the disease is more common in adults. The prevalence of infection is significantly correlated with increasing age

Gender: Epidemiological data worldwide show that the prevalence of small liver flukes in males is significantly higher than in females.

Occupation: this is also one of the risk factors related to small liver fluke infection.

1.1.5.3. Some other factors:

- Socio-economic factors.
- Lifestyle and farming habits.
- Natural environmental factors.

1.1.6. Prevention measures

- Do not eat fish salad or raw and uncooked fish.
- Avoid using human feces to raise fish, and refrain from defecating into water sources.
- Health communication and education for the community on liver fluke prevention.
- Periodically deworming dogs, cats, and pigs.

- Prevention and treatment in the community: Apply the Guidelines for liver fluke eradication in the community according to Decision No. 1931/QĐ-BYT, May 19th, Vietnamese Ministry of Health

1.1.7. Characteristics of distribution and prevalence of liver fluke infection in humans in the world and Vietnam

1.1.7.1. Distribution characteristics and prevalence of liver fluke infection in the world

In the world, the distribution of small liver fluke disease varies greatly among different countries. There are about 680 million people at

risk of infection in the world. An estimated 45 million people living in Asia and Europe are suffered from disease (about 35 million with *C. sinensis*, 10 million with *O. viverrini*, and 1.2 million with *O. felineus*).

C. sinensis is one of the most common among the three small liver fluke species that cause disease in humans, mainly in Asia, including China, Korea, northern Vietnam, and eastern Russia. Furthermore, migration or traveling from endemic areas has increased, which is a leading risk factor for transmitting the disease to other countries.

Similar to *C. sinensis*, people infected with *O. viverrini* often live in areas with many ponds, lakes, and along rivers. There are an estimated 12.39 million people infected with *O. viverrini* in four endemic countries, including Thailand (6.71 million), Lao PDR (2.45 million), Vietnam (2.07 million), and Cambodia (1 million).

O. felineus is the least common of the three liver fluke species. It is reported in European countries except for Finland, Norway, and Sweden. There are about 12.5 million people at risk of *O. felineus* infection, mainly in Russia and Eastern Europe.

1.1.7.2. Characteristics of distribution and prevalence of small liver fluke infection in Vietnam

Vietnam is a developing country, and differences in the distribution of climate zones between the North and South create favorable conditions for the appearance and distribution of two small liver fluke species: *C. sinensis*, which circulates in at least 21 northern provinces, and *O. viverrini*, which affects 11 central provinces and the Central Highlands region, with infection rates varying depending on the location.

Nam Dinh (with the highest rate ranging from 26.0% to 37.5%), Ninh Binh (with infection rates fluctuating between 23.5% and 31.0%), Phu Yen, and Binh Dinh are considered infection hotspots, where consuming fish salad is a long-standing habit

The survey data from the Institute of Malaria - Parasitology - Entomology over the past five years has shown varying rates of liver fluke infection in several provinces. In Binh Dinh, Quang Tri, Dak Lak, Phu Yen, Quang Nam, Hoa Binh, Nam Dinh, Ninh Binh, Yen Bai, and Thanh Hoa, the infection rates are 6.8%, 8.9%, 4.8%, 15.3%, 4.3%, 24.4%, 11.8%, 21%, 21.6%, and 23% to 64.7%, respectively. Only Son La has an infection rate of less than 1%. However, many provinces still have the habit of eating fish salad without evaluation or current prevalence data on liver fluke infection, hindering intervention and prevention measures.

1.1.7.3 Some characteristics and incidence of small liver fluke infection in Ninh Binh and Phu Yen communes.

- In Ninh Binh:

A study conducted by Doan Thuy Hoa (2020) in Kim Son and Yen Khanh, Ninh Binh, showed that the rate of liver fluke infection is 20.1%. The average infection rate and intensity of small liver fluke infection in men are higher than in women ($p < 0.001$; OR = 3.994). People who eat fish salad are likely to be infected with small liver flukes 5.8 times more than those who do not eat fish salad ($p < 0.001$). The majority were slightly infected (87.2%); nobody was severely infected.

- In Phu Yen:

A survey conducted by the Institute of Malaria - Parasitology - Entomology from 2015 to 2018 showed that *O. viverrini* is mainly distributed in the Central provinces and the Central Highlands region, of which Phu Yen account for 15.3%.

1.2. Liver fluke - clinical features, diagnosis, and treatment

1.2.1. Clinical characteristics of Liver fluke in human.

Majority of infected cases have no clinical symptoms. There are three levels of the disease: light, moderate, and severe.

1.2.2. Diagnose

According to instructions in Decision No. 1172/QĐ-BYT, May 13, 2022, Vietnamese Ministry of Health.

1.2.3. Liver fluke – treatment.

All patients were treated with Praziquantel (oral dose, 75mg/kg).

1.3. Methods for diagnosis of small liver fluke infection

Kato-Katz method, immunoassay for diagnose small liver flukes (ELISA reaction - enzyme-Linked immunosorbent assay), molecular biology methods.

1.4. Developing LAMP technique and researching the production of LAMP test kits to detect small liver fluke infection in humans

1.4.1. LAMP principle

The LAMP technique uses 4-6 different primers specifically designed to recognize 6-8 distinct regions on the target gene, and the reaction takes place at a unique temperature (55°C-65°C). Reaction products can be observed with the naked eye. LAMP is often used to create rapid diagnostic test kits.

