DEVELOPMENT OF SIMPLEX AND MULTIPLEX DETECTION FOR THREE MAJOR PARASITES IN POULTRY BASED ON LOOP-MEDIATED ISOTHERMAL AMPLIFICATION COUPLED WITH A LATERAL FLOW DIPSTICK ASSAY AND MICROFLUIDIC CHIP TECHNOLOGY

WASIN PANICH

Graduate School Srinakharinwirot University

2024

การพัฒนาวิธีการตรวจสอบแบบเดี่ยวและแบบผสม สำหรับตรวจหาหนอนพยาธิสามกลุ่มหลักในสัตว์ปีก โดยอาศัยเทคนิค loop-mediated isothermal amplification ควบคู่กับการตรวจสอบผลแบบ lateral flow dipstick และเทคโนโลยี microfluidic chip

วศิน พานิช

ปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปรัชญาดุษฎีบัณฑิต สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
ปีการศึกษา 2567

DEVELOPMENT OF SIMPLEX AND MULTIPLEX DETECTION FOR THREE MAJOR PARASITES IN POULTRY BASED ON LOOP-MEDIATED ISOTHERMAL AMPLIFICATION COUPLED WITH A LATERAL FLOW DIPSTICK ASSAY AND MICROFLUIDIC CHIP TECHNOLOGY

WASIN PANICH

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of DOCTOR OF PHILOSOPHY

(Biotechnology)

Faculty of Science, Srinakharinwirot University

2024

THE DISSERTATION TITLED

DEVELOPMENT OF SIMPLEX AND MULTIPLEX DETECTION FOR THREE MAJOR PARASITES IN POULTRY BASED ON LOOP-MEDIATED ISOTHERMAL AMPLIFICATION COUPLED WITH A LATERAL FLOW DIPSTICK ASSAY AND MICROFLUIDIC CHIP TECHNOLOGY

BY

WASIN PANICH

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY AT SRINAKHARINWIROT UNIVERSITY

.....

(Assoc. Prof. Dr. Chatchai Ekpanyaskul, MD.)

Dean of Graduate School

.....

ORAL DEFENSE COMMITTEE

..... Major-advisor

(Assoc. Prof. Dr.Thapana Chontananarth)

..... Chair

(Assoc. Prof. Dr.Kittisak Buddhachat)

..... Co-advisor

(Asst. Prof. Dr.Thanawan Tejangkura)

..... Committee

(Assoc. Prof. Dr.Siwaporn Longyant)

..... Co-advisor

(Asst. Prof. Dr.Chokchai Puttharugsa)

Title	DEVELOPMENT OF SIMPLEX AND MULTIPLEX DETECTION FOR THREE MAJOR PARASITES IN POULTRY BASED ON LOOP-MEDIATED ISOTHERMAL AMPLIFICATION COUPLED WITH A LATERAL FLOW DIPSTICK ASSAY AND MICROFLUIDIC CHIP TECHNOLOGY
Author	WASIN PANICH
Degree	DOCTOR OF PHILOSOPHY
Academic Year	2024
Thesis Advisor	Associate Professor Dr. Thapana Chontananarth
Co Advisor	Assistant Professor Dr. Thanawan Tejangkura
Co Advisor	Assistant Professor Dr. Chokchai Puttharugsa

The study of morphological characteristics, conducted through post-mortem and parasite egg examinations in host's digestive tract and feces, is a standard set of methods for the diagnosis of gastrointestinal helminth infections. However, these methods require parasitological knowledge and technical skills for species identification, making large-scale sample analysis time-consuming. This study sought to develop the molecular assays using loop-mediated isothermal amplification (LAMP) combined with lateral flow dipstick (LFD) and microfluidic chip technology for the simplex and multiplex detections of three major parasitic groups in poultry: *Raillietina* (*Raillietina echinobothrida*, *R. tetragona*, and *R. cesticillus*), *Ascaridia galli*, and Echinostomatidae (*Echinostoma miyagawai*, *E. mekongi*, *E. macrorchis*, and *Hypoderaeum conoideum*). Seven assays were developed consisting of three simplex LAMP-LFD assays, three duplex LAMP-LFD assays, and one colorimetric LAMP on a microfluidic chip coupled with chromatic analysis. All assays exhibited high specificity in detecting the target species without cross-amplification. Regarding the analytical sensitivities, they showed minimum detectable DNA concentrations ranging from 5×10^{-2} to 5×10^{-4} ng/reaction for the LAMP-LFD assays and 40×10^{-1} to 40×10^{-3} ng/chip for the colorimetric LAMP on a microfluidic chip. To evaluate the diagnostic performance of each assay, 30 fecal samples were examined. These assays successfully detected parasitic infections in fecal samples, with clinical sensitivities ranging from 66.67% to 100% and clinical specificities from 89.29% to 100%, showing agreement from low to high levels of agreement (K= 0.35-0.93). Moreover, McNemar's tests indicated that all developed assays were not significantly different when compared to post-mortem examinations. Therefore, these detection tools could serve as a viable alternative assays for a variety of purposes and guide decision-making regarding the use of anthelmintic drugs for treatments and farm management in the future.

Keyword : Multiplex detection, Loop-mediated isothermal amplification (LAMP), Lateral flow dipstick (LFD), Microfluidic chip, Chromatic analysis

ACKNOWLEDGEMENTS

The first, I would like to thank my thesis major advisor, Assoc. Prof. Dr. Thapana Chontananarth, for the continuous support of my parasitic knowledge and a lot of opportunities in scientific research. Thanks to my minor advisors, Asst. Prof. Dr. Thanawan Tejangkura for consulting me in molecular biology issues or had a question about my research writing, and Asst. Prof. Dr. Chokchai Puttharugsa for very kind teaching in microfluidic chip technology and color theory knowledges.

Besides my advisor, I would like to thank my lovely little brother, Dr. Sirapat Nak-on, who always side by side since day one of my doctor's degree study, as well as other members in the Applied Parasitology Research Laboratory and the laboratories in 11th floor, Department of Biology, Faculty of Science at Srinakharinwirot University, especially Tripatchara Atiratana and Terawut Prasitporn etc.

I would like to thank my thesis committees, namely Assoc. Prof. Dr. Kittisak Buddhachat and Assoc. Prof. Dr. Siwaporn Longyant, for taking the time to carefully read my thesis and advising excellent comments to further develop the future work.

Thanks to Srinakharinwirot University, the National Research Council of Thailand (NRCT) and the Science Achievement Scholarship of Thailand (SAST) for providing research funding.

Finally, I am so grateful to thank my warmly family, Potipan Panich, Tathata Panich, Chinudda Panich, and other members for kindly love, care, support and proud of me.

WASIN PANICH

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1
INTRODUCTION

Background

Poultry industry has continuously been growing every single year over the last decades. According to the Food and Agriculture Organization (FAO), about 75 % of chicken are raised in the developing countries (Ola-Fadunsin et al., 2019). Poultry are domesticated birds raised by human for the purpose of producing meat and eggs, especially chicken (*Gallus gallus domesticus*) and duck (*Anas platyrhynchos domesticus*), which are affordable sources of essential proteins and nutrition (Uhuo et al., 2013). Thus, the poultry industry has increased its economic value in several countries, including Singapore, Malaysia, and Thailand (Aini, 1990). Poultry farming are managed under different systems, namely an organic free-range, conventional free-range (housing system) and intensive indoor system (Daş et al., 2010; Gauly et al., 2002; Grafl et al., 2017). The organic free-range and conventional free-range systems are mainly conducted by small ranchers in agricultural areas, where animals eat a variety of foods that serve as intermediate or paratenic hosts for parasites in nature, such as insects, arthropods, earthworms, and snails (Ferdushy et al., 2016).

Gastrointestinal helminth parasites infection is an important issue in the commercial production process that causes economic losses, such as interfering the host metabolism, reducing feed conversion ratio, slowing growth rate, increasing weight loss, decreasing egg production, and increasing mortality (Nair & Nadakal, 1981; Ola-Fadunsin et al., 2019; Van et al., 2020). The gastrointestinal helminth parasites in poultry can be divided into three groups; (1) tapeworms (cestodes), (2) roundworms (nematodes) and (3) flukes (trematodes) (Ola-Fadunsin et al., 2019; Shifaw et al., 2021).

Cestode belonging to the genus *Raillietina*, namely *Raillietina echinobothrida*, *R. tetragona*, and *R. cesticillus*, is the most important group that spread around the world with the high prevalence as 84.40% in Zimbabwe (Butboonchoo & Wongsawad, 2017; Eshetu et al., 2001; Mukaratirwa & Hove, 2009; Ngongeh et al., 2014). Regarding nematode group, *Ascaridia galli* is one of the most common nematodes affecting poultry

health with the cosmopolitan prevalence of 10.40–96.70% in Austria, Italy, Germany, Netherlands, Tunisia, Nigeria, Nepal, and Thailand (Ben Slimane, 2016; Offiong et al., 2013; Sharma et al., 2018; Subedi et al., 2018; Thapa et al., 2015; Wuthijaree et al., 2019). In trematode group, the dominant species that frequently found in chicken and duck is trematode belonging to the family Echinostomatidae, including *Echinostoma* spp. and *Hypoderaeum* sp. (Begum et al., 2019; Butboonchoo & Wongsawad, 2017; Fu et al., 2019; Li et al., 2019; Yang et al., 2015). Moreover, several previous studies reported co-infections between those groups in poultry, leading to the necessity of the diagnostic method to discriminate each parasitic infection for appropriate anthelmintic drugs prescription (Ekpo et al., 2010; Hassan et al., 2015; Hembram et al., 2015; Khanum et al., 2021; Suhaila et al., 2015).

Conventional diagnostic methods rely on the morphological characters of adult or egg stages by post-mortem detection or copromicroscopic investigation of eggs in feces (Carney, 1991; Panich et al., 2022; Tarbiat et al., 2021; Zloch et al., 2021). Nevertheless, the morphological identification requires personal experiences, professional skills, and is time-consuming for several sample detection at the same time. In order to find a user-friendly method, molecular methods based on DNA amplification, such as high annealing temperature random amplified polymorphic DNA (HAT-RAPD) (Butboonchoo & Wongsawad, 2017), species-specific PCR (Watcharakranjanaporn et al., 2021), melting curve analysis (Buddhachat & Chontananarth, 2019), multiplex PCR (Tantrawatpan & Saijuntha, 2020) have been applied as an alternative choice since they are simpler, more convenient, and more reliable than the conventional methods. However, these techniques need scientific instruments that set-up in a laboratory to perform the reaction and result analysis, such as a thermal cycler machine and gel electrophoresis devices, which is a limitation for some laboratories and point-of-care stations, as well as the costly reagents and long reaction period.

Loop-mediated isothermal amplification (LAMP) is a feasible molecular technique, which requires at least four primers recognizing six target DNA regions and a *Bst* DNA polymerase with strand displacement activity for DNA amplification under the

constant temperature without any costly equipment. LAMP also offers higher sensitivity and specificity and more tolerant to low purity DNA sample than PCR. Thus, LAMP has become a diagnostic method with greater potential to detect clinical samples recently (Adao & Rivera, 2016; Shang et al., 2020; Wan et al., 2019; Wang et al., 2019). Consequently, LAMP has frequently been used for the detection of several species of parasites, such as *Opisthorchis viverrini*, *Clonorchis sinensis*, *Echinococcus granulosus*, *Taenia* spp., *Ascaris lumbricoides*, and *Necator americanus* (García-Bernalt Diego et al., 2021). Nevertheless, a multiplex detection using LAMP has not been reported to simultaneously detect the helminth parasites in one reaction.

Hence, the objective of this study was to develop multiplex detection methods based on LAMP integrated with a lateral flow dipstick (LFD) assay and microfluidic chip technology to simultaneously detect and discriminate the three major parasitic groups; *Raillietina* spp., *A. galli*, and Echinostomatidae in poultry.

Objectives

1. To develop simplex and duplex assays using LAMP-LFD for the detection and discrimination of *Raillietina* spp., *A. galli*, and Echinostomatidae infections in poultry.

2. To develop multiplex assay using colorimetric LAMP on a microfluidic chip coupled with a chromatic analysis for the detection and discrimination of *Raillietina* spp., *A. galli*, and Echinostomatidae infections in poultry.

Hypothesis

The developed simplex, duplex, and multiplex assays could detect and discriminate the infections of *Raillietina* spp., *A. galli*, and Echinostomatidae simultaneously in clinical samples from poultry.

CHAPTER 2
LITERATURE REVIEWS

Economic importance of chicken production

Chicken is an important and affordable source of essential protein and a chicken has the highest quantity of protein per weight when compared with another terrestrial economic animals (Agricultural Research Service, 2019). Moreover, it can generate a higher income to agriculturist than other economic animals because of the low production period and the high rate of feed conversion ratio. As a result, the production and consumption of chicken in Thailand are higher than other types of meat and consistently grow every single year as was reported by the Office of Agricultural Economics (**Figure 1**) (Kornboontritos, 2023). In 2012-2016, Thailand exports more than 6 hundred thousand tons of chicken meat per year, making it ranked the fourth of the world (Department of Livestock Development, 2022).

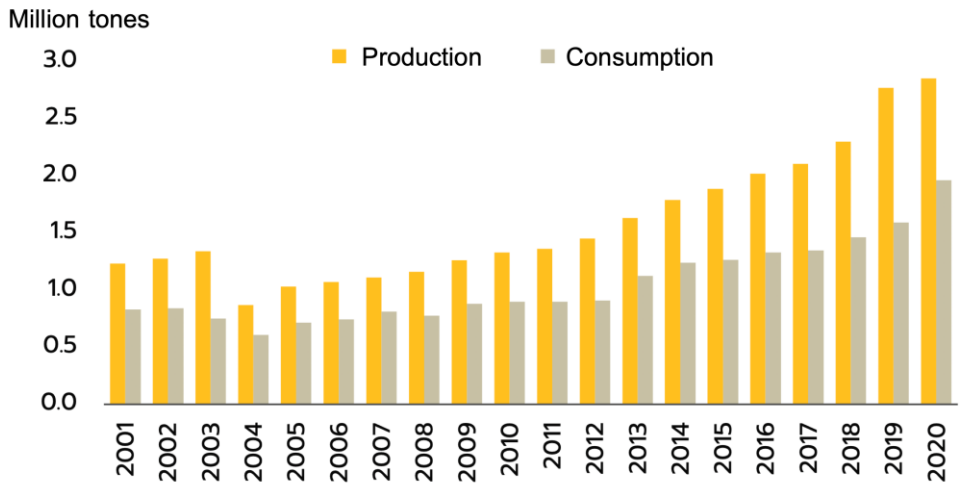


Figure 1 Chicken production and consumption in Thailand

Source: modified from Kornboontritos, S. (2023). Business/Industry outlook 2023-2025: Chilled, frozen and processed chicken industry. Krungsri Research. https://www.krungsri.com/th/research/industry/industry-outlook/food-beverage/frozen-processed-chicken/io/io-chilled-frozen-processed-chicken.

Important gastrointestinal parasites in poultry

Our study focused on several groups of gastrointestinal parasites that were frequently found and high-pathogenicity in poultry, causing animal health problem and loss of economic values. The major pathogenic parasites in poultry can be divided into three groups; 1) cestode belonging to the genus *Raillietina*, 2) the nematode, *A. galli*, and 3) trematode belonging to the family Echinostomatidae. These parasites cause damage to the intestinal mucosa leading to diarrhea, anemia, inflammation, malabsorption, and emaciation or death in case of heavy infections (Graczyk & Fried, 1998; Labony et al., 2022; McDougald, 2020; Mubarokah et al., 2019; Panich & Chontananarth, 2021).

1. Genus *Raillietina*

Cestodes belonging to genus *Raillietina*, comprising of more than 200 species, are commonly found in several species of birds and mammals. Key morphological characteristics of this genus are 1) presence of hammer-shaped rostellar hooks, 2) a single reproductive system per proglottid, consists of one ovary and more than one testis, and 3) vitelline gland always located posterior to the ovary (**Figure 2**). The genus *Raillietina* can be divided into 4 subgenera, including *Paroniella*, *Skrjabinia*, *Raillietina*, and *Fuhrmanneta* following unique characters: 1) the genital pores are unilateral (*Paroniella*) and alternating irregularly (*Skrjabinia*) in those patterns with one egg (oncosphere) per egg capsule and 2) the genital pores are unilateral (*Raillietina*) and alternating irregularly (*Fuhrmanneta*) in those patterns with several eggs (oncospheres) per egg capsule (**Figure 2**) (Butboonchoo et al., 2016; Sawada, 1964, 1965; Yamaguti, 1959). Over the past decade, several reports indicate that the three *Raillietina* species most commonly found and used in this study are *R. echinobothrida*, *R. tetragona*, and *R. cesticillus*, which can be identified to species level based on the shape of scolex and sucker, the number of row of rostellar hook, and the position of genital pore of each segment (**Figure 2**) (Begum et al., 2019; Butboonchoo & Wongsawad, 2017; Jegede et al., 2015; Ngongeh et al., 2014; Panich et al., 2021).

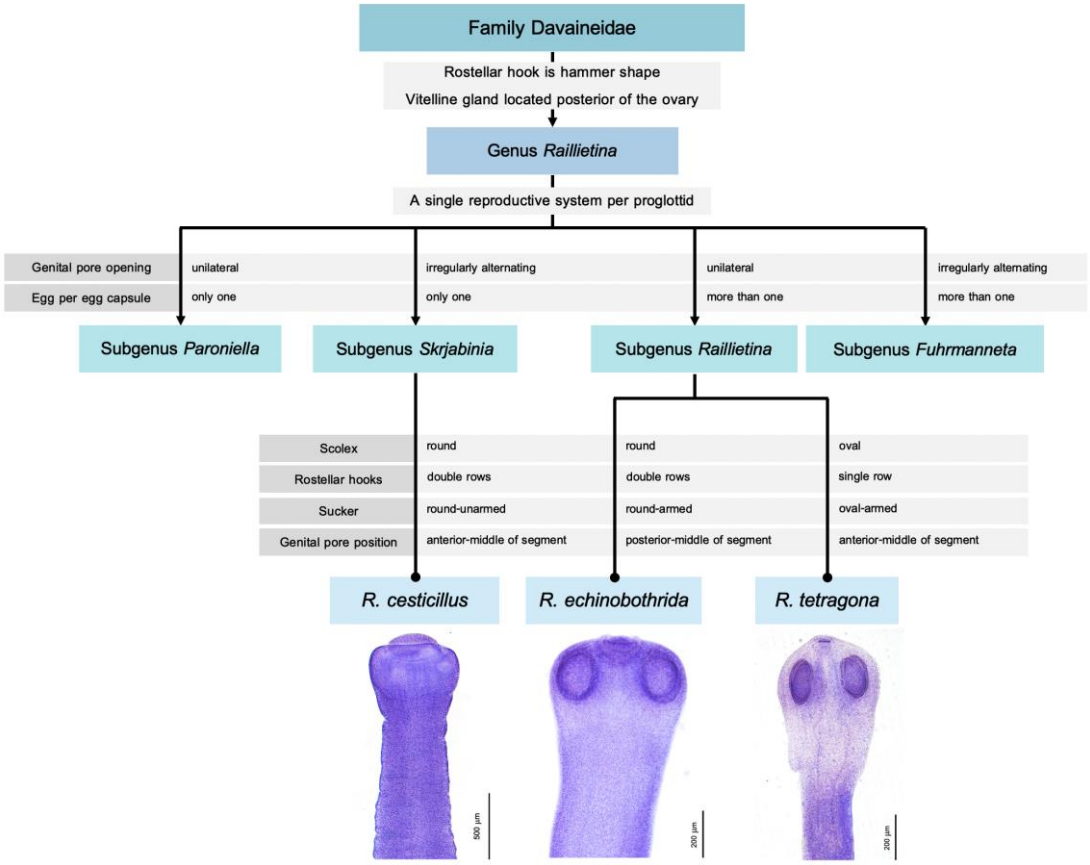


Figure 2 Classification of *R. echinobothrida*, *R. tetragona*, and *R. cesticillus*

Sources: modified from Panich, W., Tejangkura, T., & Chontananarth, T. (2021). Novel high-performance detection of *Raillietina echinobothrida*, *Raillietina tetragona*, and *Raillietina cesticillus* using loop-mediated isothermal amplification coupled with a lateral flow dipstick (LAMP-LFD). *Veterinary Parasitology*, *292*, 109396.

Life cycle of *Raillietina* spp.

The definitive hosts of tapeworm in genus *Raillietina* are birds and mammals, such as chicken, duck, pigeon, and rat (Alkharigy et al., 2018; Begum et al., 2019; Chaisiri et al., 2010; Chandler & Pradatsundarasar, 1957; Hassouni & Belghyti, 2006; Natala et al., 2009). The gravid proglottids are released from the definitive host via the fecal route and is eaten by intermediate hosts, such as ants, beetles, and flies (Bartel, 1965; Horsfall, 1938; McDougald, 2020; Reid et al., 1938). After that, the oncosphere penetrate through an alimentary canal of intermediate host and develop into

an infective stage, cysticercoid, at abdominal cavity. When the definitive hosts ingest the infected intermediate host, the cysticercoid adheres to the intestinal tract using rostellar hooks and suckers. The cysticercoid develops into an adult stage within 2 weeks (**Figure 3**) (Ackert, 1936; Reid et al., 1938). The epidemic of *Raillietina* spp. can speedily spread because a single gravid proglottid contains several egg capsules, ranging from 10-600 egg capsules. The gravid proglottids are also releasing up to 9 per day. Moreover, each egg capsule consists of 1-10 oncospheres (Baer & Sandars, 1956; Bartel & Hansen, 1964; Deardorff et al., 1976; Stunkard, 1953).

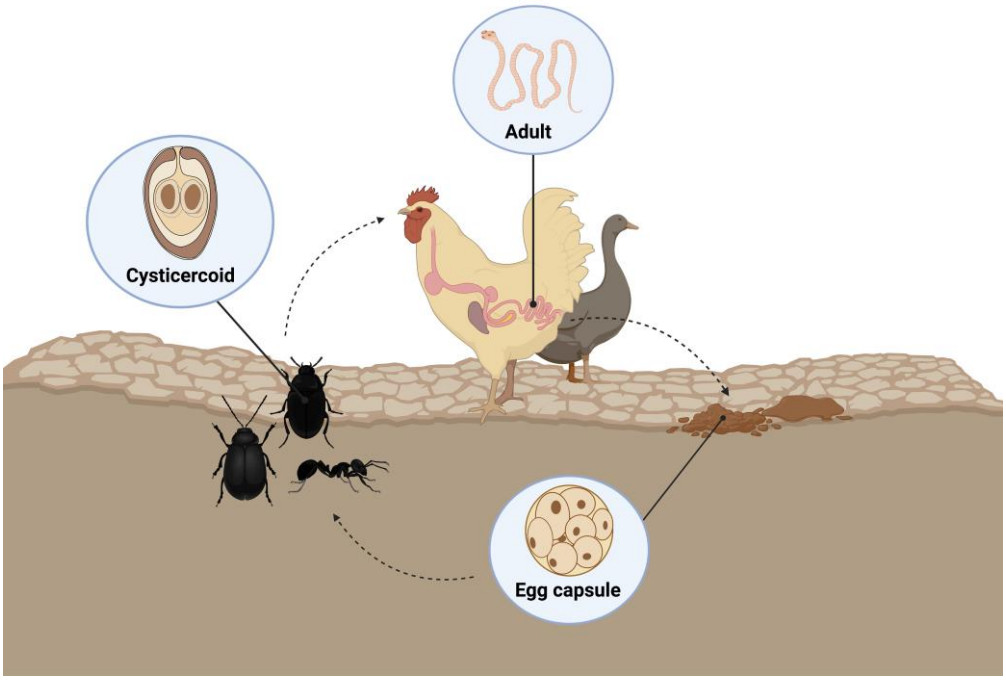


Figure 3 Life cycle of *Raillietina* spp.

Epidemiology of *Raillietina* spp. in poultry

During the past couple of decades, *R. echinobothrida*, *R. tetragona*, and *R. cesticillus* have been frequently reported in birds, especially chicken, from several countries around the world, including Thailand (**Table 1**).

Table 1 The prevalences of *R. echinobothrida*, *R. tetragona*, and *R. cesticillus* infection in chicken, duck, and pigeon around the world

Parasite	Prevalence (%)	Country	Reference
Host: chicken			
R. echinobothrida	12.0	Morocco	Hassouni & Belghyti (2006)
R. tetragona	9.3		
R. cesticillus	5.7		
R. echinobothrida	13.0	Nigeria	Luka & Ndams (2007)
R. tetragona	23.9		
R. cesticillus	9.8		
R. echinobothrida	42.0	Nigeria	Yoriyo et al. (2010)
R. tetragona	38.5		
R. cesticillus	10.5		
R. echinobothrida	33.3	Kenya	Mungube et al. (2008)
R. echinobothrida	13.2	Kenya	Kaingu et al. (2010)
R. tetragona	2.9		
R. echinobothrida	56.9	Nigeria	Attah et al. (2013)
R. echinobothrida	64.5	Nigeria	Idika et al. (2015)
R. tetragona	49.9		
R. cesticillus	4.9		
R. echinobothrida	48.3	Thailand	Butboonchoo & Wongsawad (2017)
R. tetragona	57.5		
R. cesticillus	12.5		
R. echinobothrida	29.6	Ethiopia	Molla et a. (2012)
R. tetragona	12.3		
R. cesticillus	1.1		
Host: duck			
R. echinobothrida	10.3	Nigeria	Paul et al. (2015)
R. tetragona	8.3		
R. tetragona	26.6	Iran	Shemshadi et al. (2017)

Table 1 (Continued)

Parasite	Prevalence (%)	Country	Reference
Host: duck			
R. echinobothrida	50.0	Bangladesh	Begum et al. (2019)
R. cesticillus	56.6		
R. echinobothrida	15.1	Nigeria	Ola-Fadunsin et al. (2019)
Host: pigeon			
R. echinobothrida	7.6	Nigeria	Natala et al. (2009)
R. tetragona	4.9		
R. cesticillus	3.0		
R. echinobothrida	63.0	Tanzania	Msoffe et al. (2010)
R. tetragona	6.0		
R. echinobothrida	32.0	Libya	Alkharigy et al. (2018)
R. tetragona	2.0		
R. cesticillus	4.0		

2. Genus *Ascaridia*

Family Ascaridiidae is parasitic nematodes that were classified to genus *Ascaridia*, which is only one genus in the family Ascaridiidae. The genus contains the important nematodes that are prevalent and pathogenic species in birds (Kajerova et al., 2004; Qazaz, 2020). At least 41 species of *Ascaridia* have been reported in birds. Three species are well known, including *A. galli*, *A. dissimilis*, and *A. columbae* (Griffiths, 1978). Among them, only *A. galli* is found in chicken production and is the most pathogenic species causing a major health problem affected the economic system (Biswas et al., 2021; Lalchhandama, 2010; Watcharakranjanaporn et al., 2021). Regarding the morphology, *A. galli* is the largest nematode in poultry, ranging from 50–120 mm. Typically, males are smaller than females and whole body is yellow whitish in color (Lalchhandama, 2010). In males, the perianal sucker is oval or circular and located posterior to the body; the tail is narrow-shaped with caudal alae and 10 pairs of papillae,

which are divided into the following four groups: 1) three pairs of preanal papillae, each pair located at anterior, middle, and posterior to the preanal sucker, 2) one pair of adanal papilla, 3) three pairs of postanal papillae and 4) three pairs at subterminal region. In females, the vulva is at the anterior part of the body. The eggs are oval, thick shelled, and develop outside the host (**Figure 4**) (Kajerova et al., 2004; Lalchhandama, 2010; McDougald, 2020; Tanveer et al., 2015; Tarbiat et al., 2015).

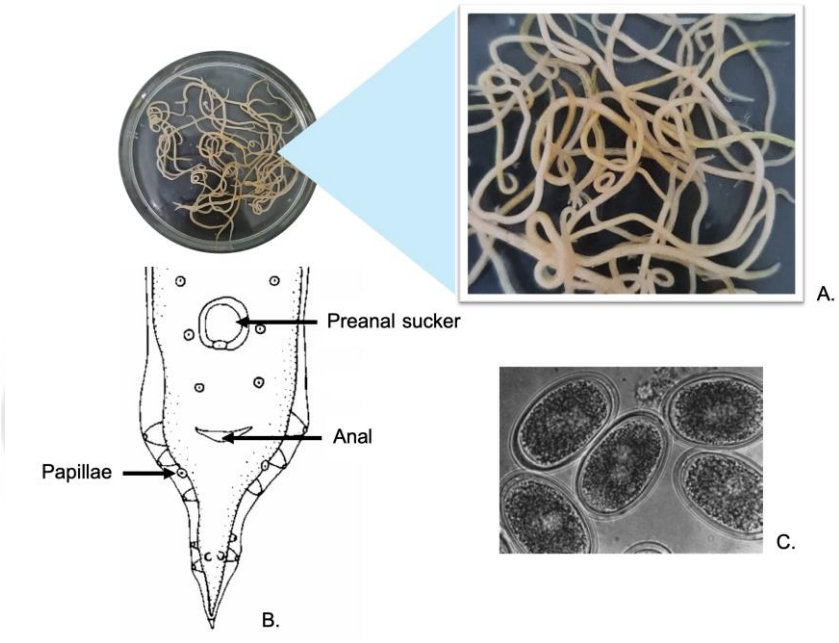


Figure 4 Morphology of *A. galli*

(A: *A. galli* freshly extracted from a chicken; B: drawing of male tail of *A. galli*; C: *A. galli* eggs from female using 400X magnification)

Sources: modified from Lalchhandama, K. (2010). On the structure of *Ascaridia galli*, the roundworm of domestic fowl. *Science Vision*, *10*(1), 20–30. McDougald, L. R. (2020). Internal parasites. *In diseases of poultry* (13th ed., pp. 1117–1146). Wiley-Blackwell.

Life cycle of *A. galli*

A. galli has a simple and direct life cycle, meaning that it can grow and develop to other stages for complete life cycle without an intermediate host. At the beginning, the eggs of *A. galli* hatch in the proventriculus or the upper intestinal tract of chickens. After hatching, the second-stage larvae live in the mucous layer of the duodenum and jejunum about 9 days after infection. Subsequently, they migrate to the spaces between the intestinal villi and eventually penetrate the mucosa, where they molt to the third larval stage. The third-stage larvae inhabit in the intestinal mucosa about 17 days before return to the intestinal lumen for molting and maturation of the fourth-stage and mature about 28 – 30 days of age. Finally, the matures reproduce sexually in the intestine. Females release fertilized eggs, which pass with the feces of host to complete their life cycle. The eggs embryonate in an environment about 5–14 days, depending on surrounding condition (**Figure 5**). Moreover, *A. galli* eggs use earthworms and grasshoppers as paratenic hosts to transport and transmit to chickens (Anderson, 2000; Chadfield et al., 2001; McDougald, 2020).

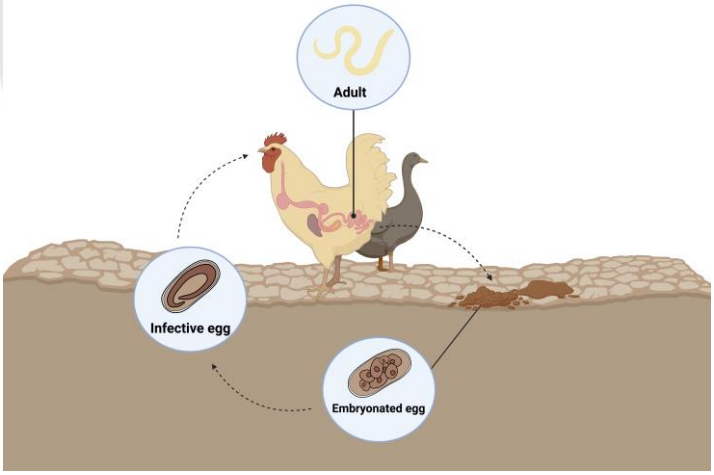


Figure 5 Life cycle of *A. galli*

Prevalence of *A. galli* in poultry

The infection of *A. galli* correlate with various factors, such as feed, age, sex, behavior, immunity, and extrinsic environments, which are affected to prevalence of

worms, range from low to high. The epidemic of *A. galli* occurred in several countries globally with the prevalence of up to 96.7% in chickens as reported by Thapa et al. (2015) from Netherlands. In Thailand, the *A. galli* infection has been reported in several regions, including northern, north-eastern, central, and southern parts with the prevalence of 21.9, 60.2, 21.5, and 8.3%, respectively (Ayudhya & Sangvaranonda, 1997; Ayudthaya & Sangvaranond, 1993; Sangvaranond, 1997; Wuthijaree et al., 2019). However, *A. galli* is not only infected in chicken, but also occurred in ducks as see in **Table 2**.

Table 2 The prevalences of *A. galli* infection in poultry

Host	Prevalence (%)	Country	Reference
Chicken	21.9	Thailand	Ayudthaya & Sangvaranond (1993)
	21.5	Thailand	Sangvaranond (1997)
	8.3	Thailand	Ayudthaya & Sangvaranond (1997)
	32.3	Tanzania	Permin et al. (1997)
	24.0	Ghana	Poulsen et al. (2000)
	30.7	India	Hange et al. (2007)
	32.6	Nigeria	Luka & Ndams (2007)
	25.6	Kenya	Kaingu et al. (2010)
	88.0	Germany	Kaufmann et al. (2011)
	53.3	Tunisia	Ben Slimane (2016)
	60.6	Austria	Thapa et al. (2015)
	54.3	Belgium	
	76.6	Denmark	
	89.5	Germany	
	96.7	Netherlands	
	72.6	Sweden	
	73.3	United Kingdom	

Table 2 (Continued)

Host	Prevalence (%)	Country	Reference
Chicken	50.8	Thailand	Butboonchoo & Wongsawad (2017)
	60.2	Thailand	Wuthijaree et al. (2019)
	36.6	Pakistan	Yousaf et al (2019)
	41.7	India	Jaiswal et al. (2020)
Duck	5.0	Egypt	AbouLaila et al. (2011)
	85.7	Nigeria	Paul et al. (2015)
	63.3	Bangladesh	Begum et al. (2019)
	72.7	Nigeria	Ola-Fadunsin et al. (2019)
	5.1	Egypt	El-Dakhly et al. (2020)
	27.5	Nepal	Shrestha et al. (2020)
	16.7	Bangladesh	Khanum et al. (2021)

3. Family Echinostomatidae

Species belonging to family Echinostomatidae are cosmopolitan intestinal parasites. Their taxonomic diversity are substantial (at least 91 genera have been described), related to a range of final hosts, including reptile, mammal, and avian (Toledo et al., 2009). Morphologically, the main characteristics feature of the adult worms of family Echinostomatidae is the presence of a circumoral collar spines arranged with one or two rows with a group of corner spines at each end, and cuticle is usually covered with tiny spines. Oral sucker, pharynx, and esophagus present obviously. Acetabulum or ventral sucker is well developed at the anterior of body. Regarding reproductive organs, testes are mostly tandem at posterior of body, genital pore is median and preacetabular, ovary is median or sub-median and located pretesticular. Vitelline follicle usually distributed laterally and postacetabular, in some genera extending into forebody (Toledo et al., 2009; Toledo & Esteban, 2016; Yamaguti,

1958). The morphological features used to identify to species level based on several characters, such as size, number, and arrangement of collar, corner spines; shape and size of testes; positions and size of oral, ventral suckers; size of cirrus sac; boundary of vitelline gland; and species of final host (Chai et al., 2021; Fried, 2001; Mohanta et al., 2019). This study is focused on two genera, including *Echinostoma* and *Hypoderaeum*, which are important parasites, having serious impacts on the poultry industry, especially chicken and duck (Anucherngchai et al., 2019; Yang et al., 2015).

Genus *Echinostoma* is easily identified by the collar, which is well developed and is interrupted ventrally, and their circumoral collar spines arranged in two rows of dorsal spines and one row of lateral spines. The number of collar spines is variable, ranging from 27 to 51 depending on species, but the corner spines are 5 at each side (formula: 2+5). Cuticle is covered with numerous spines. Ventral sucker is large, presented at anterior half of body. Testes are tandemly arranged at posterior body and ovary is located before anterior testis. The genital pore is preacetabular, and the cirrus sac is large. Vitellaria fields are postacetabular to posterior extremity. Excretory duct is usually bifurcated as Y-shaped behind the posterior testis. Overall size varies extremely among species (Fried, 2001; Huffman & Fried, 1990; Morgan & Blair, 1995; Roberts et al., 2009; Yamaguti, 1958). Members of "*E. revolutum* group" are an important and dominant species of genus *Echinostoma* because they are significant in terms of parasites of public health, medicine as well as veterinary health. This group has a unique character by 37 collar spines at their collar head and has a valid species, at least, 16 species (Anucherngchai et al., 2019; Anucherngchai & Chontananarth, 2019; Chai et al., 2020). However, the species used in this study are *E. miyagawai*, *E. mekongi*, and *E. macrorchis* as shown in **Figure 6**.

Genus *Hypoderaeum* characterized by a unique character of a head collar and collar spines, which are very weakly developed than other genera in family Echinostomatidae. *H. conoideum* is the most important outstanding species of the genus, which is frequently found in the intestines of chickens and ducks in many countries around the world, such as China, Japan, Siberia, Thailand (Anh et al., 2010;

Chai et al., 2009; Eom et al., 1984; Saijuntha et al., 2013; Tantrawatpan et al., 2016; Tantrawatpan & Saijuntha, 2020; Yang et al., 2015). Morphological characters of *H. conoideum* are a number of the collar spines range from 49-51, forebody and esophagus are very short. Cuticle is smooth without tiny spines. Sucker ratio, a ventral sucker is larger, at least 5 times, than oral sucker. Testes elongate, indented margins and tandemly arranged pattern in the middle of body. The ovary is round and located at pretesticular (**Figure 6**) (Azizi et al., 2015; Fried, 2001; Muñoz-Antolí et al., 2000; Toledo et al., 1996; Yamaguti, 1958).

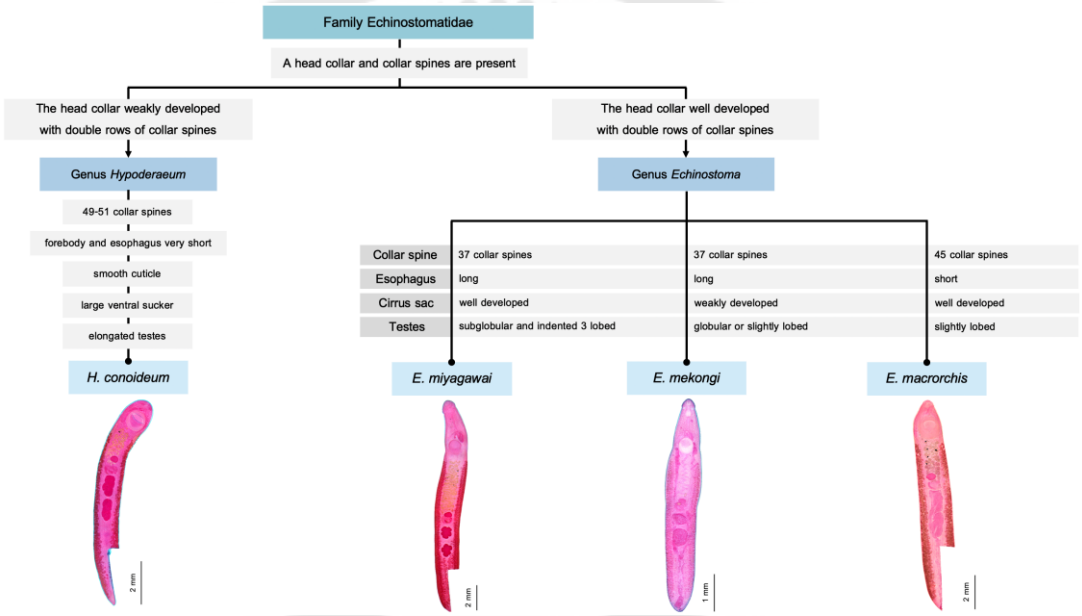


Figure 6 Classification of *E. miyagawai*, *E. mekongi*, *E. macrorchis* and *H. conoideum* used in this study

Source: modified from Glunkwamdi, P., Pakdee, S., & Raksaman, A. (2020). Epidemiological situation and risk of parasitic infections by ingesting of *Filopaludina* snails at local markets in Bangkok metropolis. Srinakharinwirot University.

Life cycle of Echinostomatidae (*E. miyagawai*, *E. revolutum* and *H. conoideum*)

Echinostomatidae species have a complicated life cycle contains seven stages, including egg, miracidium, sporocyst, redia, cercaria, metacercaria, and adult, which requires three hosts comprising the definitive, first, and second intermediate hosts. Initially, the adult worms infect the avian, particularly chickens and ducks, and release undeveloped eggs via the host feces into the water where they develop into the miracidial stage. When the miracidia hatch, they penetrate into their first intermediate hosts, several freshwater snail species, such as lymnaeid and planorbid snails, and develop into sporocysts, rediae, and finally cercariae stages, respectively. The cercariae emerge from their first intermediate hosts. After locating and entering the second intermediate hosts, namely snails, tadpoles, freshwater turtles, they encyst to develop metacercariae. Infection of the definitive hosts for the complete their life cycle, the definitive hosts become infected after ingestion of the second intermediate hosts containing the metacercariae. Finally, the metacercariae gradually mature to the adult stage in the small intestine of definitive hosts and adult worms produce eggs that are released through host feces a few weeks after infection (**Figure 7**) (Azizi et al., 2015; Chai et al., 2020; Kanev, 1994; Kanev et al., 2000; Li et al., 2019; Muñoz-Antolí et al., 2000; Nagataki et al., 2015; Roberts et al., 2009; Saijuntha et al., 2011, 2014; Toledo et al., 2009; Toledo & Esteban, 2016; Yang et al., 2015).

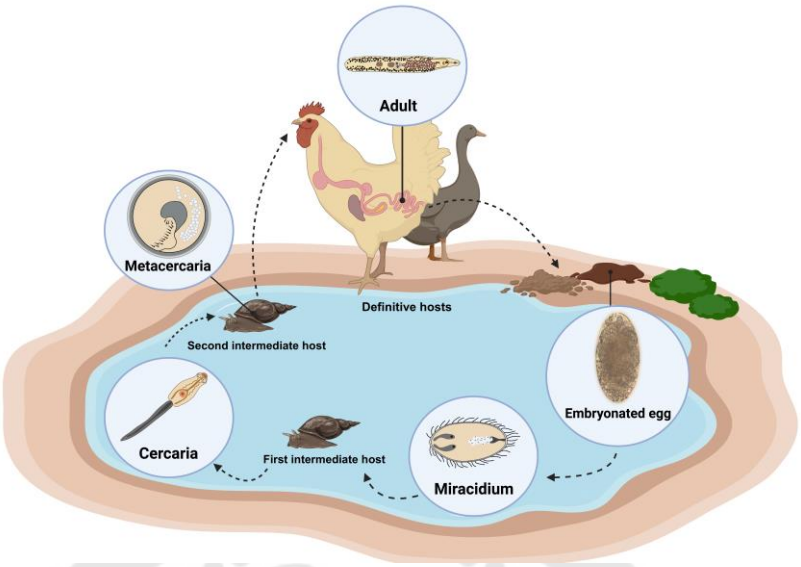


Figure 7 Life cycle of Echinostomatidae

Epidemiological reports of Echinostomatidae in poultry

Infections of Echinostomatidae species, most studies have been reported in first intermediate, second intermediate, and experimental hosts. On the other hand, the reports in natural hosts (chicken and duck) from Asia have a few studies and the prevalence have usually not been published, including Bangladesh, Thailand, Lao PDR, China, Indonesia, and Vietnam (**Table 3**).

Table 3 The prevalences of Echinostomatidae species infection in chickens and ducks

Parasite	Host	Prevalence (%)	Country	Reference
E. miyagawai	Duck	60.0	Bangladesh	Islam et al. (1988)
	Duck	NA	Thailand and Lao PDR	Nagataki et al. (2015)
	Chicken	NA	China	Fu et al. (2019)
	Duck	NA	China	Li et al. (2019)
	Duck	NA	Bangladesh	Mohanta et al. (2019)
	Duck	NA	Indonesia	Chai et al. (2021)

Table 3 (Continued)

Parasite	Host	Prevalence (%)	Country	Reference
E. revolutum	Duck	8.9	Bangladesh	Yousuf et al. (2019)
	Duck	NA	Thailand	Saijuntha et al. (2011)
	Duck	20.0	Bangladesh	Musa et al. (2012)
	Chicken	0.8	Thailand	Butboonchoo & Wongsawad (2017)
	Duck	20.0	Bangladesh	Begum et al. (2019)
	Chicken	73.3	Bangladesh	Khanum et al. (2021)
H. conoideum	Chicken	8.0	Vietnam	Anh et al. (2010)
	Duck	21.0		
	Duck	NA	Thailand	Saijuntha et al. (2011)
	Duck	35.3	Thailand	Saijuntha et al. (2013)
	Duck	NA	China	Yang et al. (2015)
H. conoideum	Duck	NA	Thailand	Tantrawatpan & Saijuntha (2020)

Co-infection of *Raillietina* spp., *A. galli*, and Echinostomatidae in poultry

Co-infections are usually found in free-range poultry system due to their food-seeking behavior associated with the different environmental habitats, such as land and water resources, which is often contaminated with parasitic eggs and various insects, earthworms, or water snails that act as paratenic and intermediate hosts of several

parasite species. **Table 4** shows previous studies reported the co-infection of each parasitic group in chickens and ducks since 2000.

