Comparative Studies on Nucleic Acid Based Biosensors for Identification of Filarial Nematode

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Background: *The microfilarial nematodes, found mainly in blood circulation, form a special group of human and veterinary parasitic diseases. Recently, the rapid nucleic acid based biosensors (NABs) have been established in our laboratory for the employment of a point-of-care diagnostic test. Herein, NABs involved in the exploitation of lateral flow dipstick (LFD), gold nanoparticle (AuNP) and turbidity in combination with PCR and LAMP amplification for detection of filarial nematodes. The validation of each NABs was investigated in comparison to that of standard detection methods using the same unknown blood specimens.*
Objective: *To compare the sensitivity, specificity and accuracy of NABs with standard detection methods.*
Material and Method: *In this study, the microfilariae of Dirofilaria immitis was used as the representative model for filarial nematode. The PCR and LAMP primers were designed and synthesized according to the specific nucleotide regions of a small subunit gene of the parasite as well as the DNA probes. The fifty unknown blood samples were sent as a gift from Prasu Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University. The samples were tested by using PCR, PCR-LFD, PCR-AuNP, LAMP, LAMP-LFD, LAMP-AuNP, LAMP-turbidity and film blood smear. The efficacy of NABs was compared to a standard film blood smear in terms of sensitivity, specificity and accuracy.*
Results: *Upon detection of fifty unknown blood samples, LAMP-LFD assay presented 100% of sensitivity, specificity and accuracy. The data revealed that sensitivity, specificity and accuracy of NABs varied from 66.67-100.00% when compared to a film blood smear stained with Giemsa dye. The data clearly indicated that LAMP-LFD was a preferred choice for the use as a point-of-care NABs.*
Conclusion: *NABs were highly sensitive performing as a selective diagnostic tool that could be applied particularly as the rapid screening tests for filarial nematodes hence as a recommended epidemiological survey.*

Keywords: *Filaria, Nucleic acid based biosensor, Lateral flow dipstick, Gold nanoparticle, Turbidity assay*

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The filarial nematodes, producing microfilariae in blood circulation, classifies as an important group of human and veterinary helminthic parasites. The diseases caused by infection with these parasites via mosquito vectors include lymphatic filariasis (LF) and onchocerciasis of humans[1], and dirofilariasis (heartworm) in dogs and cats[2]. Filarial nematodes have an indirect life cycle and use blood-feeding arthropods such as mosquitoes or blackflies as intermediate hosts and vectors[3]. The biting insects take up the microfilariae (Mf), which are circulating in the blood for years. Current tools available to monitor LF are limited to diagnostic tests targeting DNA repeats, filarial antigens, and anti-filarial antibodies[4]. At the present, the gold standard for detection filarial nematode is morphological detection in microfilarial stage with film blood smear stained with Giemsa dye. This method is relied on specialist training to accurately differentiate species of microfilariae[5,6]. Currently, various molecular methods for detection filarial nematodes are more reliable based on the highly sensitive and selective PCR[7,8] and LAMP-based methods[9,10]. Previously, the NABs have been examined in our laboratory involving the addition of DNA probe after DNA amplification and the DNA-DNA hybridization to increase specificity of the test prior to observation by using LFD[11,12] or

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gold nanoparticle (AuNP) protocols[13]. Similarly, LAMP-turbidity has been verified for detection of various infectious pathogens by observing the change of precipitation during LAMP amplification.

Herein, the PCR-LFD, PCR-AuNP, LAMP-LFD, LAMP-AuNP and LAMP-turbidity were performed and analyzed using canine filarial *Dirofilaria immitis* as the pathogen model. The sensitivity, specificity, prevalence and accuracy of NABs were achieved against standard routine methods.

Material and Method

Sample collection

The fifty unknown blood samples from dogs were gifted from Prasu Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University, Thailand. The unknown DNA were extracted by using NucleoSpin® Blood (Macherey-Nagel, Germany) prior to investigation by using NABs methods in comparison to standard routine diagnosis. The purified genomic DNA was stored in distilled water at 4°C until use.