1.4.2. LAMP reaction component KIT

Basic components of the LAMP reaction: Primer pairs, buffer solution, Bst DNA polymerase enzyme, template DNA, dye (if any).

1.4.3. Mechanism of LAMP reaction

Three main stages: generation of primary material, replication and elongation of chain, and repeating the cycle.

1.4.4. Evaluate of LAMP's results

Evaluate results with and without electrophoresis.

1.4.6. The superiority of LAMP technique

The advantage lies in its speed, simplicity, ease of performance, and lack of requirement for specialized equipment, while maintaining sensitivity and specificity equivalent to or higher than methods based on PCR technique. The results of the LAMP reaction can be observed immediately under normal light or UV light.

1.4.7. Applications of LAMP technique in diagnose for small liver fluke infection in human.

A study conducted by Le Thanh Hoa, Nguyen Van De, and colleagues (2012) found that the LAMP test is more sensitive, detecting small liver fluke *O. viverrini* 10-100 times better than PCR.

From 2017 to 2020, the Institute of Malaria - Parasitology - Entomology carried out the National Scientific Research Task titled "Research and manufacture of a LAMP kit to diagnose malaria parasites, fascioliasis, small liver flukes, and *Strongyloides stercoralis* in field research." As a result, we successfully researched and manufactured LAMP kits, including the LAMP Kit for diagnosing small liver flukes *C. sinensis* and *O. viverrini*. The sensitivity and specificity of laboratory testing are high, exceeding 95%.

1.4.9. Fabrication and standardization of LAMP kits for diagnosing small liver flukes

- Design primers to identify the desired pathogen;
- Develop a process of LAMP reactions;
- Evaluate the sensitivity, specificity, and detection threshold of the LAMP reaction;
- Create a positive standard for the kit;
- Compare with sensitivity, specificity, detection threshold;
- Packaging of biological products;
- Evaluate the stability of the biological kit on standard samples.

Chapter 2: OBJECT AND RESEARCHING METHODS

2.1. Objective 1: *Description of the current situation and some associated factors of small liver fluke infection in humans in two key communes in Ninh Binh and Phu Yen provinces (2018-2020).*

2.1.1. *Researching objects, sites and time*

- Research subjects: People over 18 years old living more than 1 year at 2 communes in Ninh Binh and Phu Yen province.

- Time: 459 people in Ninh Binh (2018,2020), 460 people in Phu Yen province (2019,2020).

Department of Molecular biology

- Research location

+ Field research: Yen Loc commune, Kim Son district, Ninh Binh province, An My commune, Tuy An district, Phu Yen province.

+ Department of parasitology (NIMPE).

+ Dang Van Ngu hospital (NIMPE).

2.1.2. *Researching methods*

2.1.2.1. *Study design*

Analytical descriptive study with cross-sectional surveys.

- *Research sample size*: Based on the formula for calculating sample size, a ratio is estimated:

$$n = Z_{1-\alpha/2}^2 \frac{p(1-p)}{d^2}$$

In this formula, n represents the minimum sample size required for research each commune in a province. $Z_{1-\alpha/2}$ denotes the reliability coefficient, corresponding to probability threshold $\alpha = 0.05$ (95% confidence), $Z_{1-\alpha/2} = 1.96$. p stands for the estimated infection rate, according to the report of the Institute of Malaria - Parasitology - Entomology, this rate is 0.21. d represents the absolute allowable error, with d chosen as 0.038. With these selected values, the calculated sample size is 441 people/site. The study conducted included 460 people in An My commune (Phu Yen) and 459 people in Yen Loc commune (Ninh Binh).

- The inclusion criteria and sampling method

Select samples using the cluster sampling method combined with the systematic sampling method for each cluster. Collect fecal samples from each individual until the research sample size is sufficient. Select subjects who are able to answer interviews, agree to participate in the study, and provide stool samples. Exclusion criteria include people with mental illness and those who have taken deworming medication within the past 6 months.

- Research content:

To determine the rate and intensity of fluke infection in humans, as well as some factors related to this disease.

- Research techniques

KAP investigation interviewing techniques, Kato-Katz technique, real time PCR technique identifies small liver flukes.

- Evaluation indicators

The rate and intensity of small liver fluke infection. Some factors related to small liver fluke infection.

- Research variables

Age, gender, occupation, education level, use of a hygienic toilet, ownership of fishing ponds, use of fresh manure for fish and crop farming, ownership of dogs/cats, and the habit of consuming fish salad (fresh fish).

- Statistical analyses: SPSS software for Windows, version 22.0, was utilized for statistical analysis of the obtained data.

2.2 Target 2: Development of a LAMP kit for testing *C. sinensis* and *O. viverrini* infections in humans at a laboratory scale.

2.2.1. Research objects, study sites and time

2.2.1.1. *Objects*

- Small liver fluke *O. viverrini* and *C. sinensis*.

- Adults or larvae of some other species of tapeworms and worms collected from humans or animals, including: *H. taichui*, *H. pumilo*, *F. gigantica*, *P. heterotremus*, *T. solium*, *A. duodenale*, *A. lumbricoides*, and *O. viverrini*.

2.2.1.2. *Research time and location*

Time: From 2018 -2020

Study sites: An My commune (Phu Yen) and Yen Loc commune (Ninh Binh), as well as the Laboratory of the Institute of Malaria - Parasitology - Entomology, conducted LAMP kit auditing at the National Institute for Control of Vaccine and Biologicals under the Ministry of Health.

