MINISTRY OF EDUCATION AND TRAINING MINISTRY OF HEALTH
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TRAN VAN HAI

CURRENT STATUS OF HUMAN FASCIOLIASIS IN FOUR COMMUNES OF THANH HOA AND NGHE AN PROVINCES, AND THE DEVELOPMENT OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) KIT FOR PATHOGEN DETECTION

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1. National library of Vietnam
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LIST OF THESIS-RELATED PUBLICATIONS OF THE AUTHOR

1. Tran Van Hai, Nguyen Thi Hong Ngoc, Tran Thanh Duong, Nguyen Thi Huong Binh, and Nguyen Thu Huong (2020), Development of A Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Human Fasciola in Vietnam. *Microbiol Infect Dis*; 4(5): 1-7.
2. Tran Van Hai, Nguyen Thi Hong Ngoc, Tran Thanh Duong (2024), Current status of human fascioliasis in Thanh Hoa and Nghe An provinces, Vietnam (2020–2022). Journal of Community Medicine, Volume 66, December 2024 (Special Issue 1), pp: 88-92.
3. Tran Van Hai, Tran Thanh Duong, Nguyen Thi Hong Ngoc (2024), Factors associated with human fascioliasis infection in Thanh Hoa and Nghe An provinces, Vietnam, 2020–2022. Journal of Community Medicine, Volume 66, December 2024 (Special Issue 1), pp: 99-104

INTRODUCTION

Human fascioliasis (HF) is a zoonotic disease caused by trematodes of the genus *Fasciola*. In 2012, it was estimated that approximately 2.6 million cases had been diagnosed across 81 countries worldwide. The prevalence of HF varies by region, with the highest rates reported in the Andes region of Latin America. Over the past three decades, HF has emerged as a significant public health concern, prompting the World Health Organization (WHO) to classify it as a re-emerging tropical disease of parasitic origin. In Vietnam, Thanh Hoa and Nghe An provinces are considered hotspots for HF, largely due to their geographical characteristics, climatic conditions, and the living habits of the local population. Epidemiological data from previous studies conducted before 2020 indicated a considerable prevalence of HF in both Thanh Hoa and Nghe An provinces. In 2019, a total of 131 cases of *Fasciola* spp. infection were reported in Thanh Hoa province. Meanwhile, during the period from 2018 to 2022, the seropositivity rate for *Fasciola* spp. among individuals attending the Nghe An Center for Disease Control (CDC) was 18.33%. Notably, in 2019 alone, the number of HF cases recorded at this unit reached 1,863.

Loop-mediated isothermal amplification (LAMP) is a high sensitivity and specificity method for RNA and DNA amplification without thermal cycling. This method offers rapid testing times, the ability to simultaneously process multiple samples, improved diagnostic accuracy, and a more efficient response to pathogen detection. Given these scientific and practical demands, an in-depth investigation is essential. Therefore, the study entitled "*Current status of human fascioliasis in four communes of Thanh Hoa and Nghe An Provinces, and the development of a Loop-Mediated Isothermal Amplification (LAMP) kit for pathogen detection*" was conducted with the following objectives:

1. To identify the current status and potential risk factors of human fascioliasis in four communes of Thanh Hoa and Nghe An provinces during the period from 2020 to 2022.

2. To develop a Loop Mediated Isothermal Amplification (LAMP) kit for detecting Fasciola spp. infection in the studied provinces.

SCIENTIFICNESS AND NOVELTY OF TOPIC

1. This study described and evaluated the current status and associated factors of *Fasciola* spp. infection in humans in Phu Lam and Tan Truong communes of Nghi Son Town, Thanh Hoa Province, as well as Nghia Thuan and Nghia My communes of Thai Hoa Town, Nghe An Province.

2. This is the first study to apply the Loop-Mediated Isothermal Amplification (LAMP) technique for the diagnosis of human fascioliasis in Vietnam, thereby contributing to the improvement of diagnostic capacity and treatment of this parasitic disease.

STRUCTURE OF THESIS 130 pages, including: Introduction with 2 pages; Overview with 31 pages; Researching object and methods 27 pages; Results 40 pages; Discussion 26 pages; Conclusion with 2 pages; and Recommendation 1 page; new contributions 1 page. The thesis includes 22 figures and 35 tables (29 result tables). There are 123 references, of which 24 documents have been published within the last 5 years.

CHAPTER 1: OVERVIEW

1.1. Concept of human fascioliasis

Human fascioliasis (HF) is a parasitic infection caused by species of Trematoda from the Fasciolidae family. These parasites can cause lesions and abscesses in the liver or other organs when they infest inappropriately.

1.1.1. Clinical features

The clinical features of HF are non-specific and vary depending on the stage of disease progression and the location of the parasitic infection. During the acute stage, the infection typically involves the liver parenchyma and is often associated with fever, right upper quadrant pain, and abdominal discomfort. In the chronic stage, the parasite invades the bile ducts, and symptoms may become less specific, making it easy to confuse with other diseases. Classic signs in the chronic stage include pain in the right upper quadrant or epigastric region, cholangitis, cholecystitis, and the presence of gallstones. The liver is invariably enlarged and may be non-tender upon palpation.

The disease can manifest in three forms: mild, moderate, and severe.

1.1.2 Paraclinical features

- Blood tests typically show a significant elevation in eosinophils (over 500/mm^3), or an eosinophil percentage greater than 8% of the total white blood cells. Blood biochemistry may reveal elevated liver enzymes, as well as increased total bilirubin and direct bilirubin levels.
- Stool or duodenal fluid examination for *Fasciola* spp. eggs is considered the 'gold standard' for diagnosis. However, *Fasciola* spp. eggs are rarely found in these samples.
- The ELISA test for antibody detection is highly valuable in diagnosing *Fasciola* spp. infection.
- In chronic HF, several methods can be used to detect parasite eggs in stool, including direct stool examination, sedimentation, and the Kato-Katz method. Additionally, molecular biology tests are becoming increasingly important for diagnosing HF.
- Abdominal ultrasound, CT, or MRI of the abdomen can reveal liver and biliary tract lesions as hypoechoic areas in the right liver. Fluid accumulation may also be observed beneath the liver capsule.

1.1.3. Diagnosis

HF was diagnosed based on the guidelines established by the Vietnamese Ministry of Health in Decision No. 1931/QD-BYT, dated May 19th.

In suspected cases: The patients who live in endemic areas and have a history of consuming raw aquatic plants and drinking unsafe water, along with clinical signs, are considered at higher risk for human fascioliasis.

In Confirmed cases: In suspected cases of the disease, stool or bile tests may detect *Fasciola* spp. eggs, while the ELISA test can identify antibodies against *Fasciola* in the serum. Focal liver lesions are often detected through ultrasound, CT, or MRI imaging, or eosinophilia was observed in patient.

1.2. Epidemiological characteristics of human fascioliasis

1.2.1. Causative agent

The causative agent: Fascioliasis is caused by *Fasciola hepatica* or *Fasciola gigantica*. *Fasciola* spp. primarily infect herbivores, including buffalo and cattle, and can subsequently cause disease in humans when they ingest contaminated undercooked aquatic vegetables and unboiled water.

The taxonomic classification of *Fasciola* spp. is as follows:

Kingdom: Animalia
Phylum: Platyhelminthes
Class: Trematoda
Order: Echinostomida
Suborder: Echinostomata
Family: Fasciolidae
Genus: Fasciola
Species: *Fasciola hepatica* and *Fasciola gigantica*

1.2.2. Hosts

Fasciola spp. require both primary and secondary hosts to complete their life cycle. The primary hosts are ruminant animals, including both domestic and wild species — primarily buffaloes, cattle, sheep, goats, as well as camels and deer. Humans are considered incidental or accidental hosts of *Fasciola* spp. The secondary hosts (intermediate hosts) are freshwater snails belonging to the family Lymnaeidae, particularly species within the genera *Lymnae*a, *Galba*, *Fossaria*, and *Pseudosuccinea*.

1.2.3. Routes of transmission and mechanisms of disease

The disease is transmitted through the digestive tract. Humans primarily become infected with *Fasciola* spp. by consuming raw aquatic plants contaminated with *Fasciola* metacercariae (larval cysts). Additionally, drinking unboiled or untreated water containing *Fasciola* metacercariae is also considered a significant route of human infection.

In humans, once the larvae reach the digestive tract, they excyst and penetrate the intestinal wall, subsequently migrating into the peritoneal cavity. After approximately three months, the parasites develop into adult flukes, which then invade the liver tissue and ultimately establish themselves in the bile ducts. In addition, *Fasciola* spp. may occasionally ectopically migrate and inhabit other organs such as muscles, joints, the abdominal cavity, eyes, and lungs.