Table 4 The occurrence of co-infection of *Raillietina* spp., *A. galli*, and Echinostomatidae in chickens and ducks

Parasite	Genus *Raillietina*			Host	Prevalence (%)	Reference
	R. echinobothrida	*R. tetragona*	*R. cesticillus*			
A. galli	+	-	-	Chicken	NA	Permin et al. (2002)
	-	+	-			
	+	-	-	Chicken	NA	Ekpo et al. (2010)
	-	+	-	Chicken	3.2	Ohaeri & Okwum (2013)
	+	-	-	Chicken	1.3	Adang et al. (2014)
	-	+	-		2.0	
	-	-	+		4.0	
	+	+	-		0.7	
	+	-	-	Chicken	1.0	Junaidu et al. (2014)
	-	+	-		6.0	
	-	+	-	Chicken	4.0	Yagoob & Mohsen (2014)
	-	+	+		1.0	
	+	-	-	Chicken	1.9	Hembram et al. (2015)
	-	+	-		14.4	
	+	+	-		1.3	
	+	-	-	Chicken	NA	Khanum et al. (2021)

Table 4 (Continued)

Parasite	Genus *Raillietina*			Host	Prevalence (%)	Reference
	R. echinobothrida	*R. tetragona*	*R. cesticillus*			
E. revolutum	+	-	-	Duck	NA	Begum et al. (2019)
	-	-	+			
	+	-	-	Chicken	NA	Khanum et al. (2021)
Parasite	*A. galli*			Host	Prevalence (%)	Reference
E. revolutum	+			Chicken	1.9	Hembram et al. (2015)
	+			Duck	NA	Begum et al. (2019)
	+			Chicken	NA	Khanum et al. (2021)

Clinical symptoms and pathogenicity

The most pathogenicity of parasitic infections is caused by the adhesive organ, such as rostellar hooks, spines, and sucker, which are used to attach and penetrate the gastrointestinal tract of the host, damaging the intestinal mucosa of hosts. These affect to the ability of nutrient absorption, growth rate, and weight depression.

Raillietiniasis (infection of tapeworm belonging to genus *Raillietina*) caused the degeneration and disintegration of villi and secretory glands, heterophilic infiltration, the necrosis of epithelial cells, nodular granulomas in intestinal surface, catarrhal enteritis, diarrhea, anemia, droopiness, and emaciation, lead to loss of nutrition, weight loss, slow growth, reduced egg production (Lalchhandama, 2010; Lalthanpuii & Lalchhandama, 2020; McDougald, 2020; Panich et al., 2022).

Ascariasis caused by the emerging and penetration of an infective stage larva from the egg. The larva migrated into the intestinal mucosa causing several damages leading to haemorrhages, anaemia, diarrhea, anorexia, emaciation and weakness. In

heavy infection, correlated with increasing worm burdens was the egg production loss, reductions in the growth rate and obstruction of the small intestine followed by eventual death of the hosts (Permin et al., 1997, 2006; Tarbiat et al., 2015).

Echinostomiasis is a food-borne parasite in wildlife and domestic animals worldwide. In human, it causes by trematodes belonging to the family Echinostomatidae. Light infections of echinostomiasis in animals do not cause important clinical symptoms and pathology, while heavy infections are related to severe pathology, disease and eventually death. The worms penetrate between the villi and feed on the tissues with their oral suckers causing desquamation of the epithelium and the villi, edema of the mucosal layer, duodenal erosions, inflammation, hemorrhagic, diarrhea and malnutrition. Finally, emaciation, weakness, and death have usually been resulted when infected with hundreds to thousands of worms (Carney, 1991; Labony et al., 2022).

Treatment and control

Nowadays, the increasing demand on poultry products from free-range production system is limited by the heavy worm burden, higher number of parasite species and increasing infection risk in such production system. Thus, the use of anthelmintic drugs has been recognized in the European Union due to the help to substantially reduce the economic losses (Abdelqader et al., 2012; McDougald, 2020). However, the kinds of anthelmintic products and dosage are efficacy difference depending on parasite group. For examples, praziquantel was suitable for *R. tetragona*, flubendazole and fenbendazole have been approved for use in chickens against *A. galli*, and rafoxanide was found to be highly effective against *E. caproni* (**Table 5**) (Abdel Aziz et al., 2018; Huffman & Fried, 1990; Rajendran & Nadakal, 1988; Tarbiat et al., 2015).

Table 5 The efficacy of drug treatments in raillietiniasis, ascariasis, and echinostomiasis

Drug	Dose (mg/kg)	Mode of application	Efficacy against disease			Reference
			raillietiniasis	ascariasis	echinostomiasis	
Praziquantel	10.0	Tablet	100%	-	-	Rajendran & Nadakal (1988)
Praziquantel	10.0	Tablet	100%	-	-	Nurelhuda et al. (1989a)
Oxfendazole	7.5	Tablet	100%	-	-	Nurelhuda et al. (1989b)
Fenbendazole	104.3	Aqueous	100%	-	-	Pote et al. (1992)
Albendazole	20.0	Aqueous	96.2%	-	-	Tucker et al. (2007)
Albendazole	5.0	Aqueous	-	100%	-	
Fenbendazole	5.0	Aqueous	-	85.5%	-	Yazwinski et al. (2013)
Levamisole	28.0	Tablet	-	99.1%	-	Feyera et al. (2021)
Piperazine	100.0	Tablet	-	96.3%	-	
Fenbendazole	10.0	Tablet	-	97.2	-	
Levamisole + Piperazine	28.0 + 100.0	Tablet	-	100%	-	
Rafoxanide	50.0	Aqueous	-	-	100%	Maurer et al. (1996)
Clorsulon	50.0	Aqueous	-	-	100%	

Table 5 (Continued)

Drug	Dose (mg/kg)	Mode of application	Efficacy against disease			Reference
			raillietiniasis	ascariasis	echinostomiasis	
Clorsulon	100.0	Aqueous	-	-	100%	Ferraz et al. (2012); Keiser et al. (2006)
Tribendimidine	125.0	Aqueous	-	-	100%	Keiser et al. (2006)

Although the anthelmintic drugs could reduce the economic losses, but the edible livestock products was accumulated the residues from the drugs, such as the appearance of levamisole in poultry egg (Rana et al., 2019). However, the interruption of the life cycle of parasites or the prevention of birds to keep them from contacting and consuming the intermediate hosts may provide additional benefits besides drug treatment. Therefore, the identification of worm species will help to accurately suggest control programs by the correctly elimination of the intermediate host (Graczyk & Fried, 1998; McDougald, 2020).

Diagnostic methods

1. **Gold standard technique**

A gold standard diagnostic method for gastrointestinal parasites is a direct diagnosis based on the morphological characters of adult or egg stages under microscope by necropsy investigations or the examination of eggs in host feces using the flotation technique for *Raillietina* spp. and *A. galli*, and sedimentation technique for Echinostomatidae (**Figure 8**) (Carney, 1991; Graczyk & Fried, 1998; Griffiths, 1978; Huffman & Fried, 1990; Oladosu et al., 2022; Panich et al., 2022; Permin et al., 2006). However, these techniques require the personal scientific skills and parasitic knowledge to discriminate and identify egg stage between each parasitic group. Moreover, the

technique is time-consuming when numerous samples are investigated simultaneously, since they require several stages for sample preparation.

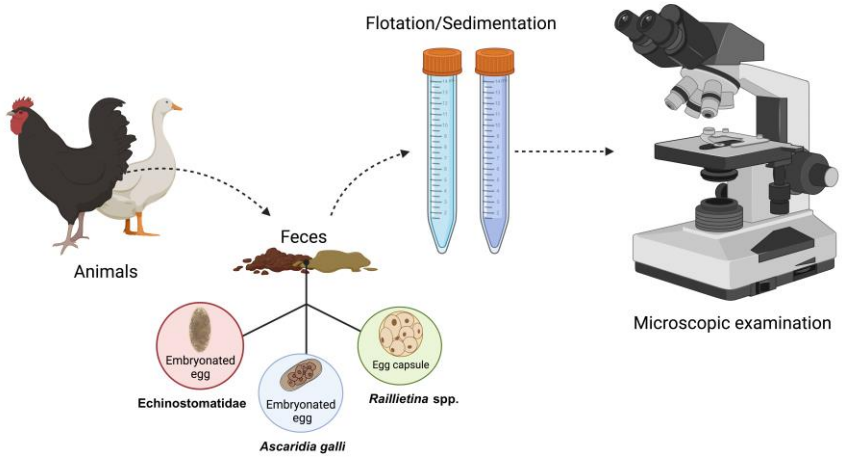


Figure 8 Schematic of egg examination via host feces using flotation and sedimentation techniques

2. **DNA amplification-based techniques**

The gold standard methods have complicated procedure and difficult to diagnose based on the morphological characteristics alone (Butboonchoo & Wongsawad, 2017). Therefore, molecular DNA amplification-based techniques have been applied as alternative diagnostic methods because they are more reliable, simple, rapid, and convenient than the morphological methods. Previously, several studies were used molecular techniques to identify or diagnose the parasitic infections using extracted DNA from eggs, which are released through host feces, or tissue from adult worm as shown in **Table 6**. Nevertheless, the methods as listed in **Table 6** need, specialized instrumentation and gel electrophoresis analysis that must be conducted in the laboratory, which is an important limitation performing in field detection (Sriworarat et al., 2015; Wang et al., 2019).

Table 6 Molecular DNA amplification-based techniques for detecting *Raillietina* spp., *A. galli*, and Echinostomatidae

Target species	Technique	Target region	Reference
Genus *Raillietina*			
R. echinobothrida	Phylogenetic analysis	Internal transcribed spacer 2 (ITS2) region	Ramnath et al. (2014)
R. echinobothrida, *R. tetragona*, *R. cesticillus*	High annealing temperature random amplified polymorphic DNA	Genomic DNA	Butboonchoo & Wongsawad (2017)
R. echinobothrida, *R. tetragona*, *R. cesticillus*	Species-specific PCR	ITS2 region	Panich & Chontananarth (2021)
	Triplex PCR	ITS2 region	Panich et al. (2022)
Genus *Ascaridia*			
A. galli	Species-specific PCR	*18S rDNA* gene	Qazaz (2020)
	Species-specific PCR	ITS1 region	Biswas et al. (2021)
	Species-specific PCR	*NADH dehydrogenase subunit 4* (*ND4*) gene	Watcharakranjanaporn et al. (2021)
	Duplex droplet digital PCR	ITS2 region	Tarbiat et al. (2021)
Family Echinostomatidae			
Artyfechinostomum malayanum	Real-time fluorescence resonance energy transfer PCR	*28S rDNA* gene	Tantrawatpan et al. (2016)

Table 6 (Continued)

Target species	Technique	Target region	Reference
E. revolutum	Species-specific PCR	*18S rDNA* gene	Anucherngchai & Chontananarth (2019)
	Species-specific PCR and Phylogenetic analysis	*Cytochrome B* (*CYTB*) gene	Anucherngchai et al. (2019)
	High-resolution melting analysis	*Cytochrome oxidase subunit 1* (*COI*) gene, *NADH dehydrogenase subunit 1* (*ND1*) gene	Buddhachat & Chontananarth (2019)
A. malayanum, *E. revolutum*, *E. ilocanum*, *H. conoideum*	Multiplex PCR	*ND1* gene	Tantrawatpan & Saijuntha (2020)

Loop-mediated Isothermal Amplification (LAMP)

To accomplish the usability of molecular techniques in field study, a loop-mediated isothermal amplification (LAMP), an isothermal amplification technique was established by Notomi et al. (2000). LAMP requires primers, at least two pairs, including a forward outer primer (F3), a backward outer primer (B3), a forward inner primer (FIP), a backward inner primer (BIP) to recognize six DNA regions of the target (**Figure 9A**), and a *Bst* DNA polymerase with strand displacement activity for DNA amplification under constant temperature without a thermal cycler machine (Wan et al., 2019). The basic principle of LAMP was divided into two steps. The first step, a dumbbell structure product is synthesized relying on the strand displacement activity of the *Bst* DNA polymerase and the self-binding ability of amplified ssDNA of FIP and BIP, which formed

loop structures at both ends called “dumbbell structure”. This is a template for the second step. In the second step, cyclic amplification is needed, using only inner primers. Both inner primers can bind the loop structures at both end and produce larger product size called “stem-loop DNA”. The mixture products of the LAMP amplification are stem-loop DNAs comprising of multiple repeats of amplified target (**Figure 9B-J**) (Shang et al., 2020).

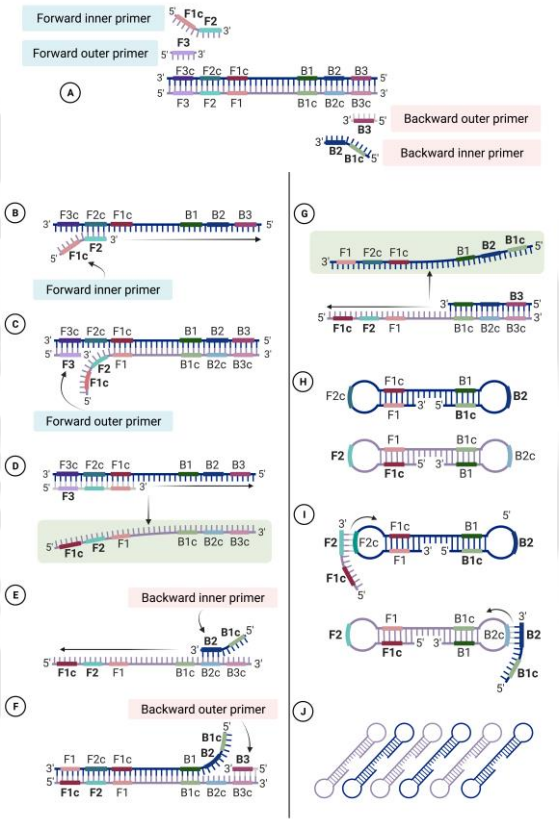


Figure 9 The basic principle of LAMP technique

In addition to the property of isothermal amplification, LAMP has higher specificity, sensitivity, and more tolerant to DNA extracted by the crude methods than PCR, which has increased the possibility to detect clinical samples for on-site diagnosis (Sriworarat et al., 2015; Xiong et al., 2020; Zhou et al., 2021). Regardless of the gel electrophoresis analysis, LAMP products can be analyzed using multiple techniques, such as turbidimetry (Mori et al., 2004), melting curve analysis (Yamamura et al., 2009),

fluorescence examination (Tsai et al., 2009), colorimetric interpretation (Nzelu et al., 2014), and lateral flow dipstick assay (Nak-on et al., 2023). For these reasons, LAMP techniques have been increasingly used to detect several parasitic species, such as Cestodes: *Echinococcus* spp., *Taenia* spp.; Nematodes: *Strongyloides stercoralis*, *Necator americanus*, *Trichuris trichiura*; Trematodes: *Fasciola* spp., *Clonorchis sinensis*, *Opisthorchis viverrini*, *Paragonimus westermani* etc (García-Bernalt Diego et al., 2021).

LAMP combined with monitoring techniques by naked eye and chromatic analysis

1. **Lateral flow dipstick assay**

Lateral flow dipstick (LFD) is an alternative immunochromatography technique to analyze LAMP products for visual interpretation or diagnostic purposes, such as clinical diagnosis, infection and contamination with specific pathogens in food or the environment. They are designed for single use at point of care testing or outside the laboratory without costly and specialized instruments. The results usually present within 10–20 minutes. The LFD has high sensitivity and selectivity by the hybridization of specific probes that can bind with some region within dumbbell structure of LAMP products to generate complex molecules composed of biotin and fluorescein isothiocyanate (FITC) or carboxyfluorescein phosphoramidite (FAM) for flowing in LFD (**Figure 10**) (Posthuma-Trumpie et al., 2009; Sajid et al., 2015).

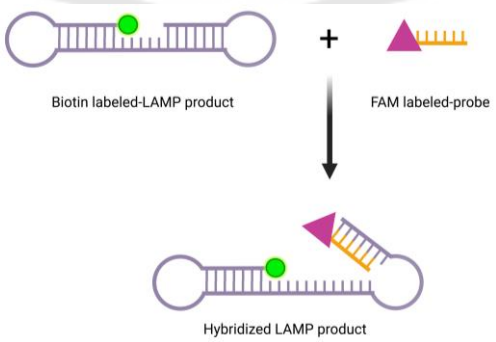


Figure 10 Single probe hybridization

Figure 11 shows the elements of the LFD analyzing LAMP products comprising of the four parts: (1) sample pad, an area where the hybridized analyte is

dropped; (2) conjugate pad, which contained anti-FITC or FAM antibody conjugated with gold nanoparticles (Anti-FITC/FAM-AuNP); (3) detection zone (usually nitrocellulose membrane), an area for the result interpretation containing test line and control line that are immobilized with bioreceptors, namely streptavidin on test line and anti anti-FITC/FAM secondary antibody on control line; and (4) absorbent pad, the end of the LFD, which helps in maintaining flow rate of the liquid over the membrane and prevents backflow of the analyte. FITC/FAM labeled-probe and biotin labeled-LAMP products form the complex molecule in probe hybridization step. This complex binds to streptavidin on the test line and remaining anti-FITC/FAM-AuNP bind to anti anti-FITC/FAM secondary antibody on the control line, thus, generated red bands over the time. For the result interpretations, the presence of test line and control line is interpreted as a positive result, while the presence of only control line is a negative result (Bahadır & Sezgintürk, 2016; Koczula & Gallotta, 2016; Sajid et al., 2015).

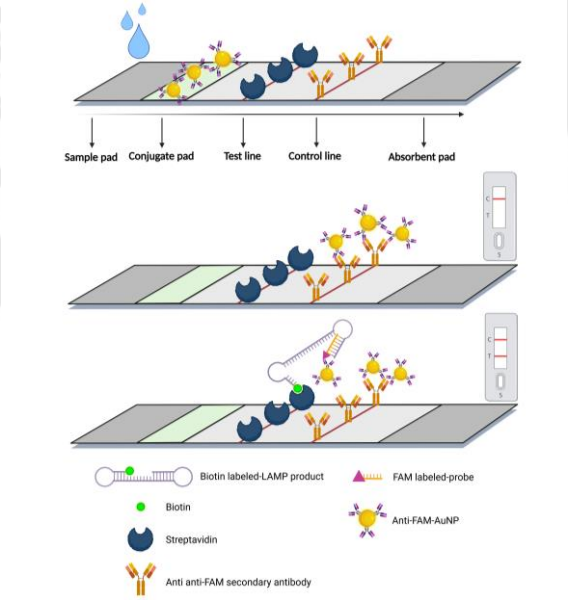


Figure 11 Schematic of LFD analysis when dropping non-target and target analytes on the sample pads

(Two lines interprets a positive result; the presence of only control line interprets a negative result)

The outstanding feature of LFD is a multiplex detection format that can detect more than one target species and can generate test lines equal to the number of target species to detect and differentiate the pathogens simultaneously. Practically, LAMP primers specific to second target species was conjugated with digoxigenin that produced digoxigenin labeled-LAMP products and, another one of FITC/FAM-probe was added to hybridize the digoxigenin labeled-LAMP products (**Figure 12**). After that, test line 2 on detection zone was immobilized with anti-digoxigenin antibody to capture digoxigenin labeled-LAMP products and visualize the red band. If both test lines appear, the result indicates a co-infection (**Figure 13**) (Sajid et al., 2015).

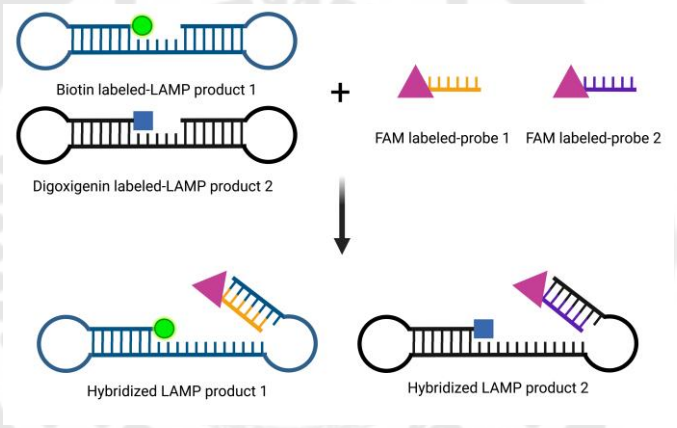


Figure 12 Dual probe hybridization

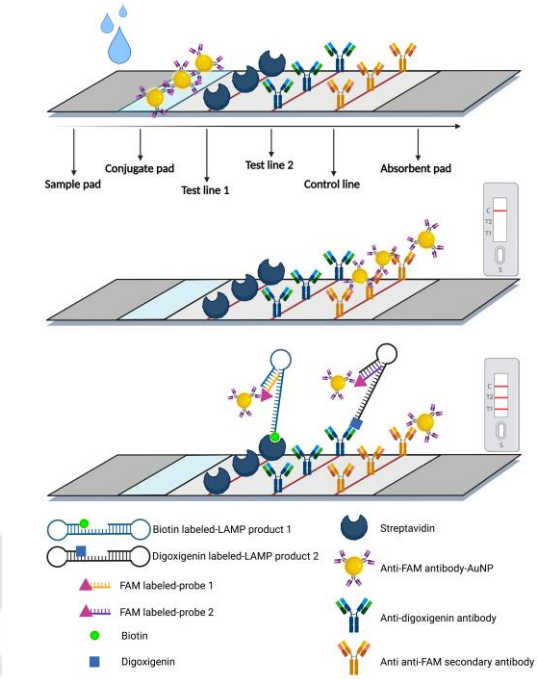


Figure 13 Schematic of LFD analysis when dropping non-target and double-target analytes on the sample pads

(The presence of three lines interprets a positive result; the presence of only control line interprets a negative result)

Hence, LAMP-LFD are very rapidly growing strategies for parasite diagnosis, such as *Babesia* spp., *Meloidogyne* spp., *Raillietina* spp., *Toxoplasma gondii*, *Paragonimus westermani*, and *Plasmodium falciparum* (Lalle et al., 2018; Niu et al., 2011; Panich et al., 2021; Xue et al., 2021; Xunhui et al., 2019; Yang et al., 2016; Yongkiettrakul et al., 2017). Nevertheless, the LFD requires the probe hybridization step before interpreting the results, lead to the risk of self-contamination, which is the limitation of the assay (Sriworarat et al., 2015; Wang et al., 2019; Xiong et al., 2020).

2. Colorimetric indicator

Colorimetric analysis using an indicator dye is one of the most convenient methods relying on color changing property in solution for monitoring the results. It can be pre-added into the mixtures without interfering the LAMP reaction (Sriworarat et al., 2015; Zhang et al., 2022). The indicator dyes can be divided into two categories

according to the cause of discoloration: (1) direct indicator dyes bind or intercalate with synthesized LAMP products, e.g. leuco crystal violet, malachite green, and methyl green; and (2) indirect indicator dyes make use of byproducts from DNA synthesis, such as hydroxynaphthol blue, calcein, phenol red, cresol red, and neutral red (Niessen & Vogel, 2010; Scott et al., 2020; Thapa et al., 2019) as listed in **Table 7**. The colorimetric LAMP assay is achieved in a single-step closed-tube, which the result is observed immediately after terminating the reaction without post-amplification handling. For this reason, the colorimetric strategy has become the most commonly used strategy to summarize the results by naked eye for commercial and field utilizations. However, the major limitations related to the colorimetric assay are a color perception that requires experienced personnel to discriminate between positive or negative colors, and colorimetric assay cannot detect more than one pathogen species in one reaction (Yin et al., 2020).

Table 7 Several dyes commonly used in LAMP detection

Direct indicator dye	Mechanism	Negative color	Positive color	Reference
Leuco crystal violet	DNA-binding dyes, intercalated with dsDNA	colorless	violet	Miyamoto et al. (2015)
Malachite green	DNA-binding dyes, intercalated with dsDNA	colorless	turquoise	Nzelu et al. (2014)
Methyl green	DNA-binding dyes, intercalated with dsDNA	colorless	turquoise	Thapa et al. (2019)

Table 7 (Continued)

Indirect indicator dye	Mechanism	Transition range (pH)	Negative color	Positive color	Reference
Hydroxynaphtol blue	Mg^{2+} indicators, the reduction of Mg^{2+}	-	purple	blue	Anupama et al. (2020)
Eriochrome black T	Mg^{2+} indicators, the reduction of Mg^{2+}	-	purple	sky blue	Neshani et al. (2023)
Calcein	Mn^{2+} indicators, the displacement of Mn^{2+} from calcein to $P_2O_7^{4-}$	-	orange	green	Tang et al. (2011)
Phenol red	Proton indicators	6.8-8.0	red	yellow	Xiong et al (2020)
Neutral red		6.8-8.0	brownish yellow	red	
Cresol red		7.2-8.8	red	yellow	

3. Chromatic analysis

Although the colorimetric interpretation with the naked eye is the simplest and fastest, but it provides only qualitative results (positive or negative results) and depends on color perception of each observer, such as experience, color blindness, and ambient lighting that could be prone to faults or the results with low confidence (Yin et al., 2019). Therefore, the method related to numerical measurement is necessary in order to eliminate the individual factors for enhancing the accuracy of the result. To numerically measure chromatic, several color spaces were used to analyze the

discoloration in colorimetric LAMP assay, including RGB (Red, Blue, Green), HSV (Hue, Saturation, Value), HSB (Hue, Saturation, Brightness), HSI (Hue, Saturation, Intensity), HSL (Hue, Saturation, Lightness), and CIELab color spaces etc.

RGB color space has the three coordinates, namely red, green, and blue (**Figure 14**), which are used to define a color in an additive color space and used as the initial chromatic value of developing the other color spaces. This color space is the most popular color space in reader or capture devices because the human visual perception works in the same way. However, RGB is a not globally standardized color space due to its response of the device to each coordinate wavelength depends on the manufacturer's devices, leading to the variable color according to the different devices(Capitán-Vallvey et al., 2015).

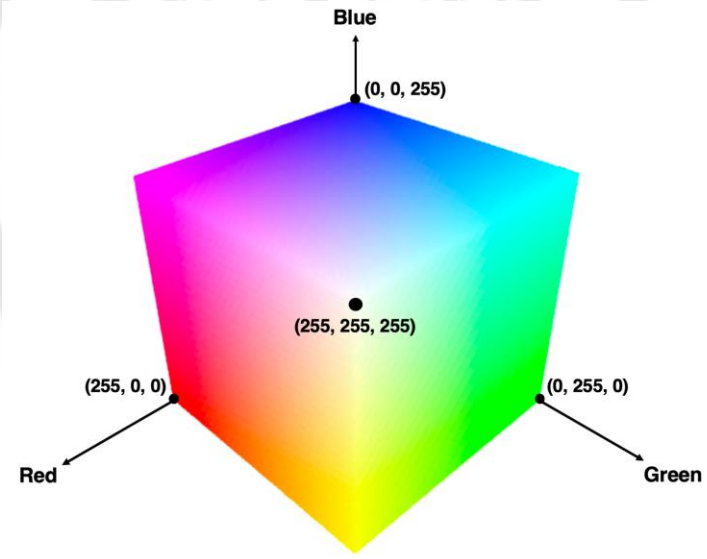


Figure 14 RGB color space

HSV, HSB, HSI, HSL color spaces completely define a color based on three different components, including (I) hue (H), (II) saturation (S), and (III) value (V) or brightness (B) or intensity (I) or lightness (L) (**Figure 15**). Hue describes the dominant wavelength of a color. Saturation describes the color purity. Value, brightness, intensity, and lightness are referred to as brightness, indicates the intensity of light level in the

color, but they are different following the definition of saturation each color space. Briefly, the saturation of HSL and HSI start from grey to saturated color, whereas HSV saturation is defined from white to saturated color (Capitán-Vallvey et al., 2015).

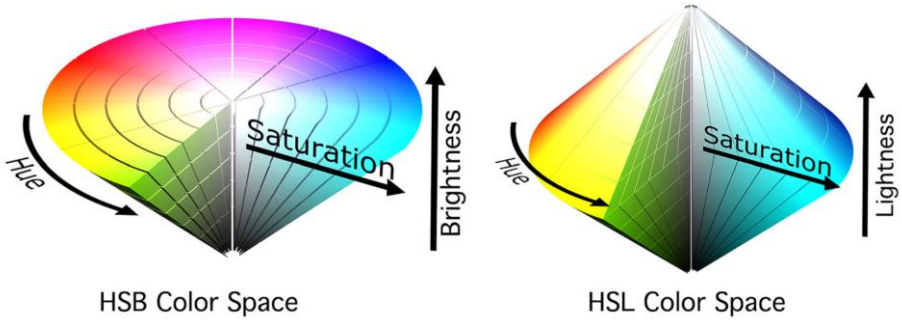


Figure 15 HSB and HSL color spaces

Source: Labrecque, L. I. (2020). Color research in marketing: Theoretical and technical considerations for conducting rigorous and impactful color research. *Psychology & Marketing*, *37*(7), 855–863.

Commission Internationale de l'Eclairage (CIE) Lab color space is perceptually uniform, meaning that a little bit change in the chromatic coordinates makes an equal change in the color perception, which is described by the three coordinates, comprising L*, a*, and b*. The L* coordinate is the luminance. The a* and b* coordinates are the green to red axis, and the blue to yellow axis, respectively (**Figure 16**). The important advantage of this space is the efficiency of differentiating between two colors (Capitán-Vallvey et al., 2015).

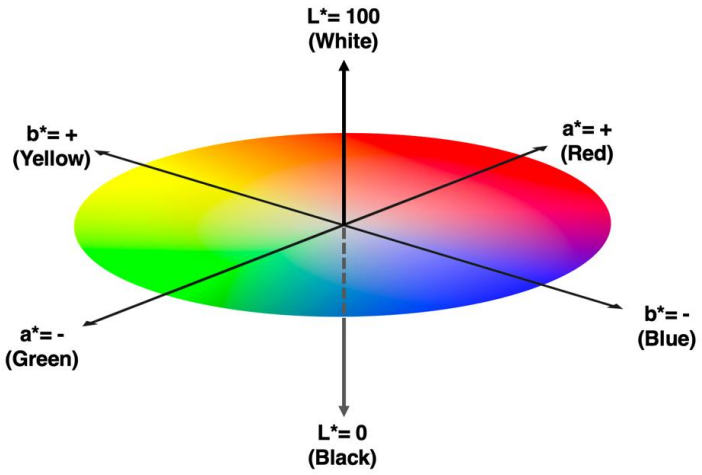


Figure 16 CIELab color space

In the last decade, color spaces as mentioned above have been applied in several studies regarding colorimetric LAMP assay. For instance, Nguyen, Nguyen, Liu & Seo (2020) used the ratio of Green value divide Red value of RGB color space to analyze between positive and negative color of eriochrome black T dye. Scott et al. (2020) used a single Hue value in HSB color space to differentiate the color results of phenol red dye. Ma, Chen & Lee (2019) distinguished the color solution of LAMP coupled with hydroxynaphthol blue by using the color distances of L*, a*, and b* in CIELab color space. Previous studies that used color spaces to analyze the results of colorimetric LAMP assay as shown in **Table 8**.

Table 8 Previous studies of colorimetric LAMP coupled with chromatic analysis

Color space (chromatic value used)	Indicator dye	Positive – Negative colors	Reference
RGB (Green/Red)	Eriochrome black T	Blue - Violet	Rodriguez-Manzano et al. (2016)
HSI (Hue)	Hydroxynaphthol blue	Blue - Violet	Yin et al. (2019)

Table 8 (Continued)

Color space (chromatic value used)	Indicator dye	Positive – Negative colors	Reference
CIELab (ΔE_{ab})	Phenol red	Yellow - Red	González-González et al. (2021)
RGB (Red)	Hydroxynaphthol blue	Blue - Violet	Scott et al. (2020)
RGB (Green)	Calcein	Green - Orange	
HSB (Saturation)	Leuco crystal violet	Crystal violet - Colorless	
	Malachite green	Turquoise - Colorless	
HSI (Hue)	Phenol red	Yellow - Red	Bokelmann et al. (2021)
HSB (Hue)	Phenol red	Yellow - Red	Layne et al. (2021)
RGB (Green-Blue)	Phenol red	Yellow - Red	Papadakis et al. (2022)
RGB (Green-Red)	Hydroxynaphthol blue	Blue - Violet	

Microfluidic chip technology

Basic of microfluidic chip concept is a device that has at least one channel with one dimension less than 1 mm and handles very small amounts of fluids to perform the reaction in several regions on a chip (also called to as lab-on-a-chip system). It has received significant attention in term of the reduction in size, low requirement of reagent quantity, low cost, easy operation and preparation, and portable testing. Thus, the microfluidic device is usually used in a variety of studies, such as chemical experiments, biological assays, environmental monitoring, medical diagnosis, and molecular

detections; and is rapidly developing and used in recent decades (Wang et al., 2017; Yuan et al., 2014).

The principles of microfluidics, the flow of a fluid in a microchannel can be characterized by the Reynolds number, which is a useful tool that predict fluid flow in microfluidic chip with different patterns for analysis how the parameters will respond to a variation in fluids and it is used to predict the transition flow from laminar to turbulent. Reynolds reported in 1883 defines the relationships via a Reynolds number value, which is defined as

$$\mathrm{Re} = \frac{\mathrm{DV}}{v}$$

Where: Re is Reynolds number;

D is diameter of microchannel (ft)

V is average velocity of the fluid (ft/s)

v is kinematic viscosity (ft^2/s)

The Reynolds number can interpret and classify into three flow-through pipe regimes, namely at values of 2,000 or below, the flow profile is the laminar flow, between 2,000 to 4,000 the flow is in the transition region, and at 4,000 or above the flow is the turbulent flow. Thus, calculation of the Reynolds number can be determined the flow velocity pattern in microchannels (Menon, 2015).

In the single target detection in LAMP usually requires at least four primers for multiplex detection, meaning duplex or triplex LAMP, needs eight and twelve primers, respectively, leading to the competition of these primers and interference the LAMP reaction. Therefore, it is difficult to optimize the reaction condition in multiplex LAMP as well as the concentration of reagents and labelled primers (biotin, digoxigenin). The expensive cost of multiplex LFD strip is also accounted for the difficulty to commercialize it in the future (C. Xie et al., 2021). Because of the many advantages, several studies have used the microfluidic chip integrated with the LAMP reaction for multiplex detection via a numerous of separate reaction chambers on the chip, each chamber containing dried primers for specific amplification of different pathogens (Huang et al., 2021; Tourlousse et al., 2012). Regarding LAMP result analysis on the

chip, chromatic observation using indicator dyes coupled with LAMP was used to monitor the results via discoloration of the solutions as mentioned above in colorimetric indicator section. In recent years, performing colorimetric LAMP on the microfluidic chips were successfully developed for multiplex detection, ranging from 3-5 targets as show in **Table 9**.

Table 9 Overview of microfluidic chips integrated with colorimetric LAMP detection

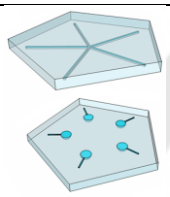
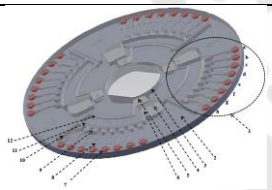
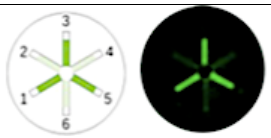
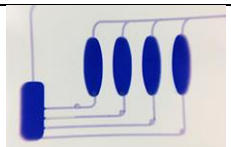
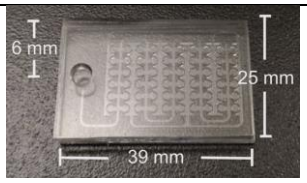
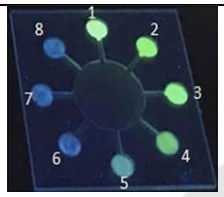
LAMP-microfluidic chip	Indicator dye	Number of targets	Reference
	Calcein	5	Jiang et al. (2016)
	Eriochrome black T	3	Seo et al. (2017)
	Neutral red	3	Yuan et al. (2018)
	Calcein	5	Li et al. (2020)
	Eriochrome black T	3	Nguyen et al. (2020)

Table 9 (Continued)

LAMP-microfluidic chip	Indicator dye	Number of targets	Reference
	Calcein	3	Xie et al. (2021)
	Calcein	4	Zhang et al. (2021)

Hence, the microfluidic chip technology integrated with colorimetric LAMP is a useful tool as an alternative platform for on-site or point-of-care screening that have great potential application in multiplex diagnosis in terms of the user-friendly, cost-effectiveness, time-saving, and easy interpretation by naked eye.

Fabrication methods for microfluidic chip

Microfabrication using machining approaches is expensive due to costly equipment and time-consuming. The manufacturing cost can be greatly decreased if the productions of microchip can be conducted using a replication. The microchips are fabricated only once as the molds or masters and duplicated via the molds. Here, we describe three methods of mold manufacture that were categorized by Scott & Ali (2021) and **Table 10** shows the summaries of achievable abilities of the fabrication methods for microfluidic chip production.

1. **Mechanical method**

Mechanical methods, consist of micro-cutting (MC) and ultrasonic machining (USM), are the processes of drilling or milling that eliminate any unwanted region in the mold using mechanical forces with sharp tools or abrasive particles. Importantly, the tools for these methods must be stronger than those of the mold materials.

2. **Energy-assisted method**

Energy-assisted methods are the processes to remove material on the chip rely on concentrated energy either through electric potential, laser, ion, or electron beams. Five energy-assisted methods were divided according to energy sources that used to erosion, namely electro-discharge machining (EDM), electrochemical machining (ECM), laser ablation, electron beam machining, and focused ion beam machining (FIB).

3. **Micro-electromechanical system**

Micro-electromechanical systems (MEMS) are methods frequently used in microfabrication with photolithography and etching. Photolithography is the photographic process to define and transfer a pattern on a thin film layer or a substrate (also called a wafer). Briefly, UV and X-ray are generally used to transfer an image from a patterned mask to a photosensitive layer (photoresist) on the wafer and then the exposed photoresist is developed with a chemical reagent (chemical developer) to determine which part of the photoresist is dissolved. Finally, the same pattern is transferred into the substrate under the photoresist layer by the etching process.

Table 10 Comparison of mold fabrication methods

Methods	Minimum size	Resolution	Roughness	Positional tolerance
Micro-cutting	6.7 μm	2 μm	65 nm	3 μm
Ultrasonic machining	5 μm	5 μm	NA	NA
Electro-discharge machining	5 μm	3 μm	100 nm	1 μm
Electrochemical machining	150 nm	5 μm	28 nm	2 μm

Table 10 (Continued)

Methods	Minimum size	Resolution	Roughness	Positional tolerance
Laser ablation	<1 μm	~1 μm	100 nm	3 μm
Electron beam machining	10 nm	20 nm	0.58 nm	NA
Focused ion beam machining	40 nm	5 nm	NA	100 nm
MEMS method	Some μm	NA	10 nm	Some μm

Source: modified from Scott, S., & Ali, Z. (2021). Fabrication methods for microfluidic devices: An overview. *Micromachines*, *12*(3), 319.

Materials for microfluidic chip fabrication

In addition to the fabrication methods, another significant factor of microfluidics is the material of the chip, which mainly determine the application of the devices. Therefore, to develop suitable chips, particular attention should be focused on selecting the right material according to the purpose of application. **Table 11** shows the overview of materials for microfluidic chip fabrication.

1. **Inorganic material**

The first-generation of microfluidic chips were commonly fabricated based on silica or glass because it is optical transparency, electrical Insulator, resistance to organic solvents, high thermal conductivity, and thermostability. However, the hardness and high cost of glass and silicon are limitation to their wide application. Furthermore, the fabrication of such chips relies on extremely condition as high pressure, high temperature and especially clean environment. By these limitations, other materials were developed, instead, to easily fabricate and apply with biological studies.

2. **Polymer-based chip**

Polymers are the most commonly used in microchip materials. They are greater flexibility than silicon/glass chips and inexpensive. According to their variety of physical properties, polymers can be classified into two groups, choosing suitable

polymer related with the purpose of use. (1) Elastomers: polydimethylsiloxane (PDMS) is the most popular because they can resist to stretch or compress from external force and return to the original shape. A major advantage of PDMS is gas permeable from a porous structure, which is broadly used for long-term cell culture, cell screening, and biochemical assays. One major problem is the absorption and evaporation of small molecules into porous walls leading to the uncertainty in concentration of solution. (2) Thermoplastics, including polymethylmethacrylate (PMMA), polycarbonate (PC), polystyrene (PS), polyethylene terephthalate (PET), and polyvinylchloride (PVC), display a better hardness and solvent compatibility than PDMS. On the other hand, they are not gas permeable, which are unsuitable for cell culture study.

3. Hydrogel

In general, the diffusion of substance and gas through PDMS is not enough to support thick layer cell culture. Hydrogels are high permeability similar to the extracellular matrix that have been used to maintain structure and deliver the solutions, cells, and other nutrients via numerous pores, helping micro- or macro-molecules diffusion for three-dimensional (3D) cell culture.

4. Paper

The usability of paper material as chip has many advantages: (1) Paper acts as built-in pump without requiring external power; (2) Paper has a large surface-to-volume ratio, and can pre-stored reagents via drying the reaction area; (3) Paper is the cheapest materials for microfluidic chips, and the fabrication process is inexpensive and convenient; (4) Paper can be stacked to multilayer microfluidic chip; (5) Paper can filter out macroparticles that may inhibit the reaction in the sample. In contrast, it has likewise the obviously drawbacks: (1) Paper can block the target molecules or reagents and dilute the sample during transportation leading to unsatisfactory clinical sensitivity; (2) The channels that fabricated by other materials are sealed, while paper-based channels do not have to be sealed; thus, the evaporation of solution from open channels also become a major problem.

Table 11 Comparison of the properties for microchip materials

Properties	Glass	PDMS	PMMA	Hydrogel	Paper
Young's modulus (GPa)	50-90	~0.0005	1.4-4.1	low	0.0003-0.0025
Smallest channel	<100 nm	<1 μm	~100 nm	~10 μm	~200 μm
Multilayer channels	hard	easy	easy	medium	easy
Thermostability	very high	medium	medium-high	low	medium
Surface charge	very stable	not stable	stable	NA	NA
Permeability to oxygen (Barrer[a])	<0.01	~500	0.05-5	>1	>1
Optical transparency	high	high	medium-high	medium-high	low

[a]1 Barrer= 10^{-10} [cm^3 O_2 (STD)] cm cm^{-1} s^{-1} $cmHg^{-1}$

Source: modified from Ren, K., Zhou, J., & Wu, H. (2013). Materials for microfluidic chip fabrication. *Accounts of Chemical Research*, *46*(11), 2396–2406.

CHAPTER 3
MATERIALS AND METHODS

1. Animal ethical approval

Experimental methods involving animals were conducted under the regulations issued by the National Research Council of Thailand and approved by the Committee of Srinakharinwirot University (License No. SWU-A-025-2562).

2. Parasite collection

Most parasite specimens (adult stage) were received from previous studies by Glunkwamdi et al. (2020) and Panich et al. (2021), which infected in gastrointestinal tract of chickens (*G. g. domesticus*), ducks (*A. p. domesticus*), and hamsters (*Phodopus* sp.). Some other related parasites were further collected from the gastrointestinal tract of chickens and hamsters from Nakhon Pathom and Bangkok Provinces, respectively. The new specimens that found in this study were divided into two sections: the first section was fixed with 10% formalin for morphological study and the second section was preserved in absolute ethanol for molecular investigation.

3. Parasite specimen identification using morphological characteristics

The specimens that fixed with formalin for at least a week were washed with distilled water and stained with haematoxylin (stained with borax carmine after dehydrated with 70% ethanol), and dehydrated using an ethanol dilution series for 30 minutes (min) in each step (10, 20, 30, 50, and 70%). Next, the specimens were destained with 1% destain reagent (1% HCl in 70% ethanol), and dehydrated using the ethanol series (70, 80, and 95%), followed by ratio ethanol: butanol (2:1, 1:1, 1:2), butanol, and ratio butanol: xylene (2:1, 1:1, 1:2), respectively. Then, specimens were cleared with xylene before being mounted with Permount. Finally, the specimens were identified according to the taxonomic keys (Butboonchoo et al., 2016, 2020; Chai et al., 2020, 2021; Heneberg et al., 2015; Kanev, 1994; McDougald, 2020; Mohanta et al., 2019; Sawada, 1964, 1965; Schultz, 1940; Tanveer et al., 2015; Yamaguti, 1958, 1959).

4. Molecular investigation

4.1 Genomic DNA extraction

The preserved tissue of each parasite in absolute ethanol were extracted using a GF-1 tissue DNA extraction kit (Vivantis), according to the manufacturer's instruction. After that, genomic DNA concentration was measured by a NanoDrop Lite spectrophotometer (Thermo Scientific™) Subsequently, genomic DNA was diluted with deionized water to adjust the final concentration to 5 ng/μL and 10 ng/μL for LAMP-LFD assay and colorimetric LAMP on a microfluidic chip assay, respectively; and then stored at -20 °C until use.

