MINISTRY OF EDUCATION AND TRAINING MINISTRY OF HEALTH
NATIONAL INSTITUTE OF MALARIOLOGY-PARASITOLOGY - ENTOMOLOGY

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TRAN THI KIM CHI

RESEARCH AND MANUFACTURE OF LAMP KIT FOR DIAGNOSIS OF INTESTINAL STRONGYLOIDIASIS *(Strongyloides stercoralis)* IN HUMANS, 2017-2020

SUMMARY OF DOCTORAL MEDICINE THESIS

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Principle supervisor: **Ass. Prof. PhD. Tran Xuan Mai**

Co – supervisor: **Ass. Prof. PhD. Nguyen Thi Huong Binh**

Reviewer 1:

Reviewer 2:

Reviewer 3:

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INTRODUCTION

Strongyloidiasis is a chronic intestinal parasitic infection in humans caused by Strongyloides spp. Vietnam is identified as an endemic area of the disease. Accurate, early diagnosis of cases is difficult and easy to miss. Fecal screening for larvae has very low sensitivity. Serological diagnosis of antibodies has high sensitivity but low specificity. The Baermann method, agar plate culture and test-tube filter paper uses a large amount of feces, specialized tools, and lots of labor and time. Isothermal ADN amplification methods (most commonly loop-mediated isothermal amplification LAMP) have sensitivity and specificity comparable to PCR. The device is simple and compact. Results can be detected with the naked eye. Time to do it fast. There is not currently commercialized lamp kit for the diagnosis of intestinal strongyloidiasis. it is necessary to research and develope the technique that can be applied to diagnose intestinal strongyloidiasis.. Therefore, we conducted a study on the topic "Research and manufacture of LAMP kit for diagnosis of intestinal strongyloidiasis (Strongyloides stercoralis) in humans 2017-2020" with the following objectives:

1. Development of a procedure and fabrication of a LAMP kit for the diagnosis of intestinal *Strongyloides stercoralis* infection in humans.

2. Evaluation of the sensitivity, specificity, and stability of the kit in the laboratory and in the field.

NOVELTY, SCIENTIFICITY AND PRACTICALITY OF STUDY

- The topic applied standard scientific methodological studies that are widely applied in Vietnam and the world

- Completing the process and manufacturing LAMP kit for detecting intestinal helminths *Strongyloides stercoralis* in Vietnam at laboratory scale.

- This is the first study to develop a LAMP kit to diagnose *Strongyloides stercoralis* in Vietnam. In the context that there is no commercialized LAMP kit for diagnosing strongyloidiasis in the world, the successful manufacture of the kit has created a breakthrough in technical solutions in diagnosing strongyloidiasis, contributing to the technology early diagnosis and timely treatment, meeting the practical requirements of intestinal strongyloides control in our country.

STRUCTURE OF THE THESIS

The thesis has 112 pages (excluding appendices) include sections: Introduction (2 pages); Chapter 1: Overview document (31 pages); Chapter 2: Objects and methods of research (19 pages); Chapter 3: Results of the study (34 pages); Chapter 4: Discussion (23 pages); Conclusion (2 pages); new contributions of the thesis (1 page); published works of authors related to the content of the thesis (1 page); references 120 (17 pages, including 26 Vietnamese documents, 94 documents in English) and the annex (30 pages). The thesis was presented with 27 tables, 28 figures.

Chapter 1: LITERATURE OVERVIEW

1.1 History of the discovery and research of *S. stercoralis*

S. stercoralis was first discovered by Normand in 1876. The genus Strongyloides has two species that have been identified as pathogenic in humans, mainly *S. stercoralis* and more rarely, *S. fuelleborni* [39].

1.2 Biological and pathological characteristics of *S. stercoralis*

S. stercoralis has a complex development cycle. It parasitizes in the small intestine, lays eggs in a very small number of eggs per day, the eggs quickly hatch into larvae right in the small intestine. During the development cycle of *S. stercoralis*, there is an autoinfection process.

Strongyloidiasis has an incubation period of about 1 month. Two main forms of strongyloidiasis are: Common diseases: abdominal pain; digestive disorders; pruritus, rash, urticaria, and severe disease [3], including hyperinfection strongyloidiasis syndrome and disseminated strongyloidiasis, are most common in patients receiving high-dose corticosteroids.

1.3 Diagnosis and treatment of strongyloidiasis

The sensitivity of microscopy based on techniques is low, especially in cases of chronic infection. Techniques such as Baermann or agar plate culture are cumbersome and time consuming. Immunity is a useful tool, but it can give results that are over the counter because it is not clear whether the disease is present or past. [31], [42]. Many molecular biology studies on strongyloides have been conducted due to the high sensitivity and specificity of these methods [44], [67], [79].