The period from larval cyst ingestion to the appearance of eggs in the stool varies depending on the host species. In sheep and cattle, this prepatent period typically averages 2 months (ranging from 6 to 13 weeks), whereas in humans, it usually takes 3 to 4 months. However, the proportion of *Fasciola* spp. successfully completing their development and producing eggs in the human host is notably low, generally estimated at less than 5%. The life span of adult of *Fasciola* spp. in humans is believed to range from 9 to 13.5 years.

1.2.4. Factors related to human fascioliasis

The factors associated with *Fasciola* spp. infection are primarily related to eating habits, lifestyle, and the use of water sources contaminated with fluke larvae. In addition, other relevant factors include the population’s knowledge, attitudes, and preventive

practices regarding HF, specifically as follows:

+ *Eating aquatic vegetables*: In individuals infected with *Fasciola* spp., the primary source of infection is the consumption of aquatic vegetables contaminated with metacercariae, such as *Ipomoea aquatica* (water spinach), watercress, knotweed, water celery, and other similar plants. In some cases, infection may also occur through the consumption of terrestrial vegetables irrigated with water contaminated by *Fasciola* larvae.

Water sources for drinking and daily activities: In endemic areas, the survival rate of *Fasciola* metacercariae in water environments is typically less than 10%. Infection can occur not only through the consumption of contaminated aquatic vegetables but also while bathing, drinking unboiled or untreated water, or through contact with kitchen utensils such as knives, cutting boards, and other surfaces contaminated with metacercariae. In regions where *Fasciola* is highly prevalent, water sources represent a significant factor contributing to the transmission of infection.

+ *Eating raw and undercooked food:*

In areas where the population does not engage in the habit of consuming raw or undercooked food and drinking untreated water, the incidence of *Fasciola* infection remains very low — even though aquatic animals and plants capable of supporting the survival of *Fasciola* larvae are still present in the environment.

1.2.5. Prevention measures

Fasciola spp. infection is strongly linked to local eating habits and cultural practices. Effective prevention requires a combination of health education, improved food hygiene, and environmental management, including the following measures:

- Avoid consuming raw and undercooked aquatic vegetables, salad, and other freshwater plants.
- Avoid drinking untreated and unboiled water.
- Avoid using human feces as fertilizer for fish farming, and refrain from defecating in water sources.
- Periodically deworming dogs, cattle, sheep, goats, and pigs to reduce the risk of *Fasciola* spp. transmission.
- Health communication and education programs for the community on the prevention of HF.

1.2.6. Prevalence of human fascioliasis in the world and in Vietnam

1*.2.6.1. Distribution and prevalence of human fascioliasis worldwide*

More than 80 countries worldwide have reported cases of HF. HF is a challenging disease to control due to its complex epidemiological characteristics. Two species of liver flukes, *F. hepatica* and *F. gigantica*, are responsible for this parasitic infection. While *F. hepatica* is found globally, *F. gigantica* is primarily observed in Africa and Asia. HF is prevalent in regions such as Oceania, Asia, Africa, the Middle East, Europe, the Caribbean, and parts of Latin America, though its distribution is not uniform across these areas.

1.2.6.2. Distribution and prevalence of human fascioliasis in Vietnam

Studies and reports conducted prior to 2020 indicated a notably high prevalence of HF in Thanh Hoa and Nghe An provinces. In a survey conducted in 2020, the Thanh Hoa CDC collected 90 stool samples and 90 blood samples from 20 communes. The results

revealed a HF prevalence rate of 0.8%. In Nghe An, from 2018 to 2022, reports from the Nghe An CDC indicated a *Fasciola* detection rate of 18.33% through laboratory tests and clinical examinations. Additionally, a survey conducted across several districts of Nghe An (2020-2021) reported an intestinal worm infection rate, including *Fasciola*, ranging from 15-20%.

1.3. Development of the Loop-Mediated Isothermal Amplification (LAMP) Technique and Application of LAMP Test Kits for the Diagnosis of Human Fascioliasis

The Loop-Mediated Isothermal Amplification (LAMP) technique was developed and first published by Notomi et al. in 2000. This innovative method of gene amplification allows for the synthesis of large DNA fragments without the need for thermal cycling. The key components of the reaction include template DNA, primers, and DNA polymerase, and the reaction mixture is incubated at a constant temperature of 65°C. LAMP is highly efficient, achieving amplification of 10^9 to 10^{10} copies within 15 to 60 minutes.

LAMP is a highly specific DNA amplification technique that occurs only when the necessary and sufficient conditions for the reaction are met. The process requires four primers: two outer primers (F3 and B3) and two inner primers (FIP, composed of F1c+F2, and BIP, composed of B1c+B2), which bind to six distinct regions on the target DNA sequence. The formation of a new circular DNA product only takes place when all primers bind specifically and function properly. If one or more primers are inactive or fail to bind to the target sequence, the circular DNA structure will not be generated. Notably, the amplification product can be visually detected by the naked eye, either through the formation of a white precipitate of magnesium pyrophosphate ($Mg_2P_2O_7$) or via fluorescence when stained with SYBR Green dye.

The basic components of the LAMP reaction:

+ Primers: Four types of primers are designed based on six distinct regions of the target gene, including F3c, F2c, and F1c at the 3' end, and B1, B2, and B3 at the 5' end.

+ Enzyme: The enzyme commonly used in the LAMP reaction is DNA polymerase derived from the bacterium *Bacillus stearothermophilus*, which possesses both DNA synthesis and strand displacement activity, and operates optimally at 66°C.

+ dNTPs and reaction buffer: similar to the PCR technique.

+ Template DNA: The template DNA used in the LAMP technique does not require high purity and can be obtained through simple extraction methods, thereby reducing preparation time.

+ Dye to indicate results.

The mechanism of the LAMP reaction comprises three main stages: (1) primer annealing, (2) amplification and strand elongation, and (3) cyclic amplification. During this process, the inner primers FIP and BIP alternately synthesize complementary DNA strands, forming characteristic loop structures at the ends of each strand. This loop formation enables continuous amplification under isothermal conditions, allowing the reaction to generate approximately 10^9 to 10^{10} copies of the target DNA within 15 minutes to 1 hour.

1.4. Principle of the LAMP Kit

The principle underlying the development of the LAMP kit is based on the amplification of target DNA or RNA under isothermal conditions, eliminating the need for thermal cycling as required in conventional PCR techniques. The LAMP reaction employs a specific primer set, typically consisting of four to six primers designed to target distinct and highly conserved sequence regions of the pathogen. The combination of a highly efficient DNA polymerase (such as Bst polymerase) and the formation of loop structures significantly enhances both the speed and efficiency of the amplification process. The development of a LAMP kit requires careful optimization of various components, including primer design, reagent concentrations, and reaction conditions. A complete LAMP kit generally comprises a ready-to-use reaction mixture, pathogen-specific primers, and appropriate positive and negative controls to ensure reliability and accuracy in diagnostic applications. Owing to its advantages of rapid detection, simplicity, and low cost, the LAMP technique has become increasingly popular in both clinical laboratories and field-based diagnostic settings.

The principles of test kit creating by LAMP technique:

- Set the target for amplification: Specific DNA or RNA sequences of the pathogen must be selected for amplification. In the case of *Fasciola* spp., commonly targeted DNA regions include the ITS-1 and ITS-2 sequences, which are highly conserved and specific to the species.
- Designing specific primers;
- Optimization of reaction conditions;
- Determination of the detection threshold, negative control, positive control;
- Evaluation of sensitivity and specificity;
- Packaging the kit;
- Testing stability;
- Publication of Baseline Standards and Technical Parameters, Including Sensitivity, Specificity, and Stability;
- Manufacturing and distribution.

1.5. General information about the research sites

Thanh Hoa and Nghe An provinces are located in Vietnam's North Central region, which is characterized by a tropical monsoon climate featuring heavy rainfall, high temperatures, and abundant sunlight. These conditions are highly favorable for the development of agriculture, forestry, and fisheries. However, the same climatic factors, combined with underdeveloped economic conditions and unhygienic living practices, have also created an environment conducive to the emergence and transmission of parasitic diseases. Furthermore, the capacity for parasitic disease prevention and control in these areas remains limited due to constraints in both professional expertise and technical equipment.

Chapter 2: RESEARCH METHODS

2.1. Objective 1: Identification of the current status and potential risk factors of human fascioliasis in four communes of Thanh Hoa and Nghe An provinces during the period from 2020 to 2022.

2.1.1. Research subjects and materials

2.1.1.1. Research subjects

- The residents living in Phu Lam and Tan Truong communes of Nghi Son town, Thanh Hoa province, and Nghia Thuan and Nghia My communes of Thai Hoa town, Nghe An province.

- *Inclusion criteria:*

+ Individuals aged 18 years and older, residing in the study area for at least 12 months.

+ Must be able to understand and respond to interviews.