4.2 Molecular confirmation

To confirm the morphological identification, all parasite species that found in this study were amplified using primers ITS3: 5'-GCATCGATGAAGAACGCAGC-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' (Barber et al., 2000) targeting the ITS2 region. The PCR protocols were demonstrated as follows: 1 cycle of 94°C for 4 min, 35 cycles of 94°C for 1 min, 50 - 60°C for 1 min, 72°C for 1 min, and 1 cycle of final extension at 72°C for 7 min. The PCR products were separated and estimated size by 1.5% agarose gel electrophoresis in TBE buffer (Tris base, boric acid, EDTA, pH 8.0), with in-gel staining using ViSafe Green (Vivantis). PCR products were subjected to sequencing before compared with available sequences in the GenBank database by Basic Local Alignment Search Tools (BLAST) using BLASTn algorithm.

5. LAMP primers and probes design

LAMP primers were manually designed together with related and co-infected parasite species via MEGA 11 software using our sequences and available sequences from the GenBank database. LAMP requires primers at least two pairs, namely a forward inner primer (FIP), a backward inner primer (BIP), a forward outer primer (F3), and a backward outer primer (B3) to recognize six different DNA regions. Probes were designed for targeting the internal LAMP products between F2 and B2 region of each LAMP primer set. A LAMP primer set for *Raillietina* spp. targeting *28S rDNA* gene was used from a previous study by Panich et al. (2021), whereas probe was newly designed

in this study. Another five LAMP primer sets were newly designed for *R. echinobothrida*, *R. tetragona*, *A. galli*, Echinostomatidae based on the ITS2 region and *R. cesticillus* targeting the *28S rDNA* gene. The LAMP assay related to LFD analysis used the LAMP primer sets for *Raillietina* spp., *A. galli*, and Echinostomatidae, while LAMP on a microfluidic chip used the LAMP primer sets for *R. echinobothrida*, *R. tetragona*, and *R. cesticillus* instead of *Raillietina* spp. The details of LAMP primers and probes are shown in **Table 12** and **Figure A1-6**.

Table 12 Details of LAMP primers and probes for all LAMP assay in this study

Primer/probe	Sequence (5'-3')	Target region	Reference
For simplex and duplex LAMP-LFD assay			
Raillietina spp.			
F3-RAI	TCGGTTGCGGCTTTTGCTAC	*28S rDNA* gene	Panich et al. (2021)
B3-RAI	GACGTGCGTGCCAGATACCAT		
FIP-RAIBi	Biotin-AGACTGGCAATACCACCGGTCGTTTTTG GCCGTCTGGTCAGTGCAC		
BIP-RAI	CTGTTGCGTGTTATCGTGCATCCACCTTTTCCT TGCACAGTCACTTTCACCACC		
Probe-RAI2	FAM-CATGGTGGGRTGYGGT		This study
A. galli			
F3-AG	GCTTGATTGCTATTGCCAT	ITS2 region	This study
B3-AG	CATTTTAGTATTCCAACGATAGGA		
FIP-AG(Bi/Di)	Biotin or digoxigenin-AACGAGCTATTATAAAA CGCTTTTATCAAGTAGTTTTTGTATGTGTATGTGCGTATGC		
BIP-AG	CAGGTACTACAATTAGATGATGATGATGATGT TTTAGCATTAGAGCTGCATTCATG		
Probe-AG	FAM-GCGTATGCGTTTGTTTACGTTG		
Echinostomatidae			
F3-ECHI	CCAAAAAGTCGTGGCTTG	ITS2 region	This study
B3-ECHI	GTATGCCGACTAGAAGCG		

Table 12 (Continued)

Primer/probe	Sequence (5'-3')	Target region	Reference
Echinostomatidae			
FIP-ECHI(Bi/Di)	Biotin or digoxigenin-CGGATACATTRGGGAAA CGCTTTTGCTGGCGTGATTTCCTCT	ITS2 region	This study
BIP-ECHI	GGAATCGTGGTTTAATRTGGCTATGCTTTTGC TCCACCCGTAKYCATAT		
Probe-ECHI	FAM-AACCACGATTCCGTCAC		
For colorimetric LAMP on a microfluidic chip assay			
R. echinobothrida (Reaction chamber 1)			
F3-RE	ATTGCTCTTGGTTGTCATCACAG	ITS2 region	This study
B3-RE	AAAGACCCTACCCCACTAA		
FIP-RE	ATACGCACACACCCACACACTTTTTTGCTGTC CTTACCGTCT		
BIP-RE	CTTAGCCCTTCTACCCTTCTGCCTTATTTTCCA CACACATCCACATTCA		
R. tetragona (Reaction chamber 2)			
F3-RT	GACATCTTGAACGCATATTG	ITS2 region	This study
B3-RT	ATTCACCAACATACCTTACC		
FIP-RT	GCAACCGAGAGCATTCAGATTTTGGCTTGTAA ACTATCACTGC		
BIP-RT	TGGGTGGGTGGGTGCATATTTTTTAAGGCGAA TCAGGGGTAGA		
R. cesticillus (Reaction chamber 3)			
F3-RC	GGAATAAGATGTGGTAGTCC	*28S rDNA* gene	This study
B3-RC	AACAGTGCGGAGACACCATTA		
FIP-RC	CCCAACAGATGAATAGCACGCTTTTTGGTTGC GGCTTTTGCTAC		
BIP-RC	GGTCAAGTCGTGTCTTTCTGATGGTTTTCCCAA ACAGTCATTATCCTCC		

Table 12 (Continued)

Primer/probe	Sequence (5'-3')	Target region	Reference
A. galli (Reaction chamber 4)			
F3-AG	GCTTGATTGCTATTGCCAT	ITS2 region	This study
B3-AG	CATTTTAGTATTCCAACGATAGGA		
FIP-AG	AACGAGCTATTATAAAACGCTTTTATCAAGTAG TTTTTGTATGTGTATGTGCGTATGC		
BIP-AG	CAGGTACTACAATTAGATGATGATGATGATGTT TTAGCATTAGAGCTGCATTCATG		
Echinostomatidae (Reaction chamber 5)			
F3-ECHI	CCAAAAAGTCGTGGCTTG	ITS2 region	This study
B3-ECHI	GTATGCCGACTAGAAGCG		
FIP-ECHI	CGGATACATTRGGGAAACGCTTTTGCTGGCGT GATTTCCTCT		
BIP-ECHI	GGAATCGTGGTTTAATRTGGCTATGCTTTTGCT CCACCCGTAKYCATAT		

Degeneracy bases are underlined (R= A, G; K= G, T; Y= C, T)

6. Simplex LAMP-LFD assays

6.1 Simplex LAMP validations

The optimal LAMP reactions were performed at 66 °C and varied durations to evaluate the minimum reaction times. The concentrations of LAMP reagent were optimized, including outer primers, inner primers, betaine (Sigma-Aldrich), deoxynucleotide triphosphates (dNTPs) (Vivantis), magnesium sulfate ($MgSO_4$) (New England Biolabs), and *Bst* 2.0 DNA polymerase (New England Biolabs). To confirm the target LAMP products, 1 μL of the LAMP products was digested with different restriction enzyme (New England Biolabs) for each LAMP primers set (**Table 13; Figure A1-6**).

The analytical specificity evaluation of each primer set was tested together with parasites that found in this study and definitive hosts. Deionized water was used as a template in negative control. Regarding the analytical sensitivity test, genomic DNA

was initially 5 ng/reaction and then was diluted with 10-fold serial dilution ranging from 5 to 5×10^{-6} ng/reaction with three replicates to determine the minimum DNA detectable concentration. The LAMP results were analyzed by 1.5% agarose gel electrophoresis.

Table 13 Details of restriction enzymes for LAMP product confirmations

Species	Restriction enzyme	Cut site	Expected amplicon
***Raillietina* spp. LAMP**			
R. echinobothrida	*Ms*II	CAYNN/NNRTG	294, 500 bp
R. tetragona			289, 490 bp
R. cesticillus			319, 550 bp
***R. echinobothrida* LAMP**			
R. echinobothrida	*Hin*dIII-HF	A/AGCTT	214 bp
***R. tetragona* LAMP**			
R. tetragona	*Hpy*CH4III	ACN/GT	115, 191 bp
***R. cesticillus* LAMP**			
R. cesticillus	*Hpy*CH4V	TG/CA	114, 163, 213 bp
***A. galli* LAMP**			
A. galli	*Dra*I	TTT/AAA	123 bp
Echinostomatidae LAMP			
E. miyagawai	*Ms*II	CAYNN/NNRTG	202, 311 bp
E. mekongi			205, 317 bp
E. macrorchis			
H. conoideum			

Degeneracy bases are underlined (R= A, G; Y= C, T; N= A, T, C, G)

6.2 Simplex LAMP-LFD developments

To analyze the LAMP products via LFD assay with the naked eye, FIP primer and probe of each parasitic group were labelled with biotin and FAM at 5' end, respectively. To estimate the optimal concentration of probe, the quantities of probe (2, 20, and 200 pmol) were added into the 8 μL LAMP products and incubated at 66 °C for varying durations. After incubation, 8 μL of the hybridized LAMP products were mixed with 92 μL of assay buffer and the LFD strip (Milenia Genline HybriDetect) was dipped into the mixture. Then the color was observed at the test line and control line after flowing 5 min. For results interpretation, the presence of test and control lines indicates a positive result, whereas only the control line is visible demonstrate a negative result.

7. Duplex LAMP-LFD assays

7.1 Duplex LAMP validations

The LAMP primers of each parasitic group were combined for three combinations of duplex LAMP, comprising of *Raillietina* - *A. galli*, *Raillietina* - Echinostomatidae, and *A. galli* – Echinostomatidae LAMP assays. Each duplex LAMP assay was newly validated for the reagents concentration and reaction time to create the suitable environment for simultaneous amplification of two LAMP primer sets in a single reaction, including inner primers, $MgSO_4$, dNTPs, and *Bst* 2.0 DNA polymerase. Duplex LAMP products were digested/double-digested with *Msl*I or/and *Dra*I enzymes and separated by 1.5% agarose gel electrophoresis for simultaneous amplification confirmation. The analytical specificity and sensitivity tests were performed the same with simplex LAMP process in the section 6.1 as mentioned above. Moreover, the analytical sensitivity was further tested in case of co-infection.

7.2 Duplex LAMP-LFD developments

To differentiate duplex LAMP products, two FIP primers in duplex LAMP reaction were labelled differently with the biotin and digoxigenin at 5' end. Meanwhile, two probes were conjugated with FAM at 5' end. Initially, the quantities of probe for analyzing on test line 2 were firstly hybridized and then were dual-hybridized with probe for observing on test line 1. The quantities of probes yielding the most intensity color on

test lines were selected as the optimal quantity for each duplex LAMP-LFD assay. Hybridization procedure was performed as mentioned above in the section 6.2. The presence of red band at only one test line interprets a positive result for single infection, while the presence of red band on both test lines interprets a positive result for co-infection.

8. Microfluidic chip design and fabrication

Due to the multiple LAMP-LFD assay has a several limitations, such as it needs primer at least four pairs in one reaction, leading to the competition of these primers and interference the LAMP reaction, and the LFD assay requires the post-amplified analysis before interpreting the results prone to the contamination from previous reaction, which are a DNA template for next reaction resulting the false positive results (Wang et al., 2019; Xiong et al., 2020). In addition, it is difficult to validate the optimal condition in multiplex LAMP reaction as well as expensive cost of the multiplex LFD strip and label reagents, namely biotin, digoxigenin, and FAM. Therefore, the colorimetric LAMP reaction performing on a microfluidic chip was developed for LAMP amplification separately of each reaction chamber and reducing the cost of devices and reagents.

Microfluidic chip pattern was designed using 123D design program and the pattern was transferred to a polymethyl methacrylate or PMMA (thickness 2.0 mm). Engraving parameters that are shown in **Table 14**, were generated the G-Codes by Estlcam V11 program and engraved on the PMMA surface by using the Mini-computer numerical control (CNC) W349 machine (Winner prototype Co., Ltd.) coupled with Openbuilds controller program. The chip has a single layer, comprising of the microchannel (depth 0.3 mm), six reaction chambers (depth 2.0 mm), one inlet (depth 2.0 mm), and six outlets (depth 2.0 mm) (**Figure 17**). The fabricated chips were cleaned with isopropanol for 5 min and thereafter scrubbed with a soft brush in DNase-free water. After that, the chips were air-dried at room temperature and stored in a clean container.

Table 14 Details of engraving parameters for microfluidic chip fabrication

Component	Diameter of end mill	Toolpath depth	Plunge angle	Feedrate (x, y axis)	Feedrate (z axis)	Rotational speed
Microchannel	0.5 mm	0.3 mm	90˚	25 mm/min	25 mm/min	24000 rpm
Reaction chamber	1.5 mm	2.0 mm	90˚	50 mm/min	50 mm/min	24000 rpm
Inlet/Outlet hole	0.9 mm	2.0 mm	90˚	50 mm/min	50 mm/min	24000 rpm

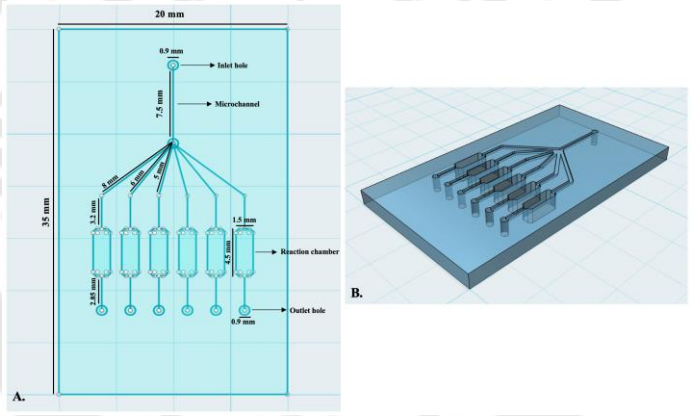


Figure 17 2D and 3D microfluidic chip pattern designed by 123D design program

(A: 2D image; B: 3D image)

To setup multiplex detection on a microfluidic chip, a bottom side of the chip was sealed with a pressure sensitive film (ThermalSeal RTS™). Second, each LAMP primer set was manually loaded by pipetting into different reaction chambers; the reaction chamber No. 1, 2, 3, 4, and 5 were coated the LAMP primer set for detecting *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and Echinostomatidae, respectively. While the reaction chamber No. 6 as a negative control chamber was uncoated in order to maintain a negative color for comparison of reaction chamber discoloration in

colorimetric assay, and then the chip was dried at room temperature in the dark for 60 min. Finally, the chip was sealed the top side with the pressure sensitive film (ThermalSeal RTS™) and immediately used (**Figure 18A-C**). For usability the microfluidic chip, a sterilized needle was used to drill the inlet and outlet holes via the bottom side of microfluidic chip for the injection and ventilation, respectively; after that, LAMP mixture was injected by micropipette with 200 μL tip via inlet hole. After loading the mixture into the chip, the small pieces of adhesive film were sealed on the holes to prevent the evaporation of the mixture and dipped in water bath (Thermo Scientific™) to perform the LAMP reaction further (**Figure 18D-F**).

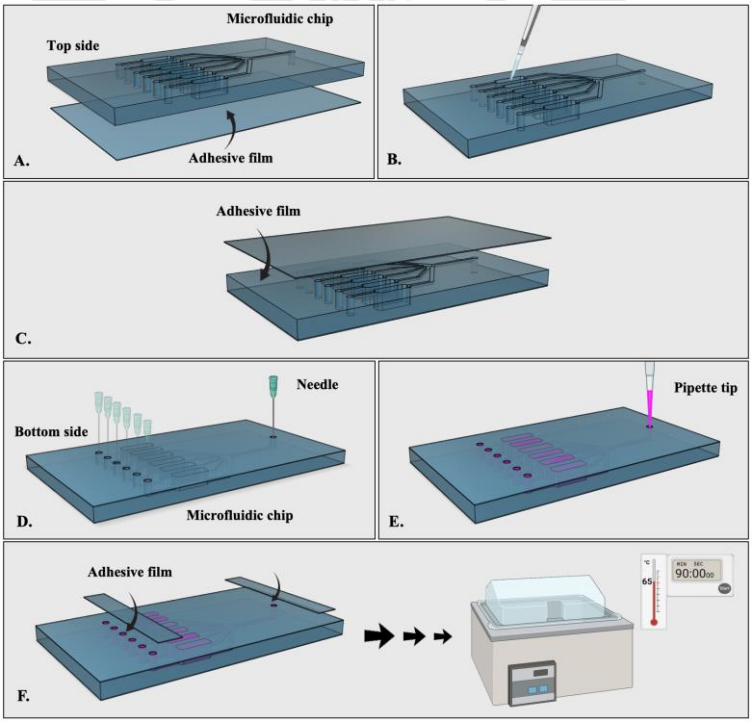


Figure 18 Schematics of microfluidic chip fabrication and usability

(A: an adhesive film is covered at bottom side of microfluidic chip; B: the LAMP primers were loaded in five reaction chambers and air-dried in the dark at room temperature for 60 min; C: a top side of the chip is sealed by adhesive film before using; D: a sterilized needle punched to the inlet and outlet holes at a bottom side; E: a sample loading by micropipette; F: the final sealing of holes before reacting in water bath)

9. **Colorimetric LAMP on a microfluidic chip coupled with a chromatic analysis via image processing**

9.1 Colorimetric LAMP on a microfluidic chip development

Colorimetric LAMP reaction was developed by using phenol red dye (Sigma-Aldrich) to monitor the results via the discoloration in the solution, which can add into the reaction mixture before starting without interference the reaction. Phenol red dye is an indirect indicator dye relies on the accumulation of H^+ ion that is a byproduct of DNA amplification (Scott et al., 2020). The significant reduction of pH in the reaction offers resulting the discoloration of phenol red from red to yellow (Tanner et al., 2015). Consequently, the LAMP mixture for colorimetric readout method was newly prepared as a weakly buffer by adjusting the final concentration of Tris-HCl from 20 mM to 3.75 mM. Regarding the DNA amplification on a microfluidic chip, a previous study also found that the PMMA compound could inhibit the amplification of PCR method (Kodzius et al., 2012). To overcome this issue, Taylor et al. (1997) recommended adding bovine serum albumin (BSA) (Sigma-Aldrich) into the reaction to compete with polymerase for adsorption on the surfaces of chip to enhance the performance of DNA amplification, which corresponded to the results by Kodzius et al. (2012). Hence, the BSA was also added in the LAMP mixture to prevent the adsorption of PMMA surface for performing the colorimetric LAMP reaction on a microfluidic chip. A total colorimetric LAMP mixture that injecting in the chip was 100 μL, while the LAMP reaction was performed in the reaction chambers with full volume approximately 12.8 μL (without bubble). The loaded chip was incubated in water bath to maintain the temperature at 65 °C for 90 min. The results can be interpreted by naked eye after the chip was cooling down. *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and Echinostomatidae LAMP products in the reaction chambers were digested with *Hin*dIII-HF, *Hpy*CH4III, *Hpy*CH4V, *Dra*I, and *Ms*I enzymes, respectively, and separated by 1.5% agarose gel electrophoresis for specific target confirmation.

9.2 Image processing and chromatic analysis

The amplified chips were placed under stereomicroscope (Olympus) with minimum magnification and maximum level of bottom light to control the light stability

along the experiment. After that, the images were captured using the camera of iPhone version 13 with auto white balance, and were extracted the red (R), green (G), and blue (B) values by using ImageJ software. The region of interest (ROI) that extracted chromatic value from acquired image was square (25×25 pixels) to determine and measure the solution color. A major problem that occurred in acquired images was an inconstancy of light in the same frame affected the variability of RGB color space. To fix this problem, a hue (H) value in HSL (Hue, Saturation, Lightness) color space was used instead of RGB values to analyze the color solution in reaction chambers because the hue was measured from the dominant wavelength of the spectral radiance of the color, which was not affected by the inconstant light and provided ease of users when compared to other color spaces. (Capitán-Vallvey et al., 2015; Nguyen et al., 2020; Yin et al., 2020). The R, G, and B values were calculated and converted to hue degree (H°) for each reaction chamber according to the equation 1-4 reported by Saravanan et al. (2016). In addition, the distance of hue degree (ΔH°) as shown in **Figure 19** was computed based on the difference of H° between reaction chamber and negative control chamber in the same chip following to the equation 5:

$$R' = \frac{R}{255}; G' = \frac{G}{255}; B' = \frac{B}{255} \quad (1)$$

$$\Delta = C_M - C_m \begin{cases} C_M = \max(R, G, B) \\ C_m = \min(R, G, B) \end{cases} \quad (2)$$

$$H = \begin{cases} \text{undefined, if } C_M = 0 \\ \left(\frac{G'-B'}{\Delta}\right), \text{if } C_M = R' \\ \left(\frac{B'-R'}{\Delta} + 2\right), \text{if } C_M = G' \\ \left(\frac{R'-G'}{\Delta} + 4\right), \text{if } C_M = B' \end{cases} \quad (3)$$

$$H° = H\, x\, 60;\ H° = H° + 360, \text{if } H° < 0 \quad (4)$$

$$\Delta H° = \begin{cases} (360 - H°_n) + H°_r, \text{if } 0° < H°_r < 60° \\ H°_r - H°_n, \text{if } 300° < H°_r < 360° \end{cases} \quad (5)$$

where $H°_n$ and $H°_r$ are the hue degrees of negative control chamber and reaction chamber, respectively.

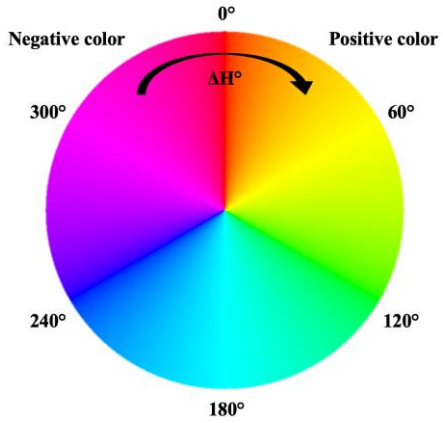


Figure 19 Hue color wheel by degree

(ΔH°: distance of hue degree)

9.3 Validation of colorimetric LAMP on a microfluidic chip coupled with chromatic analysis assay

For analytical sensitivity evaluation, each parasitic target DNA, comprising of *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and *E. miyagawai* was individual carried out with initial 40 ng of genomic DNA per chip, and thereafter diluted 10-fold with serial dilutions ranging from 40 to 40×10^{-5} ng/chip with three replicates per species; furthermore, the sensitivities were tested in case of their co-infection. The interpretation by naked eye provides the qualitative results (infected or uninfected) relying on the color perception of an individual, which needs experienced personnel to determine the color between positive and negative results. Thus, the readout method related to numerical value was developed by using the ΔH° to define a cut-off value for chromatic analysis with high confidence. The cut-off value was calculated based on mean $\Delta H^{\circ}_{\text{negative result}}$ plus three-fold standard deviation (3SD) of the $\Delta H^{\circ}_{\text{negative result}}$ from the analytical sensitivity section, meaning that a ΔH° above the cut-off value was interpreted a positive result, while the lower value was interpreted a negative result. The analytical specificity was tested with other parasites and definitive hosts as well as the section 6.1.

9.4 Shelf life of primers loaded microfluidic chip

The primers loaded chips were stored at ambient temperatures (25–30 °C) in the darkness to check the effect of storage durations and were taken out every week to simultaneously detect the multi-target DNA. The target DNAs of each parasitic group, including *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and *E. miyagawai*, were used the initial DNA concentration of 40 ng/chip and diluted with 10-fold serial dilutions to the lowest DNA detectable dilution.

10. Clinical sample detection

To evaluate the efficiency of each assay, a total of 30 fecal samples was individually sampled from the cloaca of dead chickens, ranging from 0.25 to 0.45 g; thereafter, the gastrointestinal tracts were longitudinally dissected and examined under a stereomicroscope (as a reference method) in order to confirm the infection via a presence or absence of adult worms. The fecal samples were subjected to the genomic DNA extraction using a GF-1 Tissue DNA Extraction Kit. After that, the extracted DNA was tested with the seven developed LAMP assays. The clinical sensitivity and specificity of each assay were computed according to the equation 6 and 7 reported by Lalkhen & McCluskey (2008). For statistical analyses, degrees of agreement in clinical sample detection was evaluated by using Cohen's kappa (K) ranging from 0 to 1, which were indicated as different degrees of agreement according to McHugh (2012), including K$\leq$ 0.39 as poor, K= 0.40–0.79 as moderate, and K$\geq$ 0.80 as high. Finally, McNemar's test was used to compare the difference between developed assays and reference method, considering $p< 0.05$ as the level of significance, by using an online tool(https://www2.ccrb.cuhk.edu.hk/stat/confidence%20interval/McNemar%20Test.htm).

$$\%\ \text{clinical sensitivity} = \frac{\text{True positive}}{\text{True positive+False negative}} x100 \qquad (6)$$

$$\%\ \text{clinical specificity} = \frac{\text{True negative}}{\text{True negative+False positive}} x100 \qquad (7)$$

CHAPTER 4
RESULTS AND DISCUSSION

1. Parasite species

Fourteen parasite species were used to validate the LAMP assays in this study, including *A. galli*, *Heterakis gallinarum*, *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *Cotugnia* sp., *Fimbriaria* sp., *Diorchis* sp., *Hymenolepis nana*, *E. miyagawai*, *E. macrorchis*, *E. mekongi*, *H. conoideum*, and *Prosthogonimus* sp. (**Table 15**). The morphological characteristics of target species, including *Raillietina* spp., *A. galli*, and Echinostomatidae species are shown in **Figure A7-11**. The results of molecular confirmation were performed based on ITS2 region by using the BLASTn algorithm tool to compare with available sequences in the GenBank database as shown in **Table A1**.

Table 15 Parasite species that were found and used in this study

Parasite	Host	Source
Nematode		
A. galli	*G. g. domesticus* (natural host)	This study
H. gallinarum		
Cestode		
R. echinobothrida	*G. g. domesticus* (natural host)	Panich et al. (2021)
R. tetragona		
R. cesticillus		
Cotugnia sp.		
Fimbriaria sp.	*A. p. domesticus* (natural host)	
Diorchis sp.		
H. nana	*Phodopus* sp. (natural host)	Panich et al. (2022)
Trematode		
E. miyagawai	*G. g. domesticus* (natural host)	This study
E. macrorchis		
E. mekongi	*Phodopus* sp. (experimental host)	Glunkwamdi et al. (2020)
H. conoideum	*A. p. domesticus* (natural host)	This study
Prosthogonimus sp.		Panich et al. (2021)

2. Simplex LAMP-LFD assay

2.1 *Raillietina* LAMP-LFD assay

2.1.1 Optimal LAMP reaction

The *Raillietina* LAMP primers recognized to *28S rDNA* gene of *R. echinobothrida*, *R. tetragona*, *R. cesticillus* (Panich et al., 2021) and the optimal reaction was 66 °C for 50 min. The reaction was terminated after reaching 85 °C for 5 min. The optimal reagents of LAMP reaction are shown in **Table 16**. For confirmation of LAMP products, 1 μL of LAMP products of three species yielded under the optimal reaction were digested using *Ms*II enzyme at 37 °C for 180 min, which produced two band of 294, 500 bp for *R. echinobothrida*, 289, 490 bp for *R. tetragona*, and 319, 550 bp for *R. cesticillus* as shown in **Figure 20**.

Table 16 Optimal concentration of reagents for *Raillietina* LAMP reaction

Reagent	Final concentration
10X isothermal amplification buffer	1X
$MgSO_4$	4 mM
F3-RAI	0.2 μM
B3-RAI	0.2 μM
FIP-RAIBi	1.6 μM
BIP-RAI	1.6 μM
dNTPs	1.4 mM
Betaine	1 M
Bst 2.0 DNA polymerase (8,000 U/mL)	4 U
Raillietina DNA template (5 ng/μL)	1 μL
Nuclease-free water	adjusted to 12.5 μL

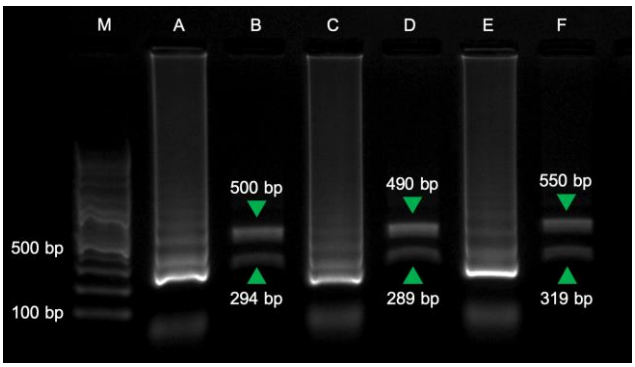


Figure 20 LAMP product confirmations of *Raillietina* spp. by *Msl*I enzyme digestion

(M: molecular marker 100 bp; A: LAMP products of *R. echinobothrida*; B: digested LAMP products of *R. echinobothrida*; C: LAMP products of *R. tetragona*; D: digested LAMP products of *R. tetragona*; E: LAMP products of *R. cesticillus*; F: digested LAMP products of *R. cesticillus*)

2.1.2 *Raillietina* LAMP-LFD development

To examine probe in hybridization step, probe was newly designed in this study to improve the efficient hybridization between LAMP products and probe leading to the obvious color on the test line. The concentration of 2, 20, and 200 pmol of *Raillietina*-probe (Probe-RAI2) were added into the 8 μL of *R. cesticillus* LAMP products (as a representative species) and incubated at 66 °C for 5 min. The results revealed that the 20 pmol of new probe that designed in this study provided the higher intensity color on the test line than Probe-RAI from the original report by Panich et al. (2021). Thus, the 20 pmol Probe-RAI2 was selected for LAMP-LFD development of *Raillietina* spp. (**Figure 21**).

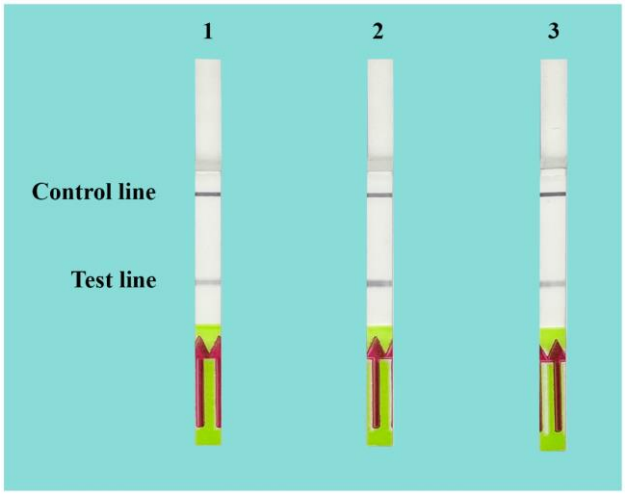


Figure 21 *Raillietina* LAMP-LFD development of various probe quantities

(1: 2 pmol; 2: 20 pmol; 3: 200 pmol)

2.1.3 Analytical specificity of *Raillietina* LAMP-LFD assay

The specificity of *Raillietina* LAMP primers was tested by amplification together with the parasites that co-infected with *Raillietina*. The results showed that *Raillietina* LAMP primers were specifically amplified only *Raillietina* species without cross-reaction of other parasites and their hosts (**Figure 22**), as was reported in previous study by Panich et al. (2021). Furthermore, the newly tested species in this study, including *H. gallinarum*, *H. nana*, *E. macrorchis*, and *E. mekongi* were not detected by *Raillietina* LAMP primers as well.

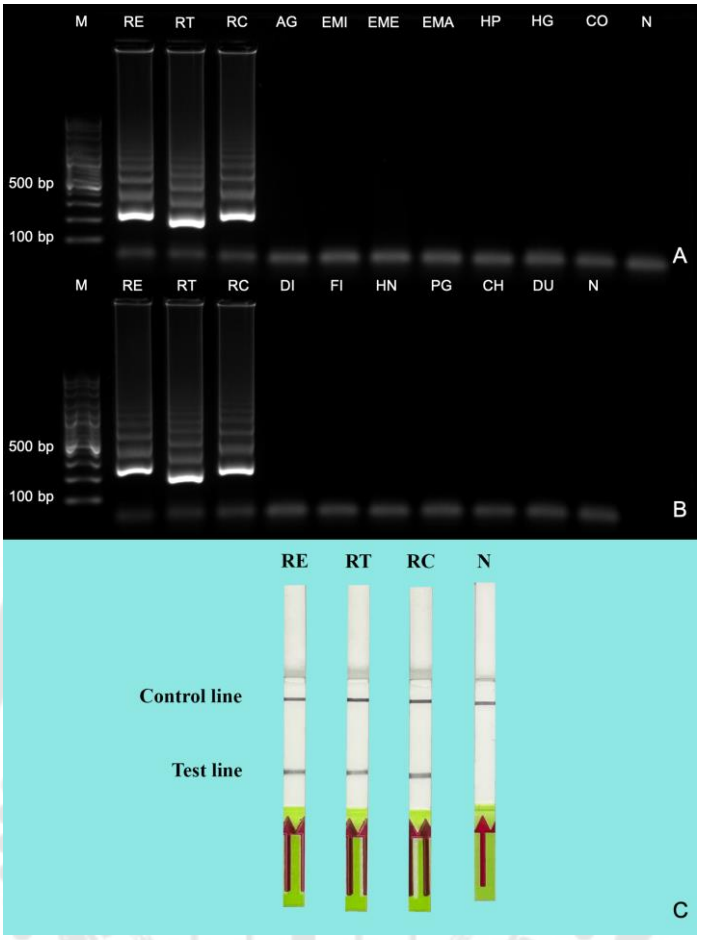


Figure 22 Analytical specificity of *Raillietina* LAMP primers for detecting *R. echinobothrida*, *R. tetragona*, and *R. cesticillus*

(A, B: gel electrophoresis results; C: LFD result; M: molecular marker 100 bp; RE: *R. echinobothrida*; RT: *R. tetragona*; RC: *R. cesticillus*; AG: *A. galli*; EMI: *E. miyagawai*; EME: *E. mekongi*: EMA: *E. macrorchis*: HP: *H. conoideum*; HG: *H. gallinarum*; CO: *Cotugnia* sp.; DI: *Diorchis* sp.; FI: *Fimbriaria* sp.; HN: *H. nana*; PG: *Prosthogonimus* sp.; CH: chicken; DU: duck; N: negative control)

2.1.4 Analytical sensitivity of *Raillietina* LAMP-LFD assay

For the analytical sensitivity tests, the DNA concentration of each *Raillietina* species was initially 5 ng/reaction and diluted 10-fold with serial dilutions. The lowest detectable DNA concentration from each species was found to be 50 pg/reaction for *R. tetragona* and *R. cesticillus*, and 5 pg/reaction for *R. echinobothrida*, which were consistent with the gel electrophoresis. Therefore, the analytical sensitivity of *Raillietina* LAMP-LFD assay that could detect all three species was 50 pg/reaction (**Figure 23**). When compared with the original report by Panich et al. (2021) showed that the new LAMP condition and probe-RAI2 in this study could enhance the analytical sensitivity and reduce the reaction time of LAMP up to 10 min. A previous study reported that multiplex PCR technique of three *Raillietina* species detected the lowest DNA concentration of 0.5 pg, 5 pg, and 50 fg/reaction for *R. echinobothrida*, *R. tetragona*, and *R. cesticillus*, respectively (Panich et al., 2022). Nevertheless, PCR-based technique still requires complicated instruments, such as a thermal cycler machine and gel electrophoresis equipment as well as operation time of at least 180 min. On the other hand, developed LAMP-LFD assay completed in 60 min (LAMP amplification= 50 min; LFD assay= 10 min) and can interpret the results with the naked eye.

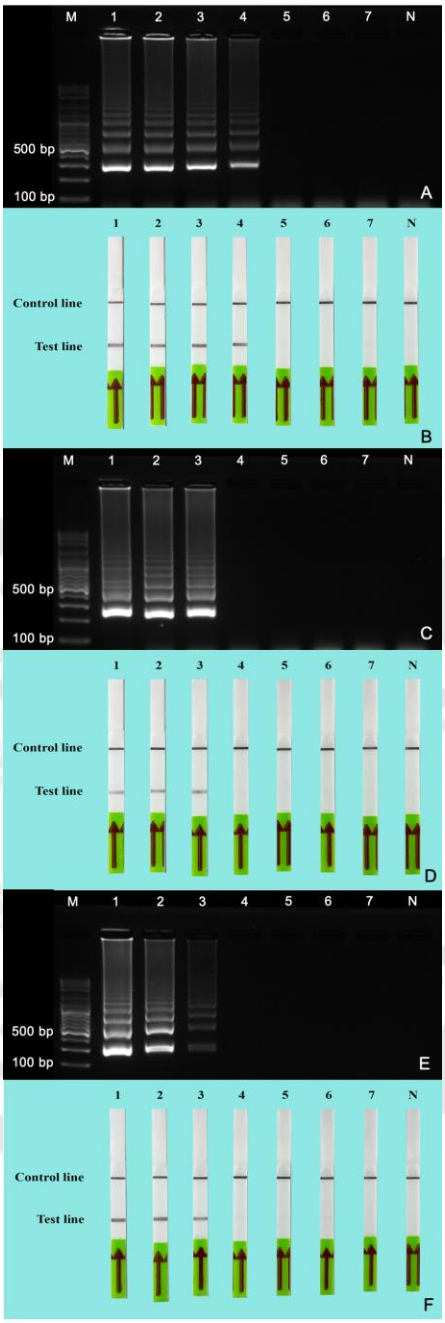


Figure 23 Analytical sensitivity of *Raillietina* LAMP visualized by gel electrophoresis and LFD assay

(A, B: *R. echinobothrida* results; C, D: *R. tetragona* results; E, F: *R. cesticillus* results; M: molecular marker 100 bp; 1-7: 5 to 5 x 10^{-6} ng/reaction, respectively; N: negative control)

2.2 *A. galli* LAMP-LFD assay

2.2.1 LAMP optimization and analytical specificity testing

Table 17 shows the optimum reagents of LAMP reaction that was conducted at 66 °C for 50 min and was terminated the reaction at 85 °C for 5 min. To confirm the expected LAMP products, 1 μL of LAMP products were digested using *Dra*I enzyme at 37 °C for 180 min, which produced single band of 123 bp as shown in **Figure 24**.

Table 17 Optimal reagents for *A. galli* LAMP reaction

Reagent	Final concentration
10X isothermal amplification buffer	1X
$MgSO_4$	4 mM
F3-AG	0.2 μM
B3-AG	0.2 μM
FIP-AGBi	1.6 μM
BIP-AG	1.6 μM
dNTPs	1.4 mM
Betaine	1 M
Bst 2.0 DNA polymerase (8,000 U/mL)	4 U
A. galli DNA template (5 ng/μL)	1 μL
Nuclease-free water	adjusted to 12.5 μL

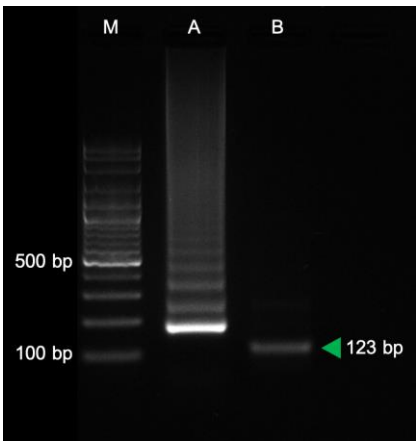


Figure 24 *A. galli* LAMP product confirmation

(M: molecular marker 100 bp; A: LAMP products of *A. galli*; B: digested LAMP products)

2.2.2 *A. galli* LAMP-LFD development

For the optimal quantity of *A. galli*-probe (Probe-AG), the results demonstrated that *A. galli* LAMP products were incubated with 2 pmol of Probe-AG at 66 °C for 5 min giving the most intense color at the test line. Therefore, the suitable condition for LFD development was 2 pmol of Probe-AG and incubated at 66 °C for 5 min (**Figure 25**).

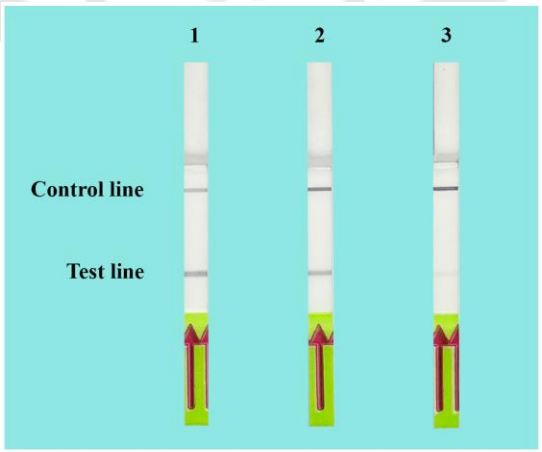


Figure 25 *A. galli* LAMP-LFD development of various probe quantities

(1: 2 pmol; 2: 20 pmol; 3: 200 pmol)

2.2.3 Evaluation of analytical specificity

The developed assay specifically detected only *A. galli* without any cross-amplifications with other species (**Figure 26**), which corresponded with previous reports by Biswas et al. (2021) and Tarbiat et al. (2021) that have been successfully established the molecular methods for *A. galli* targeting the ITS region. These supported that the characteristic of ITS region was suitable to establish a molecular assay for a wider range of parasites of veterinary importance (Tarbiat et al., 2021).

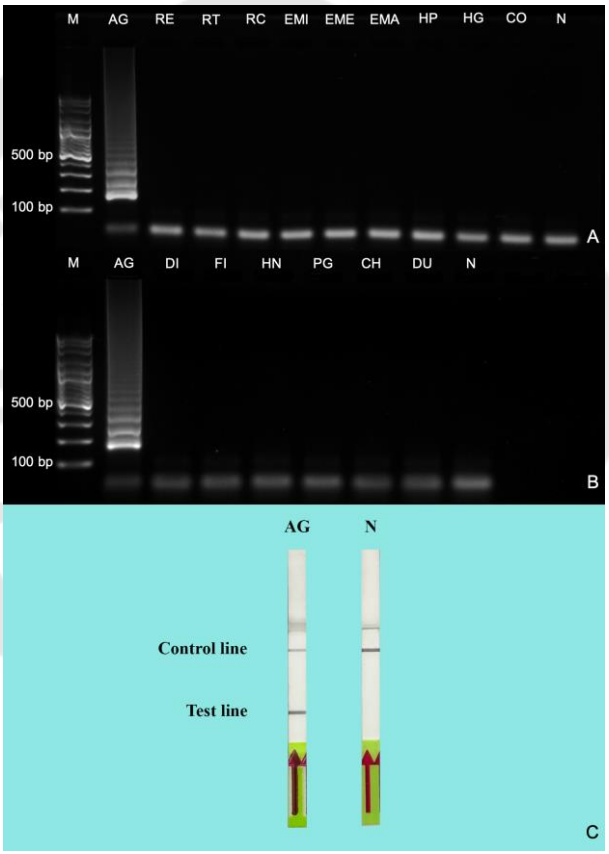


Figure 26 Analytical specificity assessment of *A. galli* LAMP reaction

(A, B: gel electrophoresis results; C: LFD result; M: molecular marker 100 bp; AG: *A. galli*; RE: *R. echinobothrida*; RT: *R. tetragona*; RC: *R. cesticillus*; EMI: *E. miyagawai*; EME: *E. mekongi*: EMA: *E. macrorchis*: HP: *H. conoideum*; HG: *H. gallinarum*; CO: *Cotugnia* sp.; DI: *Diorchis* sp.; FI: *Fimbriaria* sp.; HN: *H. nana*; PG: *Prosthogonimus* sp.; CH: chicken; DU: duck; N: negative control)

2.2.4 Evaluation of analytical sensitivity

The *A. galli* LAMP-LFD assay was evaluated with initial DNA concentrations from 5 to 5×10^{-6} ng/reaction to define the limit of detection. Based on the results, the analytical sensitivity of *A. galli* LAMP-LFD could detect the DNA concentration as low as 50 pg/reaction (**Figure 27**), which is more sensitive than a previous report by Watcharakranjanaporn et al. (2021), where the lowest DNA concentration was 156.3 pg/reaction based on the *NADH dehydrogenase subunit 4* (*ND 4*) gene by using the specific primer PCR method.

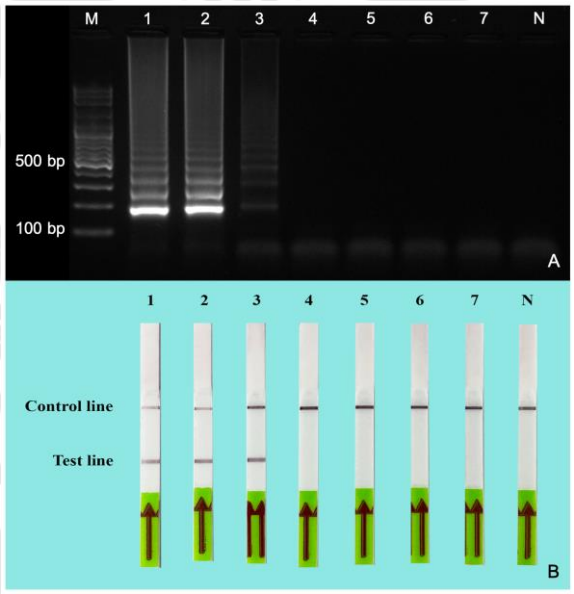


Figure 27 Analytical sensitivity test of *A. galli* LAMP detected by gel electrophoresis and LFD assay

(A: gel electrophoresis result; B: LFD result; M: molecular marker 100 bp; 1-7: 5 to 5×10^{-6} ng/reaction, respectively; N: negative control)

2.3 Echinostomatidae LAMP-LFD assay

2.3.1 LAMP condition and analytical specificity investigation

A total volume of LAMP reaction was 12.5 μL and was carried out at 66 °C for 60 min and then the reaction was stopped at 85 °C for 5 min. The details of optimal reagents are shown in **Table 18**. To verify the LAMP products of Echinostomatidae that were amplified, the four LAMP products were digested using the *Msl*I enzyme at 37 °C for 180 min. The results indicated that the *Msl*I enzyme could digest Echinostomatidae-LAMP products yielding two amplicons of 202, 311 bp for E. miygawai and 205, 317 bp for *E. mekongi*, *E. macrorchis*, and *H. conoideum* (**Figure 28**).

