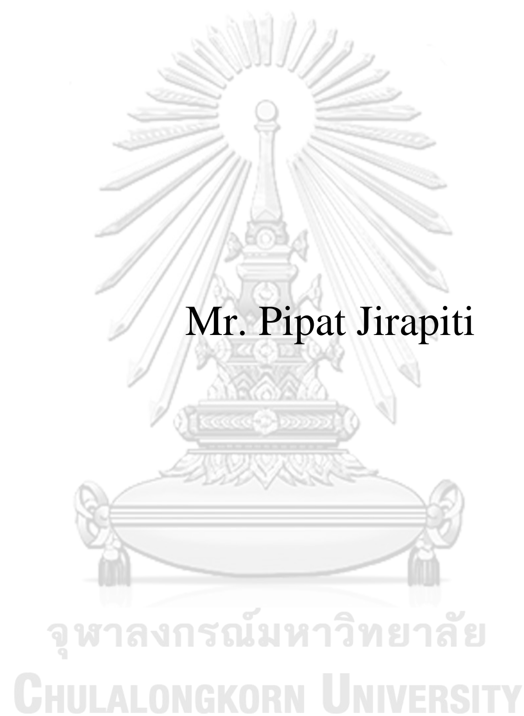
Validation of LAMP-LFD *Ehrlichia canis* DNAsensor Kit



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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Biosciences
Department of Veterinary Anatomy
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Chulalongkorn University
Academic Year 2018
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การประเมินประสิทธิภาพแลมป์-แอลเอฟดี เออร์ลิเคีย เคนิส ดีเอนเอเซนเซอร์คิท

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CHULALONGKORN UNIVERSITY

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวศาสตร์ทางสัตวแพทย์ ภาควิชากายวิภาคศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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การวินิจฉัยโรคในสุนัข Canine Monocytic Ehrlichiosis (CME) ขึ้นอยู่กับหลายปัจจัย ได้แก่ การพิจารณาประวัติการติดโรค, การปรากฏของ cytoplasmic morulae ภายใต้กล้องจุลทรรศน์, อาการทางคลินิกและผลการทดสอบในห้องปฏิบัติจากการนับเกล็ดเลือด ซึ่งปัจจัยดังกล่าวไม่ใช่การยืนยันโรคที่แน่นอน ดังนั้นเรามักใช้เทคนิค PCR ในการยืนยันโรค CME หรือการติดเชื้อ *Ehrlichia canis* อยู่เสมอ อย่างไรก็ตาม ข้อเสียของการทดสอบด้วยเทคนิค PCR คือการพึ่งอุปกรณ์ต่างๆ ในห้องปฏิบัติการเป็นสำคัญ ดังนั้นจึงมีการพัฒนาเทคโนโลยีทางดีเอ็นเอใหม่ๆ ขึ้นเพื่อลดการใช้อุปกรณ์และเครื่องมือ เช่น thermocycler และ gel electrophoresis LAMP-LFD ถือเป็นนวัตกรรมที่ใช้เพิ่มปริมาณดีเอ็นเอโดยใช้อุณหภูมิคงที่ร่วมกับการใช้ Lateral Flow Device เพื่อแสดงผลการตรวจสอบผลิตภัณฑ์ดีเอ็นเอเป็นแบบแถบ LAMP-LFD จะรวมอยู่ในชุดทดสอบ DNAsensor ที่ได้รับการพัฒนาขึ้นเพื่อทดแทนความต้องการใช้อุปกรณ์พิเศษสำหรับ PCR และ gel electrophoresis งานวิจัยนี้มีวัตถุประสงค์เพื่อประเมินประสิทธิภาพของ LAMP-LFD เปรียบเทียบกับ nested PCR (nPCR) และ single PCR (sPCR) ผลการตรวจสอบพบว่า nPCR, LAMP-LFD และ sPCR มีค่าความจำเพาะ (specificity) เท่ากันที่ 100 % ในขณะที่ วิธีทั้งสามมีความแตกต่างกันในด้านความไว (sensitivity) ในการทดสอบ nPCR เป็นวิธีที่ความไวมากที่สุดจึงใช้เป็นการบ่งชี้สุนัขในกลุ่มที่เป็นโรค CME อย่างไรก็ตาม, LAMP-LFD มีความไวเทียบเคียงกับ nPCR ที่ 78.57% (95% Confident Interval, CI = 63.19%-89.70%) ส่วน sPCR อยู่ที่ระดับ 40.48% (95% CI 25.36% -56.72%) ค่าสัมประสิทธิ์ของ Cohen เมื่อเปรียบเทียบ nPCR กับ LAMP-LFD ถือว่ามีความสอดคล้องกันสูง (0.785, 89.28% observed agreement) แต่มีความสอดคล้องต่ำกับ sPCR การประยุกต์ใช้ LAMP-LFD จึงอาจใช้เป็นวิธีทางเลือกสำหรับการตรวจวินิจฉัยโรค CME และมีความเป็นไปได้ที่จะถูกนำมาใช้ในห้องปฏิบัติการรวมถึงสถานพยาบาลสัตว์ (point-of-vet care)

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สาขาวิชา	ชีวศาสตร์ทางสัตวแพทย์	ลายมือชื่อนิสิต ....
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5975512631 : MAJOR VETERINARY BIOSCIENCES
KEYWORD: Ehrlichia canis DNAsensor kit LAMP-LFD

Pipat Jirapiti : Validation of LAMP-LFD *Ehrlichia canis* DNAsensor Kit. Advisor: Assoc. Prof. Meena Sarikaputi, D.V.M., M.S., Ph.D. Co-advisor: Asst. Prof. Nareerat Viseshakul, D.V.M., M.S., Ph.D.

Diagnosis of Canine Monocytic Ehrlichiosis is in light of anamesis, visualized cytoplasmic morulae, clinical signs and laboratory test result of platelet counts. The PCR technique is always a definitive confirmatory test of *Ehrlichia canis* infection. However, the drawback of PCRs test is crucially needed of laboratory equipments. This drawback is somehow enabled newly DNA based technology to be developed in order to eliminate laborious thermocycler and gel electrophoresis. The LAMP-LFD is on focus by using a single temperature to amplify DNA and no gel electrophoresis involved to detect DNA product. LAMP-LFD is included in DNAsensor test kit that recently developed to replace the special equipment requirement for PCRs. This work is aimed to validate LAMP-LFD against nested PCR and single PCR. The validation results showed that nPCR, LAMP-LFD and sPCR are equally in specificity test meanwhile, they have different degrees of sensitivity. nPCR is determined to be the most sensitive method. However, sensitivity of LAMP-LFD and sPCR when compare to nPCR are 78.57% (95% CI= 63.19%-89.70%) and 40.48% (95% CI 25.63% to 56.72%) respectively. The Cohen's kappa coefficient revealed that nPCR and LAMP-LFD is in substantial agreement (0.785, 89.28% observed agreement) but not sPCR. The application of LAMP-LFD is hence capable of being an alternative method of molecular diagnostic test for CME and a point-of-veterinary care device.

Field of Study:	Veterinary Biosciences	Student's Signature ....
Academic Year:	2018	Advisor's Signature ....
		Co-advisor's Signature ....

ACKNOWLEDGEMENTS

First of all, I would love to thank Assoc. Prof. Meena Sarikaputi to giving me a great opportunity to be your student. I am grateful to all my beloved classmates for your help that supports me both physically and mentally. I really appreciate everyone in Biochemistry Unit, Faculty of Veterinary Science, Chulalongkorn University for your all guidance, advice and kindness.

In addition, I am grateful to receive CHULALONGKORN UNIVERSITY GRADUATE SCHOLARSHIP TO COMMEMORATE THE 72nd ANNIVERSARY OF HIS MAJESTY KING BHUMIBOL ADULYADEJ for tuition fee of my Master program.

Importantly, I thank my parents for their love for me.

Last but not least, I love to sincerely thank my co-advisor Assist. Prof. Nareerat Viseshakul, you are my best of all time.