+ Participants must agree to take part in the study.

- *Exclusion criteria:*

+ Individuals with mental illness.

+ Individuals who have been treated for HF within 6 months prior to the start of the study.

2.1.1.2. Research materials

- The "Fasciola Antibody Detection Test Kit" from Scimedx uses the ELISA technique to detect IgG antibodies specific to *Fasciola* spp. in serum.

2.1.2. Research duration and location

- Study period: From December 2020 to December 2022.
- *Study sites:* Phu Lam and Tan Truong communes, Nghi Son town, Thanh Hoa province, and Nghia Thuan and Nghia My Communes, Thai Hoa town, Nghe An.
- *Laboratory:* Department of Parasitology and the Laboratory of Department of Molecular Biology, National Institute of Malaria Parasitology and Entomology.

2.1.3. Study method

- Study design: A cross-sectional study
- Sample size: The sample size for this study was determined using the formula

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

n: The minimum sample size for research at the 4 communes.

$Z_{1-\alpha/2}$: Denotes the reliability coefficient, corresponding to a probability threshold of $\alpha = 0.05$ (95% confidence level), where $Z_{1-\alpha/2} = 1.96$.

p: Represents the estimated infection rate by ELISA, with p estimated to be 0.03.

d: Denotes the absolute allowable error, with d chosen as 0.008.

With these selected values, the calculated minimum sample size is 1,747 individuals per site.

A supplementary 15% contingency was added to the sample size to account for random sampling cases where selected subjects could not be reached, resulting in a total expected sample size of 2,010 individuals. Ultimately, we surveyed a total of 2,014 individuals across the four communes: Phu Lam and Tan Truong communes (Thanh Hoa Province), and Nghia Thuan and Nghia My communes (Nghe An Province), with 503, 501, 498, and 512 participants, respectively.

- *Inclusion criteria and participant selection methods:*

The study was purposively conducted in four communes: Phu Lam and Tan Truong (Nghi Son town, Thanh Hoa province), and Nghia Thuan and Nghia My (Thai Hoa town, Nghe An province). Within each commune, study participants were selected using a simple random sampling method.

2.1.4. Research content

To determine the intensity of *Fasciola* spp. infection in humans by examining fecal

samples from the study subjects using the sedimentation method and the ELISA technique with the *Fasciola* Antibody Detection Test Kit (Appendix 1).

2.1.5. Variables and research indicators

- Variables in the study: The main variable is the presence of *Fasciola* spp. infection, determined through stool examination using the sedimentation technique or by a positive result from the serum Ab-ELISA test. Additional variables include demographic characteristics such as age, gender, occupation, and education level; as well as sanitation-related factors, such as the use of hygienic toilets. Other variables involve behavioral and environmental factors that may be associated with *Fasciola* spp. infection among the study subjects.

- Indicators Research

+ The prevalence of *Fasciola* spp. infection among the population based on two diagnostic methods and associated risk factors.

+ The odds ratio (OR) was used to analyze the association between risk factors and *Fasciola* spp. infection, where OR ≠ 1 indicates a potential association. The OR was calculated using the formula OR = (ad)/(bc).

2.1.6. Research techniques

- Interview and data collection were conducted using structured questionnaires (Appendix 1).

- Sample collection and preservation were conducted in accordance with SOP NIMPE.HD 03.PP/25. The ELISA technique for detecting antibodies against *Fasciola* spp. was performed using the "Fasciola Antibody Detection Test Kit" (Scimedx, USA), following the procedures outlined in SOP NIMPE.HD 07.PP/34.

- Stool examination using the sedimentation method to detect *Fasciola* spp. eggs on slides under a light microscope: according to SOP NIMPE.HD 07.PP/36.

2.2. *Objective 2:* To develop a Loop Mediated Isothermal Amplification (LAMP) kit for detecting *Fasciola* spp. infection in the studied provinces

2.2.1. Research subjects

- Samples of *Fasciola* spp.
- LAMP kit for diagnosing *Fasciola* spp. infections.

2.2.2. Research time and location

- Time: development Kit phase: from January 2018 to December 2020.

Application phase of the LAMP kit for diagnosis in Thanh Hoa and Nghe An provinces: from January 2021 to December 2022.

- Research location: At the Laboratory/Department of Molecular Biology and Dang Van Ngu Hospital - Institute of Malaria - Parasitology and Entomology.

Field sites: Thanh Hoa and Nghe An provinces.

2.2.3. Research methods

2.2.3.1. Research design

Theory and practical research

+ Design a LAMP kit for diagnosing the *Fasciola* spp. and test its accuracy and functionality.

+ Create a positive control sample for the kit using a plasmid carrying the DNA sequence specific to *Fasciola* spp.

+ Development of the LAMP reaction process and optimization of reaction conditions.

+ Evaluation of sensitivity, specificity, and detection threshold of the LAMP reaction.
+ Finalizing the LAMP kit.
- Manufacturing the LAMP Kit according to production standards.
- Testing the LAMP Kit.

2.2.3.2. Research sample size

- Standard positive samples for technical standards: A minimum of 3 samples at each stage, with each sample tested 3 times.

- Samples used to compare the sensitivity and specificity of the test kit: Purposefully collected samples on a small scale in the laboratory, based on the number of available positive samples. These were collected at Dang Van Ngu Hospital and supplemented with field samples to create a clinical trial sample set, assuming an infection rate (p) of 25%. The calculated sample size included 25 true positive (+) samples and 75 true negative (-) samples. A total of 130 samples were collected, including 30 fecal samples infected with *Fasciola* spp. and 100 fecal samples not infected with *Fasciola* spp.

- Sample size used to evaluate the stability of the kit: 3 positive control samples, including 1 positive sample at the concentration used as the detection threshold for the LAMP technique, and 2 positive samples collected from patients, for a total of 5 samples.

- Sample size used to assess the *Fasciola* spp. infection rate in Nghe An and Thanh Hoa provinces: A total of 2,014 samples were collected from the field.

2.2.4. Study techniques

- Sample collection and preservation followed SOP NIMPE.HD 03.PP/25: 'Procedure for collecting, preserving, and transporting stool and serum samples to detect *Fasciolas* spp. infection in humans in the field."

- Sample processing and DNA extraction were performed using the alcohol precipitation method according to SOP NIMPE.HD 03.PP/51..

- Primer design was carried out using specialized bioinformatics software such as Primer Explorer v.5, Primer Blast, Mega7, etc., to identify conserved regions in target gene areas, design the primers, and then evaluate the properties of the designed oligonucleotides, following SOP NIMPE.HD 03.PP/31: "Procedure for designing, evaluating, and selecting primers for the LAMP Kit for diagnosing *Fasciola* spp." (Appendix 2).

- Stool examination technique to detect *Fasciolas* spp.: sedimentation technique. qPCR technique to identify *Fasciola* spp. (Samer Alasaad et al., 2011); sequencing technique using the automatic sequencer 3500.

2.3. Methods for controlling bias and confounding in research

Strictly adhere to all research guidelines. All data and results must be recorded on the original forms. Standard control samples (-) and (+) should be used to ensure consistency in the results.

2.4. Statistical methods and data analysis

- Input and analyze data using Excel and STATA 12.0. Qualitative variables are described as percentages. The relationship between *Fasciola* spp. infection status and factors is evaluated by calculating the odds ratio (OR) with 95% confidence intervals (CI).

- Data analysis is also performed using bioinformatics software: AB7500 version 2.06, Primer Explorer v.5, Primer Blast, and Mega 7. Sensitivity, specificity, and the Kappa coefficient are calculated using MedCalc software.

2.5. The Ethics of Research

This study complies with the ethical review guidelines and regulations for biomedical research of the Institute of Malariology, Parasitology, and Entomology, as outlined in Decision No. 182/QĐ-VSR, dated February 24th, 2020, and has received approval from the local authorities. It only includes individuals who voluntarily participate in the study.

CHAPTER 3. RESEARCH RESULTS

3.1. Objective 1*:* To identify the current status and potential risk factors of human fascioliasis in four communes of Thanh Hoa and Nghe An provinces during the period from 2020 to 2022.

A total of 2,014 people participated in the study, with the majority in the 21-40 age group (40.42%), followed by the 41-60 age group (39.18%). The group over 60 years old accounted for 16.53%, while the smallest group was those aged 18-20 years (approximately 3.87%). Females represented a higher percentage than males, at 53.67% and 46.33%, respectively. Farmers constituted the largest occupational group (61.82%), followed by other professions (21.35%). In each commune, farming was the primary occupation of the study participants, fluctuating between approximately 50-70%.

The educational level of the study participants was predominantly secondary school (55.31%), followed by high school (22.44%). The rates of illiteracy and those with higher secondary education were both low, at 3.33% and 5.51%, respectively. The percentage of participants aware of HF was 35.30%. The proportion of study subjects with access to a latrine/toilet was 94.14%, of which 83.71% had hygienic latrines/toilets. Additionally, 87.39% of participants reported consuming raw aquatic vegetables.