Table 18 Final concentration of LAMP reagents for detecting Echinostomatidae

Reagent	Final concentration
10X isothermal amplification buffer	1X
$MgSO_4$	4 mM
F3-ECHI	0.2 μM
B3-ECHI	0.2 μM
FIP-ECHIBi	1.6 μM
BIP-ECHI	1.6 μM
dNTPs	1.4 mM
Betaine	1 M
Bst 2.0 DNA polymerase (8,000 U/mL)	4 U
Echinostomatidae DNA template (5 ng/μL)	1 μL
Nuclease-free water	adjusted to 12.5 μL

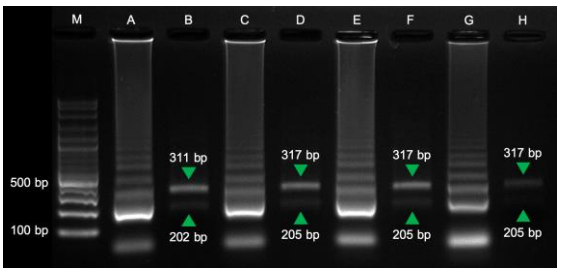


Figure 28 Confirmations of Echinostomatidae LAMP products by *Msl*I enzyme digestion

(M: molecular marker 100 bp; A: *E. miyagawai* LAMP products; B: digested *E. miyagawai* LAMP products; C: *E. mekongi* LAMP products; D: digested *E. mekongi* LAMP products; E: *E. macrorchis* LAMP products; F: digested *E. macrorchis* LAMP products; G: *H. conoideum* LAMP products; H: digested *H. conoideum* LAMP products)

2.3.2 Echinostomatidae LAMP-LFD development

To validate the optimal probe used, a various Echinostomatidae-probe (Probe-ECHI) quantity of 2, 20, and 200 pmol were mixed with the *E. miyagawai* LAMP products and hybridized at 66 °C for 5 min. To select the optimal probe quantity, the most intense color on test line based on interpretation with the naked eye was appeared when 2 pmol of probe was used. Consequently, the Probe-ECHI of 2 pmol was chosen for LAMP-LFD development of Echinostomatidae (**Figure 29**).

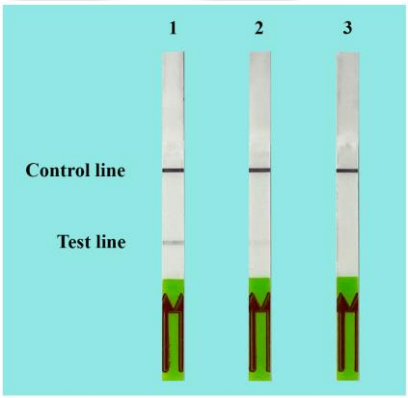


Figure 29 Echinostomatidae LAMP-LFD development of various probe quantities

(1: 2 pmol; 2: 20 pmol; 3: 200 pmol)

2.3.3 Determination of analytical specificity

For analytical specificity test, Echinostomatidae-specific LAMP reaction was amplified only parasites belonging to the family Echinostomatidae, comprising of *E. miyagawai*, *E. mekongi*, *E. macrorchis*, and *H. conoideum* (**Figure 30**). The LAMP reaction was specific without cross-reactions of other non-target species. In several previous studies, the *18S ribosomal DNA* (*18S rDNA*) gene, *ND1* gene, *Cytochrome B* (*CYTB*) gene, and *Cytochrome oxidase subunit 1* (*COI*) gene were used as molecular markers to develop diagnostic methods for detection and discrimination between members of the genus *Echinostoma* (Anucherngchai et al., 2019; Anucherngchai & Chontananarth, 2019; Buddhachat & Chontananarth, 2019; Chai et al., 2020; Tantrawatpan & Saijuntha, 2020). Based on the results in this study, the ITS2 region of trematodes belonging to family Echinostomatidae has a highly conserved sequence and suitable for detecting in terms of family level. Hence, this study is the first report for the detection of Echinostomatidae species using the ITS2 region based on LAMP-LFD assay.

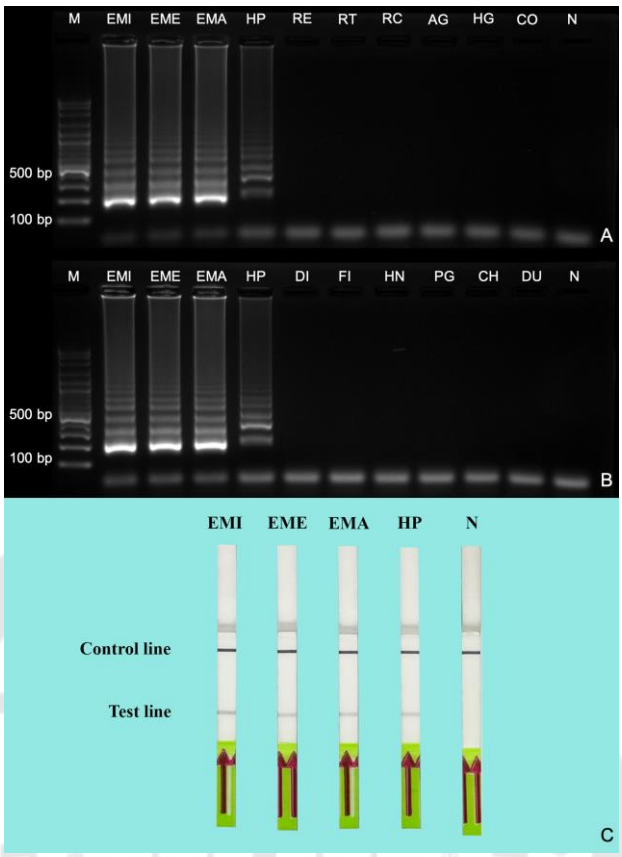


Figure 30 Analytical specificity of Echinostomatidae LAMP amplification

(A, B: gel electrophoresis results; C: LFD result; M: molecular marker 100 bp; EMI: *E. miyagawai*; EME: *E. mekongi*: EMA: *E. macrorchis*: HP: *H. conoideum*; RE: *R. echinobothrida*; RT: *R. tetragona*; RC: *R. cesticillus*; AG: *A. galli*; HG: *H. gallinarum*; CO: *Cotugnia* sp.; DI: *Diorchis* sp.; FI: *Fimbriaria* sp.; HN: *H. nana*; PG: *Prosthogonimus* sp.; CH: chicken; DU: duck; N: negative control)

2.3.4 Determination of analytical sensitivity

The 10-fold serial dilutions of each Echinostomatidae species from 5 to 5×10^{-6} ng/reaction was evaluated to set the analytical sensitivity for Echinostomatidae LAMP-LFD assay. Based on the results, the detection limit of this assay was 5×10^{-2} ng or 50 pg/reaction for *E. miyagawai*, *E. mekongi*, *E. macrorchis*, and *H. conoideum* (**Figure 31**). As previous studies, Anucherngchai and Chontananarth (2019) reported the analytical sensitivity of *E. revolutum* based on PCR method as low as 90 pg/reaction

amplifying the *18S rDNA* gene, and Tantrawatpan and Saijuntha (2020) developed multiplex PCR based on the *ND1* gene for detecting the lowest DNA of *E. revolutum*, *E. ilocanum*, *A. malayanum*, and *H. conoideum* was 50 pg/reaction, whereas developed LAMP-LFD assay in this study showed to be better or equal the detection limit than the previous studies, respectively.

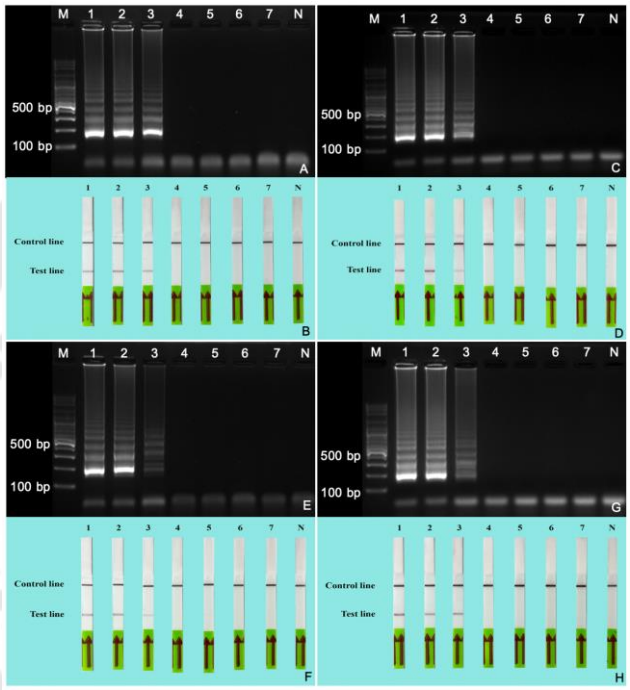


Figure 31 Analytical sensitivity determination of Echinostomatidae species detected by gel electrophoresis and LFD assay

(A, B: *E. miyagawai* results; C, D: *E. mekongi* results; E, F: *E. macrorchis* results; G, H: *H. conoideum* results; M: molecular marker 100 bp; 1-7: 5 to 5×10^{-6} ng/reaction, respectively; N: negative control)

2.4 Simplex LAMP-LFD conclusions

The conclusions of developed simplex LAMP-LFD assays in this study consisted of three assays, comprising of *Raillietina* spp., *A. galli*, and Echinostomatidae specific detection. The details of simplex LAMP-LFD assays are shown in **Table 19**. The LAMP primers and probes of simplex LAMP-LFD assays for *A. galli* and Echinostomatidae were published in Panich et al. (2023) and Panich et al. (2024), respectively.

Table 19 Summaries of *Raillietina* spp., *A. galli*, and Echinostomatidae LAMP-LFD assays

Simplex LAMP-LFD assay	Target species	Operation time	Analytical sensitivity
Raillietina spp.	*R. echinobothrida*	60 min	50 pg/reaction
	R. tetragona		
	R. cesticillus		
A. galli	*A. galli*	60 min	50 pg/reaction
Echinostomatidae	*E. miyagawai*	70 min	50 pg/reaction
	E. mekongi		
	E. macrorchis		
	H. conoideum		

3. Duplex LAMP-LFD assays

3.1 *Raillietina* - *A. galli* LAMP-LFD assay

3.1.1 Optimal duplex LAMP reaction

Duplex LAMP reaction was to combine LAMP primers between *Raillietina* LAMP primers and *A. galli* LAMP primers. *R. cesticillus* and *A. galli* were chosen as the representatives of each parasitic group to determine the optimal concentration of reagents and optimum time period for duplex LAMP assay. The optimal reagents of duplex reaction are shown in **Table 20**. The *Raillietina* - *A. galli* LAMP reaction was conducted at 66 °C for 60 min because it was a minimum reaction time for the simultaneous amplification and was terminated after reaching at 85 °C for 5 min.

Table 20 Final concentration of LAMP reagents for *Raillietina* - *A. galli* LAMP reaction

Reagent	Final concentration
10X isothermal amplification buffer	1X
$MgSO_4$	6 mM
F3-RAI, B3-RAI	0.2 μM
F3-AG, B3-AG	0.2 μM
FIP-RAIBi, BIP-RAI	0.8 μM
FIP-AGDi, BIP-AG	1.6 μM
dNTPs	2 mM
Betaine	1 M
Bst 2.0 DNA polymerase (8,000 U/mL)	4 U
Raillietina DNA template (5 ng/μL)	1 μL
A. galli DNA template (5 ng/μL)	1 μL
Nuclease-free water	adjusted to 12.5 μL

To check the duplex reaction, genomic DNAs of *R. cesticillus* and *A. galli* were mixed as DNA template for simultaneous amplification, and then the duplex LAMP products were digested using the *Msl*I and *Dra*I enzymes at 37 °C for 180 min. The results found that the *Msl*I enzyme can digest the *R. cesticillus* products yielding two amplicons of approximately 319 and 550 bp size, while the *Dra*I enzyme digested *A. galli* products resulting only one amplicon of approximately 123 bp size (**Figure 32**). Therefore, the results demonstrated that the quantity of reagents in duplex LAMP reaction can detect two targets of parasitic groups in single reaction simultaneously.

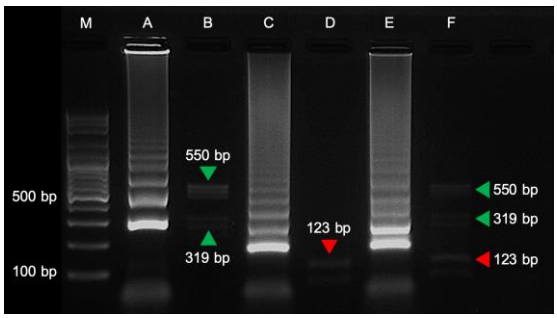


Figure 32 Confirmations of duplex LAMP products by using restriction enzymes

(M: molecular marker 100 bp; A: *R. cesticillus* LAMP products; B: digested LAMP products of *R. cesticillus*; C: *A. galli* LAMP products; D: digested LAMP products of *A. galli*; E: duplex LAMP products of *R. cesticillus* and *A. galli*; F: digested duplex LAMP products of *R. cesticillus* and *A. galli*)

3.1.2 *Raillietina* - *A. galli* LAMP-LFD development

To validate the appropriate quantity of probes for visual interpretation, the FIP primers of *Raillietina* spp. and *A. galli* were labeled with biotin and digoxigenin at 5' end, respectively. The duplex LAMP products were initially incubated with a varying quantity of *A. galli*-probe (Probe-AG), including 2, 20, and 200 pmol, at 66 °C for 5 min. The results showed that at 2 pmol of Probe-AG displayed the highest intensity color on *A. galli* line. Subsequently, 2 pmol of Probe-AG and a variety of Probe-RAI2 concentrations at 2, 20, and 200 pmol were simultaneously hybridized with the duplex LAMP products at 66 °C for 5 min. The results indicated that at 2 pmol of both probes yielded the most intense red bands on the *A. galli* and *Raillietina* lines (**Figure 33**). Consequently, the optimal condition of LFD development was 2 pmol of each probe for dual-hybridization at 66 °C for 5 min.

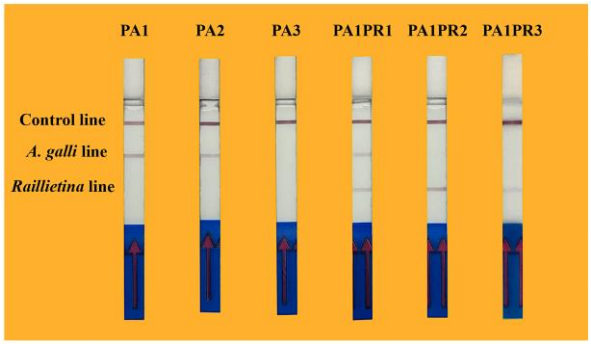


Figure 33 Determination of the optimal concentration of probes used in the *Raillietina* - *A. galli* LAMP-LFD assay

(PA1-3: 2, 20, 200 pmol of Probe-AG, respectively; PA1PR1: 2 pmol of Probe-AG + 2 pmol of Probe-RAI2; PA1PR2: 2 pmol of Probe-AG + 20 pmol of Probe-RAI2; PA1PR3: 2 pmol of Probe-AG + 200 pmol of Probe-RAI2)

3.1.3 Assessment of analytical specificity

The analytical specificity of *Raillietina* - *A. galli* LAMP-LFD assay was evaluated by testing together with the parasites and definitive hosts as shown in **Table 14**. This assay detected *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and mixed DNAs of *R. cesticillus* and *A. galli* simultaneously without cross-amplifying other related species (**Figure 34**). Therefore, the duplex LAMP-LFD assay was still highly-specific for the detection of *Raillietina* spp. and *A. galli*, which corresponded to the simplex LAMP-LFD assays. Moreover, the result interpretation via LFD analysis revealed that it can detect and clearly differentiate between infections of *Raillietina* spp. and *A. galli* by naked eye without time-consuming and complicated protocols, such as gel electrophoresis, which is a limitation for on-site screening (Sriworarat et al., 2015; Wang et al., 2019). Currently, the available methods have not been reported for the simultaneous detection of *Raillietina* spp. and *A. galli*. Hence, this study is the first duplex LAMP-LFD assay used for the detection of *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, and *A. galli* in a single reaction.

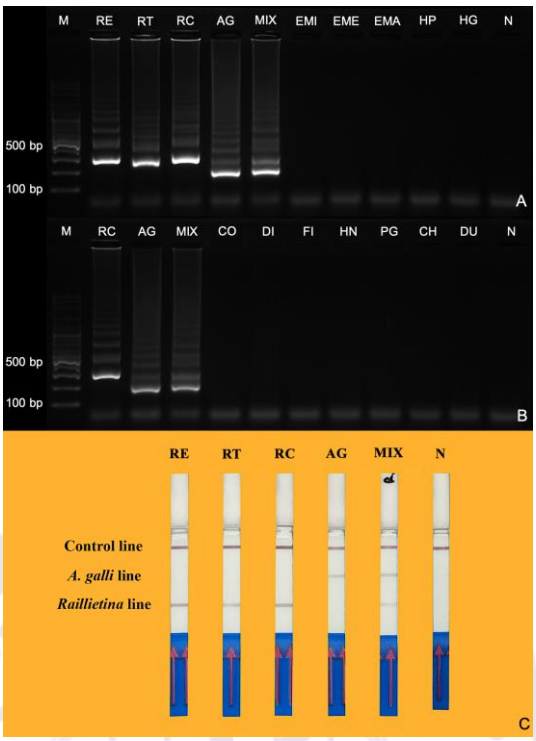


Figure 34 Analytical specificity of *Raillietina* - *A. galli* LAMP-LFD assay

(A, B: gel electrophoresis results; C: LFD result; M: molecular marker 100 bp; RE: *R. echinobothrida*; RT: *R. tetragona*; RC: *R. cesticillus*; AG: *A. galli*; MIX: mixed DNAs of *R. cesticillus* and *A. galli*; EMI: *E. miyagawai*; EME: *E. mekongi*; EMA: *E. macrorchis*; HP: *H. conoideum*; HG: *H. gallinarum*; CO: *Cotugnia* sp.; DI: *Diorchis* sp.; FI: *Fimbriaria* sp.; HN: *H. nana*; PG: *Prosthogonimus* sp.; CH: chicken; DU: duck; N: negative control)

3.1.4 Evaluation of analytical sensitivity

The 10-fold serial dilution methods were performed at ranges from 5 to 5×10^{-6} ng/reaction of genomic DNA. Genomic DNA of *R. cesticillus* was selected as a target representative for *Raillietina* group to assess the analytical sensitivity. The results showed that the sensitivities of *R. cesticillus* and *A. galli* were detected at the lowest DNA concentrations of 5×10^{-3} ng/reaction or 5 pg/reaction for both species, which was an equal to the sensitivity in term of co-infection between *R. cesticillus* and *A. galli* (**Figure 35**). As the result, the simultaneous amplification in a single reaction did not

impact the efficient in amplification of each primer set when compared to the individual sensitivity. Regarding the analytical sensitivity comparison, the analytical sensitivities of *R. cesticillus* and *A. galli* were better than the original report by Panich et al. (2021) up to 100 times and our simplex LAMP-LFD of *A galli* was to be 10 times, respectively.

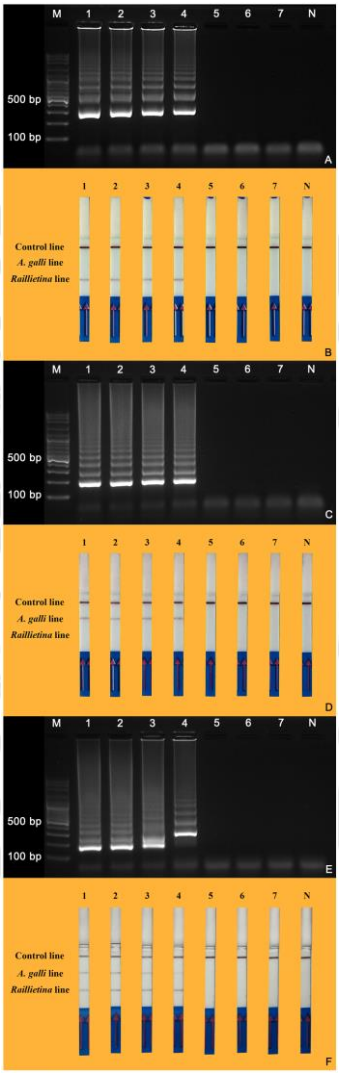


Figure 35 Analytical sensitivity of *Raillietina* - *A. galli* LAMP analyzed by gel electrophoresis and LFD assay

(A, B: *R. cesticillus* results; C, D: *A. galli* results; E, F: *R. cesticillus* + *A. galli* results; M: molecular marker 100 bp; 1-7: DNA concentrations of 5 to 5×10^{-6} ng/reaction, respectively; N: negative control)

3.2 *Raillietina* - Echinostomatidae LAMP-LFD assay

3.2.1 Optimal duplex LAMP reaction

The duplex LAMP reaction was developed and performed at 66 °C for 80 min and terminated the reaction at 85 °C for 5 min. This assay was combined LAMP primers between *Raillietina* (Panich et al., 2021) and Echinostomatidae LAMP primer sets. The representatives of *Raillietina* and Echinostomatidae groups, *R. cesticillus* and *E. miyagawai*, were selected to validate the optimal concentration of reagents. The details of optimal reagents for *Raillietina* - Echinostomatidae LAMP reaction are shown in **Table 21**.

Table 21 Optimal reagents for *Raillietina* - Echinostomatidae LAMP reaction

Reagent	Final concentration
10X isothermal amplification buffer	1X
$MgSO_4$	6 mM
F3-RAI, B3-RAI	0.2 μM
F3-ECHI, B3-ECHI	0.2 μM
FIP-RAIBi, BIP-RAI	0.8 μM
FIP-ECHIDi, BIP-ECHI	1.6 μM
dNTPs	2 mM
Betaine	1 M
Bst 2.0 DNA polymerase (8,000 U/mL)	4 U
Raillietina DNA template (5 ng/μL)	1 μL
Echinostomatidae DNA template (5 ng/μL)	1 μL
Nuclease-free water	adjusted to 12.5 μL

To confirm the concurrent amplification, the *Msl*I enzyme was used to digest the duplex LAMP products that were amplified from the target DNA of each parasitic group, including *R. cesticillus* and *E. miyagawai* by using optimal duplex LAMP condition as mentioned above. **Figure 36** shows the *Msl*I enzyme can digest *R. cesticillus* and *E. miyagawai* LAMP products at 37 °C for 180 min that yielded two

amplicons of each species approximately 319, 550 bp size and 202 and 311 bp size, respectively. The results indicated that the developed *Raillietina* - Echinostomatidae LAMP reaction can amplify the mixed target-DNAs of both parasitic groups simultaneously in one reaction. Thus, the optimum duplex LAMP reaction of *Raillietina* - Echinostomatidae was 66 °C for 80 min.

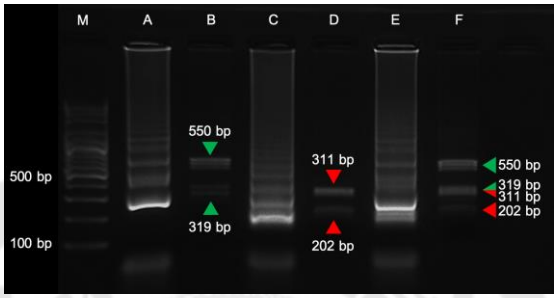


Figure 36 Confirmations of duplex LAMP products by using *Msl*I enzyme digestion

(M: molecular marker 100 bp; A: LAMP products of *R. cesticillus*; B: digested LAMP products of *R. cesticillus*; C: LAMP products of *E. miyagawai*; D: digested LAMP products of *E. miyagawai*; E: duplex LAMP products of *R. cesticillus* and *E. miyagawai*; F: digested duplex LAMP products of *R. cesticillus* and *E. miyagawai*)

3.2.2 *Raillietina* - Echinostomatidae LAMP-LFD development

To visual interpretation by LFD, two probes of each parasitic group were assessed the quantities of 2, 20, and 200 pmol by incubating at 66°C for 10 min. The quantity of probes that yield the highest intense color was selected as the optimal hybridization for *Raillietina* - Echinostomatidae LAMP-LFD development. The FIP primers of *Raillietina* spp. and Echinostomatidae were conjugated with biotin and digoxigenin at 5' end, respectively. Initially, the duplex LAMP products of *R. cesticillus* and *E. miyagawai* were incubated with a various quantity of Probe-ECHI (Echinostomatidae probe) comprising of 2, 20, and 200 pmol. The results showed that at 2 pmol of Probe-ECHI, the color of the Echinostomatidae line had obviously color (**Figure 37**). Subsequently, the 2 pmol of Probe-ECHI combined with a variety of Probe-RAI2

concentrations (2, 20, and 200 pmol) were hybridized with the duplex LAMP products for result interpretation by naked eye. The results revealed that at 2 pmol of both probes presented the most intense color on Echinostomatidae and *Raillietina* lines (**Figure 37**). Therefore, 2 pmol of each probe that incubated at 66°C for 10 min was the optimal condition of *Raillietina* - Echinostomatidae LAMP-LFD development. The major concern related to *Raillietina* - Echinostomatidae LAMP-LFD that found in this study was color-fading of Echinostomatidae line when dual-probe hybridization. This may be indicated that the Probe-RAI2 affected the binding between Echinostomatidae-LAMP products with Probe-ECHI.

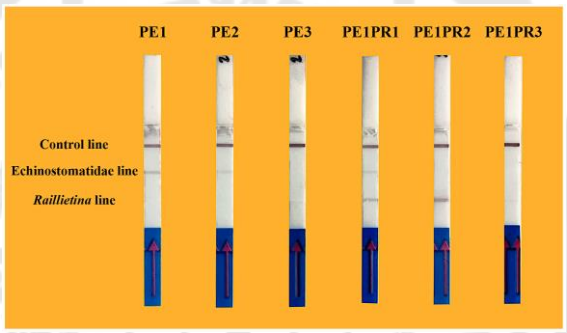


Figure 37 Determination of optimal probe quantities used in *Raillietina* - Echinostomatidae LAMP-LFD assay

(PE1-3: 2, 20, 200 pmol of Probe-ECHI, respectively; PE1PR1: 2 pmol of Probe-ECHI + 2 pmol of Probe-RAI2; PE1PR2: 2 pmol of Probe-ECHI + 20 pmol of Probe-RAI2; PE1PR3: 2 pmol of Probe-ECHI + 200 pmol of Probe-RAI2)

3.2.3 Assessment of analytical specificity

Analytical specificity of *Raillietina* - Echinostomatidae LAMP-LFD was validated with parasites and their definitive hosts that obtained in this study at DNA concentration of 5 ng/reaction, which were amplified at 66°C for 80 min and hybridized at 66°C for 10 min. Based on the results, this assay was specifically detected only species belonging to genus *Raillietina* (*R. echinobothrida*, *R. tetragona*, and *R. cesticillus*) and family Echinostomatidae (*E. miyagawai*, *E. mekongi*, *E. macrorchis*, and

H. conoideum) and simultaneously amplified the mixed of *R. cesticillus* and *E. miyagawai* DNAs in single reaction (**Figure 38**). Thus, the mix of two LAMP primer sets did not affect the analytical specificity of both parasitic groups. This duplex LAMP-LFD assay can complete within 95 min (LAMP amplification 80 min, LFD analysis 10 min and results observation 5 min), excluded DNA extraction, which is not enough yet in terms of rapid detection. However, performing a simplex LAMP-LFD consumes twice the cost, labor, and time compared with duplex LAMP-LFD assay. To decrease the reaction time in the future, the usage of loop primers might be reducing the LAMP duration (Nagamine et al., 2002).

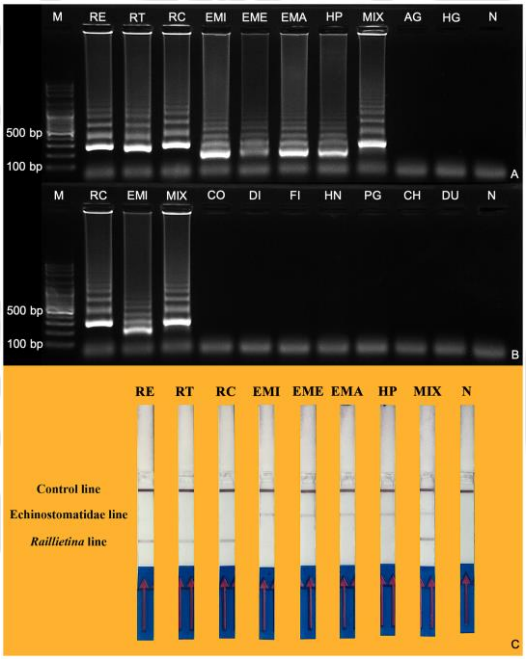


Figure 38 Analytical specificity of *Raillietina* - Echinostomatidae LAMP-LFD assay

(A, B: gel electrophoresis results; C: LFD result; M: molecular marker 100 bp; RE: *R. echinobothrida*; RT: *R. tetragona*; RC: *R. cesticillus*; EMI: *E. miyagawai*; EME: *E. mekongi*; EMA: *E. macrorchis*; HP: *H. conoideum*; MIX: mixed DNAs of *R. cesticillus* and *E. miyagawai*; AG: *A. galli*; HG: *H. gallinarum*; CO: *Cotugnia* sp.; DI: *Diorchis* sp.; FI: *Fimbriaria* sp.; HN: *H. nana*; PG: *Prosthogonimus* sp.; CH: chicken; DU: duck; N: negative control)

3.2.4 Evaluation of analytical sensitivity

The analytical sensitivity, *R. cesticillus* and *E. miyagawai* were selected as the representatives from each parasitic group. Moreover, the mixture DNAs of *R. cesticillus* and *E. miyagawai* were further tested to determine and evaluate the simultaneous sensitivity in case of co-infection. The mixed DNAs were initial 5 ng per species and then were diluted to 5×10^{-6} ng/reaction for determining the lowest DNA detectable concentration. The results demonstrated that the individual analytical sensitivities of *R. cesticillus* and *E. miyagawai* were detected as low as 0.5 pg/reaction and 50 pg/reaction, respectively, which coincided with the sensitivity for simultaneously amplifying of their mixed DNAs (**Figure 39**). However, the detection limit of *Raillietina* LAMP was higher than Echinostomatidae LAMP up to 100 times; although, the quantity of *Raillietina* inner primers was adjusted to a half of Echinostomatidae inner primers or extended the reaction time. This indicated that the *Raillietina* LAMP primers could be higher efficiency than Echinostomatidae LAMP primers. Panich et al. (2021) reported that *Raillietina* LAMP primer set detected the lowest DNA concentration of *R. cesticillus* at 0.5 ng/reaction, whereas developed assay is higher than original report up to 1,000 times. It should be noted that our developed assay has not been assessed for the analytical sensitivity with all species belonging to both groups. However, *R. cesticillus* was chosen as a DNA representative due to the worst sensitivity out of three species following the original report (Panich et al., 2021), while *E. miyagawai* was selected due to it had a high prevalence in poultry. Finally, the major advantages of developed assay are the simultaneous amplification in single reaction and discrimination the results between *Raillietina* and Echinostomatidae groups via the naked eye.

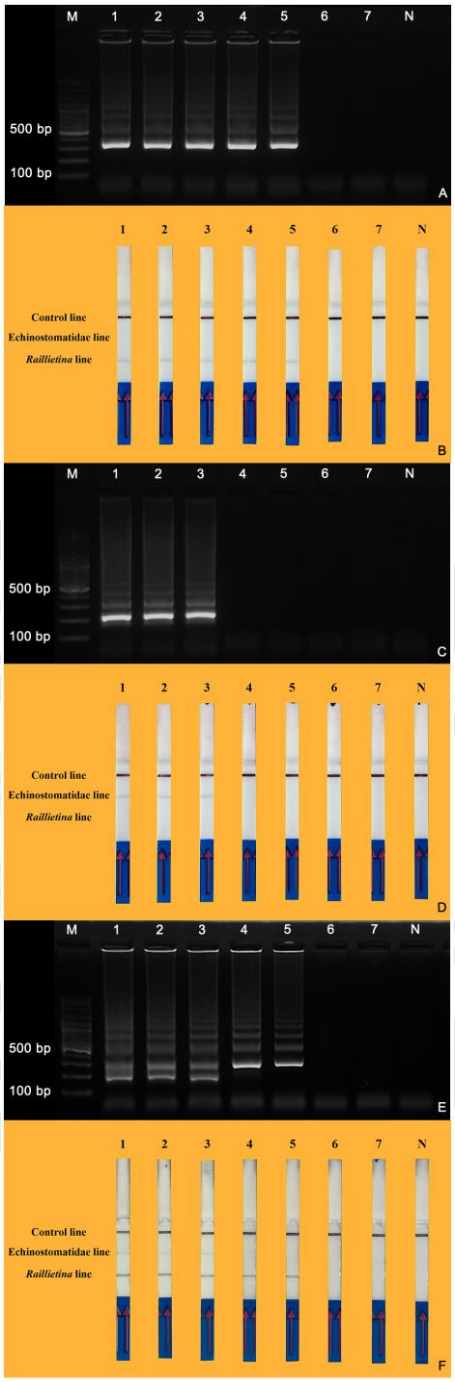


Figure 39 Analytical sensitivity of *Raillietina* - Echinostomatidae LAMP analyzed by gel electrophoresis and LFD assay

(A, B: *R. cesticillus* results; C, D: *E. miyagawai* results; E, F: *R. cesticillus* + *E. miyagawai* results; M: molecular marker 100 bp; 1-7: DNA concentrations of 5 to 5×10^{-6} ng/reaction, respectively; N: negative control)

3.3 *A. galli* - Echinostomatidae LAMP-LFD assay

3.3.1 Optimal duplex LAMP reaction

The LAMP primer sets and probes of *A. galli* and Echinostomatidae groups from each simplex LAMP-LFD were used to combine in the duplex LAMP-LFD assay. Genomic DNAs of *A. galli* and *E. miyagawai* were used as the DNA template for each parasitic group to validate the optimal concentration of reagents and reaction duration. Total volume of *A. galli* - Echinostomatidae LAMP reaction was 12.5 μL, the details of reagents are shown in **Table 22.** The reaction was performed at 66 °C for 80 min and terminated the reaction after reaching at 85 °C for 5 min.

Table 22 Final concentration of LAMP reagents of duplex LAMP reaction for *A. galli* and Echinostomatidae group

Reagent	Final concentration
10X isothermal amplification buffer	1X
$MgSO_4$	6 mM
F3-AG, B3-AG	0.2 μM
F3-ECHI, B3-ECHI	0.2 μM
FIP-AGBi, BIP-AG	0.96 μM
FIP-ECHIDi, BIP-ECHI	1.6 μM
dNTPs	2 mM
Betaine	1 M
Bst 2.0 DNA polymerase (8,000 U/mL)	4 U
A. galli DNA template (5 ng/μL)	1 μL
Echinostomatidae DNA template (5 ng/μL)	1 μL
Nuclease-free water	adjusted to 12.5 μL

To preliminarily verify the expected duplex LAMP products, 1 μL of duplex LAMP products were double-digested using *Dra*I and *Msl*I enzymes at 37 °C for 180 min. The results showed that the *Dra*I and *Msl*I enzymes could simultaneously digest

duplex LAMP products, which yielded a single amplicon of 123 bp size for *A. galli*, and two amplicons of 202 and 311 bp size for *E. miyagawai* in single reaction (**Figure 40**).

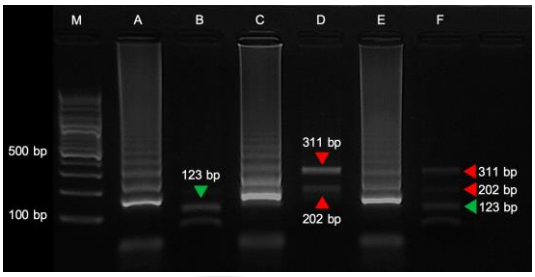


Figure 40 Confirmations of duplex LAMP products by using *Dra*I and *Msl*I enzyme digestions

(M: molecular marker 100 bp; A: LAMP products of *A. galli*; B: digested LAMP products of *A. galli*; C: LAMP products of *E. miyagawai*; D: digested LAMP products of *E. miyagawai*; E: duplex LAMP products of *A. galli* and *E. miyagawai*; F: digested duplex LAMP products of *A. galli* and *E. miyagawai*)

3.3.2 *A. galli* - Echinostomatidae LAMP-LFD development

To define the appropriate quantity of probes for observation by naked eye, the FIP primers of *A. galli* and Echinostomatidae groups were attached with biotin and digoxigenin at 5' end, respectively. The duplex LAMP products of *A. galli* and *E. miyagawai* were initially incubated with a various quantity of Echinostomatidae-probe (Probe-ECHI), including 2, 20, and 200 pmol, at 66 °C for 10 min. Based on the results, the 2 pmol of Probe-ECHI presented the obvious color on Echinostomatidae line. After that, the Probe-ECHI at 2 pmol was used to simultaneously incubate with a varying of *A. galli*-probe (Probe-AG) at 2, 20, and 200 pmol. The results demonstrated that 2 pmol of Probe-ECHI and Probe-AG can be used to observe the color on the Echinostomatidae and *A. galli* line with the naked eye (**Figure 41**). Hence, the optimal hybridization of LFD development was 2 pmol of each probe at 66°C for 10 min.

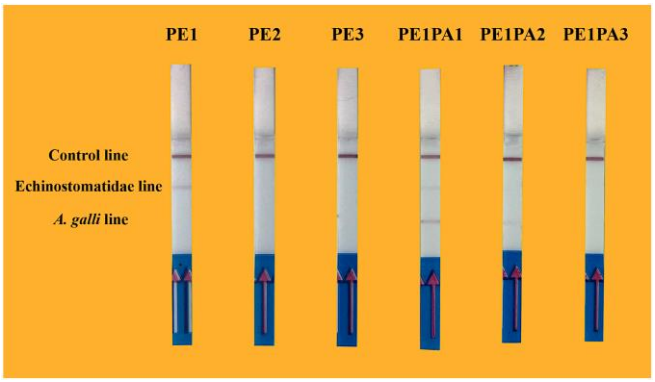


Figure 41 Determination of the optimal concentration of probes used in the *A. galli* - Echinostomatidae LAMP-LFD assay

(PE1-3: 2, 20, 200 pmol of Probe-ECHI, respectively; PE1PA1: 2 pmol of Probe-ECHI + 2 pmol of Probe-AG; PE1PA2: 2 pmol of Probe-ECHI + 20 pmol of Probe-AG; PE1PA3: 2 pmol of Probe-ECHI + 200 pmol of Probe-AG)

3.3.3 Assessment of analytical specificity

After amplification, the results were preliminarily verified by gel electrophoresis, and then the positive ladder-like LAMP product samples were processed in the LFD assay for visual observation. The results presented that duplex LAMP reaction could amplify *A. galli*, *E. miyagawai*, *E. mekongi*, *E. macrorchis*, *H. conoideum*, and mixed DNAs of *A. galli* and *E. miyagawai* without cross-reaction of other related species by gel electrophoresis. Although, all positive samples could be analyzed by gel electrophoresis, but it could not differentiate between A. galli-LAMP products and Echinostomatidae-LAMP products; in contrast, the LFD strip achieved to discriminate the results with the naked eye via the presences of red color on each test line (**Figure 42**). The *A. galli* - Echinostomatidae LAMP reaction was highly specific for *A. galli* and species belonging to family Echinostomatidae, and the result verification of LFD assay was easier than other methods in terms of operating time, simple equipment and data analysis for identifying the multiple LAMP products for each target pathogen, including restriction enzyme digestion, melting curve analysis, and fluorescent-dye-

conjugation etc. (Aonuma et al., 2010; Dong et al., 2019; Fan et al., 2018; Iseki et al., 2007; Prompamorn et al., 2011; Tone et al., 2017).

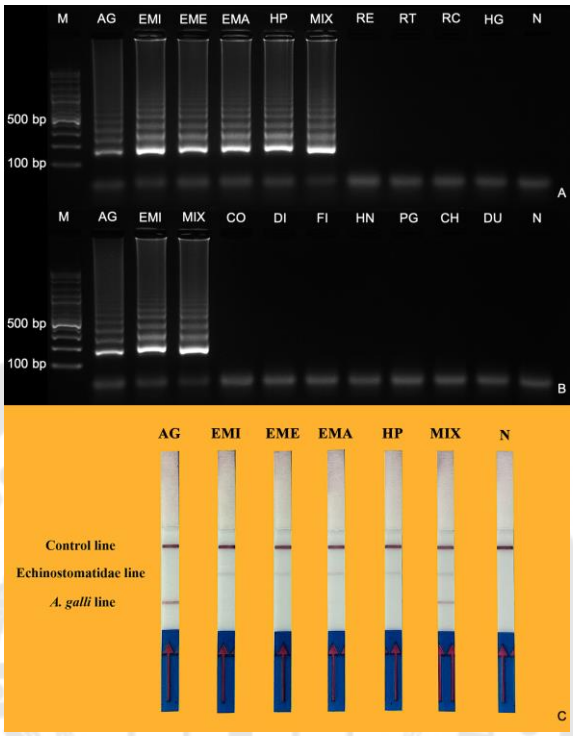


Figure 42 Analytical specificity of *A. galli* - Echinostomatidae LAMP-LFD assay

(A, B: gel electrophoresis results; C: LFD result; M: molecular marker 100 bp; AG: *A. galli*; EMI: *E. miyagawai*; EME: *E. mekongi*; EMA: *E. macrorchis*; HP: *H. conoideum*; MIX: mixed DNAs of *A. galli* and *E. miyagawai*; RE: *R. echinobothrida*; RT: *R. tetragona*; RC: *R. cesticillus*; HG: *H. gallinarum*; CO: *Cotugnia* sp.; DI: *Diorchis* sp.; FI: *Fimbriaria* sp.; HN: *H. nana*; PG: *Prosthogonimus* sp.; CH: chicken; DU: duck; N: negative control)

3.3.4 Evaluation of analytical sensitivity

After assessing the specificity of *A. galli* - Echinostomatidae LAMP-LFD assay, we evaluated its analytical sensitivity by using DNAs of *A. galli* and *E. miyagawai* from seven 10-fold dilutions, ranging from 5 to 5×10^{-6} ng/reaction. As presented in **Figure 43**, the developed assay detected both targets as low as 0.5 pg/reaction and 50 pg/reaction for *A. galli* and *E. miyagawai*, respectively, which were consistent with the gel electrophoresis results. Moreover, mixed DNAs of them was further tested in terms of simultaneous detection. The results indicated that the analytical sensitivity did not differ from the single target-DNA in the reaction (**Figure 43**). Based on the reaction time periods from simplex LAMP assays, the simplex LAMP for Echinostomatidae group consumed the reaction duration longer than *A. galli* about 10 min, meaning that the efficiency of Echinostomatidae primers lower than *A. galli* primers. This is corresponded with the analytical sensitivity of duplex LAMP assay, where the sensitivity of *A. galli* was superior than Echinostomatidae as high as 100 times.