Treatment for strongyloidiasis is recommended for all infected individuals, whether symptomatic or not, with either ivermectin or albendazole regimens. [3].

1.4 The situation of strongyloidiasis

Strongyloidiasis occurs in many countries, the degree of infection various from region to region. Africa, South and Central America, and Southeast Asia are endemic areas. The rate of S. stercoralis is underestimated compared to actual infection. According to a survey by Hanoi Medical University and the National Institute of Malaria - Parasitology - Entomology, the prevalence of strongyloidiasis in the North is often below 1%[6]. In the southern provinces of Vietnam, the number of patients with strongyloidiasis detected, diagnosed and treated in recent years is relatively high [12], [18]. In addition, Vietnam has several reports of severe strongyloidiasis.

1.5 Loop-mediated isothermal amplication technique LAMP

Kỹ thuật LAMP LAMP is a gene cloning method that can synthesize large DNA fragments without the need for thermoregulation. LAMP uses 4-6 different primers specifically designed to recognize 6-8 distinct regions on the target gene. LAMP occurs only when all four primer chains bind to the target sites of the template, yielding a cyclic DNA product. The process takes place at 55oC-65oC, the amplification efficiency is high. The products of the reaction are visible to the naked eye. LAMP is commonly used to create rapid diagnostic kits [9], [110] and has been built to diagnose a number of protozoa, the commercialization of this application is also considered very effective [70]. In Vietnam, there are currently no published studies on the application of LAMP in the diagnosis of S. *stercoralis* infection. There is no commercialized and clinically applied LAMP kit on the market to diagnose *S. stercoralis*.

Chapter 2: SUBJECTS AND METHODS

2.1 Objective 1: Develop a procedure and fabricate a LAMP kit for the diagnosis of intestinal *S. stercoralis* infection in humans

Research subjects: *S. stercoralis* larvae as standard control and positive control.

Place and time of research: from September 2017 to May 2019 at the Molecular Biology Laboratory of the National Institute of Malaria, Parasitology and Entomology, Department of Microbiology and Parasitology, Faculty of Medicine, UMP Ho Chi Minh City.

Research design: Experiment and describe in the laboratory

Sample size: Positive standard samples: at least 03 samples of *S. stercoralis* larvae at all stages of the study.

Research content

- Building a process consists of 4 steps:

+ Step 1: Primer set design: Load 30 strongyloidyloides 18S rRNA gene sequences, determine the conservation region, and put it into LAMP primer design software to select primer sets. Investigation of primer specificity.

+ Step 2: Investigate optimization of LAMP reaction conditions.

+ Step 3: Investigate the detection threshold of the primer set.

+ Step 4: Generate positive controls by recombinant DNA technology.

- Packing the kit.

Techniques used in the study :

Sample collection and preservation, sample processing and DNA extraction, using specialized bioinformatics software such as Primer Explorer v.5, Primer Blast, Mega 7, qPCR technique – Taqman Probe, electrophoresis method, method cloning and sequencing methods.

Rating Indicators

- Specificity of primer sets with S. stercoralis
- Reaction conditions.
- Detection threshold
- Quality of positive control
- Process of making kit and packaging: 2000 tests.

2.2 Objective 2: Evaluation of the sensitivity, specificity, and stability of the kit in the laboratory and in the field

Research subjects: LAMP kits from objective 1, stool samples and serum samples were collected from individuals with confirmed, suspected and uninfected intestinal strongyloidiasis.

Place and time of research: from September 2017 to August 2020 at Duc Hoa – Long An province, Pham Ngoc Thach University of Medicne, The Molecular Biology Laboratory and The Parasitology of the NIMPE, Department of Microbiology and Parasitology, Faculty of Medicine, UMP Ho Chi Minh City.

Research design: Experimental research in the laboratory, the field.

Sample size: Evaluation of the sensitivity and specificity of the kit: based on the sample calculation formula [105], the sample size was calculated as 73 samples, of which 19 true positive samples were needed. We used a set of 132 samples including: 100 samples (-) and 32 samples (+) actually. Evaluation of the stability of the kit: 07 samples. Field kit evaluation: purposeful collection of 300 samples. Comparing the kit with another primer set for the same purpose requires 50 samples including 25 positive and 25 negative samples. Based on 141 samples stored and collected at the Department of Examination during the study period to compare the kit with stool smear and ELISA.

