Original Article

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Development of On-site Molecular Diagnostics of *Nosema* diseases in Honeybee

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Abstract

Nosema is a parasitic microsporidia fungal disease. There are now two different types of *Nosema: N. apis* and *N. ceranae*. *Nosema* affects adult bees only by infecting epithelial cells lining the mid-gut after spores are ingested. In this study, the LAMP (Loop-mediated Isothermal amplification) detection method againsts *Nosema* diseases which can be easily recognized its result by naked eye instead of electrophoresis was developed. Nucleic acid fluorescent dye, SYBR Green-and Gene-Finder^{TM}, were adopted in this method and real-time thermal cycler and UF-PCR equipment were also used. The new method can perform the amplification of target DNA successfully within 30 minutes and againsts each pathogen by a successfully amplification from 1×10^2 molecules of template DNA with quantitative manner in real-time. The new method was demonstrated to be a specific, sensitive and easy tool for on-site detection. Thus, it may be useful for the monitoring of natural infection in *N. apis* and *N. ceranae*.

Key words: *Nosema apis*, *Nosema ceranae*, LAMP, Detection

INTRODUCTION

Nosemosis is caused by the obligate intracellular and spore-forming parasites, *Nosema apis* and *Nosema ceranae*, which attack the lining of the middle intestine of the worker, queen, and drones of honeybee (Bailey, 1955). *N. apis* infecting *Apis mellifera* was first described in 1907 by Enock Zander and to be considered as the perpetrator of *Nosema* disease in honeybee *A. mellifera* for a long time (Higes *et al*., 2007). *N. apis* is generally considered to be not severe pathogenic, but be prevalent throughout the world (Fries *et al*., 1984; Anderson and Giacon, 1992). On the other hand, *N. ceranae* infecting *Apis cerana* was first discovered in 1994 (Fries *et al*., 1996).

Nosema is transmitted from the worker bees feed on exchanges (Smith, 2012). *Nosemosis* can induce queen supercedure (Webster *et al*., 2004), reduce pollen collection (Anderon and Giacon, 1992), and shorten bee life span (Wang and Moeller, 1970).

According to the detection result about the twelve kinds of honeybee disease published by Korea animal and plant quarantine agency in 2013, out of which the occurrence of *Nosema* disease was found the highest level (36.49%). The timing of *Nosema* diagnosis is very important, because *Nosema* spp. infestation is associated with reduced honey production and increased mortality in winter (Higes *et al*., 2006, 2007) and lead to shorten the worker life span and considerable weakening of heavily infected colonies, resulting in significant economic damage (Fries *et al*., 1984; Anderson and Giacon, 1992).

Nosema disease diagnostic methods using PCR has already been developed (Lim *et al*., 2014; Byeon *et al*.,

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2014; Yoo *et al*., 2011). However, the detection of disease in laboratory takes a long time and on-site detection is difficult because these detection methods need the expensive equipment. Also no method is available to distinguish between *N. apis* and *N. ceranae*.

Loop-mediated Isothermal Amplification (LAMP) method developed in 2000 has a high specificity and sensitivity as a new method for quickly amplifying a nucleic acid in isothermal conditions (Notomi *et al*., 2000). LAMP method has three main features. Firstly, all reactions are carried out under isothermal conditions. Compared to PCR and real-time PCR assays, LAMP is requiring only heat block or regular laboratory water bath for reaction instead of expensive equipment like thermocycler (Ushikubo, 2004). Secondly, the amplification efficiency is extremely high, resulting in a tremendous amount of amplification products (Yamazaki *et al*., 2008). Thirdly, the method has a high specificity (Yamazaki *et al*., 2009).

Genechecker™ device (Dongwoo Science Company, Korea) developed in 2013 incorporates the high level of real-time fluorescence detection technology and it is possible to examine the reaction results through a window of the above equipment. The equipment is also small and easy to carry. By using 12V DC voltage, this equipment is able to be connected to the vehicle battery or power source and on-site analysis is possible.

In this study, by combining the advantages of the LAMP method and the Genechecker™, the new on-site molecular diagnostic method was developed that is capable to detect *N. apis* and/or *N. ceranae* separately.

MATERIALS AND METHODS

Infected honeybee

All infected honeybees and larvae were collected from apiaries in whole of 2014 from Jeju and Ulsan, Korea. These samples were stored at −70°C until processed.