Pipat Jirapiti

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

TABLE OF CONTENTS

Page

.... iii

ABSTRACT (THAI) iii

.... iv

ABSTRACT (ENGLISH) iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER I INTRODUCTION 1

1.1 Background and rationale 1

1.2 Studies of the thesis 2

1.3 Objective 2

CHAPTER II LITERATURE REVIEWS 3

2.1 *Ehrlichia canis* as the etiologic pathogen 3

2.2 Life Cycle 4

2.3 Clinical signs of CME 5

2.4 Methods of *Ehrlichia canis* diagnosis 6

2.4.1 Hematological findings 6

2.4.2 Blood smear evaluation 7

2.4.3 Serology 7

2.4.4 Polymerase chain reaction (PCR) 8

2.4.4.1 Single PCR (sPCR) 8

2.4.4.2 SYBR Green Real-time PCR 12

2.4.4.3 Nested PCR (nPCR) 13

2.4.5 Loop-mediated isothermal amplification (LAMP) 13

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.4.5.1 Chromatography lateral flow dipstick E. canis DNAsensor Kit .. 14
CHAPTER III MATERIALS AND METHODS 17
3.1 Canine blood sample collection 17
3.2 DNA preparation 18
3.3 DNA-based identification 18
3.3.1 The nested PCR protocol (nPCR) 18
3.3.2 The single PCR protocol (sPCR) 19
3.3.3 *Ehrlichia canis* DNAsensor Kit 20
3.3.3.1 LAMP-LFD E. canis DNAsensor kit 20
3.3.3.2 Validation of E. canis DNAsensor Kit assay 21
CHAPTER IV RESULTS 22
4.1 Canine blood sample collection and DNA preparation 22
4.2 Three assays used in test kit validation: nPCR, sPCR and LAMP-LFD 22
4.3 The selective test and limit of detection of LAMP-LFD 31
4.4 The statistical analysis for LAMP-LFD validation 33
CHAPTER IV DISCUSSION 35
5.1 Canine blood sample collection and DNA preparation 35
5.2 Primers design in the assays used in validation of test kit 36
5.3 Test kit validation 36
REFERENCES 38
VITA 42

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

Page

Table 1. The importance of evaluating true platelet counts in E. canis suspected dogs. 7

Table 2. The distribution of Ehrlichia canis in regions widely occurred. 9

Table 3. Primer names, sequences and amplicon size used in this study to identify the DNA region of 16S rRNA of E. canis. 12

Table 4. The number of DNA samples tested with 3 different DNA methods; nPCR, LAMP-LFD and sPCR. 23

Table 5. The limit of detection comparisons between assays; nPCR, SYBR Green Real time PCR*, LAMP-LFD and sPCR. 32

Table 6. The two by two table used to calculate the sensitivity and specificity of LAMP-LFD compared to nPCR as the CME presence or absence 33

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

Page

Figure 1. The Schematic diagram showed the locations of primers of nPCR and LAMP-LFD.......14

Figure 2. The graphic scheme of Lateral Flow Device (LFD) using in the DNAsensor E. canis test kit. 15

Figure 3. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 1-14 25

Figure 4. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 15-28 26

Figure 5. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 29-42 27

Figure 6. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 43-56. 28

Figure 7. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 57-70 29

Figure 8. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 71-84 30

Figure 9. The selective test of LAMP-LFD against other commonly found blood parasites in Thai domestic dogs. 31

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER I

INTRODUCTION

1.1 Background and rationale

The general challenges of microbial infection of human and animals have introduced primarily by noncommercial Polymerase Chain Reaction methods, PCRs, and later on, other newly developed, commercializing procedures are also into the laboratory. Since methods of DNA amplification technology were extensively developed according to identify the infections, it greatly achieved the lower limit of detection in clinical pathogens. For example, single PCR, sPCR, it finally became a standard method of all.

Nowadays, in every clinic and hospital, molecular diagnostic of PCRs was applied as a routine practice. Although PCRs appeared to be the gold standard for especially viral, rickettsial and bacterial infections, on the other hand, they still have one major disadvantage as machine requirement. PCRs required equipment, thermocycler and gel electrophoresis. In the remote clinics and fundamental laboratories, PCRs are therefore hardly to be accessed.

To clinically investigate the disease, ones not only need the highly sensitive whatever tools but also the rapid, simple, cost effective, less machine depending and accurate approaches. Here in this thesis, I would like to introduce Loop- Mediated Isothermal Amplification method and Lateral Flow Device (LAMP- LFD) with a single temperature amplification, knowing the result without using thermocycle or the gel electrophoresis. If LAMP-LFD is to be used as a point-of-vet care, it should be

validated against other gold standard or routine method. The aim is to validate LAMP-LFD against sPCR and nested PCRs (nPCR). Results will also show the accuracy of detection of *Ehrlichia canis* in canine blood samples.

Ehrlichia canis is a cause of Canine Monocytic Ehrlichiosis (CME), a fatal disease transmitted by ticks, *Rhipicephalus sanguineaus*, or brown dog tick. It was always focused in veterinary attention because of its difficulty in detection also having a high risk of infection in the abundance of ticks of the tropical areas. When infected, in clinical-related phases, dogs showed severe thrombocytopenia, anemia of inflammation and it was often undetected before the induction of fatal CME.

Among the DNA assays, I enlisted herein 3 DNA amplification methods that are highly sensitive so that it is able to detect ones in a very early stage of CME. The first is the sPCR which is used worldwide, secondly, the nPCR which is a routine method in our laboratory and also the reference test of this study and lastly, LAMP-LFD, DNAsensor test kit which is recently introduced in this trial. In search of the DNA based method that is rapid, cheap, convenient, accurate LAMP-LFD can be performed at the point-of-care when dealing with veterinary diseases.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.2 Studies of the thesis

Validation study of LAMP-LFD *Ehrlichia canis* DNAsensor kit on domestic dog samples intentionally recruited from clinical cases of Veterinary Teaching Hospital Chulalongkorn University and other sources that CME is presented.

1.3 Objective

To identify, *Ehrlichia canis*, the cause of canine monocytic ehrlichiosis, can be detected by Loop-mediated isothermal amplification (LAMP) in conjunction with lateral flow device (LFD) for result detection.

CHAPTER II

LITERATURE REVIEWS

2.1 *Ehrlichia canis* as the etiologic pathogen

The genus Ehrlichia is named after a great German microbiologist, Paul Ehrlich. The first ehrlichial organism was recognized as *Ehrlichia ruminantium* in South Africa, a cause of a highly pathogenic heart-water disease in cattle during the early 19th century. Subsequently, *E. canis* was first demonstrated in Algeria in 1935. In more than three decades later, it was rediscovered in 1973, as a serious cause of 80% death in German shepherd military dogs serving in Vietnam War. Since then CME have never been eradicated, in contrast, it increases in pathogenicity and widely spreads in many areas of the world.

Ehrlichia canis is a pathogenic rickettsial agent resembling an obligate intracellular, Gram-negative-like ∝-proteo-bacterium without cell wall. The genome size of *E. canis* is 1.2 Mb. It is the most important blood parasitic bacteria found in dogs worldwide and fatal if untreated. CME is a long-term persistent illness occurring widely through a blood-sucking ticks abundant in tropical areas including Thailand (Huxsoll et al., 1969; Huxsoll et al., 1970; Hildebrandt et al., 1973). Most of the CME cases normally returned for the 2nd and 3rd visits of treatment and recheck. For example, after treatment with doxycycline, CME was asked to be rechecked with PCR. If it is persisted, the 3rd visit is also required. This disease is also complicated in term of there is no vaccine available. CME needs sensitive method to recheck the existence of *E. canis* in the blood, while blood sample is a hallmark of diagnosis.

Among the illness of CME in 3 phases, the acute phase is therefore to be the most challenging to be diagnosed accurately. In many reports, CME is diagnosed using combination of different methods i.e., visualizing a morulae of *E. canis* in the blood or buffy coat smears, serology of anti-*E. canis* IgG antibody, isolation of pathogen in the tissue culture, and demonstration of *E. canis* DNA by PCRs. (Harrus and Waner, 2011).

Amplification of *E. canis* DNA using the LAMP-LFD technique was successfully achieved by using 2 specific sets of primers in conjunction with LFD, lateral flow device, or so called, dipstick to replace gel electrophoresis in order to show the amplification products. To obtain the specific PCR amplicons, they were probed before applying to dipstick. Probe bound specific LAMP product and will give a chromatographic banding output on the LFD. This procedure may help to measure the prevalence of CME and to validate the technique in where there is no data available.

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CHULALONGKORN UNIVERSITY

Roughly 10% of pet dogs in Thailand randomly found harboring the bacteria through tick bites and 65% of the tick infested dogs found *E. canis* in the bloodstream (Ariyawutthiphan et al., 2008; Jirapattharasate et al., 2012). Moreover, *E. canis* can transmit mechanically by tick and by other mechanical procedures such as blood transfusion and contaminated surgical instruments (Ipek et al., 2017).

2.2 Life Cycle

After entering hosts' bloodstream via tick, the organisms were engulfed by phagocytic leucocytes, especially macrophages, sometime neutrophils and lymphocytes. A single cell of *E. canis* survived and became noticeable as the initial bodies, a small colony dividing within the cells. The increasing in number of *E. canis*

cells by binary multiplication within cytoplasmic membrane bound structure is known as morulae or inclusion body (Hildebrandt et al., 1973). This character of cytoplasmic morulae is then applied as a specific identification of *E. canis* since its discovery. Once morulae exit one leucocyte, they continuously enter other host cells and at the same time to another tick bites to complete the life cycle.