2.1.2. Researching methods

2.1.2.1. *Study design*

Experiments in the Laboratory and in the field research

- Research sample size: Evaluate the sensitivity and specificity of the kit at the Laboratory:

$$n_{se} = \frac{z^2_{1-\alpha/2} \text{Pse} (1 - \text{Pse})}{W^2 \cdot P} = 72,9 \quad n_{sp} = \frac{z^2_{1-\alpha/2} \text{Pse} (1 - \text{Pse})}{W^2 \cdot (1-P)} = 24,3$$

In this context: $Z_{1-\alpha/2}$ represents the reliability coefficient at 95% confidence level, with $Z_{1-\alpha/2} = 1.96$. The estimated positivity rate (p) is

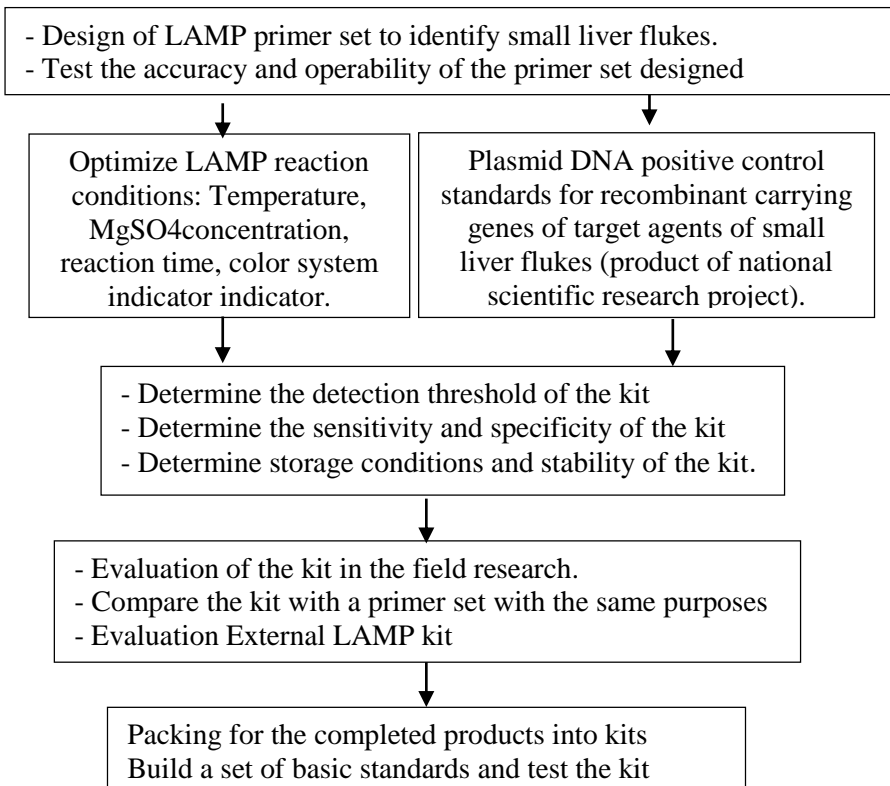
25%. The expected sensitivity of the LAMP kit (P_{se}) is 95%, and the expected specificity (P_{sp}) is 95%. The absolute allowable error of sensitivity and specificity (W) is 10%. The calculated minimum sample size for determining sensitivity is 72.9, and for specificity, it is 24.3. In fact, 100 samples were collected, with 73 positive samples and 27 negative samples.

- Evaluation the effect of kit in the field research: 150 samples/province

2.2.2.3. *Research techniques*

- Design primers using specialized bioinformatics software. Real-time PCR technique. Statistical processing using MedCalc 19.4.1 software.

2.2.2.5. *Research content: Research diagram manufacturing LAMP kits.*



2.2.2.6. *Statistical methods and data analysis*

The data analysis is performed using biomedical statistical methods and Quickcalc, and Medcalc software

2.2.2.7. The Method of control bias and confounding in research

Control interview process to research subjects and stool testing procedure. Encrypt data.

2.3. The Ethics of Research

The research outline of the topic was approved by the Ethics Committee in Biomedical Research of the National Institute of Malaria-Parasites-Entomology. Research subjects' consent is obtained. The rights and obligations of research participants and the responsibilities of researchers are described. Cases infected with parasites receiving treatment according to the protocol of the Ministry of Health.

CHAPTER 3. RESEARCH RESULTS

3.1. Description of the current situation and some associated factors of small liver fluke infection in humans in two key communes in Ninh Binh and Phu Yen provinces (2018-2020).

3.1.1. Characteristics of research subjects

Table 3.1. Demographic characteristics of the research population (n=919)

Index	Classification	Yen Loc commune Ninh Binh (459)		An My commune Phu Yên (460)		Total (919)	
		n	%	N	%	n	%
Gender	Male	218	47.5	196	42.6	414	45.0
	Female	241	52.5	264	57.4	505	55.0
Age groups	18 – 29	39	8.5	72	15.7	111	12.1
	30 – 49	212	46.2	210	4.7	422	45.9
	50 - 59 year	129	28.1	145	31.5	274	29.8
	≥ 60 years	79	17.2	33	7.2	112	12.2
Occupation	Farmer	291	63.4	322	70.0	613	66.7
	Worker	38	8.3	19	4.1	57	6.2
	State office, retirement	31	6.8	33	7.2	64	7.0
	Others: students, business, freeland	99	21.6	86	18.7	185	20.1
Education level	Primary	100	21.8	115	25	215	23.4
	Junior high school	157	34.2	175	38	332	36.1
	High school	152	33.1	135	29.3	287	31.2
	Intermediate professional school, college university	50	10.9	35	7.6	85	9.2

The study participation of the age group 30 to 49 years old accounted for the highest proportion (45.9%). Females make up 55% of the participants, with the majority being farmers (66.7%), almost all of whom have completed junior high school (36.1%) or high school (31.2%).