Genomic DNA extraction

Larvae and adult honeybee were homogenized at 6000 rpm for 60 sec by MagNa Lyser (Roche, Switzerland) and genomic DNA of each sample was extracted using AccuPrep® Genomic DNA Extraction Kit (Bioneer Inc., Korea). The experiments were carried out according to the instructions of the manufacturer. The final concentration of total genomic DNA was determined using a spectrophotometer (Effendorf, Germany) before applying to the LAMP reaction. All genomic DNA was stored at −70°C until processed.

Template DNAs preparation

pBX-Nosema apis (Lim *et al*., 2014) and pBX-Nosema (Yoo *et al*., 2008) were used as standard templates. pBX-Nosema apis includes the sequence that shows 99% homology with *N. apis* sequence (GenBank No.U97150). pBX-Nosema includes the sequence that show 99% homology with *Nosema ceranae* sequence (GenBank No.DQ486027). Each clone used to template for optimized condition and test the sensitivity of specific LAMPs.

Specific primer sets for LAMP

N. apis-specific LAMP primers were designed based on the sequence of large subunit ribosomal RNA of *N. apis* (GenBank No.U97150, 1860-2320nt). Specific primer sets were consisting of four primers: two outer primers (*N. apis* F3 and *N. apis* B3) and two inner primers (*N. apis* FIP consisting of F2 and F1c sequences and *N.apis* BIP consisting of B2 and B1c sequences) (Fig. 1 and Table 1). All primers were synthesized by Bionics Corporation, Korea.

N.ceranae-specific LAMP primers were designed by Lee *et al*. (2010). They were Nosema F3 (25nt), Nosema B3 (19nt), Nosema FIP (45nt), and Nosema BIP (48nt) (Table 1)

Optimization of temperature for LAMP reaction

Reaction were performed in a total 25μl volume in case of *N. apis*-specific LAMP, containing 20pmole each of N-apis F3, N-apis B3, 80pmole each of N-apis FIP, N-apis BIP, 10mM dNTPs, 8 units of *Bst* DNA polymerase large fragment (New England Biolabs), 10X ThermoPol Reaction Buffer (20mM Tris-HCl, 10mM KCl, 10mM $(NH4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100), and

template DNA. The mixture was incubated for 60 min at 41.5~57.0°C and the reaction was terminated by heating to 85°C for 10 min. After LAMP reaction, the amplicons were confirmed by agarose-gel electrophoresis.

Optimization of condition for LAMP reaction

In order to establish the optimal reaction conditions for *N. apis*-LAMP, the optimum conditions of the LAMP components were confirmed. The ratio of the outer primer and inner primer (1:4) showed the highest efficiency (Notomi *et al*., 2000). This ratio is maintained and the reactions were examined by changing only the absolute concentration of the primer. In other words, when the concentration of each outer primer was 5pmole, 10pmole, 15pmole, 20pmole, 25pmole, the concentration of each inner primers was 20pmole, 40pmole, 60pmole, 80pmole, 100pmole. To optimize the concentration of dNTP, the reactions were examined by using the each dNTP that was set as 2.5mM, 5.0mM, 7.5mM, 10.0mM, 12.5mM, 15.0mM, and to optimize concentration of BST polymerase, each 4U, 8U, 12U, 16U, 20U of the *Bst* polymerase was used in LAMP reaction.

Construction of the Loop primers

The addition of Loop primers might accelerate the LAMP reaction (Nagamine *et al*., 2002). Each loop primer set for *N.apis*-specific LAMP and *N.ceranae*-specific LAMP was designed. The loop primer set for *N.apis*-specific LAMP was designed based on *N. apis* large subunit ribosomal RNA gene (GenBank No.U97150). Forward loop primer called *N. apis* Loop F was designed to locate between the F2C and F1C that were complementary sequence of *N. apis* sense sequence. And reverse loop primer called *N. apis* Loop B was designed to locate between the B1C and B2C that were complementary sequence of *N. apis* anti-sense sequence (Fig. 4A).

The loop primer set for *N. ceranae*-specific LAMP was designed based on *Nosema ceranae* small subunit ribosomal RNA gene (GenBank No.DQ486027). Forward Loop primer called Nosema Loop F was designed to locate between the F2C and F1C that were complementary sequence of *N. ceranae* sense sequence. And reverse loop primer called Nosema Loop B was designed to locate between the B1C and B2C that were complementary sequence of *N. ceranae* anti-sense sequence (Fig. 4B).

Determination of reaction time through the Real-time LAMP assay

To determine the *N. apis*-specific LAMP reaction time according to the DNA molecules for *N. apis* and the *N. ceranae*-specific LAMP reaction time according to the DNA molecules for *N. ceranae*, each of the real-time LAMP was carried out using 10-fold serial dilution of each pBX-Nosema apis (Lim *et al*., 2014) and pBX-Nosema (Yoo *et al*., 2008). One cycle is set to one minute, and reactions were incubated at 54.0°C for 70 min and 85.0°C for 10 min. After the LAMP reaction, the results were confirmed by electrophoresis analysis.