Polymerase chain reaction (PCR)

PCR primers and DNA probe

The primers (petty patent application number 1603001290) were designed specifically for *D. immitis* by using Primer Explorer software version 4. (http://primerexplorer.jp/elamp4.0.0/index.html).The DNA probe for hybridization with PCR products was designed between forward primer and reverse primer (Bio Basic, Canada).

PCR amplification

The PCR reaction was carried out in 25 µl containing 2 µl of genomic DNA, 1xTaq buffer containing 0.2 mM dNTPs (New England Biolabs, Ipswich, MA, USA), 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Invitrogen, USA) and 0.3 µM of each of the 2 primers. The PCR reaction was heated at 94°C for 3 minutes, before proceeding to 30 cycles of denaturation step at 94°C for 1 minute, annealing step at 50°C for 1 minute, extension step at 72°C for 1 minute. The reaction was then incubated at 72°C for another 2 minutes. PCR products were analyzed by using 2.0% agarose gel electrophoresis in 0.5x Tris/Borate/EDTA (TBE) buffer at 100 volt. The PCR amplification profile was observed under ultraviolet (UV) light after ethidium bromide staining.

Loop mediated isothermal amplification (LAMP)

LAMP primers and DNA probe

LAMP primers and DNA probe (petty patent application number 1603001290) were designed specifically for *D. immitis* by using Primer Explorer software version 4 (http://primerexplorer.jp/elamp4.0.0/index.html). The DNA probe for hybridization with LAMP products was designed within inner forward primer. The primers and DNA probe were synthesized by Bio Basic, Canada.

LAMP amplification

LAMP amplification was performed in 25 µl reaction containing 2 µM each of FIP and BIP, 0.2 µM each of F3 and B3, 1x Isothermopol-supplied reaction buffer, 0.5 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 6 mM $MgSO_4$ (New England Biolabs, Ipswich, MA, USA), 1.6 mM dNTP mix (New England Biolabs, Ipswich, MA, USA), 8 Units of *Bst*II DNA polymerase (New England Biolabs, Ipswich, MA, USA), and 2 µL of DNA template. The LAMP reaction was incubated at 62°C for 60 minutes. Prior to analysis by using 2.0% agarose gel electrophoresis in 0.5x Tris/Borate/EDTA (TBE) buffer at 100 volts. The LAMP amplification profile was observed under UV light after ethidium bromide staining.

Lateral flow dipstick (LFD)

The DNA probes were labeled with FITC at 5' end of the sequence. After PCR and LAMP amplification, the mixture was heated at 95°C for 5 minute. The hybridization was achieved by the addition of 1 µM FITC-DNA probe into 10 µl containing biotin-labeled PCR or LAMP products and incubated at 53°C and 62°C, respectively for 10 minute. After hybridization, 10 µl of the mixture were transferred to the new tube containing 100 µl of the assay buffer. The LFD (Milenia Genline HybriDetect, Germany) was subsequently placed into the prepared solution and left for 10 minutes until the color bands were observed.

AuNP assay

For hybridization, each of PCR and LAMP products were mixed with AuNPs/DNA probe complex in ratio 1: 1 by v/v. The mixture was hybridized at 63°C for 10 minutes prior to the addition of 0.01M $MgSO_4$. The change of solution color was directly observed by eyes. The AuNPs/DNA probe complex solution was determined by NanoDrop™ 2000 Spectrophotometer (Thermo Scientific; Wilmington, DE, USA).

LAMP-turbidity assay

Turbidity assay was achieved by using the

LAMP real-time turbidimeter (Mobilis Automata, Thailand). LAMP reactions were carried out at 62°C for 60 minutes in 0.2 ml thin wall polypropylene tube. The reactions were performed in a 25 µL of total LAMP reaction mixture as described previously. Mineral oil was added onto the surface of solution to prevent evaporation. The data was achieved by observing the correlation between the absorbance of magnesium-DNA pyrophosphate complex and the reaction time.