3.1.2. The rate and intensity of small liver fluke infection in humans at the study sites

Bảng 3.3. The rate of small liver fluke infection in humans (n=919)

Commune (Province)	Method	Test for small liver fluke infection				p
		Kato-Katz		Real-time PCR		
		n	%	n	%	
Yen Loc commune- Ninh Binh (459)		75	16.33	89	19.39	< 0.05
An My commune -Phu Yen (460)		73	15.90	93	20.22	< 0.05
Total (919)		148	16.10	182	19.80	< 0.05

The overall rate of liver fluke infection tested by real-time PCR is 19.80%, with a *C. sinensis* infection rate in Yen Loc commune of Ninh Binh at 19.39% and an *O. viverrine* infection rate in An My commune in Phu Yen at 20.22%. When compared with the Kato-Katz testing technique (16.10%), including Yen Loc commune of Ninh Binh (16.33%) and An My commune in Phu Yen (15.90%), the rate was statistically significantly higher ($p < 0.05$).

Table 3.5. Proportion of patients by gender (n = 919)

Local		Testin g	Liver fluke infection		p
Commune(Province)	Gender		n	%	
Yen Loc commune (Ninh Binh)	Male	218	62	28.44	< 0.05
	Female	241	27	11.20	
	Total	459	89	19.39	
An My commune (Phu Yen)	Male	196	49	25.00	< 0.05
	Female	264	44	16.67	
	Total	460	93	20.22	
General	Male	414	111	26.81	< 0.05
	Female	505	71	14.06	
	Total	919	182	19.80	

The overall rate of liver fluke infection is 26.81% among men and 14.1% among women, which is statistically significant ($p < 0.05$). Similar results are observed in An Loc commune (Ninh Binh) and Yen My commune (Phu Yen).

Table 3.8. The average intensity of small liver fluke infection at the research sites (n =148)

Commune (Province)	Yen Loc commune (Ninh Binh)		An My commune (Phu Yen)		Total	
	n (%)	EPG	n (%)	EPG	n (%)	EPG
Light	69 (92.0)	241.9 ± 109.0	64 (87.7)	553.3 ± 226.8	133 (89.9)	395.5 ± 126.8
Moderate	6 (8)		9 (12.3)		15 (10.1)	
Severe	0		0		0	
Total	75		73		148	

Almost all patients have a light infection (89.9%), with no severe cases reported. Similar results are observed in Yen Loc commune (Ninh Binh) and An My commune (Phu Yen). The overall infection intensity in the two provinces is 395.5 ± 126.8 eggs per gram (EPG) (Ninh Binh: 241.9 ± 109.0 EPG, Phu Yen: 553.3 ± 226.8 EPG).

3.1.3. Some related factors for liver fluke infection in humans

3.1.3.1. Correlation between liver fluke infection and fish salad habits

Table 3.14. Correlation between liver fluke infection and traditional raw fish salad (n=919)

Commune (Province)	Frequency	Small liver fluke infection			OR, 95% CI	P
		Yes	No	Total		
Yen Loc commune Ninh Binh	Eating	79	194	273	7,17 (3.60-14.27)	< 0.05
	Never	10	176	186		
	Total	89	370	459	-	
An My commune (Phu Yen)	Eating	82	157	239	9.9 (5.14-13.78)	< 0.05
	Never	11	210	221		
	Total	93	367	460	-	
General	Eating	161	351	512	8.43 (5.23-13.57)	< 0.05
	Never	21	386	407		
	Total	182	737	919	-	

There is a correlation between liver fluke infection and fish salad habits at research location in An My commune (Phu Yen) and Yen Loc commune (Ninh Binh), with odds ratios (OR) and 95% confidence intervals (CI) of 7.17 (3.6 - 14.27), 9.9 (5.14 - 13.78), and 8.43 (5.23 - 13.57), respectively.

Table 3.17. Intensity of small liver fluke infection and frequency of eating raw fish (n=161)

Commune (Province)	Yen Loc commune (Ninh Binh)			An My commune (Phu Yen)			Total		
	n	%	p	n	%	p	n	%	P
At least once / a week	22	43.1	<0.05	20	48.8	<0.05	42	45.7	<0.05
At least once / a month	27	28.4		34	35.8		61	32.1	
twice-3 times/6 months	30	23.6		28	27.2		58	25.2	
Total	79	28.9		82	34.3		161	31.4	

The rate of liver fluke infection is proportional to the frequency of eating raw fish salad. Individuals who consume raw fish once a week have the highest infection rate (45.7%), nearly double that of those who eat fish salad two to three times every six months (25.2%). This difference in results is statistically significant with $p < 0.05$.

Table 3.18. Rate of small liver fluke infection and the source of fish used for eat salad (n=161)

Commune (Province)	Yen Loc commune (Ninh Binh)			An My commune (Phu Yen)			Total		
	n	%	P	n	%	p	n	%	P
Family's pond	19	20.0	<0.05	25	26.9	<0.05	44	23.4	<0.05
Neighbour's pond	36	33.3		23	25.8		59	29.9	
River/ditch	21	39.6		26	63.4		47	50.0	
Market	3	17.6		8	50.0		11	33.3	
Total	79	28.9		82	34.3		161	31.3	

The highest rate of liver fluke infection (50.0%) is observed in people who consumed fish salad originating from rivers or canals, and this result is statistically significant ($p < 0.05$).