LAMP reaction with Genechecker™

The LAMP reactions were carried out under the modified standard conditions for 10ul. All LAMP reactions were conducted on a Model UF-100 Genechecker™ system with polymer chip named Rapi:chip (Dongwoo Science, Seoul, Korea).

Sensitivity of LAMP reaction with Genechecker™

To evaluate the detection limit of *N. apis*-specific LAMP with Genechecker™, LAMP reaction was performed under the standard conditions. pBX-Nosema apis (Lim *et al*., 2014) was serially diluted 10-fold from 1×10^8 molecules and 1μl of each dilution was used as a template for the *N. apis*-specific LAMP reaction.

And also, to determine the detection limit of *N. ceranae*-specific LAMP with Genechecker™, LAMP reaction was performed under the standard conditions. pBX-Nosema (Yoo *et al*., 2008) was serially diluted 10-fold from 1×10^8 molecules and 1μl of each dilution was used as a template for the *N. ceranae*-specific LAMP reaction.

Specificity of LAMP reaction with Genechecker™

To assess the specificity of the *N. apis*-specific primer sets (*N. apis* F3, *N.apis* B3, *N. apis* FIP, *N. apis* BIP, *N. apis* Loop F, *N. apis* Loop B) and *N. ceranae*-specific primer sets (Nosema F3, Nosema B3, Nosema FIP, Nosema BIP, Nosema Loop F, Nosema Loop B), LAMP reactions were performed under a standard condition using genomic DNA from honeybee sample infected by *A. apis*, *A. flavus* and *N. ceranae*.

Fluorescent dye test

LAMP products were visualized under daylight by yellow fluorescence after the addition of Gene-Finder™ (Baygene Biotech Company Limited, China), under UV by green fluorescence after the addition of SYBR Green Ⅰ.

RESULTS AND DISCUSSION

Designing of specific primer sets for LAMP

The nucleotide sequence of the sense strand of the *N. apis* genome is shown. DNA sequences used for primer design are underlined (Fig. 1).

Optimal temperature of LAMP reaction

To determine the optimal temperature for the *N. apis*-specific LAMP assay, pBX plasmid containing a part of the *N. apis* large subunit ribosomal RNA gene was used. The each LAMP reaction was performed under isothermal conditions within 41.5, 44.7, 47.2, 50.0, 54.3, and 57.0°C for 60 min. After LAMP reaction, the amplicons were confirmed by agarose-gel electrophoresis. The amplicons were formed between 41.5°C and 54.3°C. For simultaneous diagnosis with *N. ceranae*, 54°C was chosen as the optimum reaction temperature for the subsequent LAMP assays (Fig. 2).

The optimum temperature for *N.ceranae*-LAMP was set as 54.0°C.

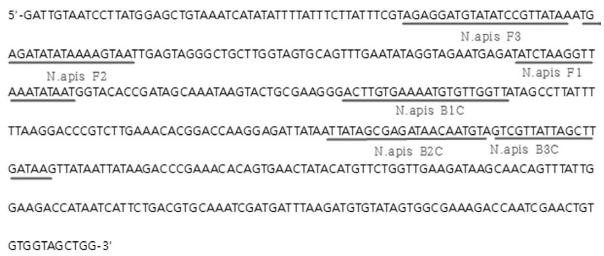


Fig. 1. Nucleotide sequences and location of primers for *N. apis*-specific LAMP.

Optimal conditions of LAMP reaction

In order to establish the optimal reaction conditions for *N. apis*-specific LAMP, several concentrations of each composition (Primers, dNTP, *Bst* polymerase) were tested. In case of primers, the strongest amplicon was formed when the inner primer was 80pmole and outer primer was 20pmole. In spite of repeated experiments, the optimum concentration of the inner primer was determined to 80pmole and the optimum concentration of the outer