Research content

- Evaluation of the sensitivity and specificity of the kit at the laboratory: qPCR as the reference method.
- Stability of the kit: conducted through 3 tests.
- Evaluation of the kit in the field.
- Compare the kit with the same target primers that have been published in reputable journals. Consensus level: Kappa coefficient.
- Compare the LAMP kit with 2 commonly used strongyloidiasis diagnostic methods today, fecal endoscopy and ELISA. Assess the level of agreement between each pair of methods: Kappa coefficient.
- Develop basic standards and register for kit testing.

Techniques used in the study: Collection and preservation of samples, sample processing and DNA extraction, ELISA techniques, fecal endoscopy techniques, qPCR techniques - Taqman Probe, agarose gel electrophoresis.

Rating Indicators:

- Sensitivity: > 95%.
- Specialty: > 95%.
- K-value when comparing LAMP kit with compatible kit
- Stability: the kit must operate stably for at least 6 months after being stored under suitable conditions.
- The standard basis is appraised by a reputable agency.

Data processing: MedCalc software, calculate sensitivity, specificity, Kappa coefficient, error by SD standard deviation.

2.3 Error control

Experimental process is based on standard procedures, complying with ISO 15189 laboratory standard, data coding.

2.4 Ethics in research

Fully comply with ethical regulations in biomedical research.

Chapter 3: RESULTS

3.1 Development of a procedure and fabrication of a LAMP kit for the diagnosis of intestinal *S. stercoralis* infection in humans

3.1.1 Result of primer design

3.1.1.1 Sequence results of primer sets

The resulting primer set has the following sequence:

Table 3.1 LAMP primer sequence designed for the diagnosis of intestinal strongyloidiasis

Od	Name	Primer sequence	Long
1	F3	AGAGGGTTTAAACCAGACATT	21
2	B3	CTTCGAACCTCTAACTTTCGTT	22
3	FIP	GCCCCCGTTTGTTCCTATTAATCA-GGTCTAGCATGGAATAACACT	45
4	BIP	TACGTTAGAGGTGAAATTCTTGGAC-CTTGATTAATGAAAACATTCTTGGC	50
5	LF	GGTCTAGCATGGAATAACACT	21
6	LB	GCCCCCGTTTGTTCCTATTAATCA	24

Table 3.4 Melting temperature and ability to create primer dimer pairing (dG <-2.34) of primer sets to identify intestinal strongyloidiasis

Od	Name	Tm	5'dG	3'dG	Tỷ lệ GC
1	F3	56.60	-5.53	-4.06	0.38
2	B3	57.89	-5.04	-4.85	0.41
3	FIP				
4	BIP				
5	LF	56.79	-4.43	-4.55	0.43
6	LB	63.00	-7.64	-3.15	0.46

Based on the results of the tables, we find:
- Designed primers F3, B3 all meet the length requirements.
- The distance between the primers is satisfactory.
- All primers have %GC within the allowable ratio (38% - 46%).
- Melting temperature of primer pairs: satisfy the condition.
- The free energy level at the 3' ends of the F2/B2 primers; F3/B3;
-The LF/LB and the 5' end of F1c/B1c were designed with ΔG from -7.64 to -4.06 < -4Kcal/mol, which is a mandatory requirement for a LAMP primer set.
- The amplification product after LAMP reaction by primer pair F3-B3 has the theoretical size of 226 bp < 250 bp.

Thus, the primer set we designed meets the requirements of a LAMP primer system.

3.1.1.2 Investigation of the specificity of the primer set

- Theory: we used the program Primer Blast on NCBI to test the characteristics of the F3-B3 couple. The obtained results showed that the primer completely paired with the 18S rRNA of the program *S. stercoralis* published in NCBI.
- Experimental survey: Check the application response of the designer through the following steps:
+ Perform PCR reaction using F3/B3 pairs with sample DNA of hookworm, *Gnathostoma sp...*
+ Electroly check the cloned product on agarose gel.
+ Required results: there is no clone product of F3/B3 couple designed with DNA of hookworm, *Gnathostoma sp...*

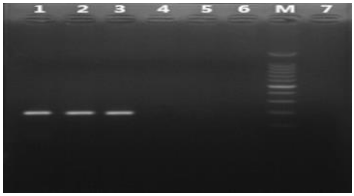

Figure 3.5: PCR products using primers F3-B3 of *S. stercoralis*

Lane 1-3: *S. stercoralis* Lane 6: *Gnathostoma sp*
Lane 4: *A. duodenale* Lane7: Negative control
Lane 5: *Necator americanus*

The results of electrophoresis on 2% agarose gel showed positive control: product line with expected size (226bp), no extra band and correct denaturation temperature as designed. Negative control has no amplification product. Thus, there is no cross-pairing of the primer pair with other species, proving that the primer works well.