2.3 Clinical signs of CME

The infected dogs could face either fatal illness or secondary complications mostly by host immunological reactions and generalized inflammation against *E. canis* (Batmaz et al., 2001). After bitten by ticks, dogs would show symptoms of high fever and lethargy within 1 to 3 weeks. The significant clinical signs can be classified into three phases by pathogenicity characteristics as following: 1) Acute phase, shown in the earliest stage after *E. canis* infection. Signs of illnesses may last for 1 to 3 weeks, for example, depression, loss of appetite, fever, lymphadenopathy and decreasing in platelet count (< 200,000/µL) and unilateral nose bleeding due to thrombocytopenia. 2) Subclinical phase, animals have no apparent signs of abnormalities. This period could take months to years without notice. There is a presumption that *E. canis* escaped from host immune system into liver, spleen and bone marrow. In the aspect of hematology, intermittent thrombocytopenia and/or hyperglobulinemia could be observed. 3) Severe clinical signs reoccur in the chronic phase. Superficial petechial hemorrhages could be seen in about 60% of canine monocytic ehrlichiosis. Furthermore, other significant abnormal characteristics include uveitis and glomerulonephritis, could also be seen. By far, dogs with chronic ehrlichiosis may not survive the systematic complications and often died of

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pancytopenia as all blood cell lineages are stopped producing cells because of the bone marrow suppression from prolong *E. canis* infection.

2.4 Methods of *Ehrlichia canis* diagnosis

2.4.1 Hematological findings

The essential constituency of CME diagnosis in the complete blood count (CBC). The moderate to severe thrombocytopenia is found during the acute stage as distinctive finding (Harrus and Waner, 2011). Moreover, the blood smear evaluation of platelet numbers is to confirm the presence of a true thrombocytopenia rather than *in vitro* pseudothrombocytopenia. In the third week after experimental infection of *E. canis*, or in the acute phase, dogs exhibited lower level of thrombocytes 20,000-52,000/μL and with mild anemia and reduction of white blood cell counts. In the sub-clinical phase, with no clinical signs, it is generally accompanied by the mild thrombocytopenia (~140,000/μL). In the chronic phase, there was a marked anemia and leukopenia and marked pancytopenia and severe thrombocytopenia.

The platelet magnitude counts were suggested as a screening test for CME in the endemic areas (Bulla et al., 2004).

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Chulalongkorn University

Table 1. *The importance of evaluating true platelet counts in E. canis suspected dogs.*

Platelets counts (per uL)	*16S rRNA* PCR Positive *Ehrlichia canis*
>200,000	1.4 %
100,000-200,000	21.0 %
<100,000	63.1 %

2.4.2 Blood smear evaluation

Demonstration of typical cytoplasmic *E. canis* morulae in monocytes in blood smears by light microscopy routinely supports a diagnosis of CME in Small Animal Teaching Hospital of Chulalongkorn University. Morulae are membrane bound vacuoles which are usually densely packed with elementary bodies containing their genetic material as seen by electron microscopy (Hildebrandt et al., 1973). Light microscopy finding is time consuming and successful rate in only 4% of cases (Woody and Hoskins, 1991). If 1000 oil immersion fields are examined, 60% sensitivity is achieved using buffy coat smears and 34% is achieved using bone marrow specimen. Time required for screening 1000 oil immersion fields is 50-60 minutes (Mylonakis et al., 2003).

2.4.3 Serology

The occurrence of CME was studied in the early 1970s using antibody-ELISA (enzyme-linked immunosorbent assay) test together with immunofluorescent (IFA). IFA test for anti-*E. canis* antibodies is considered the ‘serological gold standard’,

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indicating exposure to *E. canis*. However, IgM is not considered a reliable indicator of *E. canis* exposure due to the inconsistent development during the course of the disease (McBride et al., 2003). In contrast IgG titers ≥ 1:40 are considered positive for *E. canis* exposure. For the acute infection, two consecutive IFA tested and 7-14 days apart, are required. Moreover, four-fold increasing in antibody titers is considered as the active infection. And anti-ehrlichial IgG antibodies persisted for several months to years regardless of rickettsial elimination (Bartsch and Greene, 1996).

ELISA or enzyme-linked immunosorbent assay was developed and found useful for diagnosis (Harrus et al., 2002). All sero-methods aim to detect anti-*E. canis* IgG antibodies, some used crude *E.canis* extract and some used recombinant protein antigen. The SNAP 3Dx is made from immunodominant protein of *E. canis* p30 and p30-1 (IDEXX Laboratory) (Harrus et al., 2002). When SNAP 3Dx compared to IFA, 74.6% was overall agreement between tests and both tests are highly specific. The author recommended that SNAP 3Dx test was used to determine the clinical relevance of annual *E. canis* screening. This test alone was not considered adequate for interpretation of the clinical relevance and its results should therefore be used in combination with platelet counts and molecular results (Hegarty et al., 2009)

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2.4.4 Polymerase chain reaction (PCR)

2.4.4.1 Single PCR (sPCR)

Recently, the DNA-based PCR was introduced to replace the earlier mentioned methods. The distribution of CME in many areas of the world and the techniques of disease detection are shown in Table 2.

Table 2. The distribution of Ehrlichia canis in regions widely occurred.

The disease prevalence is mostly presented by DNA based tests including both single and nested PCRs.

Region	Country	Disease Detections	References
Asia	India	PCR	(Lauzi et al., 2016)
	Korea	ELISA with IFA	(Bell et al., 2012)
	Malaysia	PCR	(Nazari et al., 2013)
	Philippines	PCR	(Corales et al., 2014)
	Thailand	Nested PCR	(Ariyawutthiphan et al., 2008)
		PCR-FRET	(Kongklieng et al., 2014)
		PCR	(Jirapattharasate et al., 2012)
		PCR-QCM	(Bunroddith et al., 2018)
Europe	England	PCR	(Wilson et al., 2013)
	Grenada	PCR	(Lanza-Perea et al., 2014)
	Portugal	PCR	(Maia et al., 2015)
	Sicily	PCR	(Torina et al., 2013)
Latin America	Brazil	PCR	(Soares et al., 2017)
	Chile	PCR	(Lopez et al., 2012)
	Costa Rica	PCR	(Wei et al., 2014)
	Panama	PCR	(Santamaria et al., 2014)

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United States	ELISA and IFA	(Ristic et al., 1972) (Immelman, 1973) (Stephenson and Ristic, 1978) (Keefe et al., 1982)

E. canis was first discovered since 1930s by using thin blood smear with Giemsa staining samples examined under light microscope. Although morphological detection is a definitive diagnosis, sensitivity and specificity of this technique is lower than molecular approaches due to lack of pathogen amplification and disturbing an interpretation by artifacts staining sediments from the specimen preparing processes. Polymerase chain reaction (PCR) was introduced as a powerful tool to identify the causative pathogen of Ehrlichiosis (Table 2). On one hand, specific amplification of genetic material like nucleic acid of the bacterium makes it much more sensitive and specific than classical methods simultaneously. PCR requires multistep temperature for the reaction by using the expensive equipment to produce accurate results successfully and needs a few hours to complete the whole process. Because of these drawbacks, PCR might not be compatible with point-of-vet-care screening test.

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PCRs used different target genes; *16S rRNA*, *p28* and *p30*. However, *16S rRNA* based nested PCR and *p30* based PCR is most commonly used. Meanwhile, *p30* has a higher sensitivity than that of *16S rRNA* gene due to *p30* has multiple copies per genome while the *16S rRNA* gene has only one copy per genome. It is that why *16S rRNA* gene-based PCR needs to be nested amplified (Stich et al., 2002).

The primers used in this study are listed in Table 3. The primers Ehr-out-2/ECC (modified to use in this study) and ECAN5/HE3 were used as described

(Ariyawutthiphan et al., 2008). The sPCR was performed using template DNA (~100 ng of total genomic DNA), with forward primer, and reverse primer, i-Taq PCR master mix (Intron®, Korea) and sterile distill water. The PCR cycles were applied accordingly. The PCR product was electrophoresis in 1.5% agarose gel and visualized under a UV light after staining with ethidium bromide.

The advantage of PCRs over serology is the early detection of DNA before sero-conversion occurs. PCR has higher sensitivity used to detect ehrlichial DNA rather than anti-*E. canis* antibodies probably indicating active infection rather than exposure.

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Table 3. Primer names, sequences and amplicon size used in this study to identify the DNA region of 16S rRNA of E. canis.

Primer Name	Sequence 5´ -3´	Amplicon size (bp)	Reference
Ehr-out-2	GCTCGTTGCGGGACTTAACCCAA CATCTCACGAC	1049	(Breitschwerdt et al., 1998)
ECC (modified)	CAGAACGAACGCTGGCGGCAA		EU263991
ECAN5	CAATTATTTATAGCCTCTGGCTAT AGGA	389	(Murphy et al., 1998)
HE3	TATAGGTACCGTCATTATCTTCCC TAT		

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2.4.4.2 SYBR Green Real-time PCR

SYBR-Green Real-Time PCR - A standard curve was generated on the serial dilutions of *E. canis* DNA containing plasmid. These real-time PCR reactions were applied in 10 µL reaction tubes containing 1 µL of template DNA, 1 µL (10 mM) of each primer, 5 µL of 2x SensiFast SXBR No-Rox Mix (SensiMixTM, Bioline USA), and distill water. The PCR and fluorescence detection were performed using EcoTM Real Time PCR system and Eco software version 3.0.16.0 (Illumina, USA). The

optimized thermal cycler program was 95°C for 2 min, followed by 40 cycles of 30 s at 94°C, 10 s at 55°C, and 20 s at 72°C.