Table 1. Primers of *N. apis* and *N. ceranae* for LAMP

Gene	Primer name	Primer sequence (5'→3')	Lengh (mer)	Reference
N. apis	*Nosema apis* F3	AGAGGATGTATATCCGTTATA	21	This study
	Nosema apis B3	TTATAACTTATCAAGCTAATAACGA	25	
	Nosema apis FIP	GTACCATTATATTTAACCTTAG TTTT TGAGATATATAAAAGTAAT	45	
	Nosema apis BIP	GACTTGTGAAAATGTGTTGGTT TTTT ACGTTGTTATCTCGCTATAAT	47	
N. ceranae	Nosema F3	CTACGTTAAAGTGTAGATAAGATGT	25	Lee *et al*., 2010
	Nosema B3	TCCCATAACTGCCTCAGAT	19	
	Nosema FIP	ACCCGTCACAGCCTTGTTAA-TTTT-GTAAGAGTGAGACCTATCAGC	45	
	Nosema BIP	ACTTTGTAATATTCCGGAGAAGGAG-TTTT-CCATAGGTCAAGTTTCGCC	48	

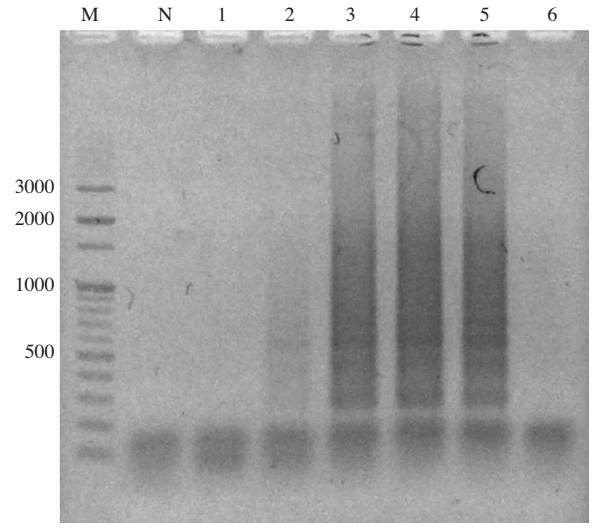


Fig. 2. Temperature gradient *N. apis*-specific LAMP. Lane M1 is 100 bp ladder Marker (Bioneer). Lane 1 to 6 were specific LAMP products from each LAMP under isothermal temperature at 41.5, 44.7, 47.2, 50.0, 54.3, and 57.0°C, respectively. Lane N is negative control without template. Optimal elongation temperature was determined at 54.0°C.

primer was determined to 20pmole (Fig. 3A).

In order to determine the optimal concentration of dNTP for *N. apis*-specific LAMP, each dNTP (2.5mM, 5.0mM, 7.5mM, 10.0mM, 12.5mM and 15.0mM) was added to each reaction solution.

After specific LAMP, agarose-gel electrophoresis was performed. The strongest amplicon was formed when the dNTP concentration was 10mM. Therefore, the optimum concentration of dNTP was determined to 10mM (Fig. 3B).

Moreover, the determination of optimal concentration of *Bst* polymerase for *N. apis*-specific LAMP was performed. Various units of *Bst* polymerase (4U, 8U, 12U, 16U and 20U) were added to each reaction. After the reaction, the amplicons were confirmed by agarose-gel electrophoresis. *N. apis*-specific LAMP products could be amplified between 4U to 16U. The various sizes of the products were observed when 8U *Bst* polymerase was added to LAMP reaction solution. Therefore, the optimal concentration of *Bst* DNA polymerase was determined to 8U (Fig. 3C).

The *N. apis*-specific LAMP standard condition was set in a total volume of 25ul containing 20pmole each of outer primers, 80pmole each of inner primers, 10mM dNTP, 8U *Bst* polymerase large fragment (New England Biolabs), 10X TermolPol Reaction buffer (20mM Tris-HCl, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100), and template DNA. Then, the mixture was incubated at 54.0°C for 60 min.