Sensitivity test of PCR and LAMP

The 10 fold serial dilutions of DNA were prepared prior to DNA amplification and hybridization. The amplicons were further analyzed using 2.0% agarose gel electrophoresis, LFD, AuNP assay and turbidity assay.

Specificity test

The specificity of each NABs were exploited by using *Babesia canis, Ehrlichia canis, Haemoplasma canis, Anaplasma platys*, all are pathogens that could be found in host blood circulation, and host DNA (dog) as negative controls.

Validation of NABs

Accuracy and prevalence of NABs were calculated according to Miks-Krajnik[14] and Anthony J. Alberg[15] as indicated in Table 2.

Results

The unknown samples test

The data of fifty unknown blood samples determined by using NABs and standard methods was shown in Table 1. The data stated that LAMP-LFD assay exhibited the 100% of sensitivity and specificity (Table 2). Among NABs, the LAMP-LFD has fulfilled all criteria of validation. It was clear that LAMP-based methods demonstrated the better precision and accuracy than those of PCR. In contrast, LAMP-Turbidity assay appeared to create the lowest sensitivity and specificity among all tested NABs since its data was correlated to those of standard film blood smear.

The whole blood-EDTA of fifty out-door dogs admitted to the clinic, without either a specific signs history of having heartworm disease, anti-microfilaria treatment or serious clinical signs. However, they were all having a slightly coughing showing a respiratory disturbance. The coughing and respiratory signs are ones of the symptoms associated with heart worm disease[2,16]. The sexes, ages and breeds of dogs

Table 1. Comparison of NABs for detection of *D. immitis*

Methods	50 unknown samples	
	Positive	Negative
Film blood smear	22	28
PCR-gel electrophoresis	25	25
LAMP-gel electrophoresis	27	23
PCR-LFD	27	23
LAMP-LFD	29	21
PCR-AuNP	25	25
LAMP-AuNP	27	23
LAMP-Turbidity assay	22	28

Table 2. The sensitivity and specificity tests in percentage compared within 4 groups of which gave the highest to lowest percent heartworm disease prevalence (n = 50) of 7 different DNA biosensor diagnostic assays and 1 gold standard film blood smear of microfilariae stained with Giemsa dye

	Group 1	Group 2	Group 3	Group 4
Assays	LAMP-LFD	LAMP-Gel electrophoresis LAMP-AuNP PCR-LFD	PCR-Gel electrophoresis PCR-AuNP	Film blood Smear LAMP-Turbidity
Prevalence[a]	58.00% (29/50)	54.00% (27/50)	50.00% (25/50)	44.00% (22/50)
Sensitivity[b]	100.00%	93.10%	86.21%	75.86%
Specificity[c]	100.00%	91.30%	84.00%	66.67%
Accuracy[d]	100.00%	92.27%	85.11%	70.72%

[a] Prevalence = [(TP+FN)/(TP+TN+FP+FN)x100]; [b] Sensitivity = [TP/(TP+FN)x100]; [c] Specificity = [TN/(TN+FP)]x100]; [d] Accuracy = [(TP+TN)/(TP+TN+FP+FN)x100]

TP = True positive; TN = True negative; FP = False positive; FN = False negative

were randomly selected. We also assumed that these animals have such high chance of heart worm infection, due to the mosquito vector of canine filariae is endemic in Bangkok and tropical areas worldwide.

All 50 samples were used in this test as the case-control study retrospectively in the year 2014-2015. We demonstrated the use of 7 DNA biosensor assays and results were compared in a simple way of field trial epidemiological investigation. Because LAMP-LFD gave the highest disease prevalence in this small group of animals (Table 2), Group 1 having the highest number of positive result (29/50) containing only the assay of LAMP-LFD. LAMP-LFD, for this reason, was to be the highest sensitive and specific assay compared to others. Using the 2 by 2 Table in the screening test model, probability value was calculated[17].