3.1.1. Current status of Fasciola infection in 4 communes of Thanh Hoa and Nghe An provinces

Table 3.1. Rate of human fascioliasis based on stool examination using the sedimentation method (n = 2,014)

Rate of HF	Thanh Hoa (1004)				Nghe An (1010)				Total (n=2014)	
	Phu Lam (503)		Tan Truong (501)		Nghia Thuan (498)		Nghia My (512)			
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Yes	1	0.20	0	0.00	2	0.40	1	0.20	4	0.20
No	502	99.8	501	100	496	99.6	511	99.8	2010	99.8

A total of 4 out of 2,014 (0.2%) study participants were infected with *Fasciola* spp., as detected by stool examination using the sedimentation method.

Table 3.2. Serum Ab-ELISA test results for *Fasciola* spp. in study participants by age group (n = 2,014)

Age group	Thanh Hoa (1004)			Nghe An (1010)			Total (2014)		
	No. tested	No. pos	(%)	No. tested	No. pos	(%)	No. tested	No. pos	(%)
18-20	42	0	0	36	1	6.67	78	1	1.28
21-40	387	6	1.55	402	11	2.74	789	17	2.15
41-60	417	5	1.20	397	9	2.27	814	14	1.72
> 60	158	1	0.63	175	2	1.14	333	3	0.90
Total	1004	12	1.20	1010	23	2.28	2014	35	1.74

The overall infection rate by serum Ab-ELISA testing is 1.74%. The highest infection rate is observed in the 21 to 40 age group, accounting for 2.15%, followed by the 41 to 60 age group at 1.72%.

Table 3.3. Serum Ab-ELISA test results for *Fasciola* spp. in study participants by gender (n=2,014)

Gender	Thanh Hoa (1004)			Nghe An (1010)			Total (2014)		
	No. tested	No. pos	(%)	No. tested	No. pos	(%)	No. tested	No. pos	(%)
Male	485	4	0.82	448	9	2.01	933	13	1.39
Female	519	8	1.54	562	14	2.49	1081	22	2.04
Total	1004	12	1.20	1010	23	2.28	2014	35	1.74

The overall positive *Fasciola* rate in the two provinces by Ab-ELISA serum testing is 1.74%, with a positive rate of 1.39% in males and 2.04% in females

Table 3.4. Ab-ELISA serum test results for *Fasciola* spp. in study participants by occupation, education level, and knowledge of *Fasciola* spp. (n = 2,014)

Factor		*Fasciola* infection		
		No. tested	No. pos	(%)
Ocupation	Farmers	1245	21	1.69
	Workders	231	3	1.30
	State office, retirement	108	2	1.85
	Other (Student, Freeland)	430	9	2.09
Education level	illiterate	67	2	2.99
	Primary school	270	6	2.22
	Secondary	1114	21	1.89
	High school	452	6	1.33
	Intermediate professsional school, college, university	111	0	0.00
Knowledge about *Fasciola* infection	Yes	711	3	0.42
	No	1303	32	2.46
Total		2014	35	1.74

The highest *Fasciola* positive rate is in the 'other professions' group (2.09%), followed by the retired group (1.85%). The illiterate group has the highest positive rate for *Fasciola* at 2.99%, followed by the primary school group at 2.22%.

3.1.2. Factors related to Fasciola infection status

There is no correlation between age group, gender, occupation, education level, and *Fasciola* infection status.

Table 3.5. Relationship between knowledge of human fascioliasis and *Fasciola* infection as detected by serum Ab-ELISA testing (n = 2,014)

Knowledge of HF	*Serum Ab-ELISA testing result*			OR (95% CI)	p-value
	No. tested	No. pos	(%)		
No	1303	32	1271	5.94 (1.81–19.47)	0.01
Yes	711	3	708		

Individuals who are unaware of HF have a 5.94 times higher risk of infection compared to those who are aware of the disease (95% CI: 1.81 – 19.47). This relationship is statistically significant ($p < 0.001$).

Table 3.6. Relationship between latrine/toilet conditions and *Fasciola* spp. infection as detected by serum Ab-ELISA testing (n=2014)

Factors	Serum Ab-ELISA Results			OR, 95% CI	p-value
	No. tested	No. pos	(%)		
No/unsanitary toilet	328	16	312	4,5, (2.29 – 8.85)	< 0.01
Sanitary toilet	1686	19	1667		

Individuals living in households without latrines or with unsanitary latrines have a 4.5 times higher risk of *Fasciola* spp. infection (95% CI: 2.29 – 8.85) compared to those living in households with sanitary latrines..

Table 3.7. Relationship between eating habits and *Fasciola* spp. infection as detected by serum Ab-ELISA testing (n=2014)

Eating equatic vegetable habit	Ab-ELISA results			OR (95% CI)	p-value
	No. tested	No. pos	(%)		
Often	531	25	506	7.28 (3.47 – 15.26)	< 0.01
sometime/hardly	1483	10	1473		

Individuals who regularly consume raw aquatic vegetables have a 7.28 times higher risk of *Fasciola* spp. infection compared to those who do not consume them (95% CI: 3.47 – 15.26).

3.2. Objective 2: To develop a Loop Mediated Isothermal Amplification (LAMP) kit for detecting Fasciola spp. infection in the studied provinces.

3.2.1. Design of LAMP primers

The ITS2 gene sequences of *F. gigantica* and *F. hepatica* were retrieved from the NCBI database (www.ncbi.nlm.nih.gov) and aligned using the Clustal W algorithm in MEGA 7 software to identify conserved regions characteristic of the *Fasciola* genus.

Table 3.8. LAMP primer sequences designed for the detection of *Fasciola* spp.

Primer name	Position 5’	Position 3’	Length	Tm (ºC)	5'dG	3'dG	% GC	Primer sequences
F3	43	61	19	56.50	-5.61	-5.84	0.53	GGTTGGACTGATAACCTGG
B3	243	260	18	57.43	-3.27	-5.23	0.50	CTTTTTGGGCGTCGTGAT
FIP			40					TGCGCTCTTCATCGACACAC-TTGACCATACGTACAACTCT
BIP			43					ACTGCTTTGAACATCGACATCTTGA-TTTATAAGCCGACCCTCG
F2	65	84	20	55.39	-5.02	-4.24	0.40	TTGACCATACGTACAACTCT
F1c	106	125	20	62.41	-7.32	-5.21	0.55	TGCGCTCTTCATCGACACAC
B2	223	240	18	55.25	-1.98	-6.47	0.50	TTTATAAGCCGACCCTCG
B1c	156	180	25	62.96	-5.63	-4.27	0.40	ACTGCTTTGAACATCGACATCTTGA
LF	88	104	17	60.28	-6.77	-6.78	0.65	AGCCGAGTGATCCACCG
LB	201	217	17	61.63	-4.93	-7.18	0.65	TTAGCCTGTGGCCACGC

Primer design: The designed primers have nucleotide lengths ranging from 17 to 25 bases, with an optimal GC content between 40% and 65%. The melting temperature (Tm) of each primer meets the standard conditions required for LAMP primers. The free energy (ΔG) at the 3' ends of primers F2/B2, F, F3/B3, LF/LB, and at the 5' ends of F1c/B1c is designed to range from –7.32 to –5.02 kcal/mol, all of which are lower than –4 kcal/mol, which is a mandatory criterion for LAMP primer stability.

The expected theoretical size of the amplified LAMP reaction product using F3-B3 primers is 218 bp.

3.2.2. The specificity and LAMP primer active Loop Mediated Isothermal Amplification (LAMP)

3.2.2.1. The specificity of LAMP primer

In theory, the PCR product amplified by the primers should correspond to the sequence of *Fasciola* species. To verify the specificity of the F3-B3 primer pair, we used the Primer-BLAST tool available on the NCBI platform. The results confirmed that the primers perfectly matched the ITS2 sequence of the *Fasciola* spp. published in the NCBI database.

3.2.2.2. Evaluation of the Functionality of LAMP Primers

To evaluate the functionality of the designed primers, we conducted LAMP reactions using the self-designed primers and DNA samples of the *Fasciola* spp., following the conditions recommended by New England Biolabs for the Bst DNA polymerase kit.

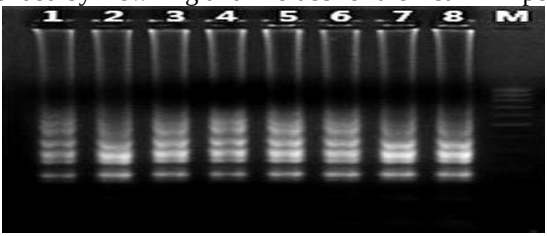


Figure 3.1. LAMP products amplified using a specific primer set for *Fasciola* spp.