3.1.2 Results of determining LAMP reaction conditions with self-designed primers

3.1.2.1 Temperature of reaction LAMP

Conduct a temperature survey in the range from 60^0C to 65^0C, only change in hybridization temperature, the components (DNA template, primer, reaction buffer...) and other conditions (equipment, time ...) of all reactions were kept the same and homogenized, the experiment was repeated 3 times. The results of electrophoresis of LAMP products showed that there was no difference between the three survey temperature points of 63^0C, 64^0C and 65^0C. We choosed a temperature point of 63°C for the LAMP reaction.

3.1.2.2 $MgSO_4$ concentration

In the LAMP reaction, the appropriate concentration of $MgSO_4$ is usually between 4mM and 8mM. We carried out the reactions with the same composition and conditions, only the difference in $MgSO_4$ concentration, the experiment was repeated three times. The results were evaluated through product electrophoresis on 2% agarose gel.

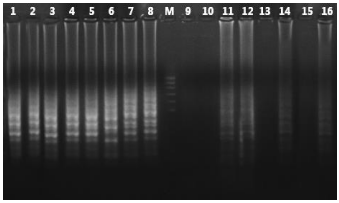

Figure 3.7A: LAMP product at a concentration of 8mM (Lane 1-8) and 6mM (Lane 10-16).

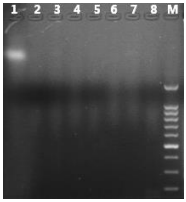

Figure 3.7B: LAMP product at a concentration of 4mM

$MgSO_4$ at a concentration of 4mM has no amplification product, $MgSO_4$ at a concentration of 6mM gives an unstable product, the yield is not high, $MgSO_4$ at a concentration of 8mM gives a LAMP product with high and stable amplification efficiency. Therefore, we chose $MgSO_4$ at the concentration of 8mM as the optimal concentration for the LAMP reaction.

3.1.2.3 LAMP reaction time

Conduct a survey on the minimum time to perform the LAMP reaction at the time conditions of 40 minutes, 60 minutes, except for the change in time, the components and other conditions of the reaction are kept the same. homogenized, the experiment was repeated 3 times. The results show that the minimum time for DNA amplification in the LAMP reaction of the kit is 60 minutes.

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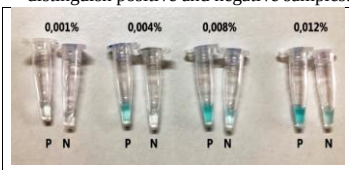
3.1.2.4 Color indicator used to read LAMP results

The concentrations of MG investigated were 0.0012%, 0.008%, 0.004% and 0.001%. Each concentration was repeated 3 times, the results were observed and recorded by 3 independent people. The results showed

100% similarity between the 3 observers and concluded that MG concentration of 0.004% was the optimal concentration that could distinguish positive and negative samples.



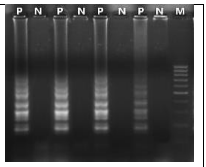


Figure 3.10 MG concentration survey results to read LAMP results

3.1.3 LAMP kit detection threshold

The test is divided into two phases.

Stage one: Perform primary cut-off point detection by performing a LAMP reaction with a sequence of 6 successive 10th-diluted recombinant plasmids concentrations (from 10-6ng/µL to 10-11ng/L). The lowest concentration at which amplification product is always present after the LAMP reaction is the primary detection threshold.

Stage two: Perform threshold detection with 95% confidence (LOD95%) by performing a LAMP reaction with a range of recombinant plasmid concentrations diluted asymptotically to the concentration of the primary threshold of detection state 1.

The results were evaluated by observing the color change of the solution in the tubes after the LAMP reaction and electrophoresis of the product on a 2% agarose gel. The results show that the primary detection threshold of the kit is 10^{-8} ng/µL, corresponding to $2.82x10^{0}$ copies/µL.

Table 3.8 Investigation of the detection threshold of intestinal strongyloidiasis diagnostic kit

Od	(ng/µL)	Copies /µL	Repeti -tions	(+)	Positive Ratio
1	$1x10^{-6}$	$2,82x10^{2}$	12	12	100,00
2	$1x10^{-7}$	$2,82x10^{1}$	12	12	100,00
3	$1x10^{-8}$	$2,82x10^{0}$	12	12	100,00
4	$7,5x10^{-9}$	$2,12x10^{0}$	12	11	91,67
5	$5 x 10^{-9}$	$1,41x10^{0}$	12	8	66,67
6	$2,5x10^{-9}$	$7,05 x 10^{-}$	12	6	50,00
7	$1,25x10^{-9}$	$3,53x10^{-1}$	12	4	33,33
8	$1x10^{-9}$	$2,82 x10^{-1}$	12	2	16,67
9	$6,25 x10^{-10}$	$1,41 x10^{-1}$	12	1	8,33
10	$3,125x10^{-10}$	$7,05x10^{-2}$	12	0	0,00
11	0	0	12	0	0,00

LOD95% of the LAMP kit for intestinal strongyloidiasis after the survey was $2.12x10^{0}$ gene copies/µL (95% CI:$1.71x10^{0}$ gene copies/µL to 2.89 $x10^{0}$ gene copies/µL). This is a good detection threshold, allowing detection of pathogens at low density.