LFD based sensing method is preferred with probe in the final step of detection following either PCR or LAMP. This method will give the best result with the highest sensitivity and specificity compared to all. Although the LAMP turbidity assay is the simplest and cost effective one however, the lower value in sensitivity (75.86%) without the specific designed probe for detection giving this assay an unreliable resulting in the indecisive method of choice.

Discussion

Currently, the standard diagnostic tool, a microscopic film blood smear, for identification of *D. immitis* are very limited in terms of their applicability to large-scale surveys and in terms of species identification. Carriers of the parasites are typically identified by the detection of microfilariae using light microscopy in stained blood smears that may be limited when the low parasitemia samples were investigated. Film blood smear assay has a limit in specification of *D. immitis* because other species of such as *D. repens* or *Acanthocheilonema reconditum* could also be found with a similar shape and length.

Serological assay has been widely used for the diagnosis of parasitic infection on the basis of the specific binding between antigen and antibody. However, the false positive may occur when the other nematodes were co-infected. Up to 1% of infected dogs are microfilaria-positive and antigen-negative[18]. In any detection based on DNA amplification, specific primers combined with specific DNA probes are very critical. PCR and LAMP are sensitive and accurate tools to discriminate microfilariae from the different filarial worms that infect dogs.

LAMP-LFD has created a higher positive value compared to the microscopic method. This is due to the specific reaction and probe could detect DNA either from the intact or unintact cells of microfilaria in bloodstream, while the film blood smear could only detect the intact microfilariae. Consequently, the comparison of accuracy among NABs assays against gold standard film blood smear revealed that LAMP-LFD assay was the most accuracy test. Any unknown blood samples had not found microfilaria by film blood smear, but could extract DNA from decay of microfilaria. Therefore, the NABs could detect positive unknown samples more than film blood smear, gold standard assay.

Regarding to the specificity test, no cross-hybridization dog such as *B. canis*, *E. canis*, *H. canis*, and *A. platys* were observed of all tests. They all showed highly specific detection to *D. immitis* due to the efficacy of primers and DNA probes that were designed base on species specific.

Conclusion

The investigation of *D. immitis* in the unknown samples using seven different NABs revealed that all detection methods were highly sensitive and selective. The major advantages of NABs are the ability to amplify a desired target DNA from the host pathogen using PCR or LAMP and consequently augment the signal generated by integration with LFD, AuNP or precipitation of magnesium pyrophosphate. This study revealed that LAMP-LFD have proven a promising method in the detection and diagnosis of filarial diseases. Integrated sample preparation with isothermal DNA amplification and detection in a robust, cheaper and user-friendly manner like LFD can be applicable as point-of-care diagnostic tool. In addition, the high stability and specificity of NABs can be promising candidates in the future for clinical diagnostic market. The reliable analytical diagnostic test kits which can perform rapid and accurate analyses will be essentially dependent on the state-of-the-art NABs.

What is already known on this topic?

The filarial nematodes, producing microfilariae in blood circulation, cause of morbidity in humans and animals. Routinely, identification of microfilariae using microscopic examination of the morphological criteria can be difficult and lead to misdiagnosis. At present, various molecular methods for detection filarial nematodes are relied on the highly sensitive and selective PCR and LAMP-based methods.

What this study adds?

In this study, the rapid NABs involving lateral flow dipstick (LFD), gold nanoparticle (AuNP) and turbidity have been established for detection of *D. immitis* microfilariae in combination with PCR and LAMP amplification. The sensitivity, specificity, accuracy, and prevalence of each methods were compared against standard microscopic technique.

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Potential conflicts of interest

None.