The electrophoresis analysis on a 2% agarose gel revealed that the reaction products displayed a distinct ladder-like pattern, which is a characteristic feature of LAMP amplification, confirming the good performance of the designed primer set.

3.2.3. Results of the positive control reactions for the kit

The steps for creating the positive control were performed in accordance with SOP NIMPE.HD 03.PP/36: "Procedure for creating positive controls for the LAMP Kit for diagnosing *Fasciola* spp. in humans using eecombinant DNA technology" (with modifications). A conserved sequence segment of the ITS2 gene from *Fasciola* spp., measuring 461 bp, was inserted into the pUC19 vector and subsequently cloned. The resulting recombinant plasmid contained the ITS2 gene fragment specific to *Fasciola* spp., with a total size of 3,147 bp. The purified plasmid yield was 5 µg, which was dissolved in 50 µl of buffer to obtain a final concentration of 100 ng/µl.

3.2.4. Survey and optimization of LAMP reaction conditions

3.2.4.1. Results of the temperature survey for optimal primer annealing.

The criteria for selecting and estimating the optimal primer annealing temperature were based on the electrophoretic analysis of LAMP products on a 2% agarose gel. The results showed that the amplification achieved the highest efficiency at an annealing temperature of 63°C.

3.2.4.2. Results of the $MgSO_4$ Concentration Survey

To evaluate the effect of $MgSO_4$ concentration on the LAMP reaction, experiments were conducted at a constant temperature of 63°C, with all reaction components kept identical except for the $MgSO_4$ concentrations, which were set at 4 mM, 6 mM, and 8 mM. The amplification results were assessed by electrophoresis on a 2% agarose gel. The findings indicated that a concentration of 8 mM $MgSO_4$ yielded LAMP products with high and stable amplification efficiency.

3.2.4.3. Evaluation of LAMP reaction time

The experiment was conducted to determine the minimum duration required for the LAMP reaction, with reaction times ranging from 40 to 60 minutes. The reaction components included template DNA, primers, reaction buffer, and Mg^{2+} at a concentration of 8 mM, while all other conditions were kept constant, with only the reaction time being varied. The results indicated that a minimum reaction time of 60 minutes was required to achieve successful DNA amplification in the LAMP assay.

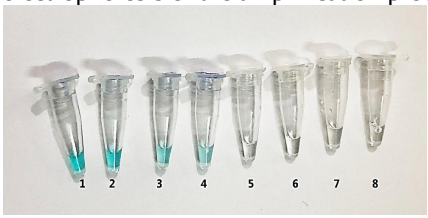
3.2.4.4. Survey of color indicators for LAMP reaction results

In this study, malachite green (MG) was selected as the color indicator for the LAMP reaction. The concentrations of MG tested were 0.0012%, 0.008%, 0.004%, and 0.001%. The results were evaluated based on the visible color change of the reaction mixture and by electrophoresis of the amplification products on a 2% agarose gel. Among the tested concentrations, 0.004% MG was identified as the optimal concentration, as it allowed for clear differentiation between positive and negative samples.

3.2.5. Survey of the detection threshold of the LAMP kit for diagnosing Fasciola spp.

Phase I: Preliminary limit testing was conducted to establish the primary detection range.

Phase II: Detection threshold testing was performed to determine the limit of detection with 95% confidence (LOD_{95}). The results were evaluated by observing the color change of the reaction mixture in the tubes after the LAMP reaction and by electrophoresis of the amplification products on a 2% agarose gel.



Tube 1: DNA concentration 10^{-6}ng/µl.
Tube 2: DNA concentration 10^{-7}ng/µl.
Tube 3: DNA concentration 10^{-8}ng/µl.
Tube 4: DNA concentration 10^{-9}ng/µl.
Tube 5: DNA concentration 10^{-10}ng/µl.
Tube 6: DNA concentration 10^{-11}ng/µl.
Tube 7: Negative control 1
Tube 8: Negative control 2

Figure 3.2. Survey of the detection limit of LAMP primers for Fasciola diagnosis

Table 3.9. Survey of the detection threshold of the LAMP primer results

Concentration (ng/µl)	**Results**		
	Time 1	**Time 2**	**Time 3**
10^{-6}	(+)	(+)	(+)
10^{-7}	(+)	(+)	(+)
10^{-8}	(+)	(+)	(+)
10^{-9}	**(+)**	**(+)**	**(+)**

10^{-10}	(-)	(-)	(-)
10^{-11}	(-)	(-)	(-)
Neg1	(-)	(-)	(-)
Neg2	(-)	(-)	(-)

The primary detection threshold of the kit was determined to be 10^{-9} ng/µl, corresponding to 2.94×10^{-1} copies/µl.

Table 3.10. Survey of the detection threshold for the Fasciola diagnostic kit.

Concentration (ng/µl)	Number of copies (copies of gen/µl)	Number of repeats	Number of positives	Positive rate (%)
$1x10^{-6}$	$2,94x10^{2}$	12	12	100,00
$1x10^{-7}$	$2,94x10^{1}$	12	12	100,00
$1x10^{-8}$	$2,94x10^{0}$	12	12	100,00
$1x10^{-9}$	$2,94x10^{-1}$	12	12	100,00
$7,5x\ 10^{-10}$	$2,21\ x10^{-1}$	12	11	91,67
$5x10^{-10}$	$1,47x10^{-1}$	12	8	66,67
$2,5x10^{-10}$	$7,35\ x10^{-2}$	12	6	50,00
$1,25x10^{-10}$	$3,68x10^{-2}$	12	4	33,33
$1x10^{-10}$	$2,94x10^{-2}$	12	1	8,33
$6,25\ x10^{-11}$	$1,84\ x\ 10^{-2}$	12	0	0,00

The LOD95% result of the kit was 2.21×10^{-1} copies of the gene/µl (95% CI: 1.79×10^{-1} to 3.01×10^{-1} copies of the gene/µl).

3.2.6. Sensitivity and specificity of the LAMP kit

An evaluation of sensitivity and specificity was conducted on 130 stool samples. The test results are presented in Appendix 4.

The sensitivity and specificity of the LAMP kit were compared with those of the gold standard method, PCR.

Table 3.11. Sensitivity and specificity of the LAMP Kit for *Fasciola* spp. diagnosis.

Fasciola-LAMP	PCR		Total
	Positive (+)	Negative (-)	
Positive	29	3	32
Negative	1	97	98
Total	30	100	130
Sensitivity and specificity	Se: 96,67% (95% CI: 82,78 - 99,92); Sp: 97,0% (95% CI: 91,48 - 99,38)		

The sensitivity and specificity of the LAMP kit for diagnosing *Fasciola* spp. were 96.97% and 97%, respectively.

3.2.7. The kit in the comparation with the bait set for the same purpose and evaluate the kit in the field.

There has not been a commercially available LAMP kit for *Fasciola* diagnosis on the market. We conducted a comparison of the LAMP kit with the LAMP primers for diagnosing *Fasciola* spp., as published by Martinez et al. in the Journal of Parasites and

Vectors in 2016. This comparison was carried out experimentally on 50 samples, including 25 positive and 25 negative samples, which were confirmed using the PCR method.

Table 3.12. Kappa coefficient between the LAMP-NIMPE Kit and the Martinez primer set.

		LAMP-NIMPE		Total
		Positive (+)	Negative (-)	
Martinez *primer set*	positive	24	0	24
	negative	1	25	26
Tông		25	25	50

Kappa = 0.96, indicating a 96% similarity between the two methods. This high Kappa value demonstrates the diagnostic detection capability of the LAMP kit, which has a detection threshold of 10^{-6} ng/µl DNA.

3.2.8. Stability of the LAMP kit for Fasciola spp. diagnosis.

The test kit is packaged and stored at a temperature of -20°C ± 5°C. The evaluation samples include three positive control samples at concentrations of 10^{-6} ng/µl, 10^{-7} ng/µl, and 10^{-9} ng/µl, two positive patient samples, and two negative samples. Experiments are conducted monthly, quarterly, and after 12 months of storage at 2-8°C and -20°C ± 5°C after the kit has been opened.

Table 3.13. The stability of the kit survey results after 12 months.