2.4.4.3 Nested PCR (nPCR)

n-PCR procedure - The first round of nested PCR assay was performed with Eher-out-2/ECC (modified) primers (Table 3). The reaction preparation and the PCR condition were conducted in the same manner as the conventional PCR. The second-round PCR mixture and PCR condition were the same as used for the first-round PCR assay, except for the use of 10 mM of ECAN5/HE3 primers. The template DNA for the second PCR was the product of the first PCR (0.1 μL of a 10 μL total volume). The PCR products were electrophoresed on a 1.5% agarose gel at 75 Volts.

2.4.5 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a DNA amplification assay which can archive the multiplication reactions of interested DNA fragments at a constant temperature (Notomi et al., 2000). This technique uses the DNA polymerase which possesses strand displacement activity like *Bst* polymerase to raise the amount of target DNA. The reaction primers were designed at the same DNA region of *16S rRNA* of *E. canis* as of sPCR and nPCR following Figure 1. I strongly recommended this primer design strategy is at its best outcome in term of method comparisons.

The specificity is increased by using four specific primers which are designed from six regions of the DNA template. Moreover, there are many studies showed that LAMP efficiency is comparable to PCR with less time consuming. In additions, LAMP has been proved that it can tolerate less purified DNA samples used in the reactions. According to its properties previous mentioned, LAMP could be a method of choice for diagnosis in field practice.

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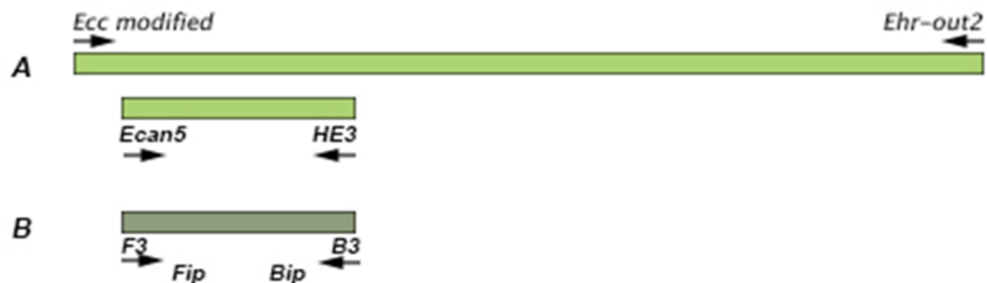

Figure 1. The Schematic diagram showed the locations of primers of nPCR and LAMP-LFD

The Schematic diagram showed the locations of primers of both nPCR (A) and LAMP-LFD DNAsensor test kit for *Ehrlichia canis* (B). In this region of *16S rRNA* gene, the primers are selective for *E. canis* only, they have no crossed reactions against other bacteria or other organisms found in the blood samples.

2.4.5.1 Chromatography lateral flow dipstick E. canis DNAsensor Kit

Lateral flow dipstick (LFD) is an alternate chromatographic method to detect products derived from DNA amplification step. Generally, LFD could finish within 10 minutes whereas agarose gel electrophoresis takes at least half an hour or more. In addition, LFD boosts the selective result and gives a lower limit of detection (LOD) due to the sequence-specific probe and probe hybridization reaction between LAMP product and conjugated gold nanoparticles. Briefly, biotinylated LAMP amplicons originated from biotin-tagged primer specifically hybridized with FITC-labelled probe forming the complexes. When the complexes reached the test line on dipstick which had embedded biotin binding molecules, streptavidin, they were fixed the tightly. Afterwards, anti-FITC coated gold nanoparticles conjugated with FITC tagged on the

complexes caused pink streak on the test line. On the other hand, if the complexes could not be formed because of lack of neither target DNA templates nor non-specific amplicons from LAMP reaction then, gold particles were not localized to the line resulting in colorless appearance over test line area.

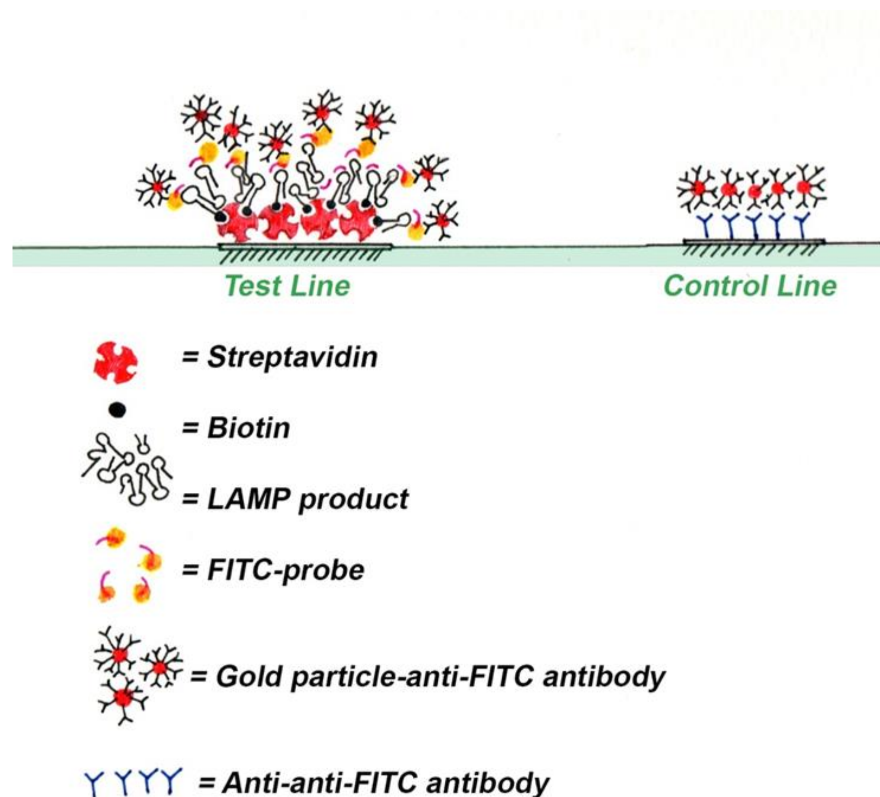

Figure 2. The graphic scheme of Lateral Flow Device (LFD) using in the DNAsensor E. canis test kit.

After the LAMP product was mixed with probe, the LFD will then be dipped into the mixture. The picture showed the test and control lines containing streptavidin and anti-anti FITC antibody. The biotinylated LAMP product with the attachment of FITC-probe will flow through the test line to the control line from left to right. The LAMP product will then traped at the test line. The gold particle conjugated with anti-FITC antibody subsequently bound to the biotinylated LAMP product making

test line in pink colored. The unbound or free gold particle conjugated with anti-FITC antibody then bound to the control line. As a result, the control line will be pink in color.

CHAPTER III

MATERIALS AND METHODS

3.1 Canine blood sample collection

In this study, all of blood specimens intentionally obtained from dogs either attending at Small Animal Teaching Hospital of Chulalongkorn University or other veterinary hospitals and shelters where canine ehrlichiosis suspected cases were prone to be found. At least 200 μL of blood sample was drawn from each specimen via cephalic or saphenous vein then collected in an EDTA containing tube. Each collected blood was kept in fridge at four degree Celsius then processed DNA isolation within 72 hours after collection. A total number of blood samples gathered in this study were 84 which could be equally divided into two groups that consisted of clinically CME suspected dogs and healthy ones. In canine monocytic ehrlichiosis suspected group, inclusive dogs were collected when they presented at least two of the most frequent apparent clinical signs of canine ehrlichiosis as following; body temperature >102.4 ºF, loss of appetite, depressed level of consciousness or weakness, number of platelet count <150,000/μL, pale mucous membrane, bleeding tendencies such as epistaxis, dermal hemorrhage, lymph nodes and/or splenic enlargements and tick infestation in the present or past. Previous doxycycline treatment prior to this study will be the exclusive criterion. In healthy group, dogs were selectively screened by having no previous illness history within three months, no noticeably clinical abnormalities from both routine physical examination and blood laboratory investigation including complete blood count, basic liver and kidney panels and thin

blood stain smear. This study was performed under Biosafety Use Protocol No. IBC 1831017 May 9, 2018- May 8, 2019 and Animal Use Protocol No. IACUC 1831008 July 2, 2018- July 1, 2019.

3.2 DNA preparation

Genetic material or DNA was isolated from blood samples by using commercial kits NucleoSpin® Blood (Macherly-Nagel GmbH & Co. KG, Germany). Briefly, 200 µL of each the blood was mixed with equal volume of Buffer B3 and 25 µL of Proteinase K from the kit then vortexed vigorously for 20 seconds. After leaving at room temperature for five minutes, the mixture was incubated at 70 ºC for 15 minutes. Afterwards, 210 µL of absolute ethanol was added into the mixture and re-vortex. Whole volume from each sample was put into NucleoSpin® Blood Column and centrifuged for a minute at 11,000 x g. resulted in DNA were fixed to silica membrane of column then the flow-through was discarded. The DNA-bound column was washed with 500 µL of Buffer BW and 600 µL of B5 respectively. When the silica membrane in the column was dry, 100 µL of Buffer BE was added onto the membrane to elute highly pure DNA ready for downstream processes. These eluted DNA were kept in freezer at -20 ºC until use in *E. canis* molecular identifications.