3.1.4 The result of positive control

The conserved sequence on the 18S rRNA gene of intestinal strongyloides with the size of 596 bp was inserted into the pUC19 vector and cloned. As a result, we obtained the recombinant Plasmid carrying the 18S rRNA gene segment specific to intestinal strongyloides with size 3282bp. The amount of Plasmid obtained was 5 µg and dissolved in 50 µL to obtain a concentration of 100ng/µL, equivalent to $2.82x10^{10}$ copies/µL.

3.1.5 Result of manufacturing LAMP kit for diagnosis of *Strongyloides stercoralis* infection

We have made the kit and packaged it with a quantity of 2000 tests at the laboratory scale for testing and verification activities. The kit is packed with 50 test/set including: Instruction sheet of the kit and 6 reagent tubes. In addition to the box there is a label with full information about the production batch, expiration date, storage conditions, place of manufacture.

3.2 Evaluation of the sensitivity, specificity, and stability of the kit in the laboratory and in the field

3.2.1 Evaluation of the sensitivity and specificity of the LAMP kit at the laboratory

Testing 132 stool samples collected in Long An province and clinical samples collected from Pham Ngoc Thach university of medincine in 2018: stool test method and real-time PCR gave the same results, including 32 positive samples and 100 negative samples. , and the LAMP kit for intestinal strongyloidiasis has 34 positive and 98 negative samples.

Table 3.11 Results of sensitivity and specificity of the LAMP kit for intestinal strongyloidiasis

Result by LAMP	**Result by qPCR**		**Total**
	Positive	Negative	
Positive	31	3	34
Negative	1	97	98
Total	32	100	132
Se: 96,88% (95% CI: 83,78% - 99.92%) Sp: 97,00% (95% CI: 91,48% - 99,38%)			

Compared with qPCR method as reference method (gold standard), LAMP kit for intestinal strongyloidiasis has high sensitivity and

specificity, meeting the requirements for a diagnostic test kit.

3.2.2 Storage conditions and stability of intestinal strongyloidiasis diagnostic LAMP kit

The kit was packaged in January 2019 and stored at -20^{0}C ± 5^{0}C. The samples used for evaluation were 3 positive standards at concentrations of 10^{-6}ng/µL, 10^{-7}ng/µL and 10^{-8}ng/µL; 2 samples were positive; 2 samples were negative. Experiments were repeated once a month, every 3 months and after 11 months at storage conditions of 2-8^{0}C and -20^{0}C ± 5^{0}C after opening the kit cap.

The test results show that the kit still works stably after 6 months of opening the lid and 1 year from the date of manufacture without opening the lid, and at the same time, do not thaw and re-freeze the LAMP kit too much 3 times.

Table 3.12 Results of testing the stability of the Kit after 6 months of storage

(ng/µL)	Storage at 2-8^{0}C after opening the cap					
	1/2019	2/2019	3/2019	4/2019	5/2019	6/20
10^{-6}	(+)	(+)	(+)	(+)	(+)	(+)
10^{-7}	(+)	(+)	(+)	(+)	(+)	(+)
10^{-8}	(+)	(+)	(+)	(+)	(+)	(+)
Pos 1	(+)	(+)	(+)	(+)	(+)	(+)
Pos 2	(+)	(+)	(+)	(+)	(+)	(+)
Neg 1	(-)	(-)	(-)	(-)	(-)	(-)
Neg 2	(-)	(-)	(-)	(-)	(-)	(-)

Table 3.15 Investigate the stability of the kit after 12 months of storage

(ng/µL)	Storage at - 20^0C ± 5^0C	
	First time: 1/2019	**Second time: 12/2019**
10^{-6}	(+)	(+)
10^{-7}	(+)	(+)
10^{-8}	(+)	(+)
Sample ADN 1	(+)	(+)
Sample ADN 2	(+)	(+)
Neg1	(-)	(-)
Neg2	(-)	(-)

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3.2.3 Evaluation and comparison of kits in the field

3.2.3.1 Results of kit evaluation in the field

Evaluation of the performance of the kit in the field in 4 communes of Duc Hoa, Long An province. The total number of samples evaluated in 2 phases is 300 samples. Stool samples were taken by 3 methods of direct fecal examination for larvae of *S. stercoralis*, using LAMP kit for intestinal strongyloidiasis and qPCR. Fecal examination did not reveal any strongyloidiasis. The LAMP and qPCR results were positive for S. stercoralis with 3 identical samples and negative for the remaining 297 samples.