3.1.3.6. *Multi-variate analysis of risk factors associated with small liver fluke infection.*

Table 3.23. Multi-variate analysis of associated risk factors for small liver fluke infection in two communes in Ninh Binh and Phu Yen province

Factors	OR (95% CI)	p-value
Toilet type Unhygienic Hygienic (ref)	1,29 (0,84 – 1,98) 1	> 0,05
Using manure aged under 6 months in agricultural activities Yes No (ref)	1,98 (1,3 – 3,02) 1	< 0,05
House with fish pond Yes No (ref)	0,93 (0,65 – 1,33) 1	> 0,05
Rasing dogs and cats Yes No (ref)	1,33 (0,93 – 1,90) 1	> 0,05
Eating raw fish Yes No (ref)	8,85 (5,46 – 14,34) 1	< 0,05
linear regression: Y (xi)= -0,028 + 0,69 (Utilizing with manure compost in agriculture less than 6 months) +2,18 (eating fish salad)		

+ People Who have eating fish salad habit are 8.85 times more likely to be infected with small liver flukes than people who do not eat fish salad. This result is statistically significant 95%CI: 5,46 – 14,34; $p < 0,05$.

+ People who utilize compost for less than 6 months in agriculture are 1.98 times more likely to be infected with small liver flukes than people who use compost for more than 6 months. The difference is statistically significant with 95%CI: 1,3 – 3,02; $p < 0,05$.

3.2. Development of a LAMP kit for testing *Clonorchis sinensis* and *Opisthorchis viverrini* infections in humans at a laboratory scale

3.2.1. Design, evaluate and select LAMP primer set design

Based on the technical criteria for evaluation, selection 2 primers sets were selected for *O. viverrini* và *C. sinensis*.

3.2.2. Valuation of the specificity and activity of the designed LAMP primer sets.

3.2.2.1. Specificity of the theoretically designed LAMP primer set

The primer sequences were blasted on the NCBI database. They specifically pair with the nad1 target gene sequences of *O. viverrini* and *C. sinensis* without cross-hybridization with other species.

3.2.2.2. Results of investigation the specificity of the designed experimentally LAMP primer set

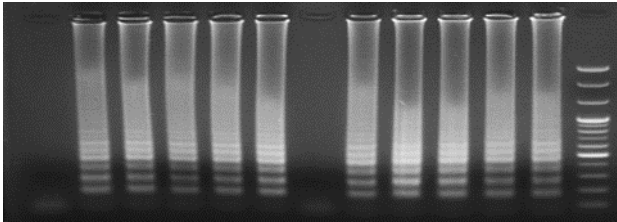


Figure 3.2. LAMP product electrophoresis on 2% agarose gel uses primer sets specifically designed for small liver flukes *O. viverrini* and *C. sinensis*

HOV1-Nad1 primer sets identify *O. viverrini*, HCS-Nad1, *C. sinensis* with good performance.

3.2.3. Survey and optimization of LAMP reaction conditions

3.2.3.1. Result of survey of optimal primer annealing temperature

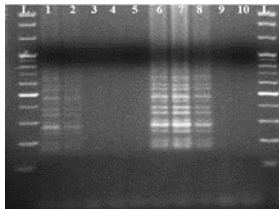


Figure 3.4. The electrophoretic image of the product investigates the primer incubation temperature of the LAMP reaction for diagnosing *O. viverrini*.

Land L: 100 bp DNA ladder; lands 1-10: Survey temperature from 59-68°C, each well is 1°C apart; land 6: LAMP with annealing temperature range of 64°C.

The survey results identified the optimal temperature for the LAMP reaction in diagnosing *O. viverrini* as 64°C and *C. sinensis* as 63°C.

3.2.3.2. Concentration survey results $MgSO_4$

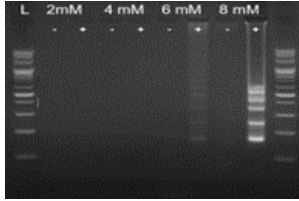


Figure 3.5. Patterns of LAMP products at different concentration of $MgSO_4$.

Land L: 100 bp DNA ladder; land (-): negative control; land (+): DNA concentration template 10^{-3} ng/ μ l.

The results shows that the $MgSO_4$ concentration of 8 mM was chosen is the optimal concentration for the LAMP kit.

3.2.3.3. The result of betaine concentration in the LAMP reaction

The results of adding an additional 0.2 M concentration of betaine to the LAMP reaction components for diagnosing *O. viverrini* and not adding betaine to the LAMP reaction components for diagnosing *C. sinensis*.

3.2.3.4. Results of surveying the total amount of DNA volume in the reaction

The result of choosing the DNA in the reaction is 4 μ l.

3.2.3.5. Survey the optimal time for the LAMP reaction

As a result, 60 minutes was chosen as the optimal time for the reaction of the LAMP kit

3.2.3.6. LAMP reaction survey results of color indicator

HNB concentration of 100mM was selected is the optimal concentration for Lam kits

3.2.3.7. Detection threshold survey of LAMP kit for diagnose small liver fluke

Phase I: The results of determining the primary detection threshold of the LAMP kit for diagnosing *O. viverrini* are 10^{-7} ng/ μ l (3.08×10^1 copies/ μ l), and for *C. sinensis*, it is 10^{-7} ng/ μ l (3.06×10^1 copies/ μ l).