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3.3 DNA-based identification

3.3.1 The nested PCR protocol (nPCR)

Briefly in reaction mixture, both rounds of nPCR will be performed under the condition in a volume of 10 µL consisted of 5 µL of i-Taq PCR master mix (Intron®, Korea) containing reaction buffer, dNTP, Taq DNA polymerase and blue loading dye, 1 µL of each 10 µM primers and 2 µL of purified DNA containing as low as 50 ng of

DNA per microliter approximately. Sterile ultrapure water was added to adjust reaction volume to 10 µL. The product of first round PCR was ten-fold diluted before adding as DNA template of the second reaction.

The nested PCR protocol used to detect *E. canis* infection is modified from previous study (Ariyawutthiphan et al., 2008). PCR reactions were incubated in thermal cycler (LongGene®, China). The touch down PCR protocol was applied to achieve high specificity and yield of amplification. Starting with heated the reaction mixture at 94 ºC for 2 minutes then followed by six rounds of touch down protocol and 30 rounds of constant annealing temperature respectively. Briefly, in each PCR round, denaturing temperature was set at 94 ºC for 30 seconds. The annealing temperature in touch down process were 60, 58, and 56 ºC respectively which were repeated twice before decrements in two-degree Celsius steps to eventually down to 54 ºC that was the constant annealing temperature. These steps were held for 15 seconds in each PCR round. According to i-Taq manual, extensional temperature was at 72 ºC for 30 seconds. After total 36 cycles of amplification, the reactions were heat to 72 ºC for 5 minutes for final extension then reaction were stopped by kept them at four-degree Celcius until product detection.

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PCR amplicons were determined by putting four microliters of each PCR mixture to analyze with 1.5% agarose gel electrophoresis in Tris-Boric-EDTA buffer and visualized under a UV light after staining with ethidium bromide. The exact amplicon size of *E. canis* DNA when detected by this nPCR was 389 base pairs

3.3.2 The single PCR protocol (sPCR)

The single PCR was performed almost similarly to nested PCR protocol except it only used forward and reverse primers of the second round of nPCR. The products

of single PCR were also detected by agarose gel electrophoresis as previous mentioned.

3.3.3 *Ehrlichia canis* DNAsensor Kit

3.3.3.1 LAMP-LFD E. canis DNAsensor kit

E. canis DNAsensor Kit is the innovation employed the principle of Loop-mediated isothermal amplification with lateral flow dipstick (LAMP-LFD). This test is developed by Kespunyavee Bunroddith, Nareerat Viseshakul, Somchai Santiwatanakul, Thongchai Kaewphinit and Kosum Chansiri then won the international innovation contest receiving a Silver Medal Award from Taipei International Invention Show and Techmart and also the Outstanding Diploma for Excellent Invention, Taiwan, 2015. The methods and materials will be employed in this study; those are following the petit patent 1503000654 signed by Srinakarintrwirot University, 1 May 2015, Department of Intellectual Property, The Ministry of Commerce, Thailand.

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LAMP was carried out in the reaction of 20 µL mixture containing 0.8 µM of each FIP and BIP primers, 0.1 µL of each outer primer, 1 mM of dNTP, 20 mM, Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1 Triton X-100, 8 units of *Bst* DNA polymerase large fragment (New England Biolabs), 2 µL of purified DNA template equal to both PCR protocols and sterile ultrapure water for adjusting reaction volume to 20 µL. Each mixture was incubated at 65 ºC of an hour in thermal cycler (LongGene®, China) then LAMP amplicons were separately analyzed by two methods. First product detection method is 1.5% agarose gel electrophoresis same as amplicons of both PCR protocols and the second is using lateral flow dip stick as amplicon detector.

3.3.3.2 Validation of E. canis DNAsensor Kit assay

Total 84 blood samples were recruited into this study. With nested PCR, all samples could be classified as 42 *E. canis* positive samples and the others were *E. canis* negative. The *E. canis* specific nested PCR was used to be gold standard of detection in this study according to its effectiveness in pathogen identification. The results of *E. canis* DNAsensor Kit were statistically evaluated in contrast to the gold standard assay by MedCalC software.

CHAPTER IV

RESULTS

4.1 Canine blood sample collection and DNA preparation

Forty-two DNA samples comprising nPCR positives and other 42 of nPCR negative *Ehrlichia canis* were recruited in the trial (50% prevalence). The nPCR was used to clarify canine groups with or without CME. To process all samples at one time manner, therefore 6 batches of experiment were performed all at once. In each batch of samples there was 3 tests; LAMP-LFD, LAMP (LAMP-GE) and sPCR gel electrophoresis (sPCR-GE). Figure 3 through 8 showed the validation of sensitivity and specificity of 84 DNA samples in comparisons among methods while nPCR was used as a gold standard assay.

4.2 Three assays used in test kit validation: nPCR, sPCR and LAMP-LFD

Figure 3 showed results of LAMP-LFD and agarose gel electrophoresis of LAMP and sPCR products. There were 3 positive samples; lanes 1, 5 and 7 gave clearly LAMP-LFD positive test bands, however, weak signals occurred with LAMP-GE and sPCR-GE. Figure 4 showed all 7 positive nPCRs that gave 7 LAMP-LFD test bands but having none positive on sPCR-GE. Figure 5-8 showed results of method comparison for sample numbers 29-84. Figure 5 showed all 7 positive nPCRs that gave 5 LAMP-LFD test bands but having only 4 positives on sPCR-GE. Figure 6 showed all 7 positive nPCRs that gave 4 LAMP-LFD test bands but having only 3 positives on sPCR-GE. Figure 7 showed all 7 positive nPCRs that gave 4 LAMP-LFD test bands but having no positives on sPCR-GE. Figure 8 showed all 7 positive nPCRs that gave 4 LAMP-LFD test bands but having no positives on sPCR-GE. Here is a conclusion in Table 4.

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Table 4. The number of DNA samples tested with 3 different DNA methods; nPCR, LAMP-LFD and sPCR.

The total number of samples are 84 and 42 are tested positive for nPCR while 33 are positive with LAMP-LFD test.

Batches	Positive nPCR	Negative nPCR	Positive LAMP-LFD	Negative LAMP-LFD	Positive sPCR	Negative sPCR
1	7	7	3	7	3	7
2	7	7	7	7	0	7
3	7	7	5	7	5	7
4	7	7	7	7	5	7
5	7	7	4	7	0	7
6	7	7	7	7	4	7
Total	42	42	33	42	17	42

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The results from Table 4 and Figure 3 through 8 indicated that all assay gave 100% specificity, with no false positives. However, LAMP-LFD (33/42) has surprisingly more sensitive outcome than that of sPCR (17/42) when both tests were compared to nPCR.

The selective test of LAMP-LFD is determined against *Babesia canis*, *Hepatozoon canis* and the other commonly found as canine-related pathogenic

rickettsia in Thailand, *Anapalsma platys*. Results were showed in Figure 9. LAMP-LFD is highly selective to those pathogens giving clear test lines on the lateral flow dipsticks.

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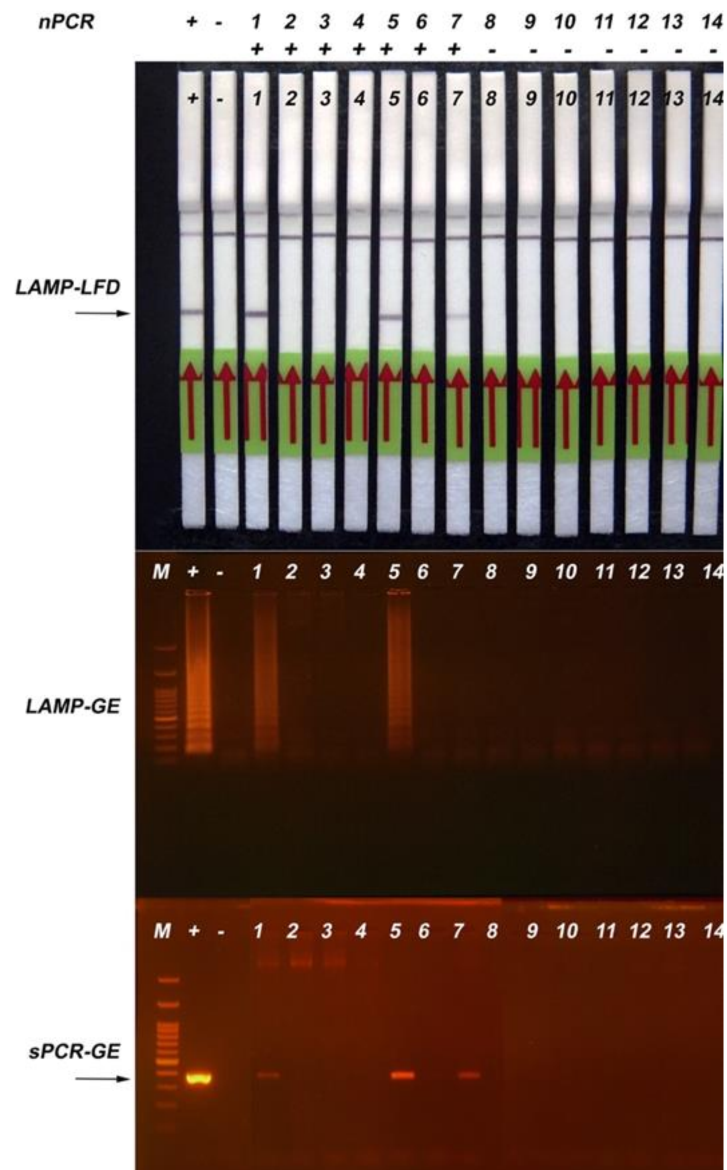