The standard condition for *N. ceranae*-LAMP was set in

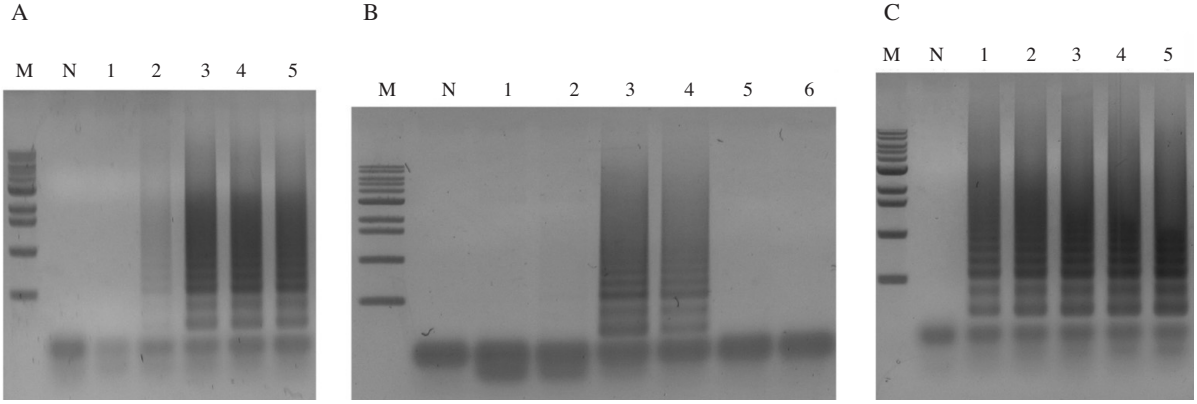


Fig. 3. The optimal concentration of reaction solutions in *N. apis*-specific LAMP. Panel A. The optimal concentration of primers. Lane M is 1kb ladder marker (Bioneer); 10.2, 8, 5.9, 5, 4, 2.9, 2, 1.6, 1, 0.5kb, respectively. Lane N, 0pmole N.apis-FIP/BIP and 0pmole N.apis-F3/B3; Lane 1, *N. apis*-specific LAMP using 20pmole *N.apis*-FIP/BIP and 5pmole *N. apis*-F3/B3; Lane 2, *N. apis*-specific LAMP using 40pmole *N. apis*-FIP/BIP and 10pmole *N. apis*-F3/B3; Lane 3, *N. apis*-specific LAMP using 60pmole *N. apis*-F3/B3 and 15pmole N. apis-F3/B3; Lane 4, *N. apis*-specific LAMP using 80pmole *N.apis*-FIP/BIP and 20pmole *N. apis*-F3/B3; Lane 5, *N. apis*-specific LAMP using 100pmole *N. apis*-FIP/BIP and 25pmole *N. apis*-F3/B3. The optimal concentration of primer was determined at 80pmole N. apis-FIP/BIP and 20pmole N. apis-F3/B3. Panel B. The optimal concentrations of dNTP. Lane M is 1kb ladder marker (Bioneer); 10.2, 8, 5.9, 5, 4, 2.9, 2, 1.6, 1, 0.5kb, respectively. Lane N is negative control. Lane 1 to 6, LAMP products using 2.5mM; 5mM; 7.5mM; 10mM; 12.5mM; 15mM dNTP. The optimal concentration of dNTP was determined at 10mM. Panel C. The optimal concentration of *Bst* polymerase. Lane M is 1kb ladder marker (Bioneer); 10.2, 8, 5.9, 5, 4, 2.9, 2, 1.6, 1, 0.5kb, respectively. Lane N is negative control. Lane 1 to 5, LAMP products using 4U; 8U; 12U; 16U; 20U *Bst* polymerase. The optimal concentration of *Bst* polymerase was determined at 8U.

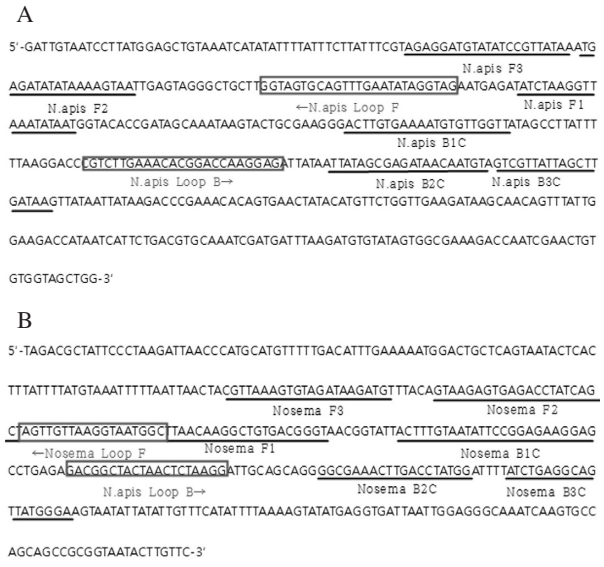


Fig. 4. Nucleotide sequences and location of primers for LAMP. Panel A. The Nucleotide sequences and location of Loop primers for *N. apis*-specific LAMP. Panel B. The Nucleotide sequences and location of Loop primers for *N. ceranae*-specific LAMP.

a total volume of 25ul containing 5pmole each of outer primers, 20pmole each of inner primers, 5mM dNTP, 4U *Bst* polymerase large fragment (New England Biolabs), 10X TermolPol Reaction buffer (20mM Tris-HCl, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100), and template DNA. Then, the mixture was incubated at 54.0°C for 60 min.