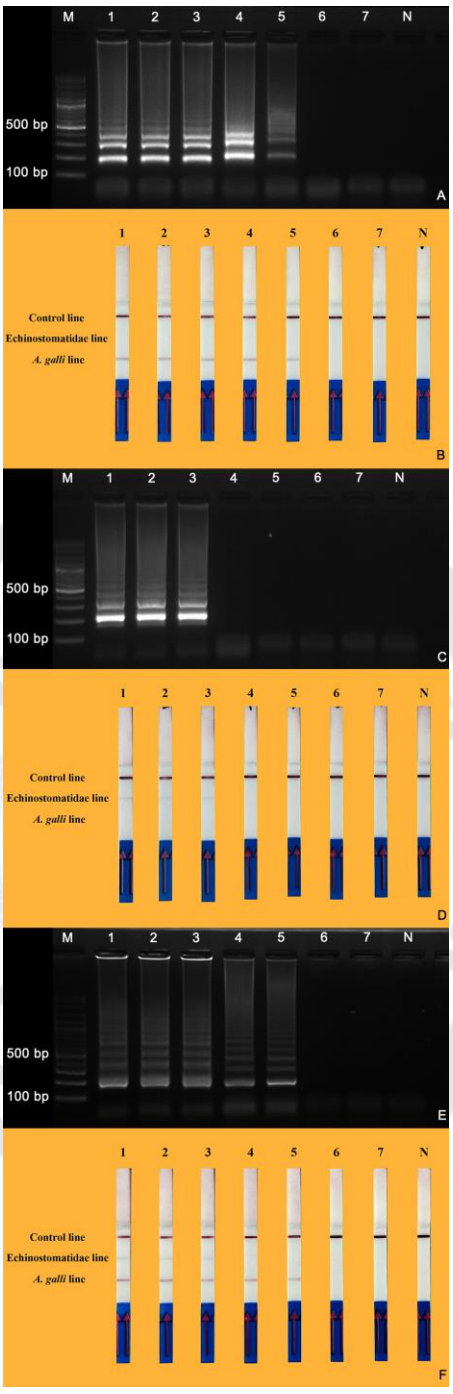


Figure 43 Analytical sensitivity of *A. galli* - Echinostomatidae LAMP analyzed by gel electrophoresis and LFD assay

(A, B: *A. galli* results; C, D: *E. miyagawai* results; E, F: *A. galli* + *E. miyagawai* results; M: molecular marker 100 bp; 1-7: DNA concentrations of 5 to 5×10^{-6} ng/reaction, respectively; N: negative control)

3.4 Duplex LAMP-LFD conclusions

Duplex LAMP-LFD assays showed that the intensity of color on test lines faded than simplex LAMP-LFD assays, especially test line of Echinostomatidae group. These phenomena might be explained the presence of two LAMP primer sets in single reaction, affecting the hybridization between probe and LAMP products was not as much as the simplex LAMP-LFD assays. To enhance the efficiency, the position of probe recognition should be selected the loop structures at both ends of dumbbell structure because of the property of single strand DNA. Overall results, highlighting target species, operation time, and analytical sensitivity are presented in **Table 23**. The duplex LAMP-LFD assays for *Raillietina* - *A. galli* and *Raillietina* - Echinostomatidae were published in Panich et al. (2024) and Panich et al.(2025), respectively.

Table 23 Summaries of duplex LAMP-LFD assays for *Raillietina*, *A. galli*, and Echinostomatidae groups

Duplex LAMP-LFD assay	Target species	Operation time	Analytical sensitivity (per reaction)		
			Raillietina	*A. galli*	Co-infection
Raillietina - *A. galli*	*R. echinobothrida*	70 min	5 pg	5 pg	5 pg, 5 pg
	R. tetragona				
	R. cesticillus				
	A. galli				
			Raillietina	Echinostomatidae	Co-infection
Raillietina - Echinostomatidae	*R. echinobothrida*	95 min	0.5 pg	50 pg	0.5 pg, 50 pg
	R. tetragona				
	R. cesticillus				
	E. miyagawai				
	E. mekongi				
	E. macrorchis				
	H. conoideum				
			A. galli	Echinostomatidae	Co-infection
A. galli - Echinostomatidae	*A. galli*	95 min	0.5 pg	50 pg	0.5 pg, 50 pg
	E. miyagawai				
	E. mekongi				
	E. macrorchis				
	H. conoideum				

4. Colorimetric LAMP on a microfluidic chip coupled with chromatic analysis

4.1 Microfluidic chip fabrication

The microfluidic chip was prepared and coated with LAMP primer sets in each different reaction chamber before using. The preparation and procedures of microfluidic chip were described above in the MATERIALS AND METHODS section. **Figure 44** shows the fabricated microfluidic chip, comprising of six reaction chambers for multiplex detection of *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, Echinostomatidae group, and negative control. Regarding the microfluidic chip design, a hydrophilic reagent did not use to coat the surface of the chip, resulting the mixture was not flowing into some reaction chambers when injection if we use a single outlet hole; consequently, we designed the outlet holes each reaction chamber separately. The separation of outlet holes becomes a notable advantage in terms of target-selected detection without full volume of the chip, meaning that the chip could be performed the reaction in only one reaction chamber with injected LAMP mixture of 50 μL (a half of full volume) for at least single target detection by punching outlet holes of one reaction chamber and negative control chamber (**Figure A12**). This advantage helps to save the cost caused by the surplus reagents in other chambers that do not want to examine. Another significant problem is the expansion of tiny air-bubble on the surface when performing the reaction at 65 °C, affecting the final concentration of coated primers in reaction chambers. To solve this problem, the reaction chamber was designed in an angle-free shape to reduce any bubble formation as much as possible, which was inspired from Nguyen et al. (2020). However, the air-bubble that occurred did not impact the chromatic analysis due to the ROI measured the homogeneous area in each reaction chamber.

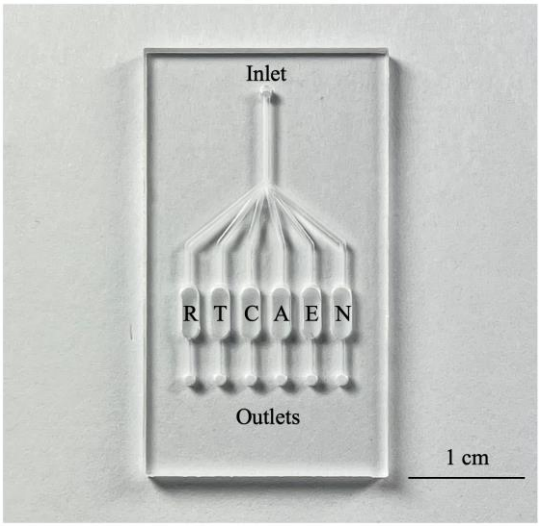


Figure 44 A fabricated microfluidic chip consists of one inlet, six reaction chambers, and six outlets

(R: *R. echinobothrida* reaction chamber; T: *R. tetragona* reaction chamber; C: *R. cesticillus* reaction chamber; A: *A. galli* reaction chamber; E: Echinostomatidae reaction chamber; N: negative control chamber)

4.2 Colorimetric LAMP reaction on a microfluidic chip

To achieve colorimetric LAMP on a microfluidic chip platform, a phenol red dye was selected as a final concentration of 100 μM to monitor the results via solution discoloration by pre-adding in the LAMP mixture before injecting in the microfluidic chip. A total volume of colorimetric LAMP mixture was 100 μL before injecting in the microfluidic chip, and then was performed at 65 °C for 90 min in water bath. The final concentrations of reagent are shown in **Table 24**.

Table 24 Details of optimal reagent for colorimetric LAMP on a microfluidic chip

Reagent	Final concentration
10X isothermal amplification weakly buffer	1X
$MgSO_4$	5.2 mM
F3 primer (pre-dried in reaction chamber)	0.2 μM
B3 primer (pre-dried in reaction chamber)	0.2 μM
FIP primer (pre-dried in reaction chamber)	1.6 μM
BIP primer (pre-dried in reaction chamber)	1.6 μM
Phenol red dye	100 μM
dNTPs	1.4 mM
BSA	0.32% w/v
Betaine	1 M
Bst 2.0 DNA polymerase (8,000 U/mL)	32 U
DNA template each species (10 ng/μL)	4 μL
Nuclease-free water	adjusted to 100 μL

Regarding the verification of LAMP products that were obtained from the colorimetric LAMP on a chip for five parasite targets, 1 μL of LAMP products in each reaction chamber was digested using *Hin*dIII-HF, *Hpy*CH4III, *Hpy*CH4V, *Dra*I, and *Ms*lI enzymes for *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and *E. miyagawai*, respectively, at 37 °C for 180 min. The results showed that each enzyme digested LAMP products for each parasitic group, which yielded a single amplicon of 214 bp size for *R. echinobothrida*, double amplicons of 115 and 191 bp size for *R. tetragona*, triple amplicons of 114, 163, and 213 bp size for *R. cesticillus*, a single amplicon of 123 bp size for *A. galli*, and double amplicons of 201 and 309 bp size for *E. miyagawai* (**Figure 45**).

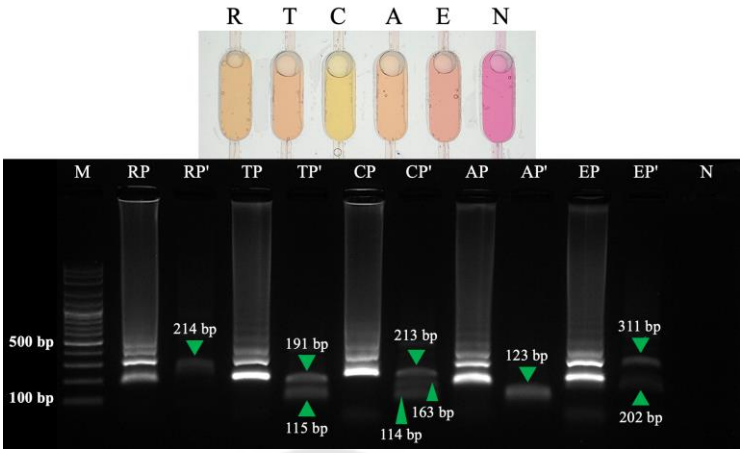


Figure 45 Verifications of colorimetric LAMP products on a chip by using restriction enzymes

(M: molecular marker 100 bp; R: *R. echinobothrida* reaction chamber; T: *R. tetragona* reaction chamber; C: *R. cesticillus* reaction chamber; A: *A. galli* reaction chamber; E: Echinostomatidae reaction chamber; N: negative control chamber; RP: *R. echinobothrida* LAMP products; RP': digested LAMP products of *R. echinobothrida*; TP: *R. tetragona* LAMP products; TP': digested LAMP products of *R. tetragona*; CP: *R. cesticillus* LAMP products; CP': digested LAMP products of *R. cesticillus*; AP: *A. galli* LAMP products; AP': digested LAMP products of *A. galli*; EP: *E. miyagawai* LAMP products; EP': digested LAMP products of *E. miyagawai*)

4.3 Colorimetric LAMP on a microfluidic chip validation

4.3.1 Analytical sensitivity and cut-off value setup for chromatic analysis

The colorimetric LAMP on a microfluidic chip was carried out three replicates with initial DNA of 40 ng/chip for each parasitic group and their mixed DNAs, and then was diluted 10-fold with serial dilutions as low as 40×10^{-5} ng/chip. Overall result divided into six analytical sensitivities, including (I) *R. echinobothrida*, (II) *R. tetragona*, (III) *R. cesticillus*, (IV) *A. galli*, (V) Echinostonatidae (*E. miyagawai* as a representative species for this group), and (VI) mixed DNAs of all five targets. The highest sensitivity out of five targets was *R. cesticillus* found to be 40 pg/chip followed by *R. echinobothrida*, *A. galli*, and *E. miyagawai* as 400 pg/chip, meanwhile the worst

sensitivity was *R. tetragona* as 4 ng/chip. Note that, the dilution 4 (40 pg/chip) of *R. echinobothrida* and *A. galli* showed an instability of positive results, leading to the overlapping between standard deviation and cut-off value (as depicted the next paragraph); therefore, the analytical sensitivities were concluded to be 400 pg/chip (**Table 25**; **Figure 46**). These results were an equal with the analytical sensitivity in case of mixed DNA for five species, which demonstrated that the contamination of non-target DNA at least four times did not impact the analytical sensitivity for each other group. However, the practical utilization with fecal sample cannot control the quantity of another DNA or inhibitory substance; therefore, the clinical detection process must be validated, which will be tested later.

However, the positive color between parasitic groups showed the yellow in shades due to the efficiency of individual LAMP set, leading to confusion in result interpretation (Xiong et al., 2020). Thus, a chromatic analysis of ΔH° between reaction chamber and negative control chamber in the same chip was generated in order to enhance the confidence in result interpretation based on cut-off value. The cut-off value of ΔH° was computed based on the reaction chamber that provided negative result in analytical sensitivities section. Our results showed that the cut-off value of ΔH° was 6.705 distance unit (d.u.), meaning that the ΔH° value above 6.705 d.u. was interpreted a positive result. Based on the chromatic analysis for evaluating the analytical sensitivity, the results showed that all positive results that analyzed by the ΔH° values were higher than the cut-off value of 6.705 d.u., ranging from 16.029 - 75.139 d.u. (**Table 25**), which were corresponding with the qualitative results observed by naked eye (**Figure 46**). Previous studies have reported that colorimetric LAMP assays by using hue value analysis could be applied to interpret the color results (Moehling et al., 2024; Nguyen et al., 2020; Yin et al., 2020).

The average analytical sensitivity from the chip was higher than LAMP-LFD assay ranging from 10 to 1,000 times for *Raillietina* group, while the sensitivity of *A. galli* LAMP-LFD assays was better than up to10 to 100 times. In contrast, Echinostomatidae LAMP-LFD assays showed that the sensitivity was an equal as the microfluidic chip.

However, the colorimetric LAMP on a microfluidic chip could offer more advantages than LFD assay in terms of the simultaneous detection and discrimination of each parasitic species infection, cost-effective, time saving, data analysis and result interpretation. Microfluidic chip technology has a wide range of integrations in molecular biology, such as sample collection, nucleic acid preparation, and molecular detection because it has the outstanding points regarding the use of very small amounts of reagents and ease of the portability which has a feasible to be used in field testing (Mahdavi Abhari et al., 2023; Mao et al., 2018).

Table 25 Chromatic values of analytical sensitivity evaluation

DNA concentration	ΔH°_{mean} ± SD values of each reaction chamber (d.u.)					
	R. echinobothrida	*R. tetragona*	*R. cesticillus*	*A. galli*	Echinostomatidae	Negative control
R. echinobothrida						
1. 40 ng/chip	55.591 ± 4.252	-1.163 ± 2.189	-3.019 ± 3.865	-1.796 ± 3.460	-1.367 ± 3.700	0
2. 4 ng/chip	57.198 ± 2.074	1.261 ± 1.241	1.786 ± 0.428	0.976 ± 0.813	2.114 ± 0.641	0
3. 0.4 ng/chip	48.803 ± 11.816	0.168 ± 0.484	0.677 ± 1.901	0.555 ± 1.596	0.283 ± 0.467	0
4. 0.04 ng/chip	8.676 ± 17.883	-1.345 ± 2.377	-1.414 ± 3.494	-0.146 ± 4.591	0.259 ± 3.614	0
5. 0.004 ng/chip	0.710 ± 0.859	0.071 ± 1.240	1.638 ± 1.958	1.596 ± 2.148	1.240 ± 0.187	0
6. 0.0004 ng/chip	0.430 ± 2.262	0.967 ± 1.926	1.023 ± 1.064	1.468 ± 0.578	1.207 ± 0.783	0
R. tetragona						
1. 40 ng/chip	1.098 ± 0.970	42.610 ± 6.904	1.460 ± 1.454	1.110 ± 1.013	0.009 ± 1.692	0
2. 4 ng/chip	0.473 ± 3.310	40.201 ± 4.273	1.606 ± 1.706	1.113 ± 3.498	1.861 ± 1.431	0
3. 0.4 ng/chip	-1.650 ± 1.392	-1.946 ± 2.214	-2.092 ± 2.144	-0.952 ± 2.176	-0.382 ± 2.030	0
4. 0.04 ng/chip	-0.597 ± 2.538	-2.689 ± 4.610	-2.225 ± 3.709	-1.689 ± 4.219	-1.154 ± 3.258	0
5. 0.004 ng/chip	1.125 ± 1.185	0.902 ± 1.004	1.973 ± 0.417	1.945 ± 0.471	1.967 ± 0.411	0
6. 0.0004 ng/chip	1.110 ± 1.042	0.455 ± 0.869	0.615 ± 1.044	1.706 ± 0.131	1.785 ± 0.203	0
R. cesticillus						
1. 40 ng/chip	-0.771 ± 2.130	-0.713 ± 1.767	68.098 ± 2.125	-0.182 ± 2.006	1.386 ± 1.197	0
2. 4 ng/chip	1.231 ± 0.358	-0.034 ± 2.194	70.478 ± 5.279	1.767 ± 0.554	2.235 ± 0.699	0
3. 0.4 ng/chip	-1.237 ± 2.691	0.013 ± 3.858	67.909 ± 2.078	-0.922 ± 0.876	-1.379 ± 1.348	0
4. 0.04 ng/chip	-1.337 ± 4.444	-1.786 ± 2.413	60.159 ± 12.650	-0.929 ± 0.875	-0.811 ± 1.122	0
5. 0.004 ng/chip	-0.982 ± 2.165	-0.539 ± 0.832	-1.702 ± 2.875	0.954 ± 0.806	1.923 ± 0.740	0
6. 0.0004 ng/chip	1.108 ± 3.830	-0.413 ± 3.398	-0.265 ± 4.859	1.050 ± 2.382	-0.841 ± 4.252	0
A. galli						
1. 40 ng/chip	2.535 ± 2.432	0.353 ± 1.729	-1.160 ± 0.422	66.169 ±2.810	-0.682 ± 2.490	0
2. 4 ng/chip	0.416 ± 1.949	-0.075 ± 2.661	0.401 ± 1.809	66.034 ±4.033	-0.705 ± 2.020	0
3. 0.4 ng/chip	0.371 ± 3.066	0.628 ± 2.037	0.459 ± 2.082	57.452 ±4.036	-0.524 ± 2.655	0
4. 0.04 ng/chip	-1.936 ± 2.642	-1.816 ± 1.475	-2.692 ± 2.893	10.915±20.055	-1.875 ± 2.783	0

Table 25 (Continued)

DNA concentration	ΔH°_{mean} ± SD values of each reaction chamber (d.u.)					
	R. echinobothrida	*R. tetragona*	*R. cesticillus*	*A. galli*	Echinostomatidae	Negative control
5. 0.004 ng/chip	0.353 ± 2.123	0.377 ± 2.169	-0.118 ± 1.348	0.428 ± 0.738	0.751 ± 1.294	0
6. 0.0004 ng/chip	1.111 ± 1.094	0.668 ± 0.652	0.770 ± 1.333	1.191 ± 1.163	0.767 ± 1.313	0
E. miyagawai						
1. 40 ng/chip	2.750 ± 0.109	0.777 ± 1.629	0.478 ± 0.851	1.755 ± 0.908	37.667 ± 6.606	0
2. 4 ng/chip	1.651 ± 3.051	-0.047 ± 1.667	0.507 ± 1.721	0.499 ± 1.671	30.518 ± 3.524	0
3. 0.4 ng/chip	0.830 ± 2.368	0.292 ± 3.251	-0.149 ± 1.563	1.141 ± 0.185	35.729 ± 13.601	0
4. 0.04 ng/chip	2.008 ± 0.663	2.416 ± 0.661	2.562 ± 0.901	2.019 ± 0.862	1.579 ± 1.631	0
5. 0.004 ng/ chip	0.903 ± 1.617	0.214 ± 2.402	-1.093 ± 0.880	-0.901 ± 1.046	0.231 ± 0.697	0
6. 0.0004 ng/chip	-2.345 ± 1.857	-1.437 ± 1.483	0.036 ± 2.558	1.031 ± 3.418	0.542 ± 3.084	0
Mixed DNA of five species						
1. 40 ng/chip	58.886 ± 1.961	57.699 ± 1.582	68.104 ± 3.476	65.031 ± 3.309	38.396 ± 5.361	0
2. 4 ng/chip	57.498 ± 0.987	24.886 ± 7.832	62.698 ± 1.283	61.000 ± 1.295	47.102 ± 3.075	0
3. 0.4 ng/chip	55.142 ± 0.872	-1.343 ± 1.573	62.875 ± 0.168	59.626 ± 1.453	43.390 ± 5.280	0
4. 0.04 ng/chip	12.841 ± 9.817	1.561 ± 0.115	56.247 ± 7.270	16.786±25.939	1.157 ± 2.512	0
5. 0.004 ng/chip	2.082 ± 0.300	1.059 ± 0.820	0.017 ± 0.199	0.649 ± 0.872	0.790 ± 1.015	0
6. 0.0004 ng/chip	-2.452 ± 2.700	-1.945 ± 1.885	-1.293 ± 2.983	-1.369 ± 3.105	0.543 ± 1.252	0

Note. Cut-off value= 6.705 d.u.

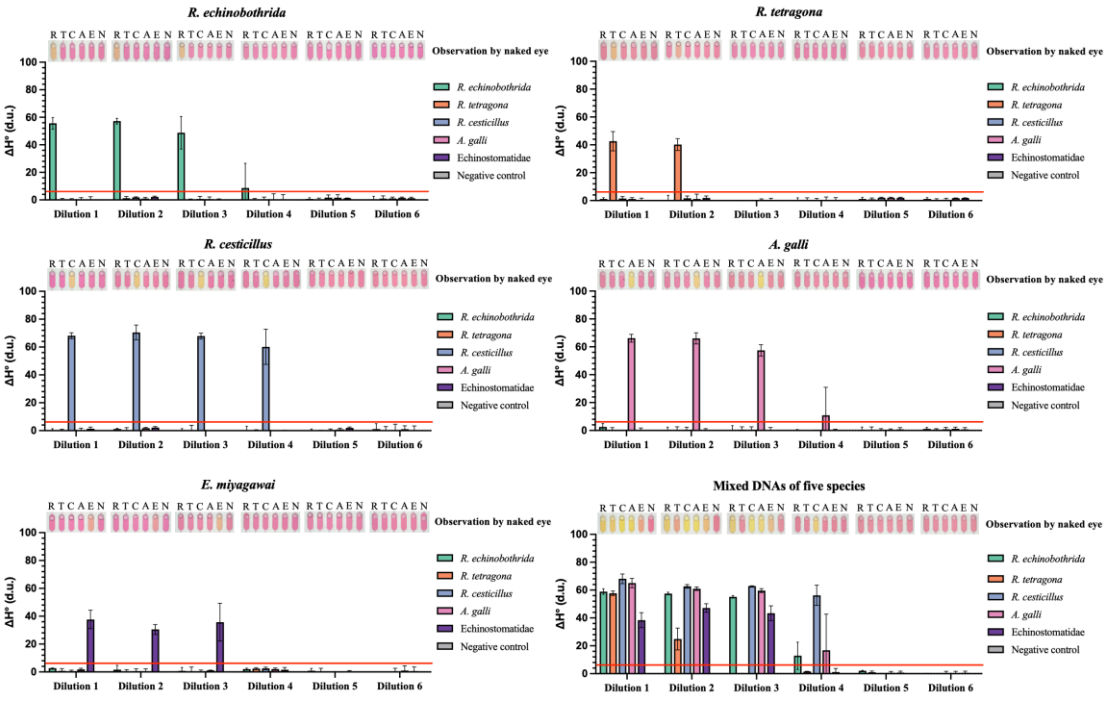


Figure 46 Analytical sensitivity of colorimetric LAMP on a microfluidic chip for detecting *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and E. miyagawai analyzed by naked eye and chromatic analysis via images

(R: *R. echinobothrida* reaction chamber; T: *R. tetragona* reaction chamber; C: *R. cesticillus* reaction chamber; A: *A. galli* reaction chamber; E: Echinostomatidae reaction chamber; N: negative control chamber; Dilution 1-6: DNA concentrations of 40 to 40×10^{-5} ng/chip, respectively; red lines: cut-off value of ΔH°)

4.3.2 Analytical specificity and multi-target detection

The developed colorimetric LAMP on a microfluidic chip was evaluated the analytical specificity with other parasites and their hosts as shown in **Table 14**. Moreover, this assay was further tested to present the readout results in terms of multi-target detection, including simplex, duplex, triplex, quadplex, and pentaplex detections. The results found that the developed assay had a high level of specificity and accuracy for detecting the five parasitic groups without false positive and false negative results as well as in case of multiplex detection (**Figure 47**). Furthermore, the chromatic results

were consistent with the color results by observing with the naked eye, but the chromatic analysis could interpret with high degree of confidence by considering the numerical results coupled with the cut-off value as shown in **Table 26**. Therefore, the developed colorimetric LAMP on a microfluidic chip integrated with chromatic analysis assay could specifically, simultaneously, and accurately detect the five parasitic groups, namely *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and Echinostomatidae in single chip. The microfluidic chips combined with LAMP assays have been employed to detect various parasitic pathogens previously, including *Trypanosoma brucei*, *Plasmodium* spp., *Toxoplasma gondii*, *Cryptosporidium* spp., *Schistosoma mansoni*, *Clonorchis sinensis*, and *Taenia solium* (Chen et al., 2023; Liu et al., 2012; Malpartida-Cardenas et al., 2019; Song et al., 2015; Wan et al., 2019). Hence, our study is the first report for detecting *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, *A. galli*, and Echinostomatidae simultaneously on a microfluidic chip.

Table 26 Chromatic values of analytical specificity evaluation

DNA template	**Δ**H° value of each reaction chamber (d.u.)					
	R. echinobothrida	*R. tetragona*	*R. cesticillus*	*A. galli*	Echinostomatidae	Negative control
R. echinobothrida	67.251	-0.173	0.725	-0.067	-0.002	0
R. tetragona	-0.026	60.468	0.229	-0.058	-1.423	0
R. cesticillus	1.645	-0.049	74.493	0.022	-0.023	0
A. galli	-0.182	-0.020	0.929	56.483	0.196	0
E. miyagawai	-0.514	-0.049	1.069	1.428	58.781	0
E. makongi	-3.056	-1.422	-2.183	-1.385	31.930	0
E. macrorchis	2.121	0.576	0.720	0.663	58.047	0
H. conoideum	-0.044	-1.551	-0.939	0.547	44.290	0
Raillietina spp.	62.072	62.608	72.103	1.553	1.570	0
Raillietina spp. + *A. galli*	59.190	61.663	67.210	64.136	2.098	0
Raillietina spp. + *E. miyagawai*	57.888	57.973	65.980	1.401	32.677	0
A. galli + *E. miyagawai*	2.832	2.286	3.979	57.999	37.646	0
Raillietina spp. + *A. galli* + *E. miyagawai*	64.719	54.448	76.663	75.116	27.883	0

Table 26 (Continued)

DNA template	**Δ**H° value of each reaction chamber (d.u.)					
	R. echinobothrida	*R. tetragona*	*R. cesticillus*	*A. galli*	Echinostomatidae	Negative control
H. gallinarum	-0.093	-1.385	-1.296	0.108	0.193	0
Cotugnia sp.	1.491	0.108	0.032	1.496	1.460	0
Diorchis sp.	1.361	-0.152	1.010	2.390	1.449	0
Fimbriaria sp.	2.485	2.305	1.121	1.155	1.153	0
H. nana	1.237	1.211	0.002	1.493	1.491	0
Prosthogonimus sp.	-1.285	-1.224	-0.657	0.122	1.538	0
G. g. domesticus	-1.407	-1.322	-1.209	-2.579	0.133	0
A. p. domesticus	1.902	-0.827	-0.588	0.893	1.003	0

Note. Cut-off value= 6.705 d.u.

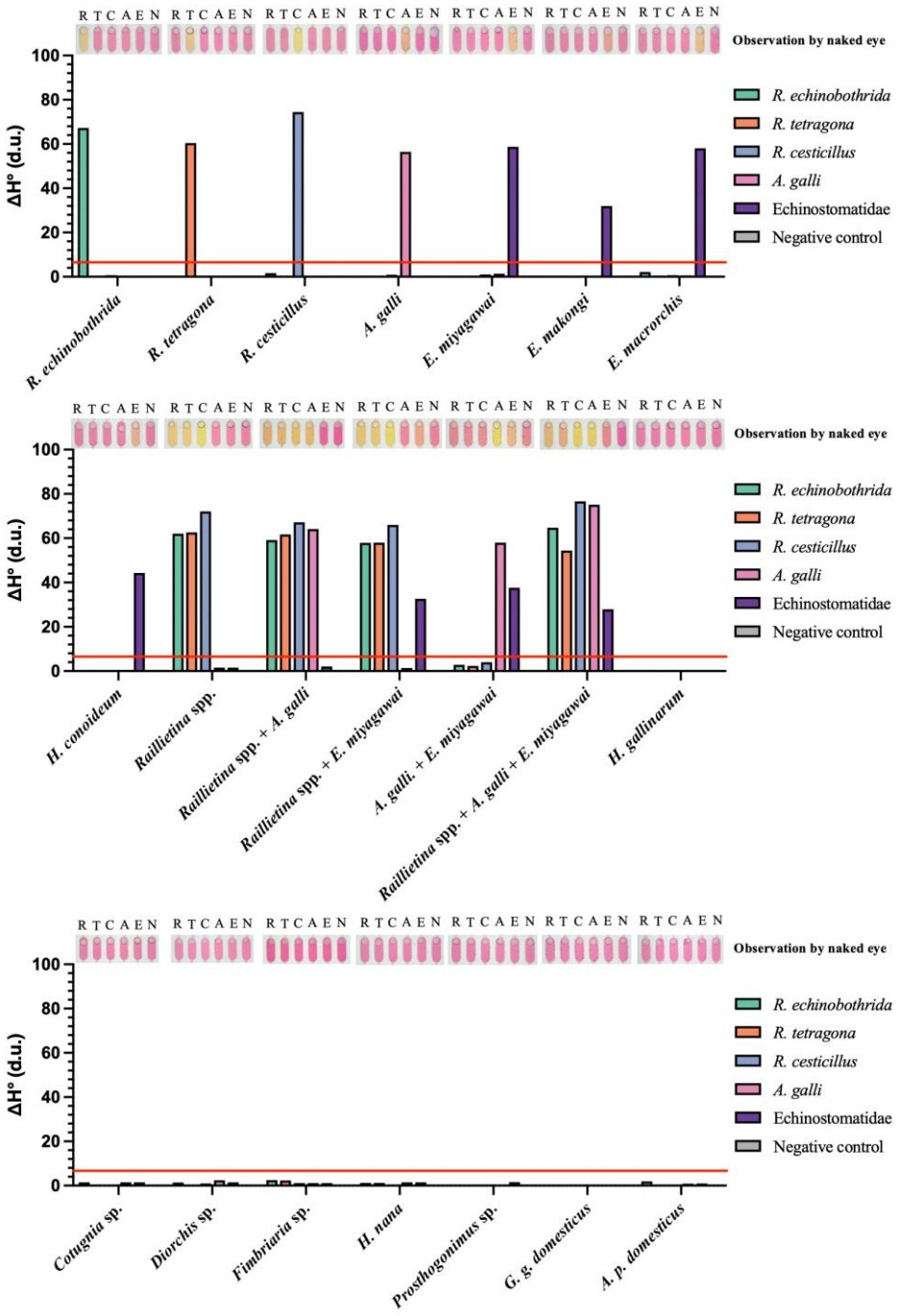


Figure 47 Analytical specificity of colorimetric LAMP on a microfluidic chip analyzed by naked eye and chromatic analysis via images

(R: *R. echinobothrida* reaction chamber; T: *R. tetragona* reaction chamber; C: *R. cesticillus* reaction chamber; A: *A. galli* reaction chamber; E: Echinostomatidae reaction chamber; N: negative control chamber; red lines: cut-off value of ΔH°)

4.3.3 Storage stability of the primers loaded microfluidic chip

To test the efficiency of LAMP amplification after storing at ambient temperatures (25–30 °C) with a variety of duration times, including 1, 2, 3, and 4 weeks, the target DNAs of each species were initially 40 ng/chip and diluted 10-fold with serial dilutions up to 40×10^{-3} ng/chip. After keeping for 1 week, the results showed that the analytical sensitivities of *R. tetragona* and *E. miyagawai* were decreased as 10 times when compared with the fresh loaded chip, remaining 40 ng/chip and 4 ng/chip, respectively. Following 3 weeks, the analytical sensitivity of *E. miyagawai* was further reduced up to 100 times found to be 40 ng/chip, which is an equal to *R. tetragona*. At the end of storage experiment for 4 weeks, the results demonstrated that the analytical sensitivities of *R. echinobothrida*, *R. cesticillus*, and *A. galli* did not impact from storage at 25–30 °C for 4 weeks, whereas the analytical sensitivities of *R. tetragona* and *E. miyagawai* were reduced to be 40 ng/chip; thus, the primers loaded microfluidic chip that was kept at 25–30 °C for 4 weeks could be detected the minimum DNA concentration of 40 ng/chip (**Table 27**; **Figure 48**). Hence, the fresh primers loaded chips were used to detect the clinical samples in next section.

In this study, other substances (excepted primers) of LAMP reaction were still stored in a refrigerator at –20 °C before using, which is a significant limitation to perform in point-of-care station. However, if all reagents were simultaneously air-dried, the LAMP reaction would not perform. A possible description for this phenomenon is the instability of the master mix because the high concentration of salts may destabilize the *Bst* DNA polymerase, which causes a loss of activity of the enzyme. Thus, the air-drying process must be separated the salt and surfactant components from other reagents (García-Bernalt Diego et al., 2019; Xie et al., 2023). A previous study used lyophilization or freeze-drying method to dry and preserve the stability of LAMP master mix (reaction buffer, dNTPs, $MgSO_4$, and *Bst* DNA polymerase) without cold condition storage. Wan et al. (2020) reported that the lyophilized LAMP reagents by using a mixture of glycine and trehalose as a stabilizer could be stored at 4 °C and ambient temperature (24–30 °C) for a duration of more than 6 months and 3 days, respectively. Therefore, the freeze-drying

of LAMP master mix on a PMMA chip can conduct following to Nguyen et al. (2022; 2024), but the storage time under the ambient condition must be further validated in the future.

Table 27 Chromatic values of storage stability assessment

DNA concentration/ species	**Δ**H° value of each reaction chamber (d.u.)					
	R. echinobothrida	*R. tetragona*	*R. cesticillus*	*A. galli*	Echinostomatidae	Negative control
1 week						
40 ng/chip	52.939	34.671	70.127	59.327	28.950	0
4 ng/chip	57.243	4.844	71.301	61.836	20.882	0
0.4 ng/chip	57.159	-1.601	67.656	55.262	1.587	0
0.04 ng/chip	0.140	1.171	67.949	1.170	2.423	0
2 weeks						
40 ng/chip	49.055	28.935	62.524	42.953	14.428	0
4 ng/chip	56.923	-1.316	69.200	53.022	12.723	0
0.4 ng/chip	49.597	-0.007	67.563	47.886	2.329	0
0.04 ng/chip	-0.128	-4.093	66.764	0.342	1.521	0
3 weeks						
40 ng/chip	52.420	43.952	68.005	51.688	34.368	0
4 ng/chip	55.458	3.918	71.028	57.544	5.340	0
0.4 ng/chip	52.192	4.356	69.168	55.768	0.170	0
0.04 ng/chip	0.007	-1.403	54.744	0.622	1.427	0
4 weeks						
40 ng/chip	54.103	41.231	66.814	46.413	17.590	0
4 ng/chip	53.333	0.072	64.312	37.568	1.183	0
0.4 ng/chip	52.106	2.199	64.079	15.490	3.760	0
0.04 ng/chip	2.462	-3.311	62.649	-0.017	3.053	0

Note. Cut-off value= 6.705 d.u.

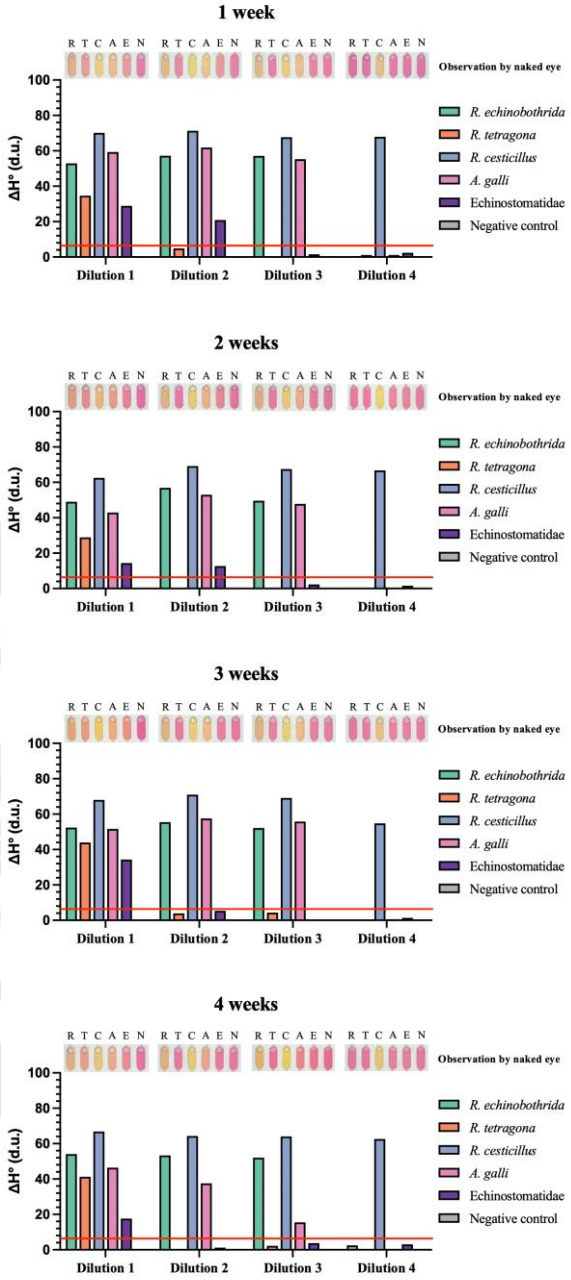


Figure 48 Validation of the primers loaded chips under different storage times

(R: *R. echinobothrida* reaction chamber; T: *R. tetragona* reaction chamber; C: *R. cesticillus* reaction chamber; A: *A. galli* reaction chamber; E: Echinostomatidae reaction chamber; N: negative control chamber; Dilution 1-4: DNA concentrations of 40 to 40×10^{-3} ng/chip, respectively; red lines: cut-off value of ΔH°)

5. Clinical detection and benchmarking of simplex LAMP assays, duplex LAMP assays, and colorimetric LAMP on a microfluidic chip coupled with a chromatic analysis assay

A total of 30 fecal samples was verified with the seven developed LAMP assays, including (I) *Raillietina* LAMP-LFD, (II) *A. galli* LAMP-LFD, (III) Echinostomatidae LAMP-LFD, (IV) *Raillietina* - *A. galli* LAMP-LFD, (V) *Raillietina* - Echinostomatidae LAMP-LFD, (VI) *A. galli* - Echinostomatidae LAMP-LFD, and (VII) colorimetric LAMP on a microfluidic chip coupled with the chromatic analysis. Based on the results of post-mortem investigation presented that 14 out of 30 samples were infected target parasitic groups in this study, comprising of *R. echinobothrida* (n= 6), *R. tetragona* (n= 4), *R. cesticillus* (n= 2), *A. galli* (n= 2), and Echinostomatidae (n= 3), while co-infection was found the only one case as *R. echinobothrida* + *R. tetragona* (n= 3) (**Table 28**). Next, the confirmed samples from post-mortem (n= 30) were applied to the seven developed assays. For assays related to the LFD development, all positive samples for post-mortem method and any samples that had the LAMP products-like pattern visualized by gel electrophoresis were further developed for visual interpretation by the LFD strip test.

Table 28 Result of clinical samples diagnosis by post-mortem examination

Clinical sample	Post-mortem result
1	*R. echinobothrida*
2	-
3	-
4	*R. tetragona*
5	-
6	-
7	-
8	-
9	-
10	-
11	-
12	-
13	*R. echinobothrida* + *R. tetragona*

Table 28 (Continued)

Clinical sample	Post-mortem result
14	*R. tetragona*
15	*R. echinobothrida* + *R. tetragona*
16	-
17	-
18	*R. echinobothrida* + *R. tetragona*
19	-
20	*R. echinobothrida*
21	*R. cesticillus*
22	-
23	*Echinostoma* sp.
24	*R. cesticillus*
25	-
26	-
27	*A. galli*
28	*Echinostoma* sp.
29	*Echinostoma* sp.
30	*A. galli*

Table 29 presents the summaries of seven developed assays highlighting operation time, cost, and clinical performances (the details of each assay are shown in **Table A2-9** and **Figure A13-20**). The colorimetric LAMP on a microfluidic chip coupled with a chromatic analysis showed the highest statistical analysis based on the kappa value as K= 0.93, whereas the lowest value found to be K= 0.35 was indicated by *A. galli* LAMP-LFD assay. This study has not been judged that colorimetric LAMP on a microfluidic chip is the best way to detect or diagnose those parasitic groups, but all developed assays were just an alternative method for parasite detection to serve a variety of users according to their purposes. The occurred clinical results showed the phenomena of false positive and false negative results. These may be elucidated by the

presence of several substances from other organisms that contaminated with feces, which lead to interference with the DNA extraction and LAMP reaction as well as cross-amplification. Previous study described other causes that provided false negative results, including (I) early infections with larva stage, (II) only male worm infection in terms of *A. galli*, (III) the irregular distribution of eggs/egg capsules in feces (Oladosu et al., 2022). To enhance the accuracy of the clinical results, before extracting the DNA should be eliminated the other substances as much as possible in feces by using filtration via gauze, which can help the egg/egg capsule concentration to improve the DNA concentration of parasites. However, the McNemar's tests demonstrated that all developed assays were not significantly different from the post-mortem examination at $p > 0.05$. Hence, the developed assays were successfully detected in naturally infected feces from the chickens and could be interpreted the results by naked eye or using chromatic analysis via image.

Table 29 Summaries of seven developed assays focusing on operation time, cost, and clinical performances compared with post-mortem examination

Diagnostic result	Operation time	Cost/sample	Clinical test		K value (degree of agreement)	McNemar's test
			Sensitivity	Specificity		
Simplex LAMP-LFD assay						
Raillietina spp.	60 min	~150 bath	69.23%	100%	0.64 (moderate)	0.067
A. galli	60 min		66.67%	93.33%	0.35 (poor)	0.500
Echinostomatidae	70 min		100%	96.43%	0.84 (high)	0.500
Duplex LAMP-LFD assay						
Raillietina spp., *A. galli*	70 min	~300 bath	84.62%	100%	0.85 (high)	0.240
Raillietina spp., Echinostomatidae	95 min		80%	100%	0.78 (moderate)	0.124
A. galli, Echinostomatidae	95 min		83.33%	89.29%	0.59 (moderate)	0.309

Table 29 (Continued)

Diagnostic result	Operation time	Cost/sample	Clinical test		K value (degree of agreement)	McNemar's test
			Sensitivity	Specificity		
Colorimetric LAMP on a microfluidic chip coupled with the chromatic analysis assay						
R. echinobothrida, *R. tetragona*, *R. cesticillus*, *A. galli*, Echinostomatidae	120 min	~210 bath	93.33%	100%	0.93 (high)	0.500

CHAPTER 5
CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

Conclusions

This study successfully developed the alternative assays based on a loop-mediated isothermal amplification (LAMP) for detecting parasitic pathogens in poultry. There are three target groups, including (I) *R. echinobothrida*, *R. tetragona*, and *R. cesticillus*, (II) *A. galli*, and (III) *E. miyagawai*, *E. mekongi*, *E. macrorchis*, and *H. conoideum*. The developed assays can be performed at the operation times from 60 min up to 120 min (excluded DNA extraction) and the results can be interpreted by naked eye without complicated or costly devices. Although some assays took a total operation time longer than the microscopic examinations, including sedimentation and flotation techniques, but the LAMP assays can simultaneously detect numerous samples and can further develop to apply in the field by using portable heat box or water bath instead of thermocycler machine. Regarding the clinical detection, all developed assays can detect the parasitic infections via fecal samples, the clinical sensitivity and specificity values ranging from 66.67-100% and 89.29-100%, respectively, comparing to the post-mortem investigation. Moreover, all developed assays were not statistically different from the post-mortem investigation based on the McNemar's tests; therefore, these assays are simple, rapid, and convenient, which has the possibility to be utilized in on-site screening in the context of epidemiological investigations, surveillance, drug treatment, and to solve a veterinary health problem along with farming management in the future.

Limitations and recommendations

For analytical sensitivities of our duplex LAMP-LFD assays, the results showed that the analytical sensitivities did not impact from the mix of two DNA targets in one reaction because this study used an equal of DNA concentration of them. On the other hand, clinical samples in natural condition cannot be adjusted the quantities of extracted DNA from egg capsule or egg stage; thus, detection limit in terms of an unequal of both target DNA that extracted from egg stage should be further tested in the

future. In addition, DNA extraction process with clinical samples has still used the commercial DNA extraction kit because simple extraction methods, such as boiling method, cannot purify the extracted DNA and cannot eliminate the large numbers of macromolecule and other substances in feces that lead to impairment DNA extraction or interference the LAMP reaction. To enhance the feasibility for point-of-care testing, the simple DNA extraction method must be further developed for application with the fecal sample purification in order to reduce the inhibitors in feces as much as possible before DNA extraction process by using gauze for simple filtration.

For user-friendliness, acquired images in this study were captured under the stereomicroscope to control the light stability, which is difficulty in setting outside the laboratory. A readout device for supporting between smartphone and microfluidic chip, and a smartphone-based chromatic analysis are required to achieve on-site performing in the future. A photography has a main advantage in terms of data recording, leads to the chip storage for post-analysis do not need, which prevents color fading, and the analytical process can be conducted wherever and whenever necessary.

To avoid and prevent the contamination, the performance of LAMP amplification is greatly sensitive to produce the large amounts of LAMP products prone to the risk of self-contamination of LAMP products from previous reaction, which is a template DNA for the next reaction affecting false positive results. A LAMP mixture preparation, addition of template DNA, and result analyses relying on open the lid of reaction tube (gel electrophoresis and LFD development) must be carried out in separate rooms along with two sets of micropipettes and filter tips. Furthermore, the reagents of each process were separately stored in refrigerators in order to prevent the contamination.