3.2.3.2 Comparison of LAMP kit with primers with similar purpose

Compared the LAMP kit in our study with the LAMP primer kit for intestinal strongyloidiasis by author Perdro- Fernandez- Soto et al. on 25 samples (+), 25 samples (-) were determined by real-time PCR. The results are shown in Appendix 4 and Table 3.19. With K = 0.91, the diagnostic detection ability of the LAMP kit in this study is in high agreement with the published primer set of Perdro.

3.2.3.3 Comparison of 3 techniques LAMP, fecal smear, ELISA in diagnosis of strongyloidiasis

The results of diagnostic tests for intestinal strongyloidiasis of 141 patients who visited the Outpatient, NIMPE are as follows:

Table 3.20 Summary of the results of diagnosis of strongyloidiasis in the Department of Medical Examination by 3 methods

	ELISA		**LAMP**	
	(+)	**(-)**	**(+)**	**(-)**
Fecal smear (+): 31 samples	20	11	30	1
Fecal smear (-): 110 samples	51	59	43	67
Total	71	70	73	68

The LAMP kit's ability to detect *S. stercoralis* diagnosis was in low agreement with fecal smear, K=0.39; in good agreement with the ELISA method, K=0.74.

3.2.4 Register to verify the basic standards of the kit.

Table 3.23 Baseline criteria of the LAMP kit for the diagnosis of *S. stercoralis.*

Od	**Evaluation criteria**	**Basic Standard**
1	Identification	LAMP reaction at 630C for 60 min Positive sample: Solution turns from dark green to light blue Negative sample: Solution turns from dark green to colorless
2	Sensitive	> 95%
3	Specific	> 95%
4	Detection threshold	3 copies /µL
5	Execution time	Less than 3 hours
6	Stability	12 months with storage conditions -20^{0}C ± 5^{0}C; 6 months from the date of

This set of basic standards is appraised by the National Institute for Accreditation of vaccines and medical biological products to meet the requirements according to the announcement.

Chapter 4 DISCUSSION

4.1 Development of a procedure and fabrication of a LAMP kit for the diagnosis of intestinal *S. stercoralis* infection in humans

4.1.1 Result of primer design

Many PCR assays have been designed to detect *S. stercoralis*, with the target gene being the mitochondrial gene cythocrome c oxidase subunit I (COX1) [71] or the nuclear gene 18S rRNA [65], [112] or 28S rRNA [73]. Among them, the amplification of the 18S small subunit has been shown to be more sensitive and is used in most qPCR assays for the diagnosis of Strongyloides stercoralis [31], [43], [56]. We selected the target gene region in our study as 18S rRNA. The results of tables 3.1, 3.2, 3.3 and 3.4 show that when evaluating the quality of the primers compared to the standard, the primers all meet the standards.

The results in Figure 3.4 show that, using Primer Blast software to check, primers F3, B3 are 100% paired on the gene bank and have amplification products with the same size of 226 bp. When performing PCR using primers F3 and B3 in the LAMP primer set with self-designed primer sets DNA templates that ensure specificity for *S. stercoralis*, there is no cross-reactivity with other worm species.

4.1.2 Determination of LAMP reaction conditions with self-designed primers

Primer temperature (Ta) is an important parameter when optimizing PCR reaction. Ta is too high, the primer cannot be coupled to the template circuit, Ta too low will lead to the formation of non-specific products. The optimal hybridization temperature fluctuates around the primer denaturation

temperature (Tm) 5ºC. Accordingly, the temperature of the LAMP reaction was investigated from 60 to 65°C. The results of electrophoresis of LAMP products showed that there was no difference between the three points of investigation temperature of 63^0C, 64^0C, and 65^0C (Figure 3.6).). We chose a temperature point of 63°C because this temperature facilitates primer annealing and enhances tolerance to inhibitors.

In the buffer solution, the Mg^{2+} ion plays the most important role, it increases the melting point (Tm) of the double-stranded DNA, forming a soluble complex with dNTPs to form a substrate that the polymerase can recognize. required for the binding of dNTPs. Through product electrophoresis on 2% agarose gel, we selected $MgSO_4$ at 8mM as the optimal concentration for LAMP reaction.