Specific Loop primers sets for LAMP

According to the result that the addition of Loop primers can accelerate the LAMP reaction of the paper (Nagamine *et al*., 2002), each loop primer set for *N. apis*-specific LAMP and *N. ceranae*-specific LAMP was designed (Fig. 4 and Table 2).

During the same time, the LAMP reactions were performed and the results were confirmed by agarose-gel electrophoresis. As a result, the lane added Loop primer was working well than the lane not added loop primer (data not shown).

Reaction time of LAMP with real-time LAMP assay

In order to determine the reaction time of *N. apis*-specific LAMP according to the number of DNA molecules, pBX-Nosema apis (Lim *et al*., 2014) was serially diluted 10-fold, and 1μl of each dilution was used as a template for the real-time LAMP reaction. As a result, at least 1×10^4 molecules of DNA could be detected within 35 min (Fig. 5A). In addition, it was confirmed again by electrophoresis analysis (data not shown).

To determine the *N. ceranae*-specific LAMP reaction time according to the DNA molecules for *N. ceranae*, the real-time LAMP was carried out using 10-fold serial dilution of pBX-Nosema (Yoo *et al*., 2008). After the LAMP reaction, at least 1×10^5 molecules of DNA could be detected within 30 min (Fig. 5B). The results were confirmed again by electrophoresis analysis (data not shown).

Detection of LAMP product with Genechecker™

After amplification, the well containing template DNA showed clearly green fluorescence signal. However, the well without template didn't show fluorescence signal (Fig. 6). The results were confirmed again by electrophoresis analysis.

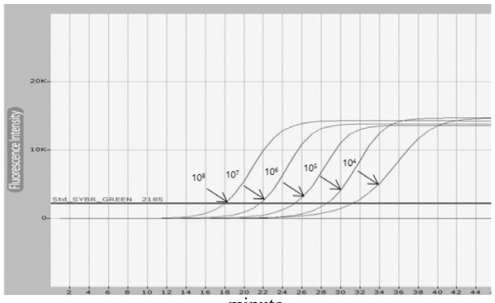
The sensitivity of LAMP assay with Genechecker™

In case of *N. apis*-specific LAMP, the fluorescence signals were gained in the range of 10^8 to 10^2 molecules of pBX-*Nosema apis* (Fig. 7A). However, in case of *N.*

Table 2. Loop primers of *N. apis*- and *N. ceranae*- specific LAMP for accelerated reaction

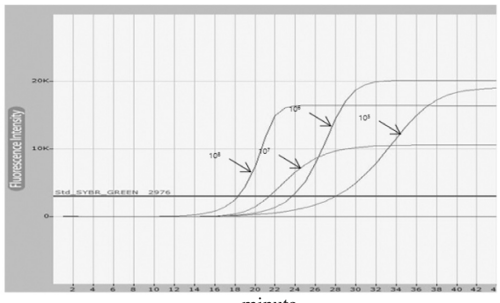
Gene	Primer name	Primer sequence (5'→3')	Lengh (mer)	Reference
N. apis	*N. apis* Loop F	CTACCTATATTCAAACTGCACTACC	25	This study
	N. apis Loop B	CGTCTTGAAACACGGACCAAGGAG	24	
N. ceranae	Nosema Loop F	GCCATTACCTTAACAACTA	19	This study
	Nosema Loop B	GACGGCTACTAAGTCTAAGG	20	

A



Molecules	10^8	10^7	10^6	10^5	10^4	10^3
time	18min	22min	26min	29min	32min	-

B



Molecules	10^8	10^7	10^6	10^5	10^4	10^3
time	19min	22min	24min	28min	-	-

Fig. 5. The LAMP result for detection time in accordance with the number of DNA molecules using Real-time fluorescent measurement. Panel A. The fluorescence results of *N. apis*-specific LAMP using the 10-fold serial dilutions from 1×10^8 molecules. It is possible to diagnose the *N. apis* 1×10^4 molecules within 35 minutes. The R^2 value of standard curve was 0.984 (data not shown) Panel B. The fluorescence results of *N. ceranae*-specific LAMP using the 10-fold serial dilutions from 1×10^8 molecules. It is possible to diagnose the *Nosema ceranae* 1×10^5 molecules within 30 minutes. The R^2 value of standard curve was 0.984 (data not shown).