REFERENCES

Abdel Aziz, A. R. A., AbouLaila, M. R., Aziz, M., Omar, M. A., & Sultan, K. (2018). *In vitro* and *in vivo* anthelmintic activity of pumpkin seeds and pomegranate peels extracts against *Ascaridia galli*. *Beni-Suef University Journal of Basic and Applied Sciences*, *7*(2), 231–234. https://doi.org/10.1016/j.bjbas.2018.02.003

Abdelqader, A., Qarallah, B., Al-Ramamneh, D., & Da**Ş**, G. (2012). Anthelmintic effects of citrus peels ethanolic extracts against *Ascaridia galli*. *Veterinary Parasitology*, *188*(1–2), 78–84. https://doi.org/10.1016/j.vetpar.2012.03.003

AbouLaila, M., El-Bahy, N., Hilali, M., Yokoyama, N., & Igarashi, I. (2011). Prevalence of the enteric parasites of ducks from Behera governorate, Egypt. *The Journal of Protozoology Research*, *21*(2), 36–44. https://doi.org/10.32268/jprotozoolres.21.2_36

Ackert, J. E. (1936). The cysticercoid of the fowl tapeworm *Raillietina cesticillus*. *Transactions of the American Microscopical Society*, *55*(1), 97. https://doi.org/10.2307/3223014

Adang, K. L., Asher, R., & Abba, R. (2014). Gastro-intestinal helminths of domestic chickens *Gallus gallus domestica* and ducks *Anas platyrhynchos* slaughtered at Gombe main market, Gombe State, Nigeria. *Asian Journal of Poultry Science*, *8*(2), 32–40. https://doi.org/10.3923/ajpsaj.2014.32.40

Adao, D. E. V., & Rivera, W. L. (2016). Loop-mediated isothermal amplification (LAMP) assay for the rapid detection of the sexually-transmitted parasite, *Trichomonas vaginalis*. *Annals of Parasitology*, *62(1)*, 25–31. https://doi.org/10.17420/ap6201.28

Agricultural Research Service. (2019). *Fooddata central*. https://fdc.nal.usda.gov/fdc-app.html#/food-details/331960/nutrients

Aini, I. (1990). Indigenous chicken production in South-east Asia. *World's Poultry Science Journal*, *46*(1), 51–57. https://doi.org/10.1079/WPS19900010

Alkharigy, F. A., El Naas, A. S., & El Maghrbi, A. A. (2018). Survey of parasites in domestic pigeons (*Columba livia*) in Tripoli, Libya. *Open Veterinary Journal*, *8*(4), 360. https://doi.org/10.4314/ovj.v8i4.2

Anderson, R. C. (2000). *Nematode parasites of vertebrates: Their development and transmission* (2nd ed.). CABI.

Anh, N. T. L., Madsen, H., Dalsgaard, A., Phuong, N. T., Thanh, D. T. H., & Murrell, K. D. (2010). Poultry as reservoir hosts for fishborne zoonotic trematodes in Vietnamese fish farms. *Veterinary Parasitology*, *169*(3–4), 391–394. https://doi.org/10.1016/j.vetpar.2010.01.010

Anucherngchai, S., & Chontananarth, T. (2019). *Echinostoma revolutum*: Development of a high performance DNA-specific primer to demonstrate the epidemiological situations of their intermediate hosts. *Acta Tropica*, *189*, 46–53. https://doi.org/10.1016/j.actatropica.2018.09.014

Anucherngchai, S., Chontananarth, T., Tejangkura, T., & Chai, J. Y. (2019). The study of Cytochrome B (CYTB): Species-specific detection and phylogenetic relationship of *Echinostoma revolutum*, (Froelich, 1802). *Journal of Parasitic Diseases*, *43*(1), 66–74. https://doi.org/10.1007/s12639-018-1057-0

Anupama, K. P., Nayak, A., Karunasagar, I., & Maiti, B. (2020). Rapid visual detection of *Vibrio parahaemolyticus* in seafood samples by loop-mediated isothermal amplification with hydroxynaphthol blue dye. *World Journal of Microbiology and Biotechnology*, *36*(5), 76. https://doi.org/10.1007/s11274-020-02851-0

Aonuma, H., Yoshimura, A., Kobayashi, T., Okado, K., Badolo, A., Nelson, B., Kanuka, H., & Fukumoto, S. (2010). A single fluorescence-based LAMP reaction for identifying

multiple parasites in mosquitoes. *Experimental Parasitology*, *125*(2), 179–183. https://doi.org/10.1016/j.exppara.2009.12.023

Attah, D. D., Danladi, Y. K., Abdullahi, K., & Ibrahim, S. (2013). A survey of gastrointestinal helminthes of chickens and guinea fowls slaughtered at Sokoto, Nigeria. *Equity Journal of Science and Technology*, *1*(1), 104–109.

Ayudhya, C. K. N., & Sangvaranonda, A. (1997). Internal parasites in the alimentary tracts of an adult native chickens in the Southern part of Thailand. *Agriculture and Natural Resources*, *31*(4), 407–412.

Ayudthaya, C. K. N., & Sangvaranond, A. (1993). Internal parasites of alimentary tracts of adult native chickens in North-eastern part of Thailand. *Agriculture and Natural Resources*, *27*(3), 324–329.

Azizi, H., Farahnak, A., Mobedi, I., & Molaei Rad, M. (2015). Experimental life cycle of *Hypoderaeum conoideum* (Block, 1872) Diez, 1909 (Trematoda: Echinostomatidae) parasite from the North of Iran. *Iranian Journal of Parasitology*, *10*(1), 102–109.

Baer, J. G., & Sandars, D. F. (1956). The first record of *Raillietina (Raillietina) celebensis* (Janicki, 1902), (*Cestoda*) in man from Australia, with a critical survey of previous cases. *Journal of Helminthology*, *30*(2–3), 173–182. https://doi.org/10.1017/S0022149X00033137

Bahadır, E. B., & Sezgintürk, M. K. (2016). Lateral flow assays: Principles, designs and labels. *TrAC Trends in Analytical Chemistry*, *82*, 286–306. https://doi.org/10.1016/j.trac.2016.06.006

Barber, K. E., Mkoji, G. M., & Loker, E. S. (2000). PCR-RFLP analysis of the ITS2 region to identify *Schistosoma haematobium* and *S. bovis* from Kenya. *The American Journal of Tropical Medicine and Hygiene*, *62*(4), 434–440.

https://doi.org/10.4269/ajtmh.2000.62.434

Bartel, M. H. (1965). The life cycle of *Raillietina* (*R.*) *loeweni* Bartel and Hansen, 1964 (Cestoda) from the black-tailed jackrabbit, *Lepus californicus melanotis* Mearns. *The Journal of Parasitology*, *51*(5), 800. https://doi.org/10.2307/3276161

Bartel, M. H., & Hansen, M. F. (1964). *Raillietina* (*Raillietina*) *loeweni* sp. n. (Cestoda: Davaineidae) from the hare in Kansas, with notes on *Raillietina* of North american mammals. *The Journal of Parasitology*, *50*(3), 448. https://doi.org/10.2307/3275854

Begum, A., Mukutmoni, M., & Akter, F. (2019). Parasite diversity in mallard: A cross sectional study on *Anas platyrhynchos* from Munshiganj, Dhaka. *Bangladesh Journal of Zoology*, *47*(1), 121–128. https://doi.org/10.3329/bjz.v47i1.42027

Ben Slimane, B. (2016). Prevalence of the gastro-intestinal parasites of domestic chicken *Gallus domesticus* Linnaeus, 1758 in Tunisia according to the agro-ecological zones. *Journal of Parasitic Diseases*, *40*(3), 774–778. https://doi.org/10.1007/s12639-014-0577-5

Biswas, P. G., Ohari, Y., Mohanta, U. K., & Itagaki, T. (2021). Molecular characterization of *Ascaridia galli* from Bangladesh and development of a PCR method for distinguishing *A. galli* from *Heterakis* spp. *Journal of Veterinary Medical Science*, *83*(4), 666–670. https://doi.org/10.1292/jvms.20-0728

Bokelmann, L., Nickel, O., Maricic, T., Pääbo, S., Meyer, M., Borte, S., & Riesenberg, S. (2021). Point-of-care bulk testing for SARS-CoV-2 by combining hybridization capture with improved colorimetric LAMP. *Nature Communications*, *12*(1), 1467. https://doi.org/10.1038/s41467-021-21627-0

Buddhachat, K., & Chontananarth, T. (2019). Is species identification of *Echinostoma revolutum* using mitochondrial DNA barcoding feasible with high-resolution melting analysis? *Parasitology Research*, *118*(6), 1799–1810.

https://doi.org/10.1007/s00436-019-06322-w

Butboonchoo, P., & Wongsawad, C. (2017). Occurrence and HAT-RAPD analysis of gastrointestinal helminths in domestic chickens (*Gallus gallus domesticus*) in Phayao province, Northern Thailand. *Saudi Journal of Biological Sciences*, *24*(1), 30–35. https://doi.org/10.1016/j.sjbs.2015.09.002

Butboonchoo, P., Wongsawad, C., Rojanapaibul, A., & Chai, J. Y. (2016). Morphology and molecular phylogeny of *Raillietina* spp. (Cestoda: Cyclophyllidea: Davaineidae) from domestic chickens in Thailand. *The Korean Journal of Parasitology*, *54*(6), 777–786. https://doi.org/10.3347/kjp.2016.54.6.777

Butboonchoo, P., Wongsawad, C., Wongsawad, P., & Chai, J. Y. (2020). Morphology and molecular identification of *Echinostoma revolutum* and *Echinostoma macrorchis* in freshwater snails and experimental hamsters in upper Northern Thailand. *The Korean Journal of Parasitology*, *58*(5), 499–511. https://doi.org/10.3347/kjp.2020.58.5.499

Capitán-Vallvey, L. F., López-Ruiz, N., Martínez-Olmos, A., Erenas, M. M., & Palma, A. J. (2015). Recent developments in computer vision-based analytical chemistry: A tutorial review. *Analytica Chimica Acta*, *899*, 23–56. https://doi.org/10.1016/j.aca.2015.10.009

Carney, W. P. (1991). Echinostomiasis-A snail-borne intestinal trematode zoonosis. *The Southeast Asian Journal of Tropical Medicine and Public Health*, 206–211.

Chadfield, M., Permin, A., Nansen, P., & Bisgaard, M. (2001). Investigation of the parasitic nematode *Ascaridia galli* (Shrank 1788) as a potential vector for Salmonella enterica dissemination in poultry. *Parasitology Research*, *87*(4), 317–325. https://doi.org/10.1007/PL00008585

Chai, J. Y., Cho, J., Chang, T., Jung, B. K., & Sohn, W. M. (2020). Taxonomy of

Echinostoma revolutum and 37-collar-spined *Echinostoma* spp.: A historical review. *The Korean Journal of Parasitology*, *58*(4), 343–371. https://doi.org/10.3347/kjp.2020.58.4.343

Chai, J. Y., Jung, B. K., Chang, T., Shin, H., Cho, J., Ryu, J. Y., Kim, H. S., Park, K., Jeong, M.H., Hoang, E.H., & Abdullah, M. B. M. (2021). *Echinostoma miyagawai* Ishii, 1932 (Echinostomatidae) from ducks in Aceh Province, Indonesia with special reference to its synonymy with *Echinostoma robustum* Yamaguti, 1935. *The Korean Journal of Parasitology*, *59*(1), 35–45. https://doi.org/10.3347/kjp.2021.59.1.35

Chai, J. Y., Shin, E. H., Lee, S. H., & Rim, H. J. (2009). Foodborne intestinal flukes in Southeast Asia. *The Korean Journal of Parasitology*, *47*(Suppl), S69. https://doi.org/10.3347/kjp.2009.47.S.S69

Chaisiri, K., Chaeychomsri, W., Siruntawineti, J., Ribas, A., Herbreteau, V., & Morand, S. (2010). Gastrointestinal helminth infections in asian house rats (*Rattus tanezumi*) from Northern and Northeastern Thailand. *The Journal of Tropical Medicine and Parasitology*, *33*, 29–35.

Chandler, A. C., & Pradatsundarasar, A. (1957). Two cases of *Raillietina* infection in infants in Thailand, with a discussion of the taxonomy of the species of *Raillietina* (Cestoda) in man, rodents and monkeys. *The Journal of Parasitology*, *43*(1), 81. https://doi.org/10.2307/3274764

Chen, Y. X., Lou, Y. R., Duan, L. J., Zhou, Q. J., Xu, Z. J., Chen, F. J., Chen, H. X., Xu, G. Z., Du, A. F., & Chen, J. (2023). Parallel detection of multiple zoonotic parasites using a real-time fluorogenic loop-mediated isothermal amplification-based quadruple-sample microfluidic chip. *Frontiers in Microbiology*, *14*, 1238376. https://doi.org/10.3389/fmicb.2023.1238376

Da**Ş**, G., Kaufmann, F., Abel, H., & Gauly, M. (2010). Effect of extra dietary lysine in

Ascaridia galli-infected grower layers. *Veterinary Parasitology*, *170*(3–4), 238–243. https://doi.org/10.1016/j.vetpar.2010.02.026

Deardorff, T. L., Schmidt, G. D., & Kuntz, R. E. (1976). Tapeworms from Philippine birds, with three new species of *Raillietina (Raillietina)*. *Journal of Helminthology*, *50*(2), 133–142. https://doi.org/10.1017/S0022149X00027619

Department of Livestock Development. (2022). *Department of Livestock Development Strategy 2018-2027*. https://planning.dld.go.th/th/index.php/th/plan-menu/1473-2566-2580

Dong, J., Xu, Q., Li, C., & Zhang, C. (2019). Single-color multiplexing by the integration of high-resolution melting pattern recognition with loop-mediated isothermal amplification. *Chemical Communications*, *55*(17), 2457–2460. https://doi.org/10.1039/C8CC09741K

Ekpo, U. F., Ogbooye, A. A., Oluwole, A. S., & Takeet, M. (2010). A preliminary survey on the parasites of free range chicken in Abeokuta, Ogun State, Nigeria. *Journal of Natural Science Engineering and Technology*, *9*(2), 123–130.

El-Dakhly, K. M., Mohamed, H., Kamel, A., Mahrous, L., El-Nahass, E. S., & Aboshinaf, A. (2020). Prevalence, distribution pattern and pathological alterations of gastro-intestinal helminthosis in domestic ducks in Beni-Suef, Egypt. *Journal of Advanced Veterinary Research*, *10*(1), 1–8.

Eom, K. S., Rim, H. J., & Jang, D. H. (1984). A study on the parasitic helminths of domestic duck (*Anas platyrhynchos* var. *domestica Linnaeus*) in Korea. *The Korean Journal of Parasitology*, *22*(2), 215. https://doi.org/10.3347/kjp.1984.22.2.215

Eshetu, Y., Mulualem, E., Ibrahim, H. M., Berhanu, A., & Aberra, K. (2001). Study of gastro-intestinal helminths of scavenging chickens in four rural districts of Amhara region, Ethiopia. *Revue Scientifique et Technique de l'OIE*, *20*(3), 791–796.

https://doi.org/10.20506/rst.20.3.1310

Fan, Q., Xie, Z., Xie, Z., Xie, L., Huang, J., Zhang, Y., Zeng, T., Zhang, M., Wan, S., Luo, S., Liu, J., & Deng, X. (2018). Development of duplex fluorescence-based loop-mediated isothermal amplification assay for detection of *Mycoplasma bovis* and bovine herpes virus 1. *Journal of Virological Methods*, *261*, 132–138. https://doi.org/10.1016/j.jviromet.2018.08.014

Ferdushy, T., Hasan, M. T., & Golam Kadir, A. K. M. (2016). Cross sectional epidemiological investigation on the prevalence of gastrointestinal helminths in free range chickens in Narsingdi district, Bangladesh. *Journal of Parasitic Diseases*, *40*(3), 818–822. https://doi.org/10.1007/s12639-014-0585-5

Ferraz, J., Souza, J., Costa-Silva, M., Torres, E., Santana, A., Lanfredi, R., Maldonado, A., & Garcia, J. (2012). Effect of praziquantel on adult *Echinostoma paraensei* worms in experimentally infected mice. *Parasitology Research*, *111*(1), 143–148. https://doi.org/10.1007/s00436-011-2810-9

Feyera, T., Ruhnke, I., Sharpe, B., Elliott, T., Shifaw, A., & Walkden-Brown, S. W. (2021). Comparative therapeutic efficacies of oral and in-water administered levamisole, piperazine and fenbendazole against experimental *Ascaridia galli* infection in chickens. *Veterinary Parasitology*, *298*, 109514. https://doi.org/10.1016/j.vetpar.2021.109514

Fried, B. (2001). Biology of echinostomes except *Echinostoma*. In *Advances in Parasitology* (Vol. 49, pp. 163–210). Elsevier. https://doi.org/10.1016/S0065-308X(01)49040-3

Fu, Y. T., Jin, Y. C., Li, F., & Liu, G. H. (2019). Characterization of the complete mitochondrial genome of the echinostome *Echinostoma miyagawai* and phylogenetic implications. *Parasitology Research*, *118*(10), 3091–3097.

https://doi.org/10.1007/s00436-019-06417-4

García-Bernalt Diego, J., Fernández-Soto, P., Crego-Vicente, B., Alonso-Castrillejo, S., Febrer-Sendra, B., Gómez-Sánchez, A., Vicente, B., López-Abán, J., & Muro, A. (2019). Progress in loop-mediated isothermal amplification assay for detection of *Schistosoma mansoni* DNA: Towards a ready-to-use test. *Scientific Reports*, *9*(1), 14744. https://doi.org/10.1038/s41598-019-51342-2

García-Bernalt Diego, J., Fernández-Soto, P., & Muro, A. (2021). LAMP in neglected tropical diseases: A focus on parasites. *Diagnostics*, *11*(3), 521. https://doi.org/10.3390/diagnostics11030521

Gauly, M., Bauer, C., Preisinger, R., & Erhardt, G. (2002). Genetic differences of *Ascaridia galli* egg output in laying hens following a single dose infection. *Veterinary Parasitology*, *103*(1–2), 99–107. https://doi.org/10.1016/S0304-4017(01)00587-8

Glunkwamdi, P., Pakdee, S., & Raksaman, A. (2020). *Epidemiological situation and risk of parasitic infections by ingesting of Filopaludina snails at local markets in Bangkok metropolis*. Srinakharinwirot University.

González-González, E., Lara-Mayorga, I. M., Rodríguez-Sánchez, I. P., Zhang, Y. S., Martínez-Chapa, S. O., Santiago, G. T., & Alvarez, M. M. (2021). Colorimetric loop-mediated isothermal amplification (LAMP) for cost-effective and quantitative detection of SARS-CoV-2: The change in color in LAMP-based assays quantitatively correlates with viral copy number. *Analytical Methods*, *13*(2), 169–178. https://doi.org/10.1039/D0AY01658F

Graczyk, T. K., & Fried, B. (1998). Echinostomiasis: A common but forgotten food-borne disease. *The American Journal of Tropical Medicine and Hygiene*, *58*(4), 501–504. https://doi.org/10.4269/ajtmh.1998.58.501

Grafl, B., Polster, S., Sulejmanovic, T., Pürrer, B., Guggenberger, B., & Hess, M. (2017).

Assessment of health and welfare of Austrian laying hens at slaughter demonstrates influence of husbandry system and season. *British Poultry Science*, *58*(3), 209–215. https://doi.org/10.1080/00071668.2017.1280723

Griffiths, H. J. (1978). *A handbook of veterinary parasitology: Domestic animals of North America*. University of Minnesota Press.

Hange, R. R., Raote, Y. V., & Jayraw, A. K. (2007). Prevalence of helminth parasites in desi fowl (*Gallus gallus domesticus*) at Parbhani. *Journal of Parasitic Diseases*, *31*(1), 61–64.

Hassan, N., Awang, A., & Rahman, M. (2015). Parasitic burden and its relation with the body weight of free range chicken in oil palm dominated Sandakan district of Malaysian Borneo. *International Journal of Livestock Research*, *5*(9), 10. https://doi.org/10.5455/ijlr.20150909073638

Hassouni, T., & Belghyti, D. (2006). Distribution of gastrointestinal helminths in chicken farms in the Gharb region-Morocco. *Parasitology Research*, *99*(2), 181–183. https://doi.org/10.1007/s00436-006-0145-8

Hembram, A., Panda, M. R., Mohanty, B. N., Pradhan, C. R., Dehuri, M., Sahu, A., & Behera, M. (2015). Prevalence of gastrointestinal helminths in Banaraja fowls reared in semi-intensive system of management in Mayurbhanj district of Odisha. *Veterinary World*, *8*(6), 723–726. https://doi.org/10.14202/vetworld.2015.723-726

Heneberg, P., Sitko, J., & Bizos, J. (2015). Integrative taxonomy of central European parasitic flatworms of the family Prosthogonimidae Lühe, 1909 (Trematoda: Plagiorchiida). *Parasitology International*, *64*(5), 264–273. https://doi.org/10.1016/j.parint.2015.02.003

Horsfall, M. W. (1938). Observations on the life history of *Raillietina echinobothrida* and of *R. tetragona* (Cestoda). *The Journal of Parasitology*, *24*(5), 409.

https://doi.org/10.2307/3272117

Huang, Q., Shan, X., Cao, R., Jin, X., Lin, X., He, Q., Zhu, Y., Fu, R., Du, W., Lv, W., Xia, Y., & Huang, G. (2021). Microfluidic chip with two-stage isothermal amplification method for highly sensitive parallel detection of SARS-CoV-2 and Measles virus. *Micromachines*, *12*(12), 1582. https://doi.org/10.3390/mi12121582

Huffman, J. E., & Fried, B. (1990). *Echinostoma* and Echinostomiasis. In *Advances in Parasitology* (Vol. 29, pp. 215–269). Elsevier. https://doi.org/10.1016/S0065-308X(08)60107-4

Idika, I. K., Obi, C. F., Ezeh, I. O., Iheagwam, C. N., Njoku, I. N., & Nwosu, C. O. (2015). Gastrointestinal helminth parasites of local chickens from selected communities in Nsukka region of South eastern Nigeria. *Journal of Parasitic Diseases*, *40*(4), 1376–1380. https://doi.org/10.1007/s12639-015-0694-9

Iseki, H., Alhassan, A., Ohta, N., Thekisoe, O. M. M., Yokoyama, N., Inoue, N., Nambota, A., Yasuda, J., & Igarashi, I. (2007). Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites. *Journal of Microbiological Methods*, *71*(3), 281–287. https://doi.org/10.1016/j.mimet.2007.09.019

Islam, M. R., Shaikh, H., & Baki, M. A. (1988). Prevalence and pathology of helminth parasites in domestic ducks of Bangladesh. *Veterinary Parasitology*, *29*(1), 73–77. https://doi.org/10.1016/0304-4017(88)90009-X

Jaiswal, K., Mishra, S., & Bee, A. (2020). Prevalence of gastrointestinal helminth parasites in *Gallus gallus domesticus* in Lucknow, U. P, India. *Advances in Zoology and Botany*, *8*(5), 422–430. https://doi.org/10.13189/azb.2020.080506

Jegede, O., Asadu, I., Opara, M., Obeta, S., & Olayemi, D. (2015). Gastrointestinal parasitism in local and exotic breeds of chickens reared in Gwagwalada Guinea

Savannah zone of Nigeria. *Sokoto Journal of Veterinary Sciences*, *13*(3), 25–30. https://doi.org/10.4314/sokjvs.v13i3.5

Jiang, X., Jing, W., Sun, X., Liu, Q., Yang, C., Liu, S., Qin, K., & Sui, G. (2016). High-throughput microfluidic device for LAMP analysis of airborne bacteria. *ACS Sensors*, *1*(7), 958–962. https://doi.org/10.1021/acssensors.6b00282

Junaidu, H., Luka, S., & Mijinyawa, A. (2014). Prevalence of gastrointestinal helminth parasites of the domestic fowl (*Gallus-gallus domesticus*) slaughtered in Giwa market, Giwa local government, area, Kaduna State, Nigeria. *Journal of Natural Sciences Research*, *4*(19), 120–125.

Kaingu, F. B., Kibor, A. C., Shivairo, R., Kutima, H., Okeno, T. O., Waihenya, R., & Kahi, A. K. (2010). Prevalence of gastro-intestinal helminthes and coccidia in indigenous chicken from different agro-climatic zones in Kenya. *African Journal of Agricultural Research*, *5*(6), 458–462.

Kajerova, V., Barus, V., & Literak, I. (2004). Nematodes from the genus *Ascaridia* parasitizing psittaciform birds: A review and determination key. *Veterinární Medicína*, *49*(6), 217–223. https://doi.org/10.17221/5698-VETMED

Kanev, I. (1994). Life-cycle, delimitation and redescription of *Echinostoma revolutum* (Froelich, 1802) (Trematoda: Echinostomatidae). *Systematic Parasitology*, *28*(2), 125–144. https://doi.org/10.1007/BF00009591

Kanev, I., Sterner, M., Radev, V., & Fried, B. (2000). An overview of the biology of Echinostomes. In B. Fried & T. K. Graczyk (Eds.), *Echinostomes as Experimental Models for Biological Research* (pp. 1–29). Springer Netherlands. https://doi.org/10.1007/978-94-015-9606-0_1

Kaufmann, F., Da**Ş**, G., Sohnrey, B., & Gauly, M. (2011). Helminth infections in laying hens kept in organic free range systems in Germany. *Livestock Science*, *141*(2–3), 182–

187. https://doi.org/10.1016/j.livsci.2011.05.015

Keiser, J., Brun, R., Fried, B., & Utzinger, J. (2006). Trematocidal activity of praziquantel and artemisinin derivatives: In vitro and in vivo investigations with Adult *Echinostoma caproni*. *Antimicrobial Agents and Chemotherapy*, *50*(2), 803–805. https://doi.org/10.1128/AAC.50.2.803-805.2006

Keiser, J., Shu-Hua, X., & Utzinger, J. (2006). Effect of tribendimidine on adult *Echinostoma caproni* harbored in mice, including scanning electron microscopic observations. *Journal of Parasitology*, *92*(4), 858–862. https://doi.org/10.1645/GE-793R.1

Khanum, H., Musa, S., Islam, M., Akter, R., & Sarker, F. (2021). Occurrence of endoparasites in domestic fowls (*Gallus gallus*) and ducks (*Anas platyrhynchos*). *Bangladesh Journal of Zoology*, *49*(1), 35–46. https://doi.org/10.3329/bjz.v49i1.53680

Koczula, K. M., & Gallotta, A. (2016). Lateral flow assays. *Essays in Biochemistry*, *60*(1), 111–120. https://doi.org/10.1042/EBC20150012

Kodzius, R., Xiao, K., Wu, J., Yi, X., Gong, X., Foulds, I. G., & Wen, W. (2012). Inhibitory effect of common microfluidic materials on PCR outcome. *Sensors and Actuators B: Chemical*, *161*(1), 349–358. https://doi.org/10.1016/j.snb.2011.10.044

Kornboontritos, S. (2023). Business/Industry outlook 2023-2025: Chilled, frozen and processed chicken industry. *Krungsri Research*. https://www.krungsri.com/th/research/industry/industry-outlook/food-beverage/frozen-processed-chicken/io/io-chilled-frozen-processed-chicken

Labony, S. S., Paul, S., Alim, Md. A., Hossain, Md. S., Inoue, T., Ritu, S. N., Alam, M. Z., Alam, M., Kawada, H., Hasan, M. M., Hatta, T., Tsuji, N., & Anisuzzaman. (2022). Research Note: Genetic analysis, pathology, and vectors of echinostomiasis, a

zoonotic helminth infection in chickens in Bangladesh. *Poultry Science*, *101*(3), 101682. https://doi.org/10.1016/j.psj.2021.101682

Labrecque, L. I. (2020). Color research in marketing: Theoretical and technical considerations for conducting rigorous and impactful color research. *Psychology & Marketing*, *37*(7), 855–863. https://doi.org/10.1002/mar.21359

Lalchhandama, K. (2010). On the structure of *Ascaridia galli*, the roundworm of domestic fowl. *Science Vision*, *10*(1), 20–30.

Lalkhen, A. G., & McCluskey, A. (2008). Clinical tests: Sensitivity and specificity. *Continuing Education in Anaesthesia Critical Care & Pain*, *8*(6), 221–223. https://doi.org/10.1093/bjaceaccp/mkn041

Lalle, M., Possenti, A., Dubey, J. P., & Pozio, E. (2018). Loop-mediated isothermal amplification-lateral-flow dipstick (LAMP-LFD) to detect *Toxoplasma gondii* oocyst in ready-to-eat salad. *Food Microbiology*, *70*, 137–142. https://doi.org/10.1016/j.fm.2017.10.001

Lalthanpuii, P. B., & Lalchhandama, K. (2020). Phytochemical analysis and *in vitro* anthelmintic activity of *Imperata cylindrica* underground parts. *BMC Complementary Medicine and Therapies*, *20*(1), 332. https://doi.org/10.1186/s12906-020-03125-w

Layne, T., Jackson, K., Scott, A., Tanner, N. A., Piland, A., Haverstick, D. M., & Landers, J. P. (2021). Optimization of novel loop-mediated isothermal amplification with colorimetric image analysis for forensic body fluid identification. *Journal of Forensic Sciences*, *66*(3), 1033–1041. https://doi.org/10.1111/1556-4029.14682

Li, R., Chen, J., Zhang, X., Cui, J., Tao, S., & Yang, L. (2020). Mini-disk capillary array coupling with LAMP for visual detection of multiple nucleic acids using genetically modified organism analysis as an example. *Journal of Agricultural and Food*

Chemistry, *68*(3), 899–906. https://doi.org/10.1021/acs.jafc.9b06979

Li, Y., Qiu, Y. Y., Zeng, M. H., Diao, P. W., Chang, Q. C., Gao, Y., Zhang, Y., & Wang, C. R. (2019). The complete mitochondrial genome of *Echinostoma miyagawai*: Comparisons with closely related species and phylogenetic implications. *Infection, Genetics and Evolution*, *75*, 103961. https://doi.org/10.1016/j.meegid.2019.103961

Liu, C., Mauk, M. G., Hart, R., Bonizzoni, M., Yan, G., & Bau, H. H. (2012). A low-cost microfluidic chip for rapid genotyping of malaria-transmitting mosquitoes. *PLoS ONE*, *7*(8), e42222. https://doi.org/10.1371/journal.pone.0042222

Luka, S. A., & Ndams, I. S. (2007). Gastrointestinal parasites of domestic chicken *Gallus-gallus domesticus* Linnaeus 1758 in Samaru, Zaria Nigeria. *Science World Journal*, *2*(1), 27–29.

Ma, Y. D., Chen, Y. S., & Lee, G. B. (2019). An integrated self-driven microfluidic device for rapid detection of the influenza A (H1N1) virus by reverse transcription loop-mediated isothermal amplification. *Sensors and Actuators B: Chemical*, *296*, 126647. https://doi.org/10.1016/j.snb.2019.126647

Mahdavi Abhari, F., Niyyati, M., Assadzadeh Aghdaei, H., & Mirjalali, H. (2023). Loop mediated isothermal amplification for detection of foodborne parasites: A journey from lab to lab-on-a-chip. *Food Control*, *143*, 109251. https://doi.org/10.1016/j.foodcont.2022.109251

Malpartida-Cardenas, K., Miscourides, N., Rodriguez-Manzano, J., Yu, L. S., Moser, N., Baum, J., & Georgiou, P. (2019). Quantitative and rapid *Plasmodium falciparum* malaria diagnosis and artemisinin-resistance detection using a CMOS Lab-on-Chip platform. *Biosensors and Bioelectronics*, *145*, 111678. https://doi.org/10.1016/j.bios.2019.111678

Mao, R., Ge, G., Wang, Z., Hao, R., Zhang, G., Yang, Z., Lin, B., Ma, Y., Liu, H., & Du, Y.

(2018). A multiplex microfluidic loop-mediated isothermal amplification array for detection of malaria-related parasites and vectors. *Acta Tropica*, *178*, 86–92. https://doi.org/10.1016/j.actatropica.2017.10.025

Maurer, K., Decere, M., & Fried, B. (1996). Effects of the anthelmintics clorsulon, rafoxanide, mebendazole and arprinocid on *Echinostoma caproni* in ICR mice. *Journal of Helminthology*, *70*(1), 95–96. https://doi.org/10.1017/S0022149X00015194

McDougald, L. R. (2020). Internal parasites. *In diseases of poultry* (13th ed., pp. 1117–1146). Wiley-Blackwell.

McHugh, M. L. (2012). Interrater reliability: The kappa statistic. *Biochemia Medica*, 276–282. https://doi.org/10.11613/BM.2012.031

Menon, E. S. (2015). Fluid Flow in Pipes. In *Transmission Pipeline Calculations and Simulations Manual* (pp. 149–234). Elsevier. https://doi.org/10.1016/B978-1-85617-830-3.00005-5

Miyamoto, S., Sano, S., Takahashi, K., & Jikihara, T. (2015). Method for colorimetric detection of double-stranded nucleic acid using leuco triphenylmethane dyes. *Analytical Biochemistry*, *473*, 28–33. https://doi.org/10.1016/j.ab.2014.12.016

Moehling, T. J., Worthington, M. D., Wong, P. Y. G., Wong, S. S., & Meagher, R. J. (2024). Development of a colorimetric loop-mediated isothermal amplification assay for the detection of *Trypanosoma cruzi* in low-resource settings. *Diagnostics*, *14*(11), 1193. https://doi.org/10.3390/diagnostics14111193

Mohanta, U. K., Watanabe, T., Anisuzzaman, Ohari, Y., & Itagaki, T. (2019). Characterization of *Echinostoma revolutum* and *Echinostoma robustum* from ducks in Bangladesh based on morphology, nuclear ribosomal ITS2 and mitochondrial nad1 sequences. *Parasitology International*, *69*, 1–7.

https://doi.org/10.1016/j.parint.2018.11.002

Molla, W., Haile, H., Almaw, G., & Temesgen, W. (2012). Gastrointestinal helminths of local backyard chickens in North Gondar Administrative Zone, Ethiopia. *Revue de Médecine Vétérinaire*, *163*(7), 353–358.

Morgan, J. A. T., & Blair, D. (1995). Nuclear rDNA ITS sequence variation in the trematode genus *Echinostoma*: An aid to establishing relationships within the 37-collar-spine group. *Parasitology*, *111*(5), 609–615. https://doi.org/10.1017/S003118200007709X

Mori, Y., Kitao, M., Tomita, N., & Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods*, *59*(2), 145–157. https://doi.org/10.1016/j.jbbm.2003.12.005

Msoffe, P. L. M., Muhairwa, A. P., Chiwanga, G. H., & Case, A. A. (2010). A study of ecto- and endo-parasites of domestic pigeons in Morogoro Municipality, Tanzania. *African Journal of Agricultural Research*, *5*(3), 264–267.

Mubarokah, W. W., Nurcahyo, W., Prastowo, J., & Kurniasih, K. (2019). *In vitro* and *in vivo* Areca catechu crude aqueous extract as an anthelmintic against *Ascaridia galli* infection in chickens. *Veterinary World*, *12*(6), 877–882. https://doi.org/10.14202/vetworld.2019.877-882

Mukaratirwa, S., & Hove, T. (2009). A survey of ectoparasites, cestodes and management of free-range indigenous chickens in rural Zimbabwe. *Journal of the South African Veterinary Association*, *80*(3), 188–191.

Mungube, E. O., Bauni, S. M., Tenhagen, B. A., Wamae, L. W., Nzioka, S. M., Muhammed, L., & Nginyi, J. M. (2008). Prevalence of parasites of the local scavenging chickens in a selected semi-arid zone of Eastern Kenya. *Tropical Animal Health and Production*, *40*(2), 101–109. https://doi.org/10.1007/s11250-007-9068-3

Muñoz-Antolí, C., Toledo, R., & Esteban, J. G. (2000). The life cycle and transmission dynamics of the larval stages of *Hypoderaeum conoideum*. *Journal of Helminthology*, *74*(2), 165–172. https://doi.org/10.1017/S0022149X00000238

Musa, S., Rahman, T., & Khanum, H. (2012). Prevalence and intensity of parasites in domestic ducks. *Dhaka University Journal of Biological Sciences*, *21*(2), 197–199.

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, *16*(3), 223–229. https://doi.org/10.1006/mcpr.2002.0415

Nagataki, M., Tantrawatpan, C., Agatsuma, T., Sugiura, T., Duenngai, K., Sithithaworn, P., Andrews, R. H., Petney, T. N., & Saijuntha, W. (2015). Mitochondrial DNA sequences of 37 collar-spined echinostomes (Digenea: Echinostomatidae) in Thailand and Lao PDR reveals presence of two species: *Echinostoma revolutum* and *E. miyagawai*. *Infection, Genetics and Evolution*, *35*, 56–62. https://doi.org/10.1016/j.meegid.2015.07.022

Nair, K. V., & Nadakal, A. M. (1981). Haematological changes in domestic fowl experimentally infected with the cestode *Raillietina tetragona* (Molin, 1858). *Veterinary Parasitology*, *8*(1), 49–58. https://doi.org/10.1016/0304-4017(81)90017-0

Nak-on, S., Tejangkura, T., & Chontananarth, T. (2023). Multi-detection for paramphistomes using novel manually designed PAR-LAMP primers and its application in a lateral flow dipstick (LAMP-LFD) system. *Veterinary Parasitology*, *317*, 109905. https://doi.org/10.1016/j.vetpar.2023.109905

Natala, A. J., Asemadahun, N. D., Okubanjo, O. O., Ulayi, B. M., Owolabi, Y. H., Jato, I. D., & Yusuf, K. H. (2009). A survey of parasites of domesticated pigeon (*Columba livia domestic*) in Zaria, Nigeria. *Nternational Journal of Soft Computing*, *4*(4), 148–150.

Neshani, A., Zare, H., Sadeghian, H., Safdari, H., Riahi-Zanjani, B., & Aryan, E. (2023). A

comparative study on visual detection of *Mycobacterium tuberculosis* by closed tube loop-mediated isothermal amplification: Shedding light on the use of Eriochrome Black T. *Diagnostics*, *13*(1), 155. https://doi.org/10.3390/diagnostics13010155

Ngongeh, L. A., Chiejina, S. N., & Lawal, A. I. (2014). Prevalence of gastrointestinal helminth infections in slaughtered chickens reared in the Nsukka area of Enugu State, Nigeria. *Journal of Agriculture and Veterinary Science*, *7*(11), 51–54.

Nguyen, H. Q., Bui, H. K., Phan, V. M., & Seo, T. S. (2022). An internet of things-based point-of-care device for direct reverse-transcription-loop mediated isothermal amplification to identify SARS-CoV-2. *Biosensors and Bioelectronics*, *195*, 113655. https://doi.org/10.1016/j.bios.2021.113655

Nguyen, H. V., Nguyen, V. D., Liu, F., & Seo, T. S. (2020). An integrated smartphone-based genetic analyzer for qualitative and quantitative pathogen detection. *ACS Omega*, *5*(35), 22208–22214. https://doi.org/10.1021/acsomega.0c02317

Nguyen, V. D., Nguyen, H. Q., Bui, H. K., Kang, Y. J., & Seo, T. S. (2024). A smartphone-controllable molecular diagnostic platform for SARS-CoV-2 detection by reverse-transcription recombinase polymerase amplification. *Sensors and Actuators B: Chemical*, *398*, 134728. https://doi.org/10.1016/j.snb.2023.134728

Niessen, L., & Vogel, R. F. (2010). Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal amplification (LAMP) assay. *International Journal of Food Microbiology*, *140*(2–3), 183–191. https://doi.org/10.1016/j.ijfoodmicro.2010.03.036

Niu, J., Guo, Q., Jian, H., Chen, C., Yang, D., Liu, Q., & Guo, Y. (2011). Rapid detection of *Meloidogyne* spp. By LAMP assay in soil and roots. *Crop Protection*, *30*(8), 1063–1069. https://doi.org/10.1016/j.cropro.2011.03.028

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase,

T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12), 63e–663. https://doi.org/10.1093/nar/28.12.e63

Nurelhuda, I. E. M., Elowni, E. E., & Hassan, T. (1989a). Anthelmintic activity of praziquantel on *Raillietina tetragona* in chickens. *Parasitology Research*, *75*(8), 655–656. https://doi.org/10.1007/BF00930965

Nurelhuda, I. E. M., Elowni, E. E., & Hassan, T. (1989b). Anticestodal action of oxfendazole on *Raillietina tetragona* in experimentally infected chickens. *British Veterinary Journal*, *145*(5), 458–461. https://doi.org/10.1016/0007-1935(89)90054-7

Nzelu, C. O., Gomez, E. A., Cáceres, A. G., Sakurai, T., Martini-Robles, L., Uezato, H., Mimori, T., Katakura, K., Hashiguchi, Y., & Kato, H. (2014). Development of a loop-mediated isothermal amplification method for rapid mass-screening of sand flies for *Leishmania* infection. *Acta Tropica*, *132*, 1–6. https://doi.org/10.1016/j.actatropica.2013.12.016

Offiong, E. E. A., Obioku, O. E., Umoh, J. U., Essien, C. A., & Idiong, N. B. (2013). A survey of gastrointestinal helminthes of local chickens in Abak local government area of Akwa Ibom State. *International Journal of Sciences: Basic and Applied Research*, *9*(1), 1–4.

Ohaeri, C. C., & Okwum, C. (2013). Helminthic parasites of domestic fowls in Ikwuano, Abia State, Nigeria. *Journal of Natural Sciences Research*, *3*(11), 1–5.

Oladosu, O. J., Hennies, M., Gauly, M., & Da**Ş**, G. (2022). A copro-antigen ELISA for the detection of ascarid infections in chickens. *Veterinary Parasitology*, *311*, 109795. https://doi.org/10.1016/j.vetpar.2022.109795

Ola-Fadunsin, S. D., Uwabujo, P. I., Sanda, I. M., Ganiyu, I. A., Hussain, K., Rabiu, M., Elelu, N., & Alayande, M. O. (2019). Gastrointestinal helminths of intensively managed poultry in Kwara Central, Kwara State, Nigeria: Its diversity, prevalence,

intensity, and risk factors. *Veterinary World*, *12*(3), 389–396. https://doi.org/10.14202/vetworld.2019.389-396

Panich, W., & Chontananarth, T. (2021). Molecular detection of three intestinal cestode species (*Raillietina echinobothrida*, *R. tetragona*, *R. cesticillus*) from poultry in Thailand. *Avian Pathology*, *50*(4), 321–326. https://doi.org/10.1080/03079457.2021.1926920

Panich, W., Jaruboonyakorn, P., Raksaman, A., Tejangkura, T., & Chontananarth, T. (2024). Development and utilization of a visual loop-mediated isothermal amplification coupled with a lateral flow dipstick (LAMP-LFD) assay for rapid detection of Echinostomatidae metacercaria in edible snail samples. *International Journal of Food Microbiology*, *418*, 110732. https://doi.org/10.1016/j.ijfoodmicro.2024.110732

Panich, W., Nak-on, S., & Chontananarth, T. (2022). High-performance triplex PCR detection of three tapeworm species belonging to the genus *Raillietina* in infected poultry. *Acta Tropica*, *232*, 106516. https://doi.org/10.1016/j.actatropica.2022.106516

Panich, W., Tejangkura, T., & Chontananarth, T. (2021). Novel high-performance detection of *Raillietina echinobothrida*, *Raillietina tetragona*, and *Raillietina cesticillus* using loop-mediated isothermal amplification coupled with a lateral flow dipstick (LAMP-LFD). *Veterinary Parasitology*, *292*, 109396. https://doi.org/10.1016/j.vetpar.2021.109396

Panich, W., Tejangkura, T., & Chontananarth, T. (2023). Feasibility of a DNA biosensor assay based on loop-mediated isothermal amplification combined with a lateral flow dipstick assay for the visual detection of *Ascaridia galli* eggs in faecal samples. *Avian Pathology*, *52*(3), 209–218.

https://doi.org/10.1080/03079457.2023.2196251

Panich, W., Tejangkura, T., & Chontananarth, T. (2024). Assay for the simultaneous detection of *Raillietina* spp. (*R. echinobothrida*, *R. tetragona*, and *R. cesticillus*) and *Ascaridia galli* infection in chickens using duplex loop-mediated isothermal amplification integrated with a lateral flow dipstick assay. *Veterinary Parasitology*, *328*, 110174. https://doi.org/10.1016/j.vetpar.2024.110174

Panich, W., Tejangkura, T., & Chontananarth, T. (2025). Development of a duplex loop-mediated isothermal amplification together with lateral flow dipstick assay for the detection and discrimination of parasitic infections in chickens between cestodes belonging to genus *Raillietina* and trematodes in family Echinostomatidae. *Research in Veterinary Science*, *185*, 105539. https://doi.org/10.1016/j.rvsc.2025.105539

Papadakis, G., Pantazis, A. K., Fikas, N., Chatziioannidou, S., Tsiakalou, V., Michaelidou, K., Pogka, V., Megariti, M., Vardaki, M., Giarentis, K., Heaney, J., Nastouli, E., Karamitros, T., Mentis, A., Zafiropoulos, A., Sourvinos, G., Agelaki, S., & Gizeli, E. (2022). Portable real-time colorimetric LAMP-device for rapid quantitative detection of nucleic acids in crude samples. *Scientific Reports*, *12*(1), 3775. https://doi.org/10.1038/s41598-022-06632-7

Paul, B. T., Lawal, J. R., Ejeh, E. F., Ndahi, J. J., Peter, I. D., Bello, A. M., & Wakil, Y. (2015). Survey of helminth parasites of free range Muscovy ducks (*Anas platyrynchos*) slaughtered in Gombe, North Eastern Nigeria. *International Journal of Poultry Science*, *14*(8), 466–470.