Figure 3. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 1-14

Lanes 1-7 showed results of sample number 1-7 positives and 8-14 negatives by nPCR. Sample numbers were written on the top of the figure including the recombinant plasmid containing 1049 bp of *16S rRNA* gene as a positive control (+). Non-template reaction is used as a blank control (-). Arrows showed the test bands of LAMP-LFD and sPCR respectively. M is 100 bp ladder DNA as a gel electrophoresis marker.

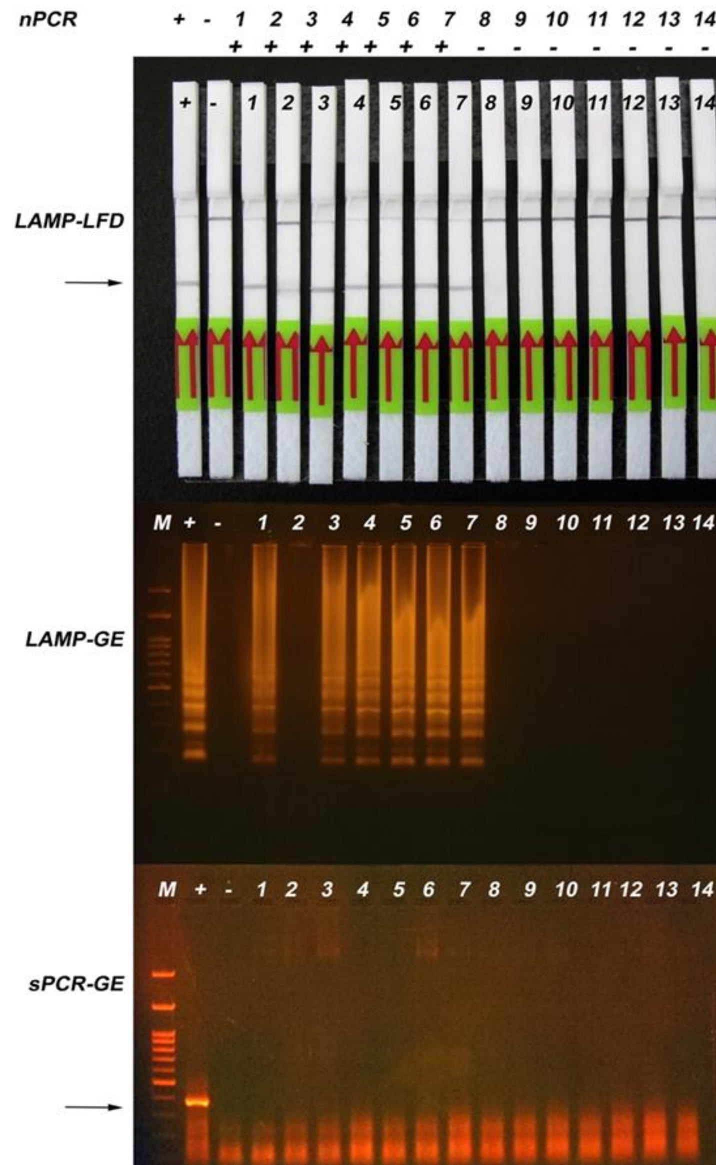

Figure 4. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 15-28

Lanes 1-7 showed results of sample number 15-21 positives and 22-28 negatives by nPCR. Sample numbers were written on the top of the figure including the recombinant plasmid containing 1049 bp of *16S rRNA* gene as a positive control (+). Non-template reaction is used as a blank control (-). Arrows showed the test bands of LAMP-LFD and sPCR respectively. M is 100 bp ladder DNA as a gel electrophoresis marker

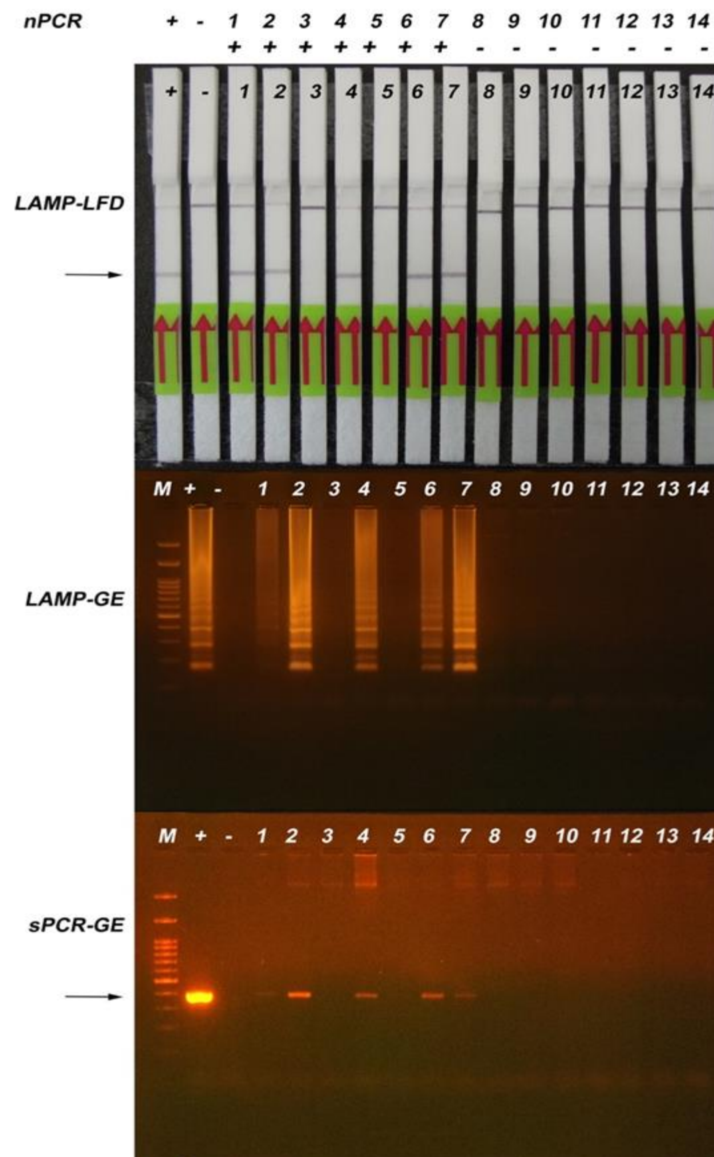

Figure 5. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 29-42

Lanes 1-7 showed results of sample number 29-35 positives and 36-42 negatives by nPCR. Sample numbers were written on the top of the figure including the recombinant plasmid containing 1049 bp of *16S rRNA* gene as a positive control (+). Non-template reaction is used as a blank control (-). Arrows showed the test bands of LAMP-LFD and sPCR respectively. M is 100 bp ladder DNA as a gel electrophoresis marker