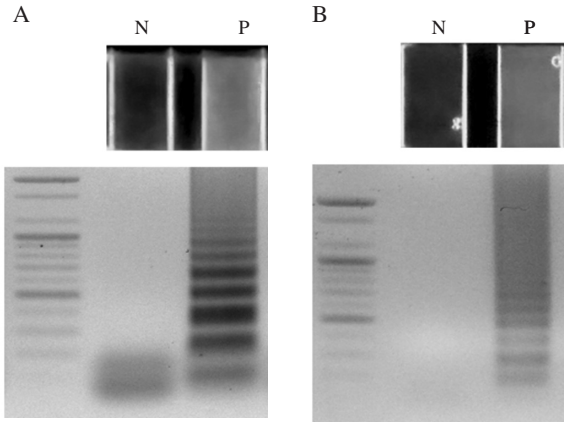


Fig. 6. The results of the *Nosema*-specific LAMP. Panel A. The *N. apis*-specific LAMP results using Geneckecker™ and agarose gel electrophoresis. Lane N is Negative control without template. Lane P is positive control using pBX-Nosema apis plasmid DNA (1ng). Panel B. The *N. ceranae*-specific LAMP results using Geneckecker™ and agarose gel electrophoresis. Lane N is Negative control without template. Lane P is positive control using pBX-Nosema plasmid DNA (1ng).

ceranae-specific LAMP, the fluorescence signals were gained in the range of 10^8 to 10^3 molecules of pBX-Nosema (Fig. 7B). The results were confirmed again by electrophoresis analysis.

Species specificity of LAMP with Genechecker™

In this study, genomic DNAs from honeybees infected by *A. apis, A. flavus* and *N. ceranae* were tested to evaluate the specificity of the *N. apis*-specific LAMP primer sets (*N. apis* F3, *N. apis* B3, *N. apis* FIP, *N. apis* BIP, *N. apis* Loop F, *N. apis* Loop B) and the *N. ceranae*-specific LAMP primer sets (Nosema F3, Nosema B3, Nosema FIP, Nosema BIP, Nosema Loop F, Nosema Loop B). The *N. apis*-specific LAMP primers demonstrated a high degree of specificity for *N. apis* by amplifying *N. apis* but yielding negative results with all of the other pathogens tested (Fig. 8A). On the other hand, the *N. ceranae*-specific LAMP primers demonstrated a high degree of specificity for *N. ceranae* by amplifying *N. ceranae* but yielding negative results with all of the other pathogens tested (Fig. 8B).

Detection of LAMP product with fluorescent dye

When Gene-Finder™ was added to the tubes after the reaction, yellow fluorescence was observed with naked dye in a positive reaction, whereas orange fluorescence was observed in a negative reaction (Fig. 9A). Meanwhile, when SYBR Green I was added to the tubes after the reaction, green fluorescence was observed with the naked eye in a positive reaction, whereas no signal was observed in a negative reaction (Fig. 9B).

Nosema spp. are a microsporidian, a small, unicellular parasite recently reclassified from protozoa to the Fungi cluster, rank Opisthokonta (Adl *et al*., 2005) that are