Permin, A., Christensen, J. P., & Bisgaard, M. (2006). Consequences of concurrent *Ascaridia galli* and *Escherichia coli* infections in chickens. *Acta Veterinaria Scandinavica*, *47*(1), 43. https://doi.org/10.1186/1751-0147-47-43

Permin, A., Esmann, J. B., Hoj, C. H., Hove, T., & Mukaratirwa, S. (2002). Ecto-, endo- and haemoparasites in free-range chickens in the Goromonzi District in Zimbabwe. *Preventive Veterinary Medicine*, *54*(3), 213–224. https://doi.org/10.1016/S0167-5877(02)00024-7

Permin, A., Magwisha, H., Kassuku, A. A., Nansen, P., Bisgaard, M., Frandsen, F., & Gibbons, L. (1997). A cross-sectional study of helminths in rural scavenging poultry in Tanzania in relation to season and climate. *Journal of Helminthology*, *71*(3), 233–240. https://doi.org/10.1017/S0022149X00015972

Posthuma-Trumpie, G. A., Korf, J., & Van Amerongen, A. (2009). Lateral flow (immuno)assay: Its strengths, weaknesses, opportunities and threats. A literature survey. *Analytical and Bioanalytical Chemistry*, *393*(2), 569–582. https://doi.org/10.1007/s00216-008-2287-2

Pote, L. M., Couvillion, C. E., Keirs, R. W., Schwartz, R. D., Taylor, R. F., Hackathorn, J. H., & Johnson, L. K. (1992). Dose-titration to confirm the level of fenbendazole for control of *Raillietina cesticillus* in broiler chickens. *Avian Diseases*, *36*(3), 722. https://doi.org/10.2307/1591772

Poulsen, J., Permin, A., Hindsbo, O., Yelifari, L., Nansen, P., & Bloch, P. (2000). Prevalence and distribution of gastro-intestinal helminths and haemoparasites in young scavenging chickens in upper eastern region of Ghana, West Africa. *Preventive Veterinary Medicine*, *45*(3–4), 237–245. https://doi.org/10.1016/S0167-5877(00)00125-2

Prompamorn, P., Sithigorngul, P., Rukpratanporn, S., Longyant, S., Sridulyakul, P., & Chaivisuthangkura, P. (2011). The development of loop-mediated isothermal amplification combined with lateral flow dipstick for detection of *Vibrio parahaemolyticus*: Detection of *V. parahaemolyticus* by LAMP-LFD. *Letters in*

Applied Microbiology, *52*(4), 344–351. https://doi.org/10.1111/j.1472-765X.2011.03007.x

Qazaz, I. A. (2020). Molecular detection of *Ascaridia galli* in local breed chickens (*Gallus gallus domesticus*) in Baghdad city. *Plant Archives*, *20*(1), 199–202.

Rajendran, M., & Nadakal, A. M. (1988). The efficacy of praziquantel (Droncit R) against *Raillietina tetragona* (Molin, 1858) in domestic fowl. *Veterinary Parasitology*, *26*(3–4), 253–260. https://doi.org/10.1016/0304-4017(88)90093-3

Ramnath, Jyrwa, D. B., Dutta, A. K., Das, B., & Tandon, V. (2014). Molecular characterization of the Indian poultry nodular tapeworm, *Raillietina echinobothrida* (Cestoda: Cyclophyllidea: Davaineidae) based on rDNA internal transcribed spacer 2 region. *Journal of Parasitic Diseases*, *38*(1), 22–26. https://doi.org/10.1007/s12639-012-0184-2

Rana, M. S., Lee, S. Y., Kang, H. J., & Hur, S. J. (2019). Reducing Veterinary Drug Residues in Animal Products: A Review. *Food Science of Animal Resources*, *39*(5), 687–703. https://doi.org/10.5851/kosfa.2019.e65

Reid, W. M., Ackert, J. E., & Case, A. A. (1938). Studies on the life history and biology of the fowl tapeworm *Raillietina cesticillus* (Molin). *Transactions of the American Microscopical Society*, *57*(1), 65. https://doi.org/10.2307/3222803

Ren, K., Zhou, J., & Wu, H. (2013). Materials for microfluidic chip fabrication. *Accounts of Chemical Research*, *46*(11), 2396–2406. https://doi.org/10.1021/ar300314s

Roberts, L. S., Janovy, J., J., & Nadler, S. (2009). *Gerald D. Schmidt & Larry S. Roberts' foundations of parasitology* (9th ed.). Michael Hackett.

Rodriguez-Manzano, J., Karymov, M. A., Begolo, S., Selck, D. A., Zhukov, D. V., Jue, E., & Ismagilov, R. F. (2016). Reading out single-molecule digital RNA and DNA

isothermal amplification in nanoliter volumes with unmodified camera phones. *ACS Nano*, *10*(3), 3102–3113. https://doi.org/10.1021/acsnano.5b07338

Saijuntha, W., Duenngai, K., & Tantrawatpan, C. (2013). Zoonotic echinostome infections in free-grazing ducks in Thailand. *The Korean Journal of Parasitology*, *51*(6), 663–667. https://doi.org/10.3347/kjp.2013.51.6.663

Saijuntha, W., Sithithaworn, P., Duenngai, K., Kiatsopit, N., Andrews, R. H., & Petney, T. N. (2011). Genetic variation and relationships of four species of medically important echinostomes (Trematoda: Echinostomatidae) in South-East Asia. *Infection, Genetics and Evolution*, *11*(2), 375–381. https://doi.org/10.1016/j.meegid.2010.11.009

Saijuntha, W., Tantrawatpan, C., Sithithaworn, P., Duenngai, K., Agatsuma, T., Andrews, R. H., & Petney, T. N. (2014). Genetic characterization and phylogenetic analysis of echinostomes. *The Southeast Asian Journal of Tropical Medicine and Public Health*, *45*(5), 1003–1010.

Sajid, M., Kawde, A. N., & Daud, M. (2015). Designs, formats and applications of lateral flow assay: A literature review. *Journal of Saudi Chemical Society*, *19*(6), 689–705. https://doi.org/10.1016/j.jscs.2014.09.001

Sangvaranond, A. (1997). Parasitic helminthes of native chickens in the Central part of Tailand. *Agriculture and Natural Resources*, *28*(3), 402–412.

Saravanan, G., Yamuna, G., & Nandhini, S. (2016). Real time implementation of RGB to HSV/HSI/HSL and its reverse color space models. *2016 International Conference on Communication and Signal Processing (ICCSP)*, 0462–0466. https://doi.org/10.1109/ICCSP.2016.7754179

Sawada, I. (1964). On the genus *Raillietina* Fuhrmann 1920 (I). *Journal of Nara Gakugei*

University, *12*, 19–36.

Sawada, I. (1965). On the genus *Raillietina* Fuhrmann 1920 (II). *Journal of Nara Gakugei University*, *13*, 5–38.

Schultz, R. L. (1940). The genus *Diorchis* Clerc 1903. *American Midland Naturalist*, *23*(2), 382. https://doi.org/10.2307/2420670

Scott, A. T., Layne, T. R., O'Connell, K. C., Tanner, N. A., & Landers, J. P. (2020). Comparative evaluation and quantitative analysis of Loop-mediated isothermal amplification indicators. *Analytical Chemistry*, *92*(19), 13343–13353. https://doi.org/10.1021/acs.analchem.0c02666

Scott, S., & Ali, Z. (2021). Fabrication methods for microfluidic devices: An overview. *Micromachines*, *12*(3), 319. https://doi.org/10.3390/mi12030319

Seo, J. H., Park, B. H., Oh, S. J., Choi, G., Kim, D. H., Lee, E. Y., & Seo, T. S. (2017). Development of a high-throughput centrifugal loop-mediated isothermal amplification microdevice for multiplex foodborne pathogenic bacteria detection. *Sensors and Actuators B: Chemical*, *246*, 146–153. https://doi.org/10.1016/j.snb.2017.02.051

Shang, Y., Sun, J., Ye, Y., Zhang, J., Zhang, Y., & Sun, X. (2020). Loop-mediated isothermal amplification-based microfluidic chip for pathogen detection. *Critical Reviews in Food Science and Nutrition*, *60*(2), 201–224. https://doi.org/10.1080/10408398.2018.1518897

Sharma, N., Hunt, P. W., Hine, B. C., McNally, J., Sharma, N. K., Iqbal, Z., Normant, C., Andronicos, N. M., Swick, R. A., & Ruhnke, I. (2018). Effect of an artificial *Ascaridia galli* infection on egg production, immune response, and liver lipid reserve of free-range laying hens. *Poultry Science*, *97*(2), 494–502.

https://doi.org/10.3382/ps/pex347

Shemshadi, B., Ranjbar-bahadori, S., & Delfan-abazari, M. (2017). Prevalence and intensity of parasitic infection in domestic ducks (*Anas platyrhynchas*) in Gilan Province, Northern Iran. *Comparative Clinical Pathology*, *26*(1), 165–167. https://doi.org/10.1007/s00580-016-2361-7

Shifaw, A., Feyera, T., Walkden-Brown, S. W., Sharpe, B., Elliott, T., & Ruhnke, I. (2021). Global and regional prevalence of helminth infection in chickens over time: A systematic review and meta-analysis. *Poultry Science*, *100*(5), 101082. https://doi.org/10.1016/j.psj.2021.101082

Shrestha, D., Subedi, J. R., & Chhetri, B. (2020). Gastrointestinal parasites of domesticated duck (*Anas platyrhynchos* Linnaeus, 1758) in Chandragiri municipality, Kathmandu, Nepal. *Ife Journal of Science*, *22*(2), 015–023. https://doi.org/10.4314/ijs.v22i2.2

Song, J., Liu, C., Bais, S., Mauk, M. G., Bau, H. H., & Greenberg, R. M. (2015). Molecular detection of schistosome infections with a disposable microfluidic cassette. *PLOS Neglected Tropical Diseases*, *9*(12), e0004318. https://doi.org/10.1371/journal.pntd.0004318

Sriworarat, C., Phumee, A., Mungthin, M., Leelayoova, S., & Siriyasatien, P. (2015). Development of loop-mediated isothermal amplification (LAMP) for simple detection of *Leishmania* infection. *Parasites & Vectors*, *8*(1), 591. https://doi.org/10.1186/s13071-015-1202-x

Stunkard, H. W. (1953). *Raillietina demerariensis* (Cestoda), from *Proechimys cayennensis trinitatus* of Venezuela. *The Journal of Parasitology*, *39*(3), 272. https://doi.org/10.2307/3273950

Subedi, J. R., Mujahid, T., & Cheetri, B. (2018). Prevalence of intestinal helminth parasites

of chicken (*Gallus gallus domesticus* Linnaeus, 1758) in Lalitpur district, Nepal. *Tribhuvan University Journal*, *32*(2), 19–28.

Suhaila, A. H., Sabrina, D. L., Nik Ahmad Irwan Izzauddin, N. H., Hamdan, A., & Khadijah, S. (2015). Study of parasites in commercial free-range chickens in northern Peninsular Malaysia. *Malaysian Journal of Veterinary Research*, *6*(2), 53–64.

Tang, M. J., Zhou, S., Zhang, X. Y., Pu, J. H., Ge, Q. L., Tang, X. J., & Gao, Y. S. (2011). Rapid and sensitive detection of *Listeria monocytogenes* by loop-mediated isothermal amplification. *Current Microbiology*, *63*(6), 511–516. https://doi.org/10.1007/s00284-011-0013-3

Tanner, N. A., Zhang, Y., & Evans, T. C. (2015). Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. *BioTechniques*, *58*(2), 59–68. https://doi.org/10.2144/000114253

Tantrawatpan, C., & Saijuntha, W. (2020). Multiplex PCR development for the differential detection of four medically important echinostomes (Trematoda: Echinostomatidae) in Thailand. *Acta Tropica*, *204*, 105304. https://doi.org/10.1016/j.actatropica.2019.105304

Tantrawatpan, C., Saijuntha, W., Manochantr, S., Kheolamai, P., Thanchomnang, T., Sadaow, L., Intapan, P. M., & Maleewong, W. (2016). A singleplex real-time fluorescence resonance energy transfer PCR with melting curve analysis for the differential detection of *Paragonimus heterotremus, Echinostoma malayanum* and *Fasciola gigantica* eggs in faeces. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, *110*(1), 74–83. https://doi.org/10.1093/trstmh/trv098

Tanveer, S., Ahad, S., & Chishti, M. Z. (2015). Morphological characterization of nematodes of the genera *Capillaria*, *Acuaria*, *Amidostomum*, *Streptocara*, *Heterakis*, and *Ascaridia* isolated from intestine and gizzard of domestic birds from

different regions of the temperate Kashmir valley. *Journal of Parasitic Diseases*, *39*(4), 745–760. https://doi.org/10.1007/s12639-013-0401-7

Tarbiat, B., Enweji, N., Baltrusis, P., Halvarsson, P., Osterman-Lind, E., Jansson, D. S., & Höglund, J. (2021). A novel duplex ddPCR assay for detection and differential diagnosis of *Ascaridia galli* and *Heterakis gallinarum* eggs from chickens feces. *Veterinary Parasitology*, *296*, 109499. https://doi.org/10.1016/j.vetpar.2021.109499

Tarbiat, B., Jansson, D. S., & Höglund, J. (2015). Environmental tolerance of free-living stages of the poultry roundworm *Ascaridia galli*. *Veterinary Parasitology*, *209*(1–2), 101–107. https://doi.org/10.1016/j.vetpar.2015.01.024

Taylor, T. (1997). Optimization of the performance of the polymerase chain reaction in silicon-based microstructures. *Nucleic Acids Research*, *25*(15), 3164–3168. https://doi.org/10.1093/nar/25.15.3164

Thapa, J., Maharjan, B., Malla, M., Fukushima, Y., Poudel, A., Pandey, B. D., Hyashida, K., Gordon, S. V., Nakajima, C., & Suzuki, Y. (2019). Direct detection of *Mycobacterium tuberculosis* in clinical samples by a dry methyl green loop-mediated isothermal amplification (LAMP) method. *Tuberculosis*, *117*, 1–6. https://doi.org/10.1016/j.tube.2019.05.004

Thapa, S., Hinrichsen, L. K., Brenninkmeyer, C., Gunnarsson, S., Heerkens, J. L. T., Verwer, C., Niebuhr, K., Willett, A., Grilli, G., Thamsborg, S. M., Sørensen, Jan. T., & Mejer, H. (2015). Prevalence and magnitude of helminth infections in organic laying hens (*Gallus gallus domesticus*) across Europe. *Veterinary Parasitology*, *214*(1–2), 118–124. https://doi.org/10.1016/j.vetpar.2015.10.009

Toledo, R., Esteban, J., & Fried, B. (2009). Chapter 3 recent advances in the biology of echinostomes. In *Advances in Parasitology* (Vol. 69, pp. 147–204). Elsevier. https://doi.org/10.1016/S0065-308X(09)69003-5

Toledo, R., & Esteban, J. G. (2016). An update on human echinostomiasis. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, *110*(1), 37–45. https://doi.org/10.1093/trstmh/trv099

Toledo, R., Muñoz-Antolí, C., Pérez, M., & Esteban, J. G. (1996). Redescription of the adult stage of *Hypoderaeum conoideum* (Bloch, 1782)(Trematoda: Echinostomatidae) and new record in Spain. *Research and Review Parasitology*, *56*(4), 195–201.

Tone, K., Fujisaki, R., Yamazaki, T., & Makimura, K. (2017). Enhancing melting curve analysis for the discrimination of loop-mediated isothermal amplification products from four pathogenic molds: Use of inorganic pyrophosphatase and its effect in reducing the variance in melting temperature values. *Journal of Microbiological Methods*, *132*, 41–45. https://doi.org/10.1016/j.mimet.2016.10.020

Tourlousse, D. M., Ahmad, F., Stedtfeld, R. D., Seyrig, G., Tiedje, J. M., & Hashsham, S. A. (2012). A polymer microfluidic chip for quantitative detection of multiple water- and foodborne pathogens using real-time fluorogenic loop-mediated isothermal amplification. *Biomedical Microdevices*, *14*(4), 769–778. https://doi.org/10.1007/s10544-012-9658-3

Tsai, S. M., Chan, K. W., Hsu, W. L., Chang, T. J., Wong, M. L., & Wang, C. Y. (2009). Development of a loop-mediated isothermal amplification for rapid detection of orf virus. *Journal of Virological Methods*, *157*(2), 200–204. https://doi.org/10.1016/j.jviromet.2009.01.003

Tucker, C. A., Yazwinski, T. A., Reynolds, L., Johnson, Z., & Keating, M. (2007). Determination of the anthelmintic efficacy of albendazole in the treatment of chickens naturally infected with gastrointestinal helminths. *Journal of Applied Poultry Research*, *16*(3), 392–396. https://doi.org/10.1093/japr/16.3.392

Uhuo, A. C., Okafor, F. C., Odikamnoro, O. O., Onwe, C. S., Abarike, M. C., & Elom, J. N.

(2013). Common gastrointestinal parasites of local chicken (*Gallus domesticus*) slaughtered in some selected eatery centres in Abakaliki, Ebonyi State: Implication for meat quality. *International Journal of Development and Sustainability*, *2*(2), 1416–1422.

Van, N. T. B., Cuong, N. V., Yen, N. T. P., Nhi, N. T. H., Kiet, B. T., Hoang, N. V., Hien, V. B., Thwaites, G., Carrique-Mas, J. J., & Ribas, A. (2020). Characterisation of gastrointestinal helminths and their impact in commercial small-scale chicken flocks in the Mekong Delta of Vietnam. *Tropical Animal Health and Production*, *52*(1), 53–62. https://doi.org/10.1007/s11250-019-01982-3

Wan, J., Guo, J., Lu, Z., Bie, X., Lv, F., & Zhao, H. (2020). Development of a test kit for visual loop-mediated isothermal amplification of Salmonella in spiked ready-to-eat fruits and vegetables. *Journal of Microbiological Methods*, *169*, 105830. https://doi.org/10.1016/j.mimet.2019.105830

Wan, L., Gao, J., Chen, T., Dong, C., Li, H., Wen, Y.-Z., Lun, Z.-R., Jia, Y., Mak, P.-I., & Martins, R. P. (2019). LampPort: A handheld digital microfluidic device for loop-mediated isothermal amplification (LAMP). *Biomedical Microdevices*, *21*(1), 9. https://doi.org/10.1007/s10544-018-0354-9

Wang, H., Zhang, Y. L., Wang, W., Ding, H., & Sun, H. B. (2017). On-chip laser processing for the development of multifunctional microfluidic chips: On-chip laser processing for the development. *Laser & Photonics Reviews*, *11*(2), 1600116. https://doi.org/10.1002/lpor.201600116

Wang, Y., Feng, J., & Tian, X. (2019). Application of loop-mediated isothermal amplification (LAMP) for rapid detection of Atlantic cod (*Gadus morhua*), Pacific cod (*Gadus macrocephalus*) and haddock (*Melanogrammus aeglefinus*). *Molecular and Cellular Probes*, *47*, 101420. https://doi.org/10.1016/j.mcp.2019.07.003

Watcharakranjanaporn, T., Sabaijai, M., Dunghungzin, C., & Chontananarth, T. (2021). Preliminary data on *Ascaridia galli* infections in *Gallus gallus domesticus* and the development of a specific primer based on the NADH dehydrogenase subunit 4. *Journal of Parasitic Diseases*, *45*(2), 293–297. https://doi.org/10.1007/s12639-020-01321-6

Wuthijaree, K., Lambertz, C., Vearasilp, T., Anusatsananun, V., & Gauly, M. (2019). Prevalence of gastrointestinal helminths in Thai indigenous chickens raised under backyard conditions in Northern Thailand. *Journal of Applied Poultry Research*, *28*(1), 221–229. https://doi.org/10.3382/japr/pfy062

Xie, C., Chen, S., Zhang, L., He, X., Ma, Y., Wu, H., Zou, B., & Zhou, G. (2021). Multiplex detection of blood-borne pathogens on a self-driven microfluidic chip using loop-mediated isothermal amplification. *Analytical and Bioanalytical Chemistry*, *413*(11), 2923–2931. https://doi.org/10.1007/s00216-021-03224-8

Xie, M., Chen, T., Cai, Z., Lei, B., & Dong, C. (2023). A digital microfluidic platform coupled with colorimetric loop-mediated isothermal amplification for on-site visual diagnosis of multiple diseases. *Lab on a Chip*, *23*(12), 2778–2788. https://doi.org/10.1039/D2LC01156E

Xiong, J., Wu, S., Liang, Y., Zou, Y., Xie, X., Huang, W., & Zhang, Y. (2020). Visual closed-tube loop-mediated isothermal amplification (LAMP) assay targeting aerolysin gene: A practical screening method for virulent *Aeromonas* species affecting cultured eels in China. *Aquaculture International*, *28*(6), 2319–2332. https://doi.org/10.1007/s10499-020-00588-z

Xue, Y., Kong, Q., Ding, H., Xie, C., Zheng, B., Zhuo, X., Ding, J., Tong, Q., Lou, D., Lu, S., & Lv, H. (2021). A novel loop-mediated isothermal amplification-lateral-flow-dipstick (LAMP-LFD) device for rapid detection of *Toxoplasma gondii* in the blood

of stray cats and dogs. *Parasite*, *28*, 41. https://doi.org/10.1051/parasite/2021039

Xunhui, Z., Qingming, K., Qunbo, T., Haojie, D., Lesheng, Z., Di, L., Jianzu, D., Bin, Z., Rui, C., Tianping, W., & Shaohong, L. (2019). DNA detection of *Paragonimus westermani*: Diagnostic validity of a new assay based on loop-mediated isothermal amplification (LAMP) combined with a lateral flow dipstick. *Acta Tropica*, *200*, 105185. https://doi.org/10.1016/j.actatropica.2019.105185

Yagoob, G., & Mohsen, H. M. (2014). Prevalence of gastrointestinal and pulmonary helminthic parasites of native chickens in Piranshahr city, Iran. *Indian Journal of Fundamental and Applied Life Sciences*, *4*(3), 324–328.

Yamaguti, S. (1958). *The Digenetic Trematodes of Vertebrates-Part II. Systema Helminthum* (1st ed.). Interscience Publishers.

Yamaguti, S. (1959). *The Cestodes of Vertebrates. Vol. II. Systema Helminthum* (1st ed.). Interscience Publishers.

Yamamura, M., Makimura, K., & Ota, Y. (2009). Evaluation of a new rapid molecular diagnostic system for *Plasmodium falciparum* combined with DNA filter paper, loop-mediated isothermal amplification, and melting curve analysis. *Japanese Journal of Infectious Diseases*, *62*(1), 20–25. https://doi.org/10.7883/yoken.JJID.2009.20

Yang, X., Gasser, R. B., Koehler, A. V., Wang, L., Zhu, K., Chen, L., Feng, H., Hu, M., & Fang, R. (2015). Mitochondrial genome of *Hypoderaeum conoideum* – comparison with selected trematodes. *Parasites & Vectors*, *8*(1), 97. https://doi.org/10.1186/s13071-015-0720-x

Yang, Y., Li, Q., Wang, S., Chen, X., & Du, A. (2016). Rapid and sensitive detection of *Babesia bovis* and *Babesia bigemina* by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Veterinary Parasitology*, *219*, 71–76.

https://doi.org/10.1016/j.vetpar.2016.02.004

Yazwinski, T. A., Tucker, C. A., Wray, E., Jones, L., & Clark, F. D. (2013). Observations of benzimidazole efficacies against *Ascaridia dissimilis*, *Ascaridia galli*, and *Heterakis gallinarum* in naturally infected poultry. *Journal of Applied Poultry Research*, *22*(1), 75–79. https://doi.org/10.3382/japr.2012-00606

Yin, K., Pandian, V., Kadimisetty, K., Ruiz, C., Cooper, K., You, J., & Liu, C. (2019). Synergistically enhanced colorimetric molecular detection using smart cup: A case for instrument-free HPV-associated cancer screening. *Theranostics*, *9*(9), 2637–2645. https://doi.org/10.7150/thno.32224

Yin, K., Pandian, V., Kadimisetty, K., Zhang, X., Ruiz, C., Cooper, K., & Liu, C. (2020). Real-time colorimetric quantitative molecular detection of infectious diseases on smartphone-based diagnostic platform. *Scientific Reports*, *10*(1), 9009. https://doi.org/10.1038/s41598-020-65899-w

Yongkiettrakul, S., Kampeera, J., Chareanchim, W., Rattanajak, R., Pornthanakasem, W., Kiatpathomchai, W., & Kongkasuriyachai, D. (2017). Simple detection of single nucleotide polymorphism in Plasmodium falciparum by SNP-LAMP assay combined with lateral flow dipstick. *Parasitology International*, *66*(1), 964–971. https://doi.org/10.1016/j.parint.2016.10.024

Yoriyo, K. P., Adang, K. L., Fabiyi, J. P., & Adamu, S. U. (2010). Helminthes parasites of local chickens in Bauchi State, Nigeria. *Science World Journal*, *3*(2). https://doi.org/10.4314/swj.v3i2.51782

Yousaf, A., Tabasam, M. S., Memon, A., Rajput, N., Shahnawaz, R., Rajpar, S., Jamil, T., & Mushtaq, M. (2019). Prevalence of *Ascaridia galli* in different broiler poultry farms of potohar region of Rawalpindi-Pakistan. *Journal of Dairy, Veterinary & Animal Research*, *8*(1), 71–73.

Yuan, D., Kong, J., Li, X., Fang, X., & Chen, Q. (2018). Colorimetric LAMP microfluidic chip for detecting three allergens: Peanut, sesame and soybean. *Scientific Reports*, *8*(1), 8682. https://doi.org/10.1038/s41598-018-26982-5

Yuan, H., Liu, Y., Jiang, X., Xu, S., & Sui, G. (2014). Microfluidic chip for rapid analysis of cerebrospinal fluid infected with *Staphylococcus aureus*. *Anal. Methods*, *6*(7), 2015–2019. https://doi.org/10.1039/C3AY41925H

Zhang, F., Gao, C., Bai, L., Chen, Y., Liang, S., Lv, X., Sun, J., & Wang, S. (2022). Dual-color blending based visual LAMP for food allergen detection: A strategy with enlarged color variation range and contrast. *Food Chemistry: X*, *13*, 100201. https://doi.org/10.1016/j.fochx.2021.100201

Zhang, M., Liu, J., Shen, Z., Liu, Y., Song, Y., Liang, Y., Li, Z., Nie, L., Fang, Y., & Zhao, Y. (2021). A newly developed paper embedded microchip based on LAMP for rapid multiple detections of foodborne pathogens. *BMC Microbiology*, *21*(1), 197. https://doi.org/10.1186/s12866-021-02223-0

Zhou, Q., Lu, J., Su, X., Jin, J., Li, S., Zhou, Y., Wang, L., Shao, X., Wang, Y., Yan, M., Li, M., & Chen, J. (2021). Simultaneous detection of multiple bacterial and viral aquatic pathogens using a fluorogenic loop-mediated isothermal amplification-based dual-sample microfluidic chip. *Journal of Fish Diseases*, *44*(4), 401–413. https://doi.org/10.1111/jfd.13325

Zloch, A., Kuchling, S., Hess, M., & Hess, C. (2021). In addition to birds' age and outdoor access, the detection method is of high importance to determine the prevalence of gastrointestinal helminths in laying hens kept in alternative husbandry systems. *Veterinary Parasitology*, *299*, 109559. https://doi.org/10.1016/j.vetpar.2021.109559

APPENDIX

Table A1 Nucleotide sequences in this study that were used to compare with available sequences in the GenBank database by using the BLASTn tool

Species	Region	Our accession number	Percent Identity
R. echinobothrida	ITS2	MN902341-MN902343	96.52-100%
R. tetragona		MN902344-MN902346	94.08-100%
R. cesticillus		MN902347	88.83-92.61%
A. galli		ON024768-ON024773	96.24-100%
E. miyagawai		OQ306491-OQ306496	99.64-99.82%
H. conoideum		PQ821047	99.40-100%

Table A2 Statistical comparison of *Raillietina* LAMP-LFD and post-mortem examination

Assay		Post-mortem examination			Clinical test (%)		K	McNemar's
		Negative	Positive	Total	Sensitivity	Specificity	value	test
Raillietina LAMP-LFD	Negative	21	4	25	69.23	100	0.63	0.067
	Positive	0	5	5				
	Total	21	9	30				

Table A3 Statistical comparison of *A. galli* LAMP-LFD and post-mortem examination

Assay		Post-mortem examination			Clinical test (%)		K	McNemar's
		Negative	Positive	Total	Sensitivity	Specificity	value	test
A. galli LAMP-LFD	Negative	26	1	27	66.67	93.33	0.35	0.500
	Positive	2	1	3				
	Total	28	2	30				

Table A4 Statistical comparison of Echinostomatidae LAMP-LFD and post-mortem examination

Assay		Post-mortem examination			Clinical test (%)		K	McNemar's
		Negative	Positive	Total	Sensitivity	Specificity	value	test
Echinostomatidae LAMP-LFD	Negative	26	0	26	100	96.43	0.84	0.500
	Positive	1	3	4				
	Total	27	3	30				

Table A5 Statistical comparison of *Raillietina – A. galli* LAMP-LFD and post-mortem examination

Assay		Post-mortem examination			Clinical test (%)		K	McNemar's
		Negative	Positive	Total	Sensitivity	Specificity	value	test
Raillietina – A. galli LAMP-LFD	Negative	19	2	21	84.62	100	0.85	0.240
	Positive	0	9	9				
	Total	19	11	30				

Table A6 Statistical comparison of *Raillietina* – Echinostomatidae LAMP-LFD and post-mortem examination

Assay		Post-mortem examination			Clinical test (%)		K	McNemar's
		Negative	Positive	Total	Sensitivity	Specificity	value	test
Raillietina – Echinostomatidae LAMP-LFD	Negative	18	3	21	80	100	0.78	0.124
	Positive	0	9	9				
	Total	18	12	30				

Table A7 Statistical comparison of *A. galli* – Echinostomatidae LAMP-LFD and post-mortem examination

Assay		Post-mortem examination			Clinical test (%)		K	McNemar's
		Negative	Positive	Total	Sensitivity	Specificity	value	test
A. galli – Echinostomatidae LAMP-LFD	Negative	22	1	23	83.33	89.29	0.59	0.309
	Positive	3	4	7				
	Total	25	5	30				

Table A8 Statistical comparison of colorimetric LAMP on a microfluidic chip coupled with the chromatic analysis and post-mortem examination

Assay		Post-mortem examination			Clinical test (%)		K	McNemar's
		Negative	Positive	Total	Sensitivity	Specificity	value	test
Colorimetric LAMP on a microfluidic chip coupled with the chromatic analysis	Negative	16	1	17	93.33	100	0.93	0.500
	Positive	0	13	13				
	Total	16	14	30				

Table A9 Chromatic values analyzed from colorimetric LAMP on a microfluidic chip of clinical results

Clinical sample	**Δ**H° value of each reaction chamber (d.u.)					
	R. echinobothrida	*R. tetragona*	*R. cesticillus*	*A. galli*	Echinostomatidae	Negative control
1	29.136	1.362	1.614	1.467	1.465	0
2	-0.348	-0.362	0.174	1.219	1.004	0
3	1.409	0.166	0.155	1.416	1.471	0
4	1.412	-0.003	-1.429	0.001	0.008	0
5	1.234	1.197	1.241	1.377	1.302	0
6	-1.268	-0.252	-1.313	0.273	-0.013	0
7	0.596	-0.247	0.957	0.020	1.573	0
8	0.490	0.218	0.059	1.305	0.970	0
9	0.848	1.163	0.374	1.626	1.191	0
10	1.261	1.541	1.580	1.571	1.336	0
11	1.321	1.308	0.122	0.592	1.012	0
12	1.247	1.482	1.203	1.241	1.181	0
13	58.301	64.182	-0.028	0.527	0.447	0
14	1.010	42.472	1.568	1.598	0.558	0
15	56.544	31.090	0.145	0.257	0.130	0
16	0.921	1.054	1.196	1.361	1.181	0
17	1.299	1.299	0.758	1.329	1.266	0
18	48.225	47.319	-0.003	0.055	-0.742	0
19	0.329	0.261	0.293	1.546	1.557	0
20	37.045	1.278	1.294	1.279	1.264	0
21	2.052	2.222	69.582	1.061	1.347	0
22	0.022	0.043	-0.036	0.042	0.065	0
23	-4.786	-1.412	-4.812	-4.810	20.446	0
24	0.730	0.114	70.324	1.537	0.147	0
25	1.324	-0.010	-0.020	1.425	1.389	0
26	1.632	0.125	0.186	1.697	0.345	0
27	-1.547	0.556	-0.002	56.653	0.008	0
28	-0.025	-0.031	1.615	1.608	39.812	0
29	3.993	3.418	1.606	1.791	51.039	0
30	-1.627	0.145	0.227	62.479	0.226	0

Note. Cut-off value= 6.705 d.u.

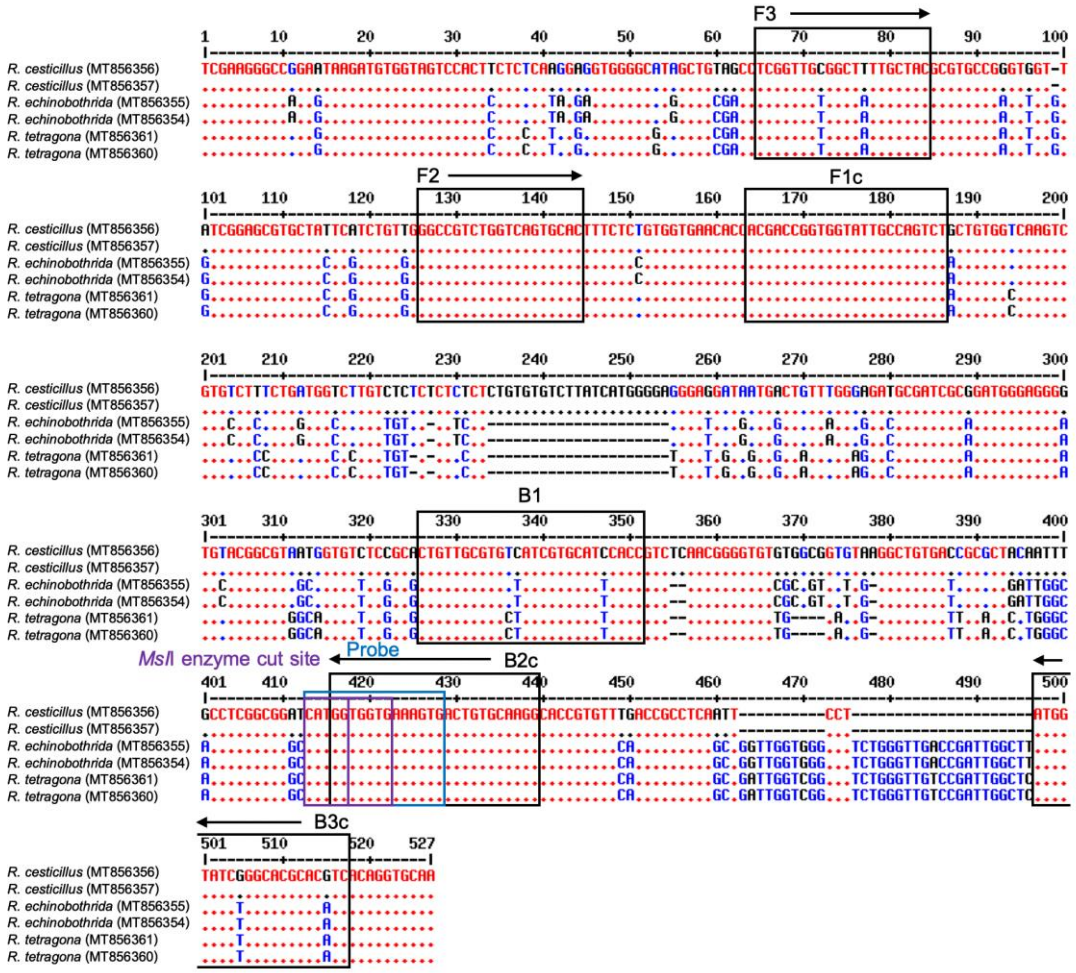


Figure A1 Multiple sequence alignment of *28S rDNA* gene for three target *Raillietina* species focused on *Raillietina* LAMP primers, probe, and restriction site of *Msl*I enzyme

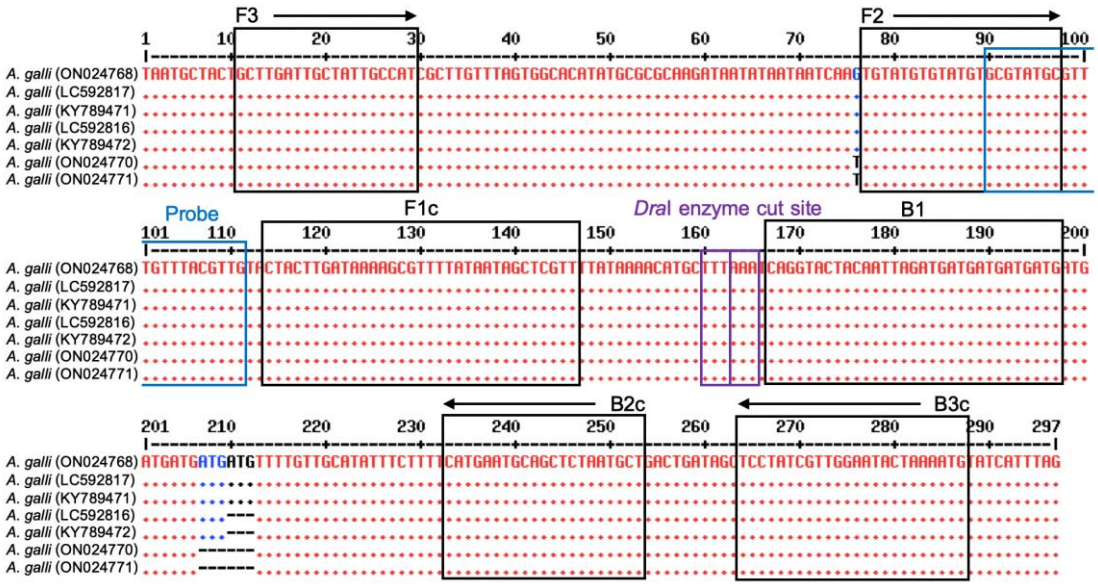


Figure A2 Multiple sequence alignment of ITS2 region for *A. galli* focused on *A*. galli LAMP primers, probe, and restriction site of *Dra*I enzyme

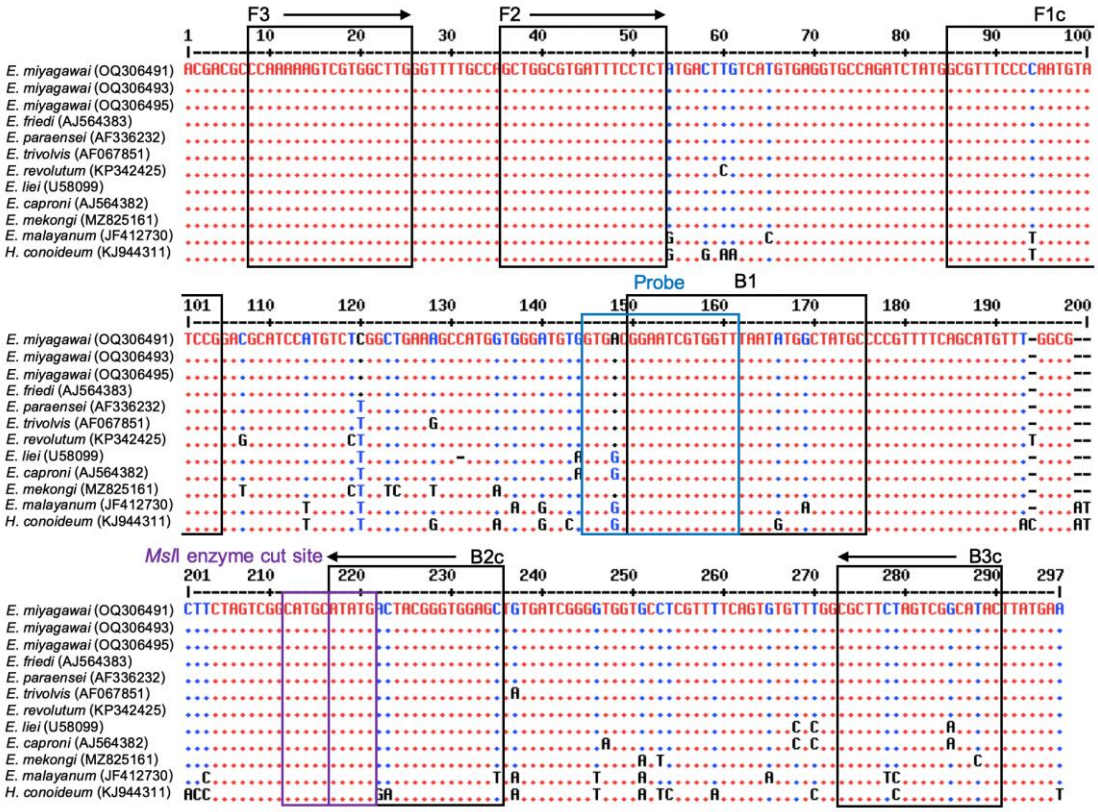


Figure A3 Multiple sequence alignment of ITS2 region for Echinostomatidae group focused on Echinostomatidae LAMP primers, probe, and restriction site of *Msl*I enzyme

Figure A4 Multiple sequence alignment of ITS2 region for *Raillietina* species focused on *R. echinobothrida* LAMP primers and restriction site of *Hin*dIII-HF enzyme

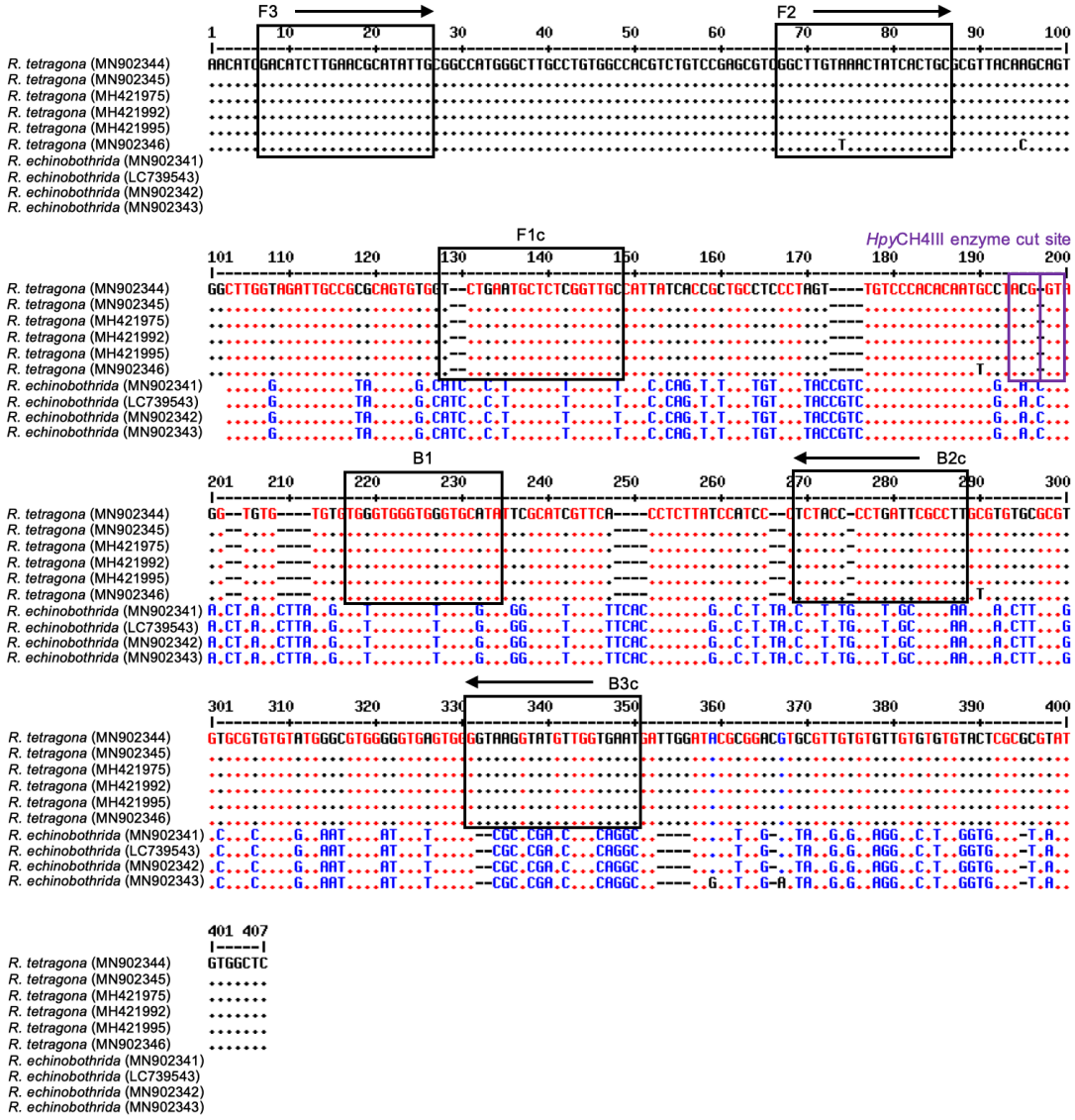


Figure A5 Multiple sequence alignment of ITS2 region for *Raillietina* species focused on *R. tetragona* LAMP primers and restriction site of *Hpy*CH4III enzyme