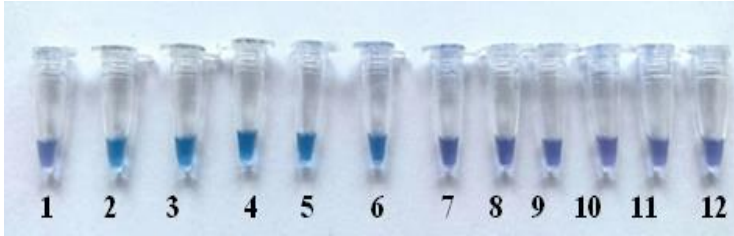


Figure 3.9. Photos of LAMP products observed with the naked eye determine the primary detection threshold of the LAMP kit

Phase II: Analysis results determined that the 95% LOD detection threshold of LAMP kit for diagnose *O. viverrini* is 2.30×10^1 gene copies/ μl (95% CI: 1.77×10^1 - 3.69×10^1 gene copies/ μl); *C. sinensis* is 2.66×10^1 gene copies/ μl (95% CI: 2.05×10^1 - 4.15×10^1 gene copies/ μl)

3.2.4. Packaged LAMP kit for diagnosing small liver flukes

Packed kits with 50 reactions per set include: 1 instruction sheet for kit preservation and usage, and 6 chemical tubes.

3.3. The results of evaluating the sensitivity and specificity of the LAMP kit for diagnosing small liver flukes were compared with those of primer sets

3.3.1. Sensitivity and specificity of the LAMP kit for diagnose *O. viverrini* in the laboratory.

Table 3.27. Sensitivity and specificity of the LAMP kit for diagnose *O. viverrini*

Real time PCR LAMP KIT	Positive	Negative	Total
Positive	72	0	72
Negative	1	27	28
Total	73	27	100
Sensitivity is 98,63% (95% CI: 91,92% - 99,99)			
Specificity is 100% (95% CI: 85,25% - 100%)			

3.3.2. Sensitivity and specificity of the LAMP kit for diagnose *C. sinensis* in the laboratory

Table 3.28. Sensitivity and specificity of the LAMP kit for diagnose *C. sinensis*.

Real time PCR LAMP Kit	Positive	Negative	Total
Positive	71	0	71
Negative	2	27	29
Total	73	27	100
Sensitivity is 97,26% (95% CI: 89,98%-99,82%)			
Specificity is 100% (95% CI: 85,24% - 100%)			

3.3.3. The results of comparing the LAMP kit with the primer set, both aimed at diagnosing small liver flukes

Both LAMP kit sets for diagnosing small liver flukes yield similar test results to the primer sets published by Le Thanh Hoa et al. (2012) and Rahman et al. (2017), with a Kappa coefficient of 1.0.

3.3.4. Evaluation of the stability of the LAMP kit for diagnose liver flukes

The results determined that LAMP kits for diagnosing *O. viverrini* and *C. sinensis* work stably after 6 months of opening and 1 year from the date of manufacture when stored unopened and undamaged, sealed at $-20\pm 5^{\circ}\text{C}$, without thawing and freezing the LAMP kit more than 3 times.

3.4. Result evaluation of the stability of the LAMP kit for diagnose small liver flukes in Phu Yen and Ninh Binh

3.4.1. Results of evaluation the LAMP kit for diagnose *O. viverrini* in Phu Yen province

The rate of *O. viverrini* infection detected by the LAMP kit is 3.21% higher than that detected by the Kato-Katz method.

The real-time PCR test results are 100% consistent with the LAMP test results, with a Kappa coefficient of 1.0.

3.4.2. Results of evaluation the LAMP kit for diagnose *C. sinensis* in Ninh Binh province

The real-time PCR test results are 100% consistent with the LAMP test results, with a Kappa coefficient of 1.0.

3.5. Developing standards and testing LAMP kits

3.5.1. Develop basic standards

Based on the obtained results, we have developed basic standards for LAMP kits.

3.5.2. Results of test LAMP kits for diagnose small liver flukes

LAMP kits for diagnosing small liver flukes have been evaluated and tested by the National Institute for control of Vaccines and Biologicals.

CHAPTER 4. DISCUSION

4.1. Description of the current situation and some associated factors of small liver fluke infection in humans in two key communes in Ninh Binh and Phu Yen provinces (2018-2020).

4.1.1. Characteristics of research subjects

The average age of the study subjects was 45.16 ± 12.26 years old. The survey proactively selected subjects over 18 years old. Previous research by authors has shown that individuals infected with small liver flukes are typically over the age of 16, which corresponds to the working age group. They often have income and frequently engage in social activities such as organizing parties, drinking alcohol, and consuming fish salad, resulting in a higher rate of liver fluke infection compared to other age groups.

Survey results indicate that the prevalence of people eating fish salad at 2 communes in Ninh Binh and Phu Yen is slightly high, accounting for 55.7% of the population. These findings are consistent with a study conducted by author Nguyen Thi Thanh Huyen (2018) in Bac Giang and Binh Dinh, which focused on the epidemiology of fascioliasis in human endemic areas.

4.1.2. Results of research on the rate and intensity of small liver fluke infection in humans

- The rate of small liver fluke infection in humans

In Vietnam, the rate of *C. sinensis* liver fluke infection at Yen Loc commune in Ninh Binh province, as determined by the Kato-Katz method, is comparable to the findings of a 2017 study (infection rate of 16.5%) conducted by author Hoang Quang Vinh in four villages of Gia Thinh commune, Ninh Binh.