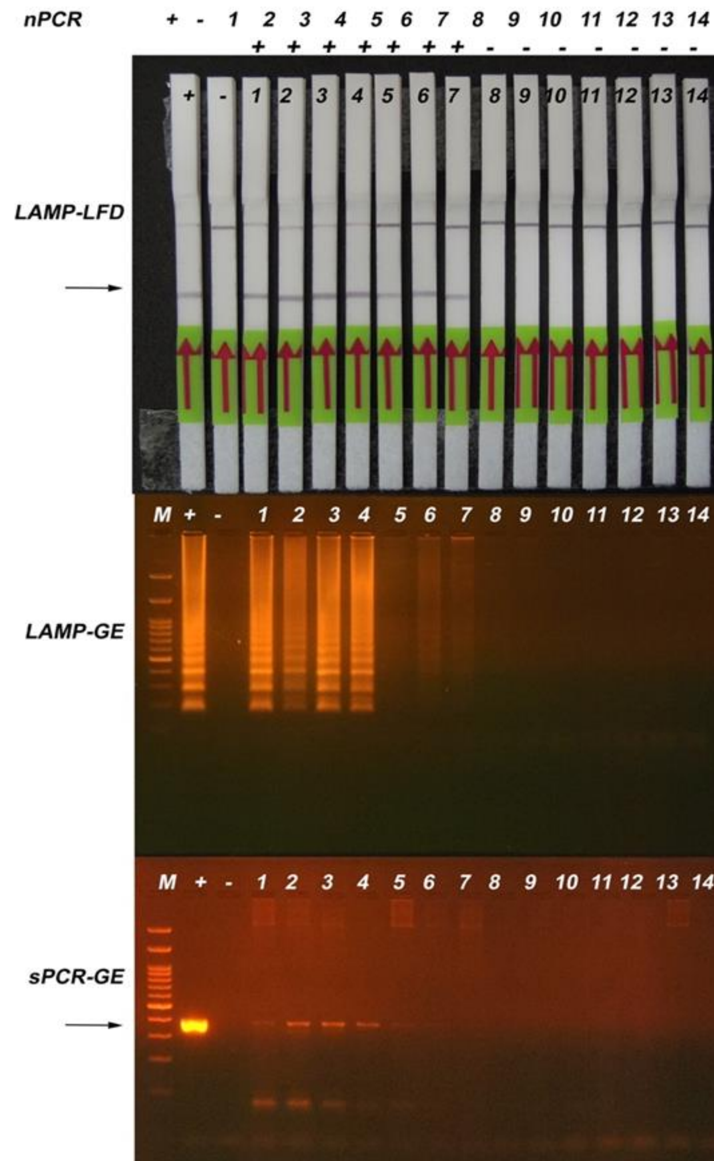

Figure 6. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 43-56.

Lanes 1-7 showed results of sample number 43-49 positives and 50-56 negatives by nPCR. Sample numbers were written on the top of the figure including the recombinant plasmid containing 1049 bp of *16S rRNA* gene as a positive control (+). Non-template reaction is used as a blank control (-). Arrows showed the test bands of LAMP-LFD and sPCR respectively. M is 100 bp ladder DNA as a gel electrophoresis marker

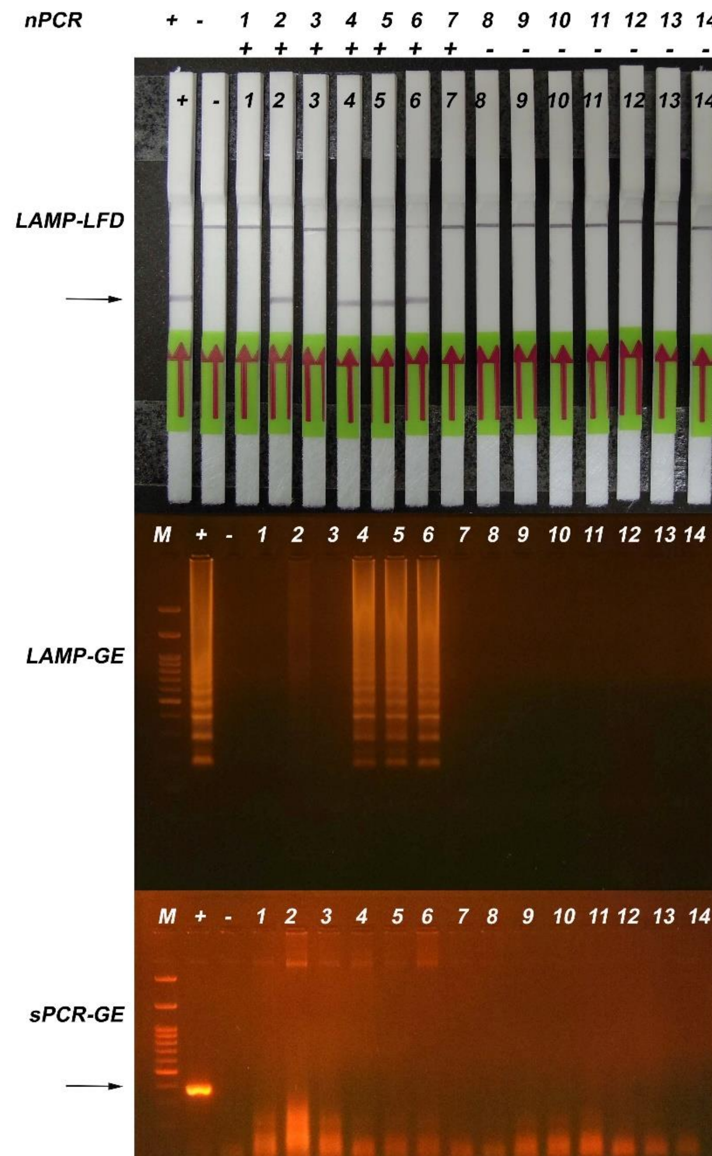

Figure 7. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 57-70

Lanes 1-7 showed results of sample number 57-63 positives and 64-70 negatives by nPCR. Sample numbers were written on the top of the figure including the recombinant plasmid containing 1049 bp of *16S rRNA* gene as a positive control (+). Non-template reaction is used as a blank control (-). Arrows showed the test bands of LAMP-LFD and sPCR respectively. M is 100 bp ladder DNA as a gel electrophoresis marker

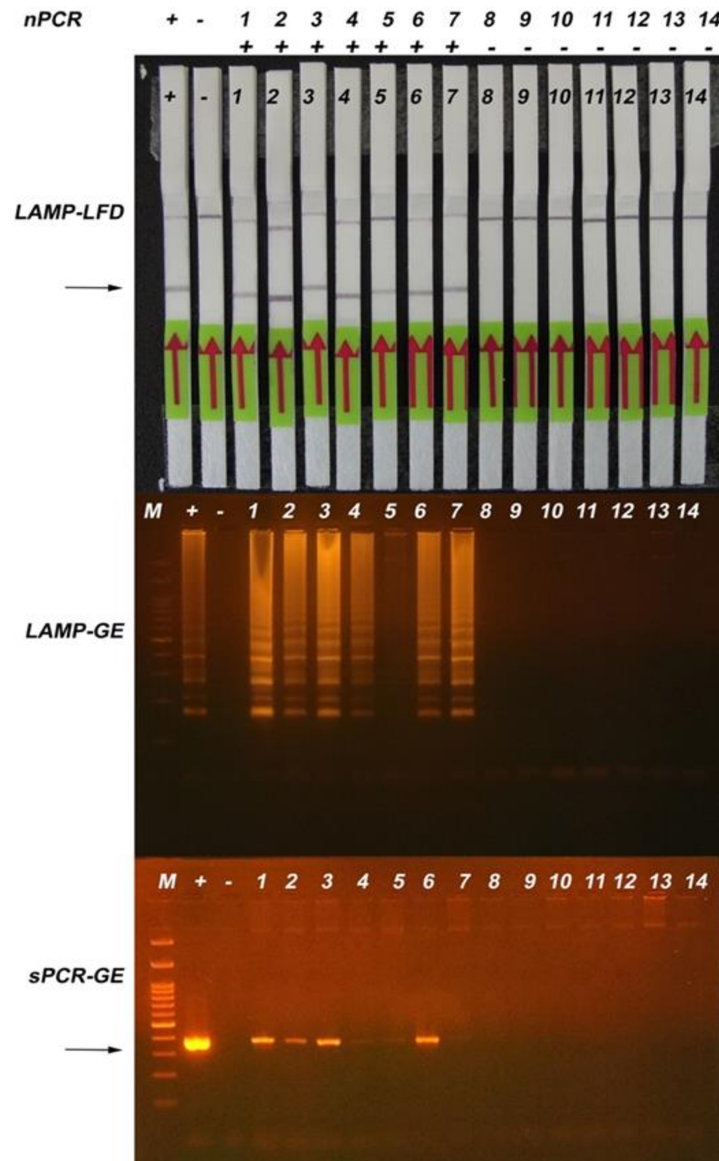

Figure 8. The detection sensitivity and specificity of assays; LAMP-LFD, LAMP-GE and sPCR-GE of samples 71-84

Lanes 1-7 showed results of sample number 71-77 positives and 78-84 negatives by nPCR. Sample numbers were written on the top of the figure including the recombinant plasmid containing 1049 bp of *16S rRNA* gene as a positive control (+). Non-template reaction is used as a blank control (-). Arrows showed the test bands of LAMP-LFD and sPCR respectively. M is 100 bp ladder DNA as a gel electrophoresis marker

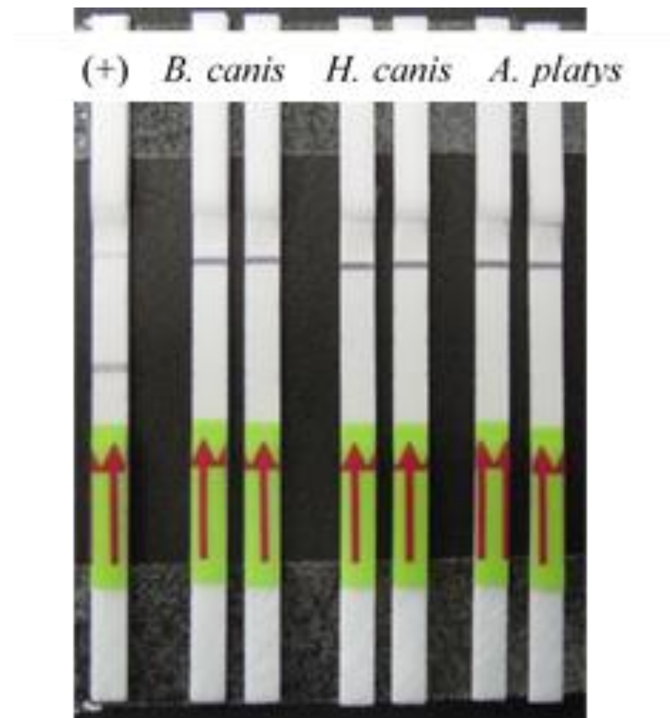

Figure 9. *The selective test of LAMP-LFD against other commonly found blood parasites in Thai domestic dogs.*

The test is determined against *Babesia canis*, *Hepatozoon canis* and *Anaplasma platys*.