Concentration (ng/µl)	Storage conditions - 20^0C ± 5^0C	
	Time 1	Time 2
10^{-6}	(+)	(+)
10^{-7}	(+)	(+)
10^{-9}	(+)	(+)
Sample DNA 1	(+)	(+)
Sample DNA 2	(+)	(+)
Neg1	(-)	(-)
Neg2	(-)	(-)

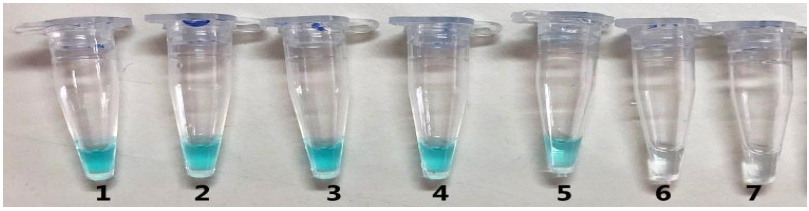


Figure 3.3. Survey of Kit stability after 12 months

The results indicated that the kit remains stable for 6 months after opening and 1 year when unopened, provided that the LAMP kit is not thawed and refrozen more than three times.

3.2.9. Packaging, baseline standard establishment, and inspection registration for the Kit

3.2.9.1. Packaging of the finished kit, user manual, and storage instructions for the kit.

The kit is packaged with 50 reactions per kit, which includes an instruction manual and 6 chemical tubes. Additionally, the kit box is labeled with complete information, including the production batch, expiration date, storage conditions, and place of manufacturing.

Figure 3.4. Image of the LAMP kit for *Fasciola* spp. diagnosis

Specific information on the tubes of the LAMP kit for *Fasciola* spp. diagnosis is provided in detail in Appendix 6. Instructions for using the LAMP kit for *Fasciola* spp. diagnosis are outlined in Appendix 7.

3.2.9.2. Establishing the baseline standards and inspection registering of the kit.

The research team is developing baseline standards for the LAMP kit used to diagnose *Fasciola* spp., based on three parameters: sensitivity, specificity, and detection threshold.

Table 3.14. Basic standards of the LAMP kit for Fasciola diagnosis.

Evaluation criteria	Basic standards
Sensitivity	≥ 95%
Specificity	≥ 95%
Limit of Detection LOD95%	2,21x10^{-1} gene copies/µl (95%CI:1,79x10^{-1} gene copies /µl to 3,01x10^{-} copies gene/µl) 2.21 x 10^-1 /µl (95% CI: 1.79 x 10^-1 /µl to 3.01 x 10^-1 gene copies/µl)

This basic standard has been evaluated by the National Institute for the Control of Vaccines and Biologicals.

3.2.10. Assessment of human fascioliasis rates in 4 communes of Thanh Hoa and Nghe An provinece (2020–2022)

Table 3.15. Results of Fasciola testing using ELISA, stool tests, and LAMP methods in 4 communes of Thanh Hoa and Nghe An provinces (2020–2022)

ELISA Results (0D > 0,1 – (+)	Test results (n=2014)						Total		
	Thanh Hoa (1004)			Nghe An (1010)					
	EISA	Phân	LAMP	ELISA	Phân	LAMP	ELISA	Phân	LAMP
0,1 – 0,5	8	0	1	13	0	0	21	0	1
> 0,5 - ≤ 1,0	3	0	2	7	0	3	10	0	5
> 1,0	1	1	1	3	3	3	4	4	4
Total (+)	12	1	4	23	3	6	35	4	10
Negative	992	1003	1000	991	1011	1008	1979	2010	2004

All positive samples (+) from the stool tests were also positive with the LAMP kit. Additionally, 5 out of 10 samples (50%) had an OD ranging from 0.5 to 1, which were positive for the LAMP kit, while only 1 case had an OD < 0.5 that was positive (+) with

the LAMP kit. The overall *Fasciola* spp. infection rate in the two provinces, based on the LAMP technique, is 0.5%.

Chapter 4: DISCUSSION

4.1. Description of *Fasciola* infection prevalence and related factors in humans at 4 communes in Thanh Hoa and Nghe An (2020–2022)

4.1.1. General characteristics of the study subjects

The study was conducted on 2,014 residents living in Phu Lam and Tan Truong communes (Nghi Son town, Thanh Hoa province) and Nghia Thuan and Nghia My communes (Thai Hoa town, Nghe An province). Participants aged 18 and older were intentionally selected, as previous studies on *Fasciola* spp. infections have shown that the infection rate in those under 18 is typically low.

The age group of study subjects was primarily 41 to 60 years (40.42%) and 21 to 40 years (39.18%), with the lowest percentage in the 18 to 20 years group (nearly 4%). The age distribution is similar to the studies by Ngo Van Thanh (2016) and Doan Thuy Hoa (2020). Females accounted for a higher percentage than males, at 53.67% and 46.33%, respectively, which is consistent with the findings of Nguyen Thu Huong and Nguyen Khac Luc. Since we randomly selected individuals from the community, the distribution of age and gender within the population may have influenced these characteristics in the study subjects.

The awareness of HF is only 35.3% among the population who are aware of the disease. Regarding the educational background of the study subjects, the majority completed only high school or less, which may explain the low level of awareness about the disease. This finding is similar to the study by Nguyễn Thu Hương in Quảng Ngãi, which reported that 34.1% of the population was aware of HF. In terms of toilet usage, the results showed that 94.14% of the population lives in houses with latrines, and 83.71% use sanitary latrines (either septic tanks or two-chamber latrines). This characteristic aligns with the study by Doan Thuy Hoa (2020) in Ninh Binh. Regarding the habit of eating raw aquatic vegetables, the majority of subjects (87.39%) have this habit, with more than a quarter regularly consuming raw aquatic vegetables.

The awareness of human fascioliasis

The rate of people regularly eating raw aquatic vegetables in two communes of Nghe An province is higher than in Thanh Hoa province, with the highest rate in Nghia My commune (nearly 35%). The most commonly consumed aquatic vegetables are lettuce (24.6%) and rice paddy herb (22.44%). Research by Ton Nu Phuong Anh and colleagues on patients visiting for examination revealed that 75.6% of patients have the habit of eating uncooked aquatic vegetables.

4.1.2. The current situation of Fasciola spp. infection in humans at 4 communes in Thanh Hoa and Nghe An from 2020 to 2022.

To determine the status of *Fasciola* spp. infection in the population, we conducted two tests: detection of *Fasciola* spp. antibodies in serum using ELISA and stool examination using the sedimentation method. Fasciola spp. eggs were found in stool samples from 4 subjects (accounting for 0.2%) using the sedimentation method. This is a very low rate, but it is consistent with the results of many other studies. Research in Malaysia and Thailand using stool microscopy to detect Fasciola spp. eggs reported positive rates of 0.3% and 0.5%, respectively.

Another study conducted on children in Pakistan in 2019 showed similar results. For the Ab-ELISA test, 35 subjects tested positive, accounting for 1.74%. The rate of Fasciola spp. infection in humans determined by the ELISA technique was statistically significantly higher compared to the stool test using the sedimentation method. The rate of Fasciola spp. infection in our study was also lower than in some other studies, both domestic and international, such as those by Nguyen Van Van and Nguyen Thu Huong.

In 2012, a study conducted in Ho Chi Minh City on 10,084 residents using the ELISA technique showed that the infection rate of Fasciola spp. was 5.9%. Compared to studies worldwide, Y. Corrales et al. (2021) investigated the status of *Fasciola* spp. infection in people living on farmhouses in the rural Andes of Venezuela. Among 34 patients, none were found to have Fasciola spp. eggs in their stool, but 29.4% tested positive by SP-ELISA.

The results of a meta-analysis report a global prevalence rate of Fasciola spp. infection at 4.5% (95% CI: 3.1 – 6.1%), with a rate of 2.0% in Asia, which is consistent with our findings.

4.1.3. Factors related to human fascioliasis in 4 communes of Thanh Hoa and Nghe An from 2020 to 2022.

Relationship between age and Fasciola spp. *infection prevalence*

We did not find a correlation between age and *Fasciola* infection status. This result differs from the study by Nguyen Khac Luc (2010) and Nguyen Thu Huong. M.A. Najib et al. (2020) conducted research in Malaysia among farmers and workers in farmhouses, showing that individuals aged 18 and older have more than three times the risk of Fasciola spp. infection compared to those under 18. Some studies suggest that age is a factor related to Fasciola infection, with higher incidence rates found in adults, which tend to increase with age and decrease in the elderly. The distribution characteristics among different age groups are mainly influenced by local habits, as immune response typically plays a less significant role in *Fasciola* spp. infection.

Relationship between gender and Fasciola spp. *infection prevalence*

We also did not find a relationship between gender and *Fasciola* spp. infection status. Our results differ from those of Nguyen Thu Huong and Nguyen Khac Luc. Toan Nguyen et al. (2016) conducted a study in Ho Chi Minh City and found that men have a 0.8 times lower risk of infection compared to women (95% CI: 0.69 – 0.96). Immune response often plays a less significant role in this disease, and the difference in disease susceptibility between the two genders is mainly attributed to behavioral risk factors.