The rate of *C. sinensis* liver fluke infection in our research is lower than that reported by Doan Thuy Hoa (2020) in Kim Son and Yen Khanh, Ninh Binh (19.5%). This variance may stem from differences in sampling methods. Moreover, Ninh Binh is a region where *C. sinensis* liver fluke

infection is traditionally prevalent. The province often implements community intervention programs aimed at reducing the incidence of fluke infection. These interventions may contribute to the divergent rates of small liver infection observed across studies.

The rate of *O. viverrini* infection detected using the real-time PCR technique in our research is comparable to the findings of Nguyen Thi Thanh Huyen (2018) in Binh Dinh (21.3%). In contrast, the rate of liver fluke infection in our study is higher than that reported by Dao Thi Thanh Ha and her colleagues (11.4%).

- Intensity of small liver fluke infection in humans

The majority of cases (89.9%) of small liver fluke infection in two communes of Ninh Binh and Phu Yen are light; there are no severe infections. Studies worldwide, as well as those in Vietnam, have shown that the intensity of small liver fluke infection in the community is usually very low, with almost all cases being mild infections.

4.2. Some factors related to small liver fluke infection in humans

4.2.1. Relationship between age and small liver fluke infection rate

The rate of small liver fluke infection tends to increase gradually with age group, with the highest prevalence observed in the group over 60 years old (33.3%). Our study results are similar to those in Thailand (2020), where the prevalence of small liver fluke is quite high, especially in the group aged > 59 years old (15.4%).

4.2.2. Relationship between gender and small liver fluke infection rate

The results showed that both the infection intensity and the rate of small liver fluke infection are associated with gender. Our research findings are consistent with studies conducted worldwide and in Vietnam.

In Vietnam, most studies indicate a significantly higher infection rate among men compared to women. For example, a survey on the rate of *O. viverrini* infection in Binh Dinh (2016) revealed an infection rate of 19.1% in men, much higher than the 2.1% rate in women. Recent studies in some northern provinces also demonstrate a higher rate of *C. sinensis* infection among men compared to women, with statistically significant differences observed.

4.2.8. Relation between the rate of small liver fluke infection and eating raw fish habit.

Most studies conducted worldwide and in Vietnam suggest that the primary factor contributing to liver fluke infection is the consumption of raw or undercooked fish. Our research findings also support this, indicating that

individuals who regularly consume fish salad are 8.43 times more likely to be at a higher risk of small liver fluke infection compared to those who do not consume fish salad (95% CI: 5.23 - 13.57).

According to a study conducted by Doan Thuy Hoa (2020), individuals who consume fish salad are 6.8 times more likely to be infected with liver fluke compared to those who do not consume fish salad ($p < 0.001$). This finding is similar to the results of our study.

4.3. Manufacturing LAMP kit to detect *Clonorchis sinensis* and *Opisthorchis viverrini* infection in humans

4.3.1. Survey the conservative region in the target gene, design and select LAMP primers

The size of the PCR product obtained corresponds to the designed size of the primers: 189 bp for *O. viverrini* and 188 bp for *C. sinensis*, with no cross-reactivity observed with other surveyed worms and flukes. Based on the results of designing and selecting LAMP primer sets in theory and practice, we have identified two optimal primer sets, HOV1-nad1 and HCS-nad1, which are ideally suited for manufacturing LAMP kits for diagnosing *O. viverrini* and *C. sinensis*

4.3.2. Optimize the composition and operating conditions of the LAMP reaction

4.3.2.1. Optimization of LAMP reaction time and primer annealing temperature.

In this study, we selected a 60-minute incubation time to develop LAMP kits. This choice aligns with the optimal time selected in many other LAMP reaction studies. For example, Rahman et al. conducted their reaction in 75 minutes, which is faster than the study by Le Thanh Hoa et al. (2012)..

4.3.2.2. Optimization the composition of substances participating in the LAMP reaction

- Investigation of the optimal Mg^{2+} concentration in the LAMP reaction

After the survey, we determined that a $MgSO_4$ concentration of 8 mM is the optimal concentration for the LAMP reaction to detect *O. viverrini* and *C. sinensis*. Our findings are similar to those of Le Thanh Hoa et al. (2012) and Rahman et al. (2017).

- Survey the total amount of initial DNA in the LAMP reaction

This study also selected 4 μ l of DNA per reaction, a quantity consistent with that used in the study by Rahman et al (2017).

4.3.3. Surveying the threshold detection of LAMP technique to diagnose small liver flukes

With a threshold detection of 2.30×10^1 gene copies/ μl , equivalent to the detection threshold of some published and commercial kits on the market, such as the LAMP kit to identify Malaria from Eiken company (Japan), which has a threshold detection of 10 - 100 gene copies/ μl .

4.3.4. Evaluate the sensitivity, specificity, and stability of the LAMP kit for diagnosing small liver flukes, comparing it with the same target primer set.

4.3.4.1. Sensitivity and specificity of the LAMP test for diagnosing small liver flukes

The sensitivity and specificity of the LAMP technique developed in this study are comparable to those reported for the detection of *C. sinensis* by Rahman et al. (2017), which demonstrated a sensitivity of 97.1% and specificity of 100%.