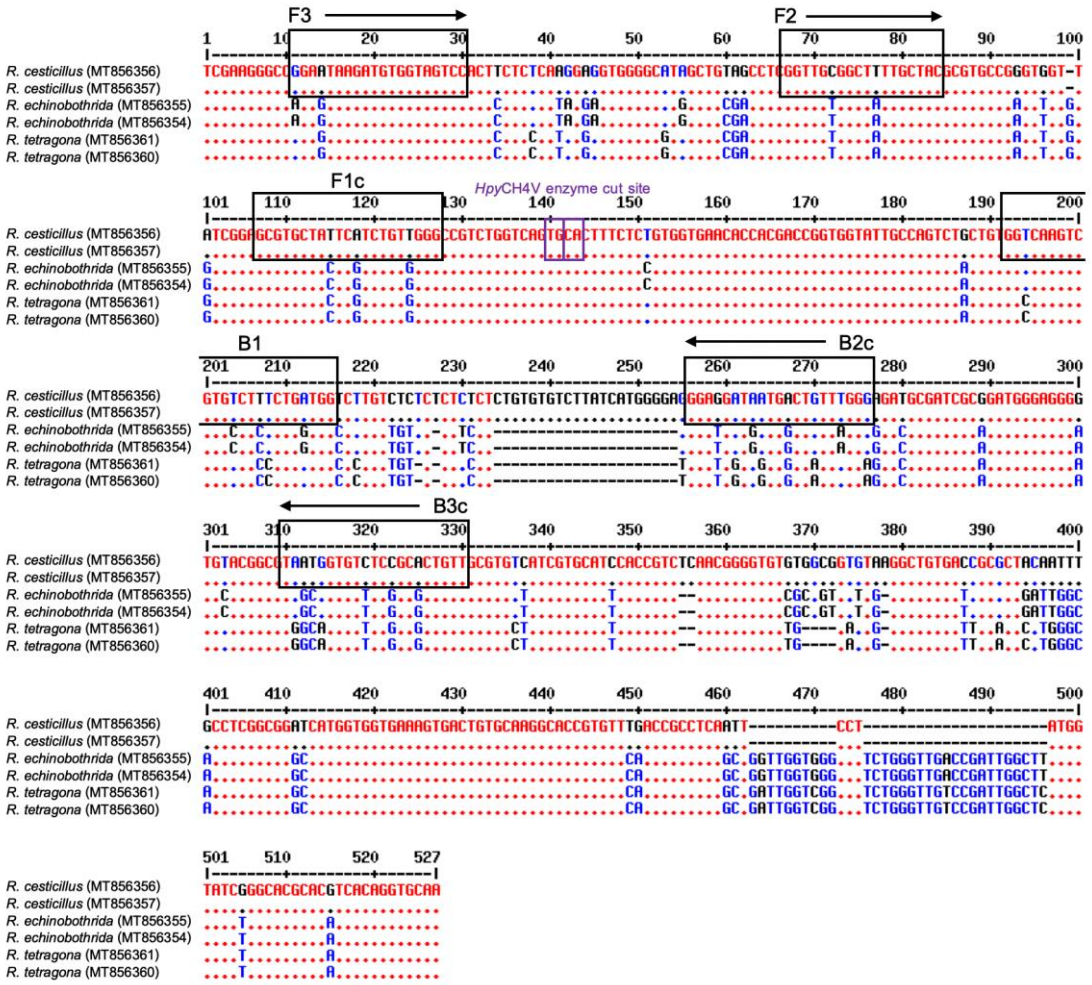


Figure A6 Multiple sequence alignment of *28S rDNA* gene for *Raillietina* species focused on *R. cesticillus* LAMP primers and restriction site of *Hpy*CH4V enzyme

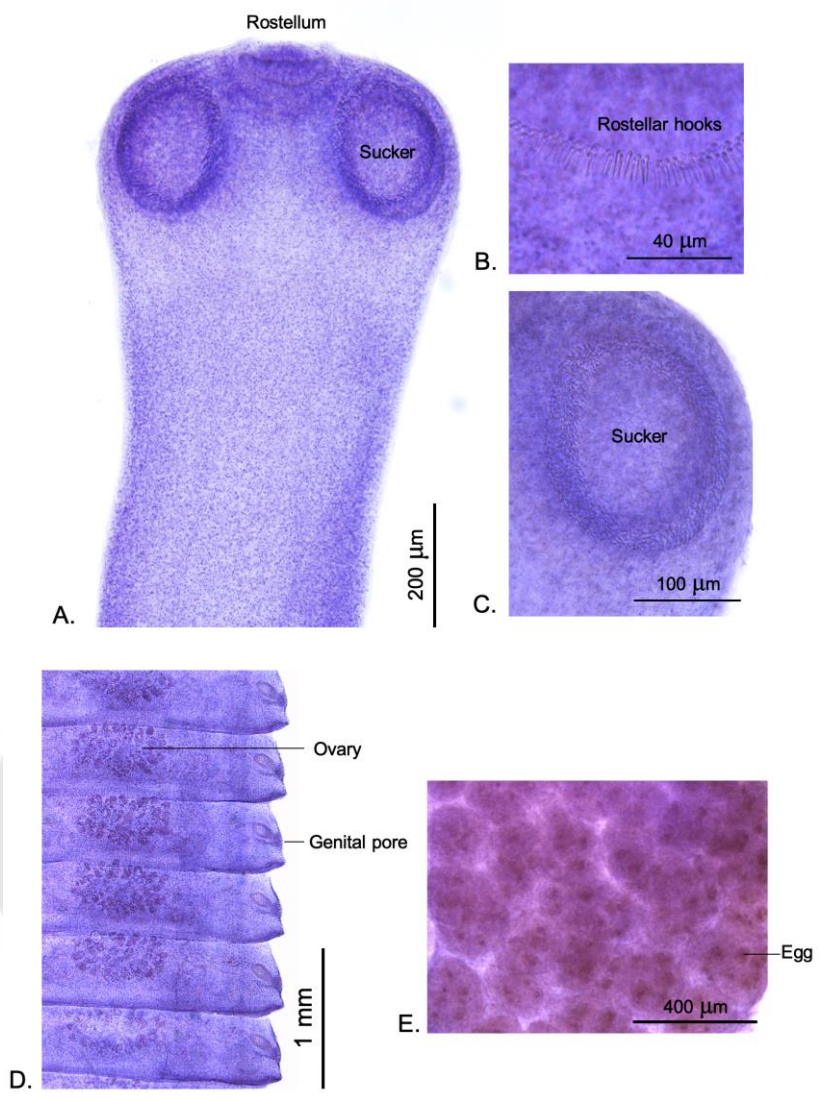


Figure A7 Morphology of *R. echinobothrida* that was used to develop the LAMP assays in this study

(A: scolex; B: rostellar hooks; C: armed sucker; D: mature proglottids presenting unilateral opening of genital pores; E: egg capsule contained several eggs in a gravid proglottid)

Sources: modified from Panich, W., Tejangkura, T., & Chontananarth, T. (2021). Novel high-performance detection of *Raillietina echinobothrida*, *Raillietina tetragona*, and *Raillietina cesticillus* using loop-mediated isothermal amplification coupled with a lateral flow dipstick (LAMP-LFD). *Veterinary Parasitology*, *292*, 109396.

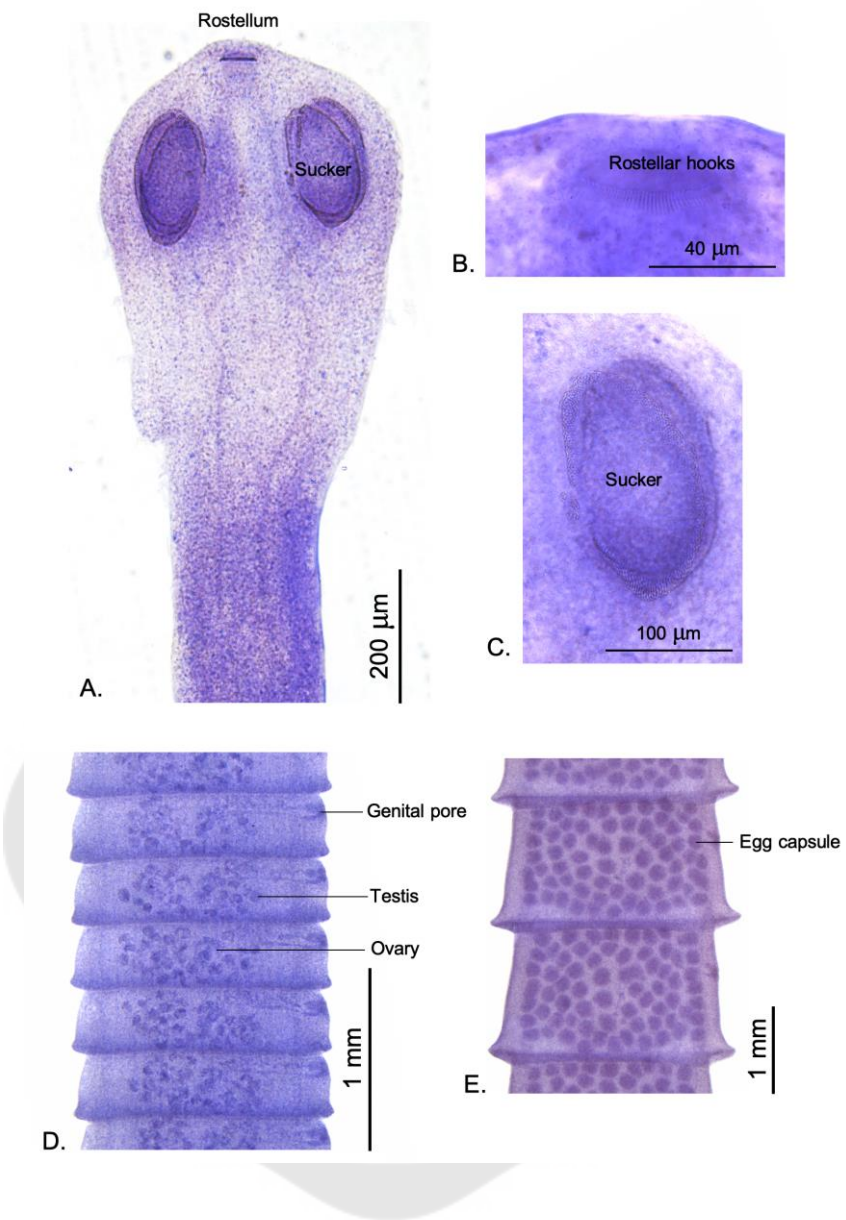


Figure A8 Morphology of *R. tetragona* that was used to develop the LAMP assays in this study

(A: scolex; B: rostellar hooks; C: armed sucker; D: mature proglottids presenting unilateral opening of genital pores; E: gravid proglottids contained egg capsules)

Sources: modified from Panich, W., Tejangkura, T., & Chontananarth, T. (2021). Novel high-performance detection of *Raillietina echinobothrida*, *Raillietina tetragona*, and *Raillietina cesticillus* using loop-mediated isothermal amplification coupled with a lateral flow dipstick (LAMP-LFD). *Veterinary Parasitology*, *292*, 109396.

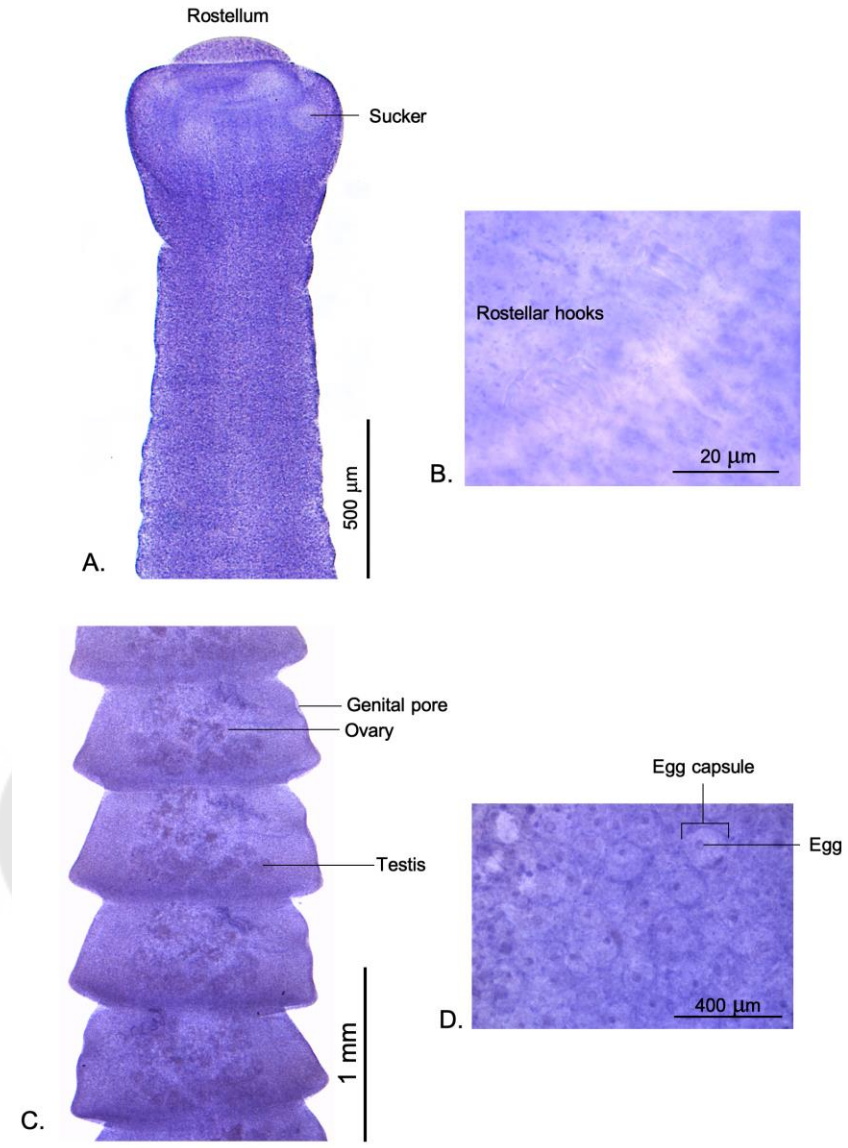


Figure A9 Morphology of *R. cesticillus* that was used to develop the LAMP assays in this study

(A: scolex; B: rostellar hooks; C: mature proglottids presenting irregularly alternating opening of genital pores; D: one egg per egg capsule in a gravid proglottid)

Sources: modified from Panich, W., Tejangkura, T., & Chontananarth, T. (2021). Novel high-performance detection of *Raillietina echinobothrida*, *Raillietina tetragona*, and *Raillietina cesticillus* using loop-mediated isothermal amplification coupled with a lateral flow dipstick (LAMP-LFD). *Veterinary Parasitology*, *292*, 109396.

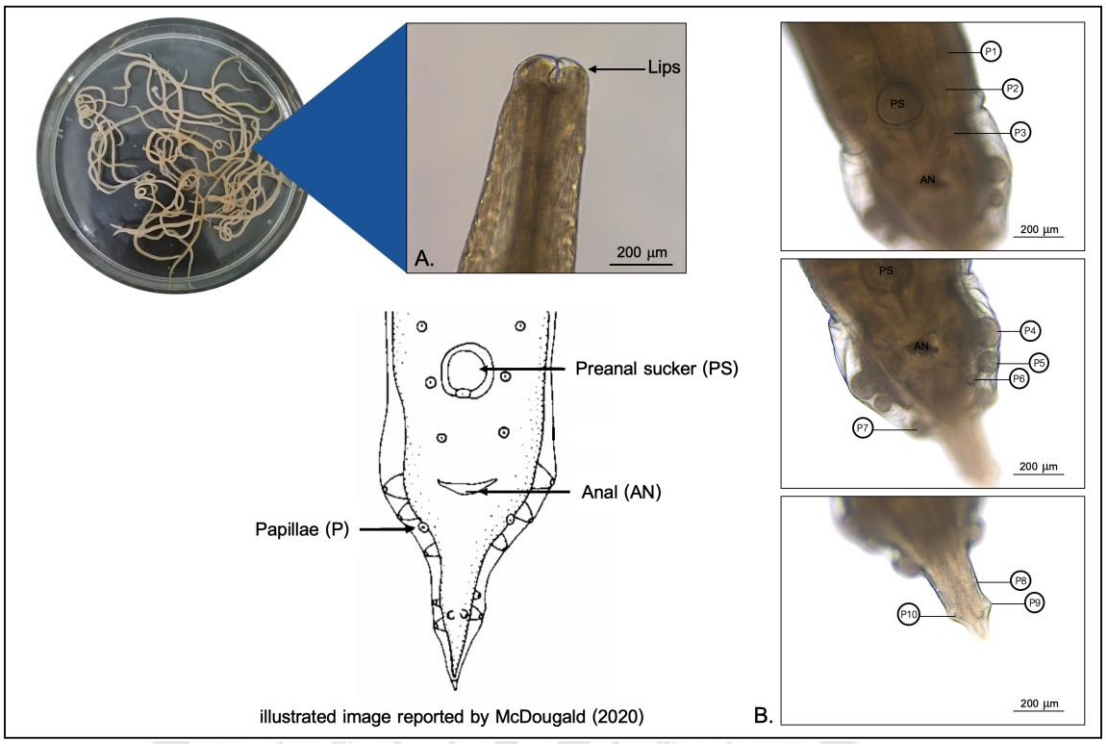


Figure A10 Morphology of male *A. galli* that was used to develop the LAMP assays in this study

(A: head; B: tail)

Sources: modified from McDougald, L. R. (2020). Internal parasites. *In diseases of poultry* (13th ed., pp. 1117–1146). Wiley-Blackwell.

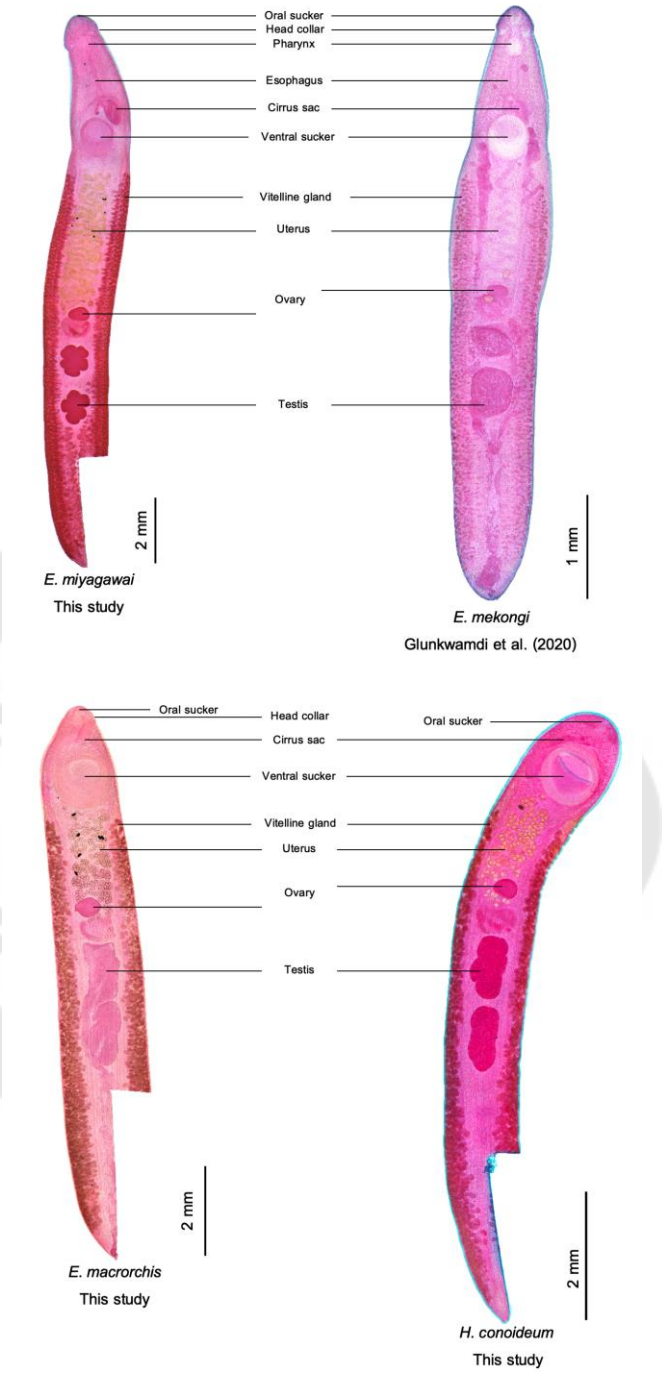


Figure A11 Morphologies of four target Echinostomatidae species that were used to develop the LAMP assays in this study

Source: modified from Glunkwamdi, P., Pakdee, S., & Raksaman, A. (2020). Epidemiological situation and risk of parasitic infections by ingesting of *Filopaludina* snails at local markets in Bangkok metropolis. Srinakharinwirot University.

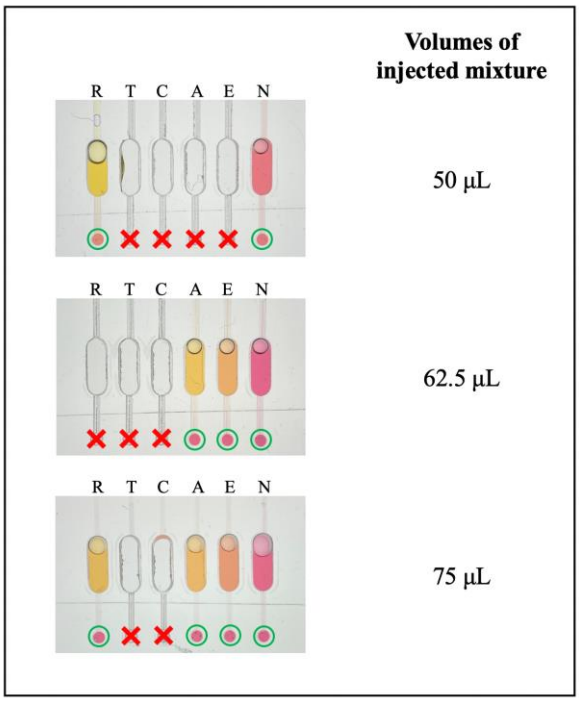


Figure A12 Testing of alternative detection on a microfluidic chip

(Green circles: punched outlet holes; Red cross signs: unpunched outlet holes)

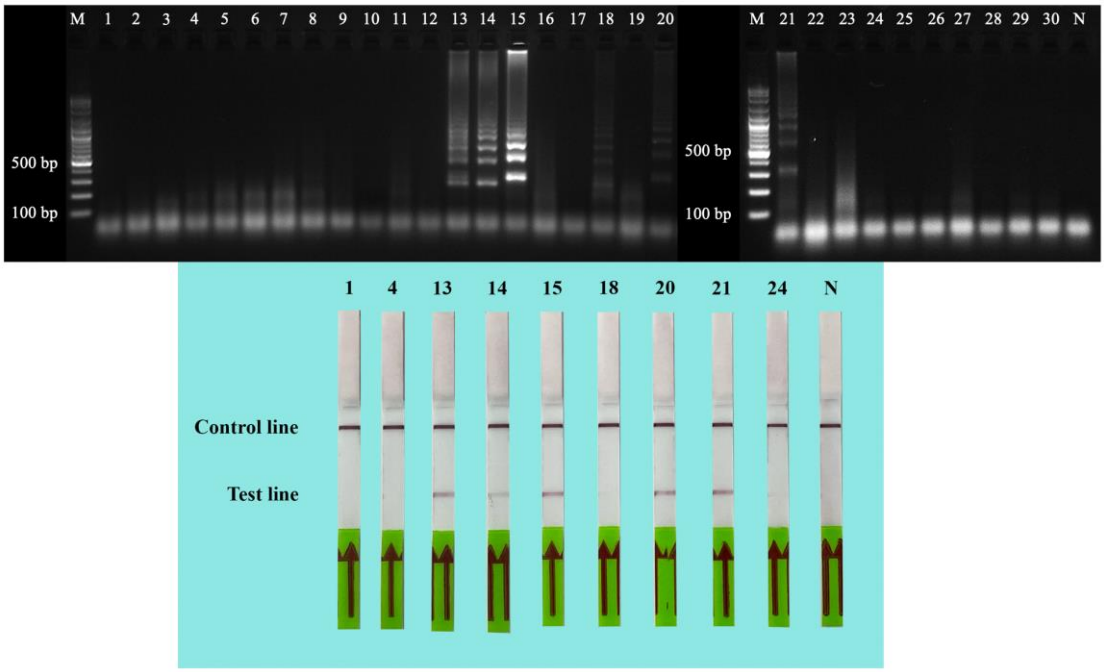


Figure A13 Clinical results of *Raillietina* LAMP assay detected by gel electrophoresis and LFD

(M: molecular marker 100 bp; 1-30: clinical samples; N: negative control)

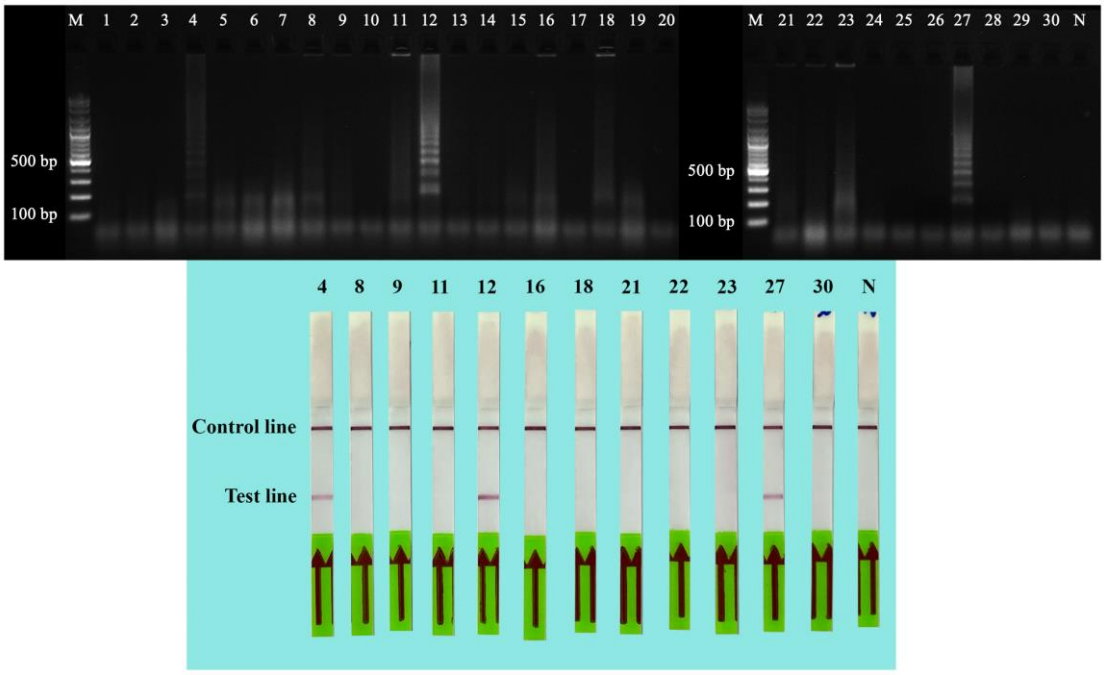


Figure A14 Clinical results of *A. galli* LAMP assay detected by gel electrophoresis and LFD

(M: molecular marker 100 bp; 1-30: clinical samples; N: negative control)

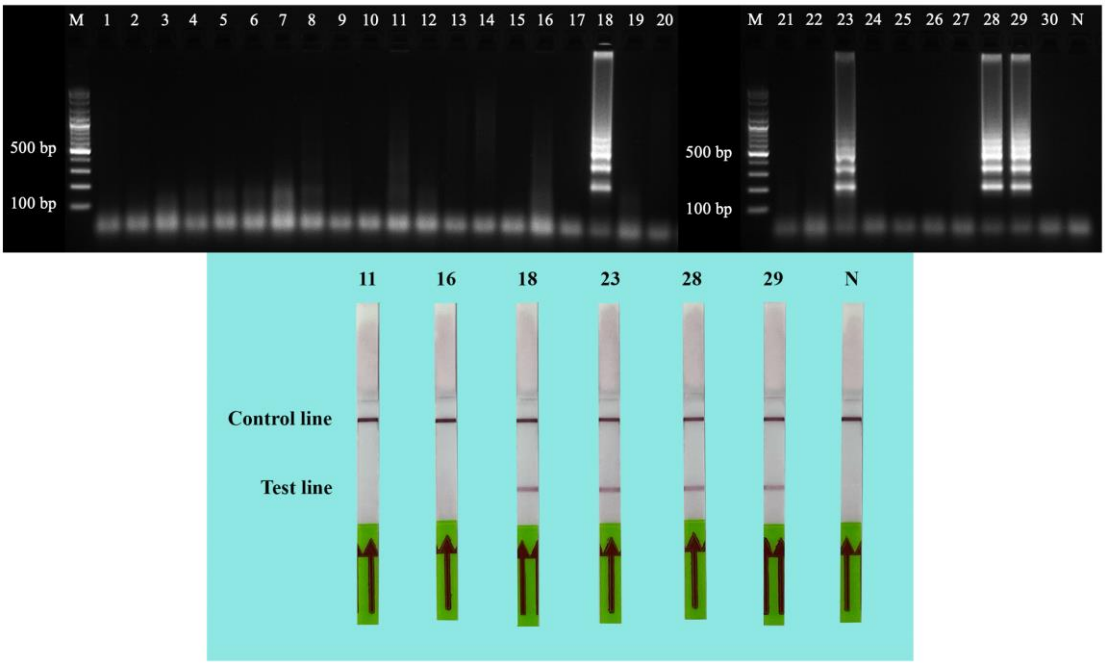


Figure A15 Clinical results of Echinostomatidae LAMP assay detected by gel electrophoresis and LFD

(M: molecular marker 100 bp; 1-30: clinical samples; N: negative control)

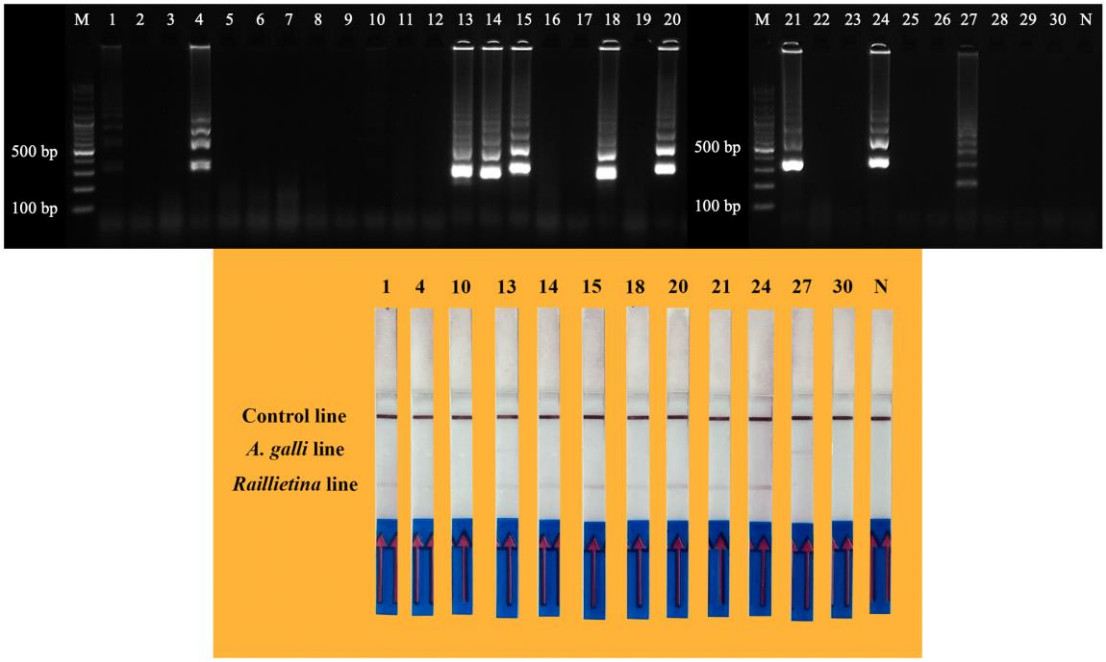


Figure A16 Clinical results of *Raillietina – A. galli* LAMP assay detected by gel electrophoresis and LFD

(M: molecular marker 100 bp; 1-30: clinical samples; N: negative control)

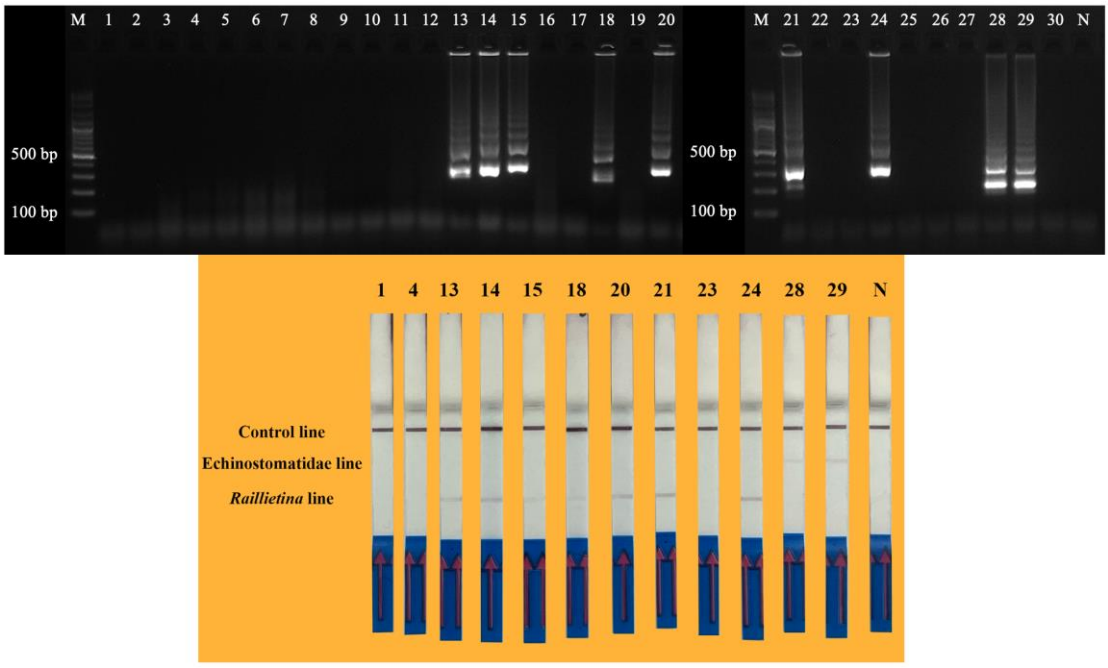


Figure A17 Clinical results of *Raillietina* – Echinostomatidae LAMP assay detected by gel electrophoresis and LFD

(M: molecular marker 100 bp; 1-30: clinical samples; N: negative control)

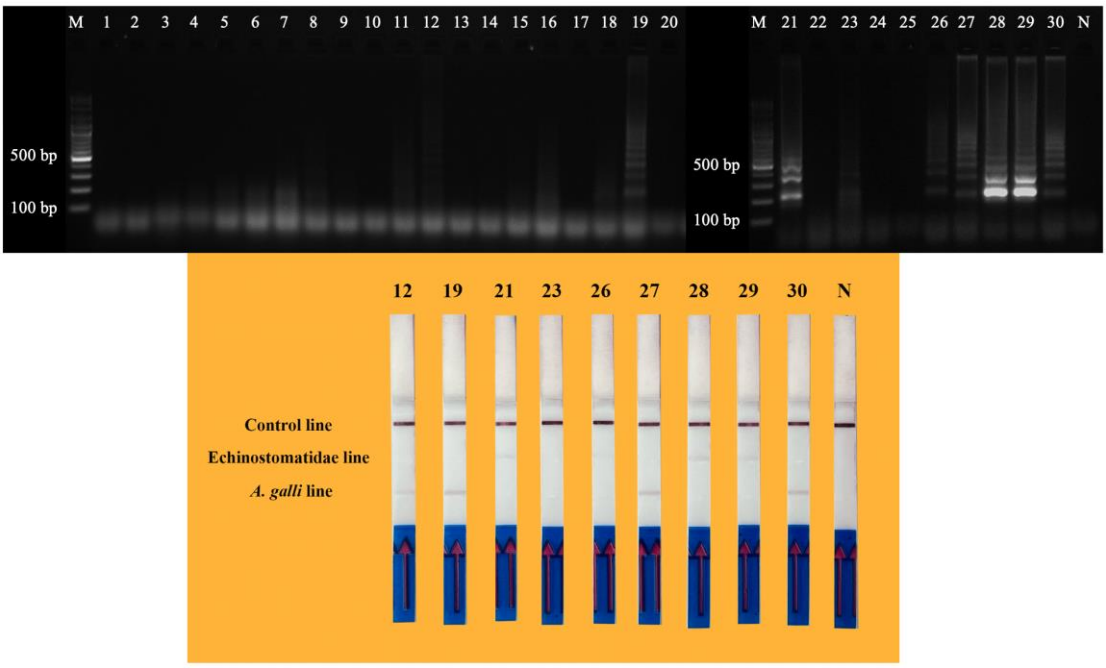


Figure A18 Clinical results of *A. galli* – Echinostomatidae LAMP assay detected by gel electrophoresis and LFD

(M: molecular marker 100 bp; 1-30: clinical samples; N: negative control)

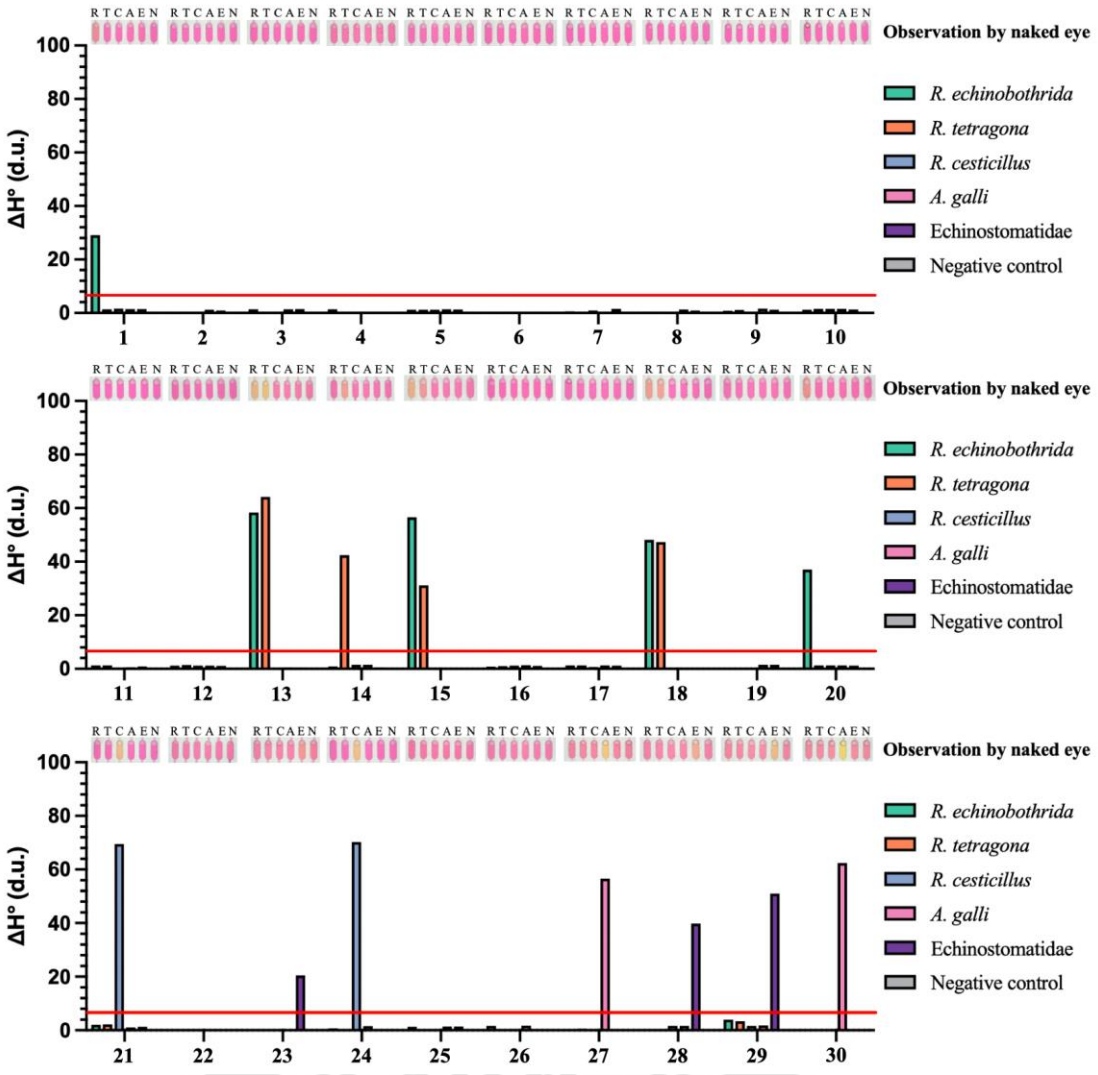


Figure A19 Clinical results of colorimetric LAMP on a microfluidic chip detected by naked eye and the chromatic analysis

(R: *R. echinobothrida* reaction chamber; T: *R. tetragona* reaction chamber; C: *R. cesticillus* reaction chamber; A: *A. galli* reaction chamber; E: Echinostomatidae reaction chamber; N: negative control chamber; 1-30: clinical samples; red lines: cut-off value of ΔH°)

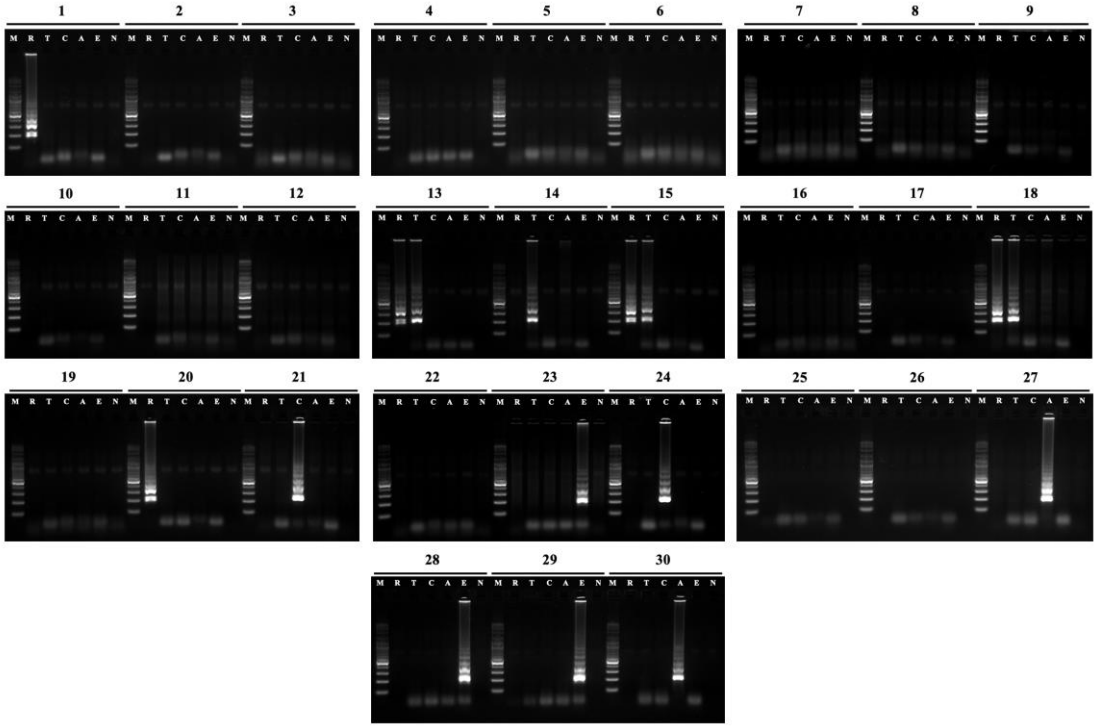


Figure A20 Clinical results of colorimetric LAMP on a microfluidic chip detected by gel electrophoresis

(M: molecular marker 100 bp; R: *R. echinobothrida* reaction chamber; T: *R. tetragona* reaction chamber; C: *R. cesticillus* reaction chamber; A: *A. galli* reaction chamber; E: Echinostomatidae reaction chamber; N: negative control chamber; 1-30: clinical samples)

VITA