Relationship between occupation and Fasciola spp. *infection status*

Farmers have a *Fasciola* spp. infection rate of 1.69%, which is similar to the infection rates in other occupational groups, including workers, officials, retirees, students, and freelancers. Our results differ from those of Nguyen Thu Huong and Nguyen Khac Luc, who reported that farmers had the highest *Fasciola* spp. infection rate. Farmers typically have the highest infection rates due to the nature of their work, which exposes them to risk factors such as increased contact with contaminated water sources, poor sanitation conditions, and livestock farming. However, we did not find a correlation between occupation and *Fasciola* spp. infection. This may be because in Vietnam, people often engage in multiple types of work. Officials, retirees, and workers frequently participate in agricultural and livestock farming activities, similar to farmers. Therefore, there is no clear distinction between these groups regarding the risk of infection.

Relationship between education level and the prevalence of Fasciola spp. infection

The prevalence of *Fasciola* spp. infection among individuals with education up to primary school is 2.37%, higher than the group with secondary school education and above, which is 1.61%. None of the 111 individuals with education above high school were infected with *Fasciola* spp. However, the difference in infection rates between educational groups is not statistically significant. This may be due to the low prevalence of *Fasciola* spp. infection. The number of infected individuals is insufficient, and therefore, the statistics do not have enough impact. Our results are consistent with those of Nguyen Khac Luc (2010) and Doan Thuy Hoa (2020).

Educational level can affect hygiene standards and personal habits. Furthermore, individuals with higher education are often more aware of how to prevent various

diseases. They may also have a better ability to understand and apply preventive measures when educated about disease prevention and health communication.

The relationship between awareness of Fasciola spp. disease and the Fasciola spp. infection prevalence.

Understanding of HF helps individuals develop better knowledge, attitudes, and practices regarding preventive measures. Statistical analysis revealed that people who have never heard of HF are nearly six times more likely to suffer from the disease compared to those who are aware of it. Those who have heard of HF tend to have higher education levels. Individuals who are aware of the disease may possess knowledge about its risk factors and preventive measures, which contributes to a lower infection rate in this group. This result also highlights the effectiveness of educational interventions and health communication in preventing *Fasciola* spp. infections.

The relationship between toilet conditions and Fasciola infection

Using sanitary latrines is one of the important factors influencing many intestinal diseases. Our results show that individuals living in households without latrines or with unsanitary latrines have a 4.5 times higher risk of Fasciola spp. infection (95% CI: 2.29 – 8.85) compared to those living in households with sanitary latrines. This finding is consistent with the study by Nguyen Thi Thanh Huyen (2018).

Septic tanks and toilets play a crucial role in waste treatment, reducing pollution and the potential spread of pathogens into water environments. Y. Huang and colleagues (2017) conducted a continuous intervention study using an integrated control strategy that included modifications to the toilet system. All old-style toilets were replaced with sanitary toilets designed for safe waste treatment. The inspection results showed that no live parasitic eggs were detected in fecal samples from the newly rebuilt toilets.

The relationship between eating habits and Fasciola spp. infection status

Eating habits play a significant role in the transmission of HF. We found that individuals who frequently consume raw aquatic vegetables have a 7.28 times higher risk of infection compared to those who occasionally or rarely eat them (95% CI: 3.47 – 15.26). Our findings are consistent with many studies both domestically and internationally. Additionally, our research indicates that people who consume watercress have a higher positive rate (3.5%) compared to those who eat rice paddy herb (2.78%), water spinach (1.2%), and lettuce (0.46%).

In the *Fasciola* spp. life cycle, after developing into cercariae, the parasite is released from the snail and attaches to suitable aquatic plants to develop into metacercariae. Humans and mammals can become infected when they consume plants containing metacercariae. Therefore, if humans frequently consume raw aquatic vegetables, it increases the risk of contracting the disease.

Regarding the habit of consuming raw or undercooked liver or poultry (such as buffalo, cattle, goats, deer, etc.), we did not find a statistically significant correlation with *Fasciola* spp. infection status. This result is consistent with the study by Nguyen Thu Huong in Quang Ngai. The risk of infection in humans will depend on the Fasciola spp. infection status in these animals and the frequency of consuming raw liver and meat from them. In this study, we focused solely on analyzing the correlation between the habit of eating raw meat and *Fasciola* spp. infection, so the results may not accurately reflect this relationship.

4.2. Development of a LAMP Kit for detecting *Fasciola* spp. infections in the studied provinces

4.2.1. Design the bait LAMP kit

Globally, many researchers have applied the LAMP technique to diagnose Fasciola spp. infections in livestock such as buffaloes, cattle, and sheep. The gene region most commonly targeted by these studies is the ITS2 region. Similarly, in this study, we also

utilized the ITS2 gene sequence to design a set of primers. As a result, a conserved sequence segment specific to *Fasciola* species was identified, with a length of 461 base pairs. Based on this conserved region, 100 different primer sets were generated.

The final selected primers meet the technical criteria regarding primer length for F3 and B3, with GC content ranging from 40% to 65%. The melting temperatures of the primer pairs comply with the standard conditions required for LAMP primers. Additionally, the free energy (ΔG) at the 3' ends of primers F2/B2, F3/B3, LF/LB, and at the 5' ends of F1c/B1c were designed to range from –7.32 to –5.02 kcal/mol, all of which are below the threshold of –4 kcal/mol, as required for LAMP primer stability. The theoretical size of the amplification product generated using the F3-B3 primer pair is 218 base pairs, which meets the technical requirement of being less than 280 base pairs.

4.2.2. Specificity and Activity of the LAMP Primers

We used the Primer-BLAST tool on NCBI to evaluate the specificity of the F3-B3 primer pair. The results indicated that the primers perfectly matched the ITS2 gene sequence of Fasciola spp. published in the NCBI database. To further validate primer specificity, we performed PCR using the F3-B3 primer pair with DNA samples extracted from *Fasciola* spp., small liver flukes, and small intestinal flukes. The PCR products were analyzed by electrophoresis on a 2% agarose gel to confirm the primer binding to the ITS2 gene of each species.

In addition, to verify that the amplified product obtained using the F3-B3 primer pair specifically belonged to Fasciola spp., we sequenced the PCR product. The sequencing results showed 100% identity with the ITS2 gene sequence of *Fasciola* spp. available in the NCBI database. Our E result was 2×10^{-108}, demonstrating a highly reliable match between the obtained sequence and the reference sequence.

4.2.3. Survey and optimization of primer annealing temperature in LAMP reaction.

The annealing temperature (Ta) is a critical factor that directly influences both the specificity and amplification efficiency of PCR and related techniques derived from PCR, including LAMP. The electrophoresis results of the LAMP products indicated that the optimal reaction temperature was 63°C, which is closely aligned with the melting temperature (Tm) of the primers (approximately 50°C ± 2°C).

This finding is consistent with previous studies by Ai et al. (2016) and Martinez et al. (2015), both of which identified 63°C as the optimal annealing temperature. Theoretically, 63°C is considered an ideal temperature, as it enhances resistance to potential inhibitors commonly present in biological samples. Furthermore, this temperature is optimal for the catalytic activity of bacterial enzymes, especially when compared to the conditions found in wild-type Bacillus strains. Based on these results, 63°C was selected as the standard temperature for conducting subsequent experiments.

4.2.4. $MgSO_4$ Concentration survey

The concentration of $MgSO_4$ plays an important role in determining both the efficiency and specificity of PCR and LAMP reactions. In this study, the optimal $MgSO_4$ concentration for the LAMP reaction was identified as 8 mM. This result is consistent with the findings reported by Martinez (2016).

4.2.5. The time LAMP reaction survey

The LAMP reaction was conducted at two incubation time points: 40 minutes and 60 minutes, while keeping the reaction components constant. At the 40-minute mark, only a small amount of amplified product was detected. In contrast, after 60 minutes of incubation, a significantly higher quantity of DNA product was observed, and the electrophoresis bands were clear and distinct.

Based on these findings, a 60-minute incubation time was selected as the optimal condition for the development of the LAMP diagnostic kit. This duration is comparable to, though slightly longer or shorter than, that reported in previous studies. For example,

Ai et al. applied the LAMP technique for the diagnosis of *Fasciola* spp. infections with a reaction time of 45 minutes, while Chen et al. also employed a 45-minute protocol for detecting *Paragonimus westermani*. In contrast, Le Thanh Hoa and colleagues established a longer reaction time of 75 minutes for the LAMP-based diagnosis of small liver fluke infections.

4.2.6. Color indicator of LAMP reaction survey

In the present study, MG was selected as the colorimetric indicator for the LAMP reaction. The results demonstrated that a concentration of 0.004% MG was optimal for clearly distinguishing between positive and negative samples. Higher MG concentrations were associated with an increased risk of false-positive results, whereas lower concentrations failed to provide a clear distinction between negative and positive reactions. These findings are consistent with previous studies that have also employed MG as a color indicator in LAMP assays.