We tested at the reaction time levels of 40 minutes and 60 minutes, with the reaction conditions and components kept the same and uniform, the experiment was repeated 3 times. The results show that the minimum time for DNA amplification in the LAMP reaction of the kit is 60 minutes (Figure 3.8).

The turbidity due to the white precipitate produced after the LAMP reaction is detectable to the naked eye but it has a short precision (about 5-10 seconds) after the sample is removed from the incubator. Using fluorescent dyes such as calcein or SYBR Green I as in the study of other authors [86], [87] are expensive, requiring a UV lamp system to read the results. Furthermore, calcein can combine with Mg2+ ions to inhibit DNA polymerase activity and reduce the overall sensitivity of the test. Malachite Blue (MG) indicator has been successfully used as a pH sensitive indicator for the detection of LAMP products and studies have demonstrated its effectiveness [81], [96]. The addition of MG to the LAMP buffer prior to the reaction did not affect the activity of Bst DNA polymerase, while eliminating the risk of cross-sample contamination. Furthermore, this technique

can be used to rapidly screen a substantial number of samples with consistent, reproducible results using simple and inexpensive equipment. The concentrations of MG investigated were 0.0012%, 0.008%, 0.004% and 0.001%. Each concentration was repeated 3 times, the results were observed and recorded by 3 independent people. The results showed that there was 100% similarity between the 3 observers (table 3.6) at MG concentration 0.004%.

From the results of the primary detection threshold of the primer set, we determined the detection threshold with 95% confidence (LOD95%). This detection threshold result is equivalent to other studies on the application of molecular biology to diagnose strongyloidiasis by authors around the world, which is the basis for developing the application of the LAMP kit with good sensitivity for diagnosis intestinal strongyloidiasis in Vietnam.

4.1.3 The result of positive control

Positive controls in *S. stercoralis* infection detection LAMP kits were constructed using recombinant DNA technology according to NIMPE.HD 03.PP/44 guidelines. We obtained the recombinant plasmid carrying the 18S rRNA gene fragment specific to intestinal strongyloides with size 3282bp. The amount of plasmid recovered was 5 µg and dissolved in 50 µL to obtain a concentration of 100 ng/µL, equivalent to $2.82x10^{10}$ copies/µL, meeting the standard control for 2000 tests.

4.2 Evaluation of the sensitivity, specificity, and stability of the kit in the laboratory and in the field

4.2.1 Evaluation of the sensitivity and specificity of the LAMP kit at the laboratory

We choosed qPCR as the reference method because these are two molecular biology methods with equivalent sensitivity on different matrices. This qPCR method amplifies also the 18S rRNA gene fragment,

which has been determined to have a specificity higher than 99% when compared with the Harada-Mori stool culture method, which has 100% sensitivity for infections. moderate and severe infections [116]. The results show that the LAMP kit for intestinal strongyloidiasis has high sensitivity and specificity, meeting the requirements for a diagnostic test kit as well as the high concordance between the two methods.

4.2.2 Storage conditions and stability of intestinal strongyloidiasis diagnostic LAMP kit

The stability of the kit was conducted through 3 tests, the results showed that if stored at - 20^0C ± 5^0C, the kit will still work stably after 1 year, and if it has been thawed and preserved Stored at 2-8^0C, the kit still works stably after 6 months, longer time is not investigated. So if the kit is applied in the field, it is completely feasible. In large medical facilities, where storage conditions with deep negative refrigerators (-20^0C ± 5^0C) are available, the kit has a shelf life of up to 1 year. In contrast, at local medical facilities, which do not have storage conditions with deep freezers, after defrosting, the kit is stored in the cooler of a regular refrigerator with a temperature of 2-8^0C. Kit still has a shelf life of 6 months. In addition, the results also show that do not thaw and refreeze the kit more than 3 times. Thus, when there is a need to travel far, the kit will still work well after that (even though it is then stored at - 20^0C ± 5^0C or 2-8^0C) but it is necessary to avoid thawing and then re-freezing a lot. times will make the kit no longer give accurate results

4.2.3 Evaluation and comparison of kits in the field

Fecal smear did not detect any intestinal strongyloidiasis (0%) and qPCR and LAMP methods both gave 100% similar results with 3 positive cases (1%). In terms of the prevalence of strongyloidiasis in our 300 field samples, this rate is much lower than the research results in 5 communes

and towns of Duc Hoa district by author Le Duc Vinh (2017 - 2018). 25] is 6.64%. The difference in rates is due to the author Le Duc Vinh uses many different methods of stool testing (including stool culture technique), the author's research is also conducted in other areas, leading to different results. The differences in local epidemiological characteristics as well as the difference in sampling time and the larger sample size in our study should lead to the dissimilarity in the prevalence of strongyloidiasis.