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4.3 The selective test and limit of detection of LAMP-LFD

To ensure the selectivity of LAMP-LFD against most commonly found blood-borne pathogens of canine in Thailand. *Babesia canis*, *Hepatozoon canis* and *Anaplasma platys* were tested.

It was found that LAMP-LFD can only be selective for *Ehrlichia canis*, no other blood parasites. In this experiment, total genomic DNA of dogs infected with *Babesia canis*, *Hepatozoon canis* and *Anaplasma platys* was previously shown be PCRs to be positive.

Table 5. The limit of detection comparisons between assays; nPCR, SYBR Green Real time PCR, LAMP-LFD and sPCR.*

The recombinant plasmid containing 1049 bp fragment of *16S rRNA* is used to determine the copy number per microliter (µL) and the molecular weight of DNA template in the amplification reactions.

Copy numbers/µL	Weight (g)	nPCR	SYBR Green qPCR*	LAMP-LFD	sPCR
$1.3x10^{9}$	$4.6x10^{-9}$ ng	+	+	+	+
$1.3x10^{8}$	$4.6x10^{-10}$	+	+	+	+
$1.3x10^{7}$	$4.6x10^{-11}$	+	+	+	+
$1.3x10^{6}$	$4.6x10^{-12}$ pg	+	+	+	+
$1.3x10^{5}$	$4.6x10^{-13}$	+	+	+	+
$1.3x10^{4}$	$4.6x10^{-14}$	+	+	+	+
$1.3x10^{3}$	$4.6x10^{-15}$ fmg	+	+	+	+/-
$1.3x10^{2}$	$4.6x10^{-16}$	+	+	+	-
$1.3x10^{1}$	$4.6x10^{-17}$	+	+	-	-
$1.3x10^{0}$	$4.6x10^{-18}$ attg	+	+	-	-
$1.3x10^{-1}$	$4.6x10^{-19}$	-	-	-	-

Table 5 showed the efficiency of nPCR and SYBR Green real-time PCR is significantly equal. Both assays can detect the target DNA as low as 1 copy. When limit of detection of LAMP-LFD was focused, I found that although it has less

efficiency than nPCR however, it is more powerful than sPCR that can detect the target DNA as low as 100 copy numbers or in a femto gram (10^{-15} gram) range of molecular weight.

4.4 The statistical analysis for LAMP-LFD validation

The diagnostic test evaluation calculation is measured by Medcalc online free software program when results retrieved from Table 4 were applied. The sensitivity and specificity of the methods were determined.

Table 6. The two by two table used to calculate the sensitivity and specificity of LAMP-LFD compared to nPCR as the CME presence or absence

	CME Presence	CME Absence	Total
LAMP-LFD Positive	True Positive a =33	False Positive c =0	a+c =33
LAMP-LFD Negative	False Negative b =9	True Negative d =42	b+d =51
Total	a+b =42	c+d =42	a+b+c+d = 84

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The validation results showed that nPCR, LAMP-LFD and sPCR are equally in specificity value meanwhile, they have different degrees of sensitivity. nPCR is routinely used in our laboratory to identify the presence or absence of CME, because it is the most sensitive method. However, in the aspect of *E. canis* DNA-based detection, LAMP-LFD is capable of comparing with nPCR with 78.57% sensitivity (95% CI= 63.19%-89.70%). Conversely, sPCR is incomparable to nPCR at the level of 40.48% sensitivity (95% CI 25.63% to 56.72%).

The Cohen's kappa coefficient (κ) is also calculated. The function of κ revealed that nPCR and LAMP-LFD is in a substantial agreement (0.785, 89.28% agreement) which is surprisingly good in relation, but not sPCR. This means that LAMP-LFD was very likely to identify CME correctly as the disease presence. However, the level of LAMP-LFD sensitivity is lower than that of nPCR.

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CHAPTER IV

DISCUSSION

5.1 Canine blood sample collection and DNA preparation

Finding *Ehrlichia canis* DNA in the blood stream of canine with CME is the most commonly procedure for veterinary clinics and hospitals. Prior to PCRs detection became a standard assay of this disease, visualization of cytoplasmic morulae was applied. Blood sample was then a sample of choice for CME constantly. Blood sample was used as the source of CME prevalence which applied to not only the serology but also the DNA detection. Canine blood samples were collected during the year 2017-2018. The recruitment criteria of CME are based on the patient history examination, especially the tick harboring records and platelet counts. CME is often found in dogs with history of tick bites. CME suspected samples were collected from Small Animal Teaching Hospital – Chulalongkorn University, Suwannachard Animal Hospital, Police K-9 and K-9 Unit of Royal Thai Air Force. Sra Pathum and Suan Pathum Royal Palace were places to gather the negative group of CME-free samples.

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More than a total of 150 DNA samples were carefully purified and determined as the presence or absence of only CME no other blood parasites such as *Babesia canis*, *Hepatozoon canis* or *Anaplasma platys*. Those mentioned could be co-contaminated with CME. A single infection of CME was included in the trial, and co-infection was eliminated by using nPCR assay. Eighty-four total DNA purified samples were identified as 42 positives and 42 negatives to achieve 50% prevalence. This prevalence figure is statistically significant good for diagnostic test validation.

5.2 Primers design in the assays used in validation of test kit

When molecular detection using PCRs targeted *16S rRNA* gene instead of *p30* to detect *E. canis* DNA in the blood, ones should consider 2 important criteria. First is the limit of detection and second is the selectivity of the test. Some research groups used *p30* as an sPCR target because its appearance as multiple copies in the genome giving good limit of detection in femtogram level of DNA. Amplification of *p30* gene might enhance the molecular detection of CME (Stich et al., 2002; Felek et al., 2003). Some used *16S rRNA* as a target gene although this gene appears to be a single copy gene in a genome, but this gene is more selective than *p30*. Nested PCR, the reference method, targeted *16S rRNA* for the reasons of its high selectivity in species differentiation and identity of sequences of this gene in acute as well as severe chronic CME (Siarkou et al., 2007). Double amplification of PCR will increase the limit of detection. This procedure again is to enrich the PCR product and increasing the power of limit of detection.

Because these three DNA amplification assays were used in the trial and nPCR were the method of CME identification, primers design is therefore on *16S rRNA* gene. Whatever that is the nPCR, LAMP-LFD or sPCR, they must be on the same region of *16S rRNA* as shown in Figure 1. All primers were located on the 5' end of *16S rRNA* gene. This primer design is intentional to emphasize all measures of validation across the test assays, the limit of detection, sensitivity and specificity.

5.3 Test kit validation

Validation of LAMP-LFD was performed as the test to identify the presence or absence of CME. Figure 3 through 8 showed that LAMP-LFD is a more sensitive assay than sPCR. Not only sensitive, when limit of detection of LAMP-LFD was

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

experimented, it can identify target DNA as low as 2 orders or 100 times less than that of sPCR (Table 5).

This is the first time ever that DNA assays of CME is in comparisons across different platforms of enzymatic assay such as Taq polymerase of PCRs over *Bst* polymerase of LAMP assay. LAMP showed lower limit of detection in numerous reports however, there were no records of how many orders of target copy numbers can be amplified by LAMP-LFD. In this study, it is confident that LAMP-LFD can replace the application of sPCR in aspects regarding of both the powerful limit of detection and the superior sensitivity.

The determination of Cohen's Kappa statistics is to express the reliability of the diagnostic test. LAMP-LFD gave a good agreement with nPCR with $\kappa = 0.785$. The meaning κ as high as 0.785 is in the level of substantial agreement with the gold standard nPCR. LAMP-LFD can similarly identify the presence of CME as well as nPCR, only less sensitive than the former test (Table 6) (Landis and Koch, 1977). If other described criterion is used to determine the reliability of LAMP-LFD, it will give $\kappa=0.785$ as the excellent agreement (Fleiss et al., 2003).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Taken all mentioned together of test kit validation measures, The application of LAMP-LFD is hence capable of being not only an alternative method of molecular diagnostic test for CME but a point-of-veterinary care device also.

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