In terms of color stability after amplification, the results showed that the blue color in positive reaction tubes remained stable for up to six weeks when stored at room temperature.

4.2.7. Threshold detection of the LAMP kit for diagnosing Fasciola spp. survey

The detection threshold of the LAMP technique for identifying Fasciola spp. was determined by conducting LAMP reactions using a series of diluted DNA concentrations from a recombinant plasmid containing the ITS2 gene segment specific to Fasciola spp. The results established the 95% limit of detection (LOD_{95}) of the kit as 22.21×10^{-1} gene copies/µl (95% CI: 1.79×10^{-1} gene copies/µl to 3.01×10^{-1} gene copies/µl). Additionally, the detection threshold was further validated by performing tests with self-designed primers on a range of diluted total DNA concentrations extracted from mature Fasciola spp. tissue samples.

The results indicate that the detection threshold reached 10^{-6} ng/µl, which is consistent with the study by Ai et al. (2010), who reported a detection threshold of 10^{-5} ng/µl when using the LAMP technique to diagnose and distinguish between *F. hepatica* and *F. gigantica* infections in sheep. Furthermore, the detection threshold in our study is higher than that reported by Martinez et al. (2016), who used the LAMP technique to identify *Fasciola* spp. infections in sheep in the field, with a detection threshold of 10^{-3} ng/µl.

4.2.8. Sensitivity and specificity of the LAMP, comparing the kit with a primer set for diagnosing Fasciola spp.

Our study evaluated over 130 stool samples collected from Binh Dinh and Quang Nam provinces, as well as clinical samples obtained from Dang Van Ngu Hospital and the Institute of Malariology, Parasitology, and Entomology. These included 30 positive samples and 100 negative samples. All samples were initially tested using the sedimentation method to detect eggs in the stool and were subsequently re-evaluated using the qPCR method. The results obtained from the LAMP technique revealed 33 positive samples and 97 negative samples.

This result demonstrates that the LAMP kit has higher sensitivity and detects more positive cases (33/30 positive samples) compared to the sedimentation technique for detecting eggs in feces (27/30 positive samples). This finding is both reasonable and consistent with other studies on *Fasciola* spp.

Compared to the qPCR reference method, the results of the LAMP technique showed 1 false negative and 3 false positives. In the case of the false negative sample, this sample was not detected by the sedimentation method for finding eggs in feces but was positive when using the qPCR technique. This suggests that the sample has a low DNA concentration, and the LAMP technique's detection threshold is insufficient to identify it at this concentration. The sensitivity of the LAMP kit for diagnosing *Fasciola* spp. in the

laboratory is 96.67% (95% CI: 82.78 - 99.92), and the specificity is 97.0% (95% CI: 91.48 - 99.38).

Compared to the LAMP diagnostic kit for *Fasciola* spp. developed by Martinez et al., published in the Journal of Parasites and Vectors in 2016, the LAMP diagnostic kit for *Fasciola* spp. approved by the Institute of Malariology, Parasitology, and Entomology (with a detection threshold of 10^{-6} ng/µl DNA) demonstrates a high diagnostic agreement, with a Kappa coefficient of 0.96.

4.2.9. Evaluate the prevalence of Fasciola spp. infection in humans in Thanh Hoa and Nghe An using the LAMP kit

Fasciola spp. are among the most important parasitic pathogens affecting humans and animals, particularly in tropical and subtropical regions. Accurate diagnosis plays a crucial role in disease control and treatment, thereby minimizing the impact on public health and livestock productivity.

In the present study, the LAMP method identified 10 positive cases of *Fasciola* spp. out of the total samples tested in Nghe An and Thanh Hoa, indicating that the actual infection rate is significantly lower than that observed with traditional methods, such as egg morphology examination and ELISA. Compared to the stool examination method, LAMP offers advantages as it is not dependent on the density of eggs in the stool—a major limitation of traditional methods. In patients with mild infections or in the early stages, the number of eggs excreted is often very low and irregular, leading to a limited detection rate by stool examination, with only 4 positive cases identified in this study.

In addition, the ability to identify *Fasciola* spp. eggs under the microscope is also limited by the risk of confusion with eggs from other types of flukes or with unrelated structures in the fecal sample.

A total of 35 positive cases were detected by ELISA, but most of these were not confirmed by LAMP, indicating a high risk of false positives. The main reason is that ELISA detects antibodies against *Fasciola* spp., which can persist in the body long after the infection has been treated or resolved on its own. Additionally, ELISA is prone to cross-reactivity with antigens from other types of flukes. In contrast, LAMP directly targets the DNA of *Fasciola* spp., allowing for accurate detection of the pathogen regardless of the infection stage. This method is not dependent on the density of eggs or the presence of antibodies. Furthermore, it has the significant advantage of being able to detect DNA from any component of *Fasciola* spp., including parasite fragments excreted in feces, not just eggs, thereby enhancing diagnostic capabilities, especially in samples with low egg counts.

The research results indicate that the LAMP method should be widely implemented for diagnosing *Fasciola* spp. in endemic areas, particularly in epidemiological surveillance programs, to replace or complement traditional methods. This would provide more accurate data, aiding in the development of more effective prevention and treatment strategies. Furthermore, combining LAMP with stool examination or ELISA can capitalize on the strengths of each method: while stool examination and ELISA are useful for initial screening, LAMP can serve as a final confirmation to identify true positive cases.

CONCLUSION

1. Prevalence of human fascioliasis and associated factors in four communes of Thanh Hoa and Nghe An provinces, 2020–2022

An investigation of *Fasciola* spp. infection was conducted through stool tests and serum ELISA in 2,014 residents from Phu Lam and Tan Truong communes (Nghi Son Town – Thanh Hoa), and Nghia Thuan and Nghia My communes (Thai Hoa Town – Nghe An):

- Stool test (Sedimentation method): The infection rates of *Fasciola* spp. in Phu Lam,

Tan Truong, Nghia Thuan, and Nghia My were 0.2%, 0%, 0.4%, and 0.2%, respectively, with an overall infection rate of 0.2%.

- Serum Ab-ELISA Test: The infection rates of *Fasciola* spp. through serum Ab-ELISA testing in the four communes were 1.59%, 0.8%, 2.61%, and 1.95%, respectively, with an overall infection rate of 1.74%.

- By age group: The highest rate of *Fasciola* spp. infection through serum Ab-ELISA testing was observed in the 21-40 age group (2.15%), followed by the 41-60 age group (1.72%), the 18-20 age group (1.28%), and the lowest rate in the over 60 age group (0.9%).

- By gender: The infection rate of *Fasciola* spp. through serum Ab-ELISA testing was 1.39% in males and 2.04% in females.

- By occupation: The infection rate of *Fasciola* spp. through serum Ab-ELISA testing was 1.85% for officials and retirees, 1.69% for farmers, 1.3% for workers, and 2.09% for other professions.

- Individuals who are unaware of the disease have a 5.94 times higher infection rate compared to those who are aware.

- Individuals from families without latrines or with unsanitary latrines are at a 4.5 times higher risk of infection compared to those living in households with sanitary latrines.

- Individuals who frequently consume raw aquatic vegetables are at a 7.28 times higher risk of infection with *Fasciola* spp. compared to those who occasionally or rarely consume them.

- No correlation was found between age group, gender, education level, occupation and *Fasciola* spp. infection.

2. Development of a Loop-Mediated Isothermal Amplification (LAMP) kit for detecting *Fasciola* spp. infections in the studied provinces

- Successfully developed a LAMP kit for diagnosing *Fasciola* spp. infection. Evaluation results, laboratory tests, and kit appraisal were conducted at the National Institute for Control of Vaccines and Biologicals (NIMPE).

- The LAMP kit for diagnosing *Fasciola* spp. has a sensitivity of 96.67%, specificity of 97.0%, and a minimum detection limit of 2.21×10^{-1} copies/µl. The kit remains stable for 12 months when stored at -20°C ± 5°C, and 6 months when stored at 20°C ± 8°C after opening.

- The infection rate of human fascioliasis in Thanh Hoa and Nghe An, as detected by the LAMP kit, is 0.5%.

RECOMMENDATIONS

1. It is essential to enhance communication and health education to raise public awareness and improve attitudes toward the risk of *Fasciola* spp. infections. Comprehensive and effective preventive measures should be implemented within the community, addressing key factors such as constructing and renovating sanitary latrines, ensuring food safety, consuming cooked food, boiling water, and limiting the consumption of raw aquatic vegetables. These efforts will help reduce the rate of *Fasciola* spp. infections in the community.

2. Widespread deployment of the LAMP method in diagnosing *Fasciola* spp. in endemic areas, particularly in epidemiological surveillance programs, will complement traditional methods. This approach will provide more accurate data, supporting the development of more effective prevention and treatment strategies.