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การศึกษาเปรียบเทียบการตรวจวินิจฉัยหนอนพยาธิฟิลาเรียด้วย Nucleic Acid Based Biosensors

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ภูมิหลัง: หนอนพยาธิไมโครฟิลาเรียเป็นปรสิตที่พบในกระแสเลือดเป็นหลักมีความสำคัญในด้านการแพทย์และด้านสัตวแพทย์ ในปัจจุบันผู้นิพนธ์ได้พัฒนาวิธี Nucleic Acid Based Biosensors (NABs) มาประยุกต์ใช้ในการตรวจหนอนพยาธิฟิลาเรียได้อย่างรวดเร็ว เพื่อใช้เป็นชุดตรวจแบบ point-of-care โดยการเพิ่มจำนวนของกรดนิวคลีอิกด้วยวิธีพีซีอาร์ และวิธีแลมป์ร่วมกับแผ่นตรวจวัดแบบแถบสีอนุภาคนาโนทองคำ และการวัดความขุ่นของปฏิกิริยา พร้อมทั้งเปรียบเทียบผลการตรวจด้วยวิธี NABs กับผลที่ได้จากการตรวจด้วยวิธีมาตรฐานในตัวอย่างเดียวกัน

วัตถุประสงค์: เพื่อเปรียบเทียบวิธี NABs กับวิธีการตรวจแบบมาตรฐานในด้านความไว ความจำเพาะ และความถูกต้อง

วัสดุและวิธีการ: งานวิจัยนี้ใช้ Dirofilaria immitis ระยะไมโครฟิลาเรียเป็นตัวแทนของหนอนพยาธิฟิลาเรีย ชุดไพรเมอร์ของพีซีอาร์แลมป์ และดีเอ็นเอตรวจจับถูกออกแบบให้มีความจำเพาะต่อดีเอ็นเอเป้าหมายในส่วนของจีน small subunit ตัวอย่างสุนัขจำนวน 50 ตัวอย่าง ได้รับจากโรงพยาบาลสัตว์ประสุอาทร คณะสัตวแพทย์ มหาวิทยาลัยมหิดล ตัวอย่างเลือดถูกนำมาทดสอบด้วยวิธีการเพิ่มปริมาณดีเอ็นเอแบบพีซีอาร์และแลมป์ร่วมกับวิธีตรวจวัดแบบแถบสี อนุภาคนาโนทองคำและการวัดความขุ่นของปฏิกิริยาและการตรวจด้วยวิธีสไลด์ฟิลม์เลือด ทำการเปรียบเทียบประสิทธิภาพของการตรวจด้วยวิธี NABs กับการตรวจด้วยวิธีสไลด์ฟิลม์เลือดในด้านความไว ความจำเพาะ และความถูกต้อง

ผลการศึกษา: ผลจากการตรวจเลือดจำนวน 50 ตัวอย่าง ด้วยวิธี NABs เปรียบเทียบกับวิธีสไลด์ฟิลม์เลือดพบว่าวิธี LAMP-LFD มีความไว ความจำเพาะและค่าความถูกต้อง 100% จากผลการทดลองวิธี NABs มีความไว ความจำเพาะ และค่าความถูกต้องอยู่ในช่วง 66.67-100.00% จากผลการเปรียบเทียบพบว่าการเพิ่มปริมาณดีเอ็นเอแบบแลมป์ร่วมกับแผ่นตรวจวัดแบบแถบสี เป็นวิธีการตรวจที่มีความเหมาะสมในการพัฒนาเป็น point-of-care NABs เพื่อตรวจหนอนพยาธิฟิลาเรีย

สรุป: วิธี NABs มีความไวและความจำเพาะสูง จึงเหมาะสมที่จะนำไปประยุกต์ใช้เป็นเครื่องมือตรวจหนอนพยาธิเท้าช้างในห้องปฏิบัติการและการสำรวจการระบาด