4.3.4.2. Stability of the LAMP kit to diagnose small liver flukes

The storage time and usage protocol are similar to many diagnostic biological kits, including the LAMP kit for diagnosing malaria parasites from Eiken company (Japan), which has been commercialized and utilized in many countries worldwide.

4.3.4.3. Comparison of the LAMP kit with the primer sets targeting the same

The results showed that both sets of LAMP kits for diagnosing small liver flukes yielded similar test results to the primer sets published by Le Thanh Hoa et al. (2012) and Rahman et al. (2017), with a Kappa coefficient of 1.0.

4.3.5. Evaluation of the LAMP kit for diagnosing small liver flukes in Phu Yen and Ninh Binh provinces

4.3.5.1. Evaluation of the LAMP kit in Phu Yen province

The test results using the LAMP technique in the field study showed that the *O. viverrini* infection rate was 17.95% higher than the results obtained using the Kato-Katz technique (3.21%). Furthermore, the test results using the LAMP kit exhibited 100% similarity with those obtained using real-time PCR in the laboratory (Kappa coefficient = 1.0).

4.3.5.2. Evaluation of the LAMP kit in Ninh Binh province

The LAMP test in the field study has the same results (100%) with the real-time PCR test, Kappa coefficient = 1.0

CONCLUSION

1. Prevalence and some factors related to human fascioliasis infection in two main priority communes in Ninh Binh and Phu Yen (2018-2020)

Real time polymerase chain reaction (PCR) was utilized for a study on 459 people at Yen Loc commune (Ninh Binh) and 460 people at An My commune (Phu Yen):

- The rate of infection with *C. sinensis* in humans at Yen Loc commune (Ninh Binh) is 19.39%, while the rate of *O. viverrini* infection in humans at An My commune (Phu Yen) is 20.22%. The overall rate of liver fluke infection in the two provinces is 19.8%.

Using the Kato-Katz technique, the rates of liver fluke infection at Yen Loc commune (Ninh Binh) and An My commune (Phu Yen) are 16.33% and 16.0%, respectively. The rate of small liver fluke infection in humans was determined by real-time PCR technique is significantly higher than that determined using the Kato-Katz method ($p < 0.05$).

- The majority of people (89.8%) exhibit light infections. The median egg count is 395.5 ± 126.8 eggs per gram (EPG), with An My commune (Ninh Binh) at 241.9 ± 109.0 EPG and An My commune (Phu Yen) at 553.3 ± 226.8 EPG.

- The rate of small liver fluke infection in males is 26.81%, significantly higher than that in females, which is 14.06% ($p < 0.05$).

- The rate of people infected with liver flukes depends on age at An My commune (Phu Yen). The highest rate is observed in the age group over 60 (33.3%), followed by the group aged 50-59 years (23.4%), and the lowest in the age group between 18 and 29 years old (11.1%).

- People who use fresh human or animal feces in planting, as food for fish and animal cultivation, are 1.96 times more likely to be infected with small liver flukes than those who do not use these practices ($p < 0.05$).

- People who consume raw fish salad are 8.43 times more likely to be infected with small liver flukes than those who do not consume raw fish salad ($p < 0.05$). The rate of liver fluke infection in people who consume fish salad at least once a week is significantly higher than in those who consume it at least once a month or 2-3 times a month ($p < 0.05$).

2. Development of a LAMP kit for testing *Clonorchis sinensis* and *Opisthorchis viverrini* infections in humans at a laboratory scale.

- Successfully manufactured two types of LAMP kits to detect small liver flukes. The results of evaluation, laboratory testing, and kit verification and validation at the Institute for Control of Vaccines and Medical Biologicals are as follows:

+ The LAMP kit for diagnosing *O. viverrini* has a sensitivity of 97.26% (95% CI: 89.98%-99.82%) and a specificity of 100% (95% CI: 85.24%-100%). The minimum detection limit is 23 gene copies/ μ l. The stability of the kit under storage conditions of $-20^{\circ}\text{C}\pm 50^{\circ}\text{C}$ for 12 months and up to 6 months after opening it.

+ The LAMP kit for diagnosing *C. sinensis* has a sensitivity of 98.63% (95% CI: 91.92%-99.99%) and a specificity of 100% (95% CI: 85.25%-100%), with a minimum detection limit of 26 gene copies/ μ l. The stability of the kit under storage conditions of $-20^{\circ}\text{C}\pm 50^{\circ}\text{C}$ for 12 months and up to 6 months after opening it.

- The LAMP kit for diagnosing *C. sinensis* and *O. viverrini* infection in humans has been evaluated and tested in the community with accurate test results, which is similar to the results of stool testing using real time PCR technique in the laboratory.

RECOMMENDATIONS

1. The research findings indicate a high prevalence of liver fluke infection among humans in Ninh Binh and Phu Yen. Therefore, it is imperative to enhance communication and health education efforts to increase public awareness regarding the risk factors of infection and effective preventive measures against small liver fluke. This includes addressing key factors such as livestock management, waste management (both human and livestock waste), and adopting behaviors that reduce the transmission of small liver flukes. Additionally, ensuring food safety and hygiene practices, such as consuming thoroughly cooked food and boiled water, while avoiding the ingestion of raw or undercooked freshwater fish, can significantly contribute to reducing the prevalence of small liver fluke infection within the community.

2. Further research is needed to evaluate the effectiveness of the LAMP kit in detecting *C. sinensis* and *O. viverrini* on larger clinical samples in field studies. Once validated, the LAMP kit can be commercialized and made available on the market, providing an effective tool for diagnosing small liver fluke infections in the community.