We searched for available kits, but there is no LAMP kit for intestinal strongyloidiasis on the market. Therefore, we compared the LAMP kit in the study with the results of using the LAMP primer to diagnose intestinal strongyloidiasis by author Perdro- Fernandez- Soto et al., published in the journal Plos Neglected tropical diseases in 2016 [86]. The Kappa coefficient of 0.92 shows that our LAMP kit is highly compatible with Perdro's published primers, meeting the standards and reliability for use in evaluation studies or applying LAMP techniques.

Appendix 5, tables 3.20 and 3.21 show that the diagnostic detection ability of the LAMP kit is in low agreement with the direct fecaloscopy method. As we know, fecaloscopy has low sensitivity in the diagnosis of strongyloidiasis [31], while LAMP is more sensitive. Appendix 5, tables 3.20 and 3.22 show that the diagnostic detection ability of the LAMP kit is quite consistent with the ELISA method. There were 18 cases that were only positive for either LAMP or ELISA techniques. These two methods use two different mechanisms, so in cases where serum antibodies and strongyloidiasis DNA do not appear together, there will be different results between ELISA and LAMP results.

4.2.4 Register to verify the basic standards of the kit.

The research team has developed the baseline criteria for the LAMP kit for intestinal strongyloidiasis, which meets the criteria set out in our study and is eligible to be widely deployed than in the field.

CONCLUSION

From the results of research on manufacturing LAMP kit for diagnosis of intestinal strongyloidiasis (Strongyloides stercoralis) in humans 2017-2020, we have the following conclusions:

5.1 Develop a procedure and manufacture a LAMP kit for the diagnosis of intestinal *S. stercoralis* infection in humans

5.1.1 The manufacturing process of LAMP kit for diagnosis of intestinal strongyloides Strongyloides stercoralis has been developed, including four steps:

- Step 1: Design a specific primer set for intestinal strongyloides on the 18S rRNA gene region including 6 primers.
- Step 2: The reaction conditions are optimized as follows:

+ The primer pairing temperature is 63^0C
+ Reaction time is 60 minutes
+ Mg^{2+} concentration is 8mM
+ The color indicator used is malachite green 0.004%.

- Step 3: Determine the 95% LOD detection threshold of the kit as $2.12x10^0$ gene copies/µL
- Step 4: Prepare positive controls by creating recombinant plasmids carrying conserved regions on the 18S rRNA gene of intestinal strongyloides using cloning vector pUC19.

5.1.2. From the above procedure, we havesSuccessfully manufacture 2000 LAMP tests packed in 40 kits for the diagnosis of intestinal strongyloidiasis Strongyloides stercoralis at the laboratory scale.

5.2 Evaluation of the sensitivity, specificity, and stability of the kit in the laboratory and in the field

- The sensitivity of the LAMP kit to diagnose intestinal strongyloides reached 96.88%. (95% CI: 83.78% to 99.92%), specificity of 97.00% (95% CI: 91.48%-99.38%).
- The kit is stable after 12 months of storage at $-20^0C \pm 5^0C$ and 6 months after opening when stored at $2\text{-}8^0C$.
- Evaluation of the kit in the field: 100% similarity with qPCR.
- Compare the kit with the primer set with the same purpose (Perdro-Fernandez- Soto primer set): similarity coefficient K = 0.91; high fit.
- LAMP kit for the diagnosis of intestinal infection *Strongyloides stercoralis* has been accredited and certified by the National Institute for Accreditation of Vaccines and Medical Biologicals as satisfactory according to the published baseline standards.

RECOMMENDATIONS

- Continue to evaluate the LAMP kit for the diagnosis of intestinal Strongyloides stercoralis on clinical samples in a broader range to supplement the effectiveness of the kit at the site.
- Research and improve the way the software package is packaged to match the requirements of the user units
- Registering intellectual property and technology to be able to produce a set of materials to supply to hospitals at the provincial and district levels, and for PhD students and teachers throughout the country.

LIST OF PUBLISHED ARTICLES RELATED TO THE THESIS

1.Tran Thi Kim Chi, Nguyen Thi Huong Binh, Tran Xuan Mai et al (2021), "Development LAMP assay for the diagnosis of *Strongyloides stercoralis* infection in human", *Journal of malaria and parasite diseases control*, 1 /2021.

2. Tran Thi Kim Chi, Nguyen Thi Huong Binh, Tran Xuan Mai et al (2021), "Evaluation of the sensitivity, specificity, and stability of the *Strongyloides stercoralis* diagnostic LAMP kit", *Journal of malaria and parasite diseases control*, 1 /2021.