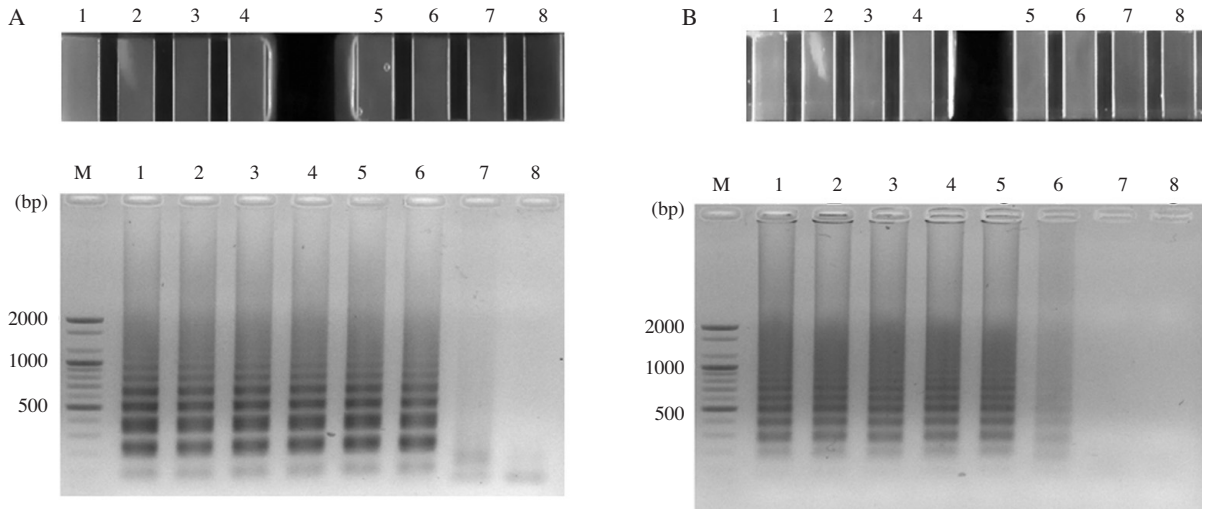


Fig. 7. Sensitivity test of the *Nosema*-specific LAMP. Panel A. The *N. apis*-specific LAMP results using Genechecker^{TM} (up) and agarose gel electrophoresis (down). Lane M is 100bp DNA ladder (Bioneer). Lane 1, *N. apis*-specific LAMP using 1×10^8 molecules. Lane 2, *N. apis*-specific LAMP using 1×10^7 molecules. Lane 3, *N. apis*-specific LAMP using 1×10^6 molecules. Lane 4, *N. apis*-specific LAMP using 1×10^5 molecules. Lane 5, *N. apis*-specific LAMP using 1×10^4 molecules. Lane 6, *N. apis*-specific LAMP using 1×10^3 molecules. Lane 7, *N. apis*-specific LAMP using 1×10^2 molecules. Lane 8 is negative control without template. Panel B. The *N. ceranae*-specific LAMP results using Genechecker^{TM} (up) and agarose gel electrophoresis (down). Lane M is 100bp DNA ladder (Bioneer). Lane 1, *N. ceranae*-specific LAMP using 1×10^8 molecules. Lane 2, *N. ceranae*-specific LAMP using 1×10^7 molecules. Lane 3, *N. ceranae*e-specific LAMP using 1×10^6 molecules. Lane 4, *N. ceranae*-specific LAMP using 1×10^5 molecules. Lane 5, *N. ceranae*-specific LAMP using 1×10^4 molecules. Lane 6, *N. ceranae*-specific LAMP using 1×10^3 molecules. Lane 7, *N. ceranae*-specific LAMP using 1×10^2 molecules. Lane 8 is negative control without template.

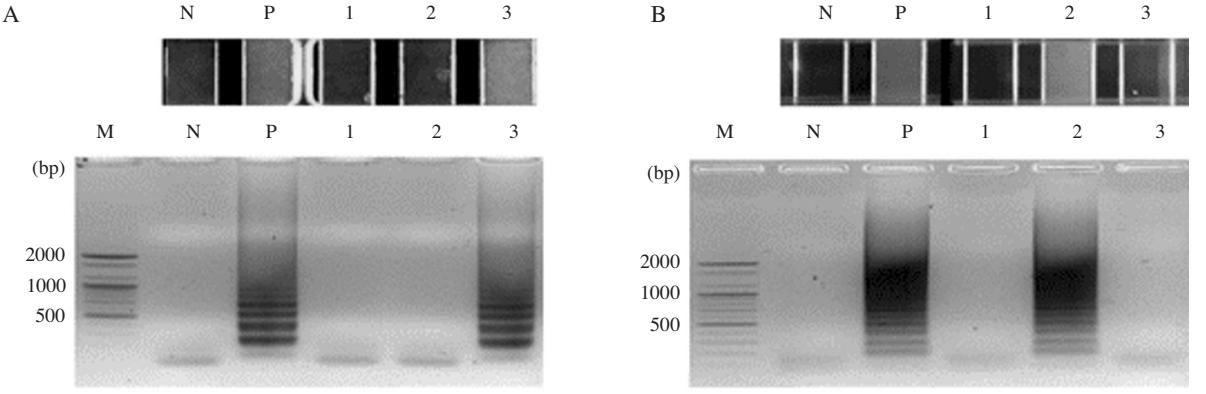


Fig. 8. Detection of *N. apis*-specific LAMP and *N. ceranae*-specific LAMP. As target templates genomic DNA isolated from honeybee sample. Each sample diagnosed *A. apis, A. flavus*, and *N. ceranae* infection were used for the experiment. Panel A. LAMP reaction with *N. apis* LAMP primer sets to Honeybee genomic DNA. Lane M is DNA size marker; 2, 1.6, 1.2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1kb, respectively. Lane N is no template as a negative control, Lane P is reacted with pBX-Nosema apis, Lane1 is reacted with Honeybee genomic DNA that was infected by *A. apis* and *A. flavus*. Lane 2 is reacted with Honeybee genomic DNA that was infected by *N. ceranae*. Lane 3 is reacted with artificial Honeybee genomic DNA that was infected by *N. apis*. Panel B. LAMP reaction with *Noseme ceranae* LAMP primer sets to Honeybee genomic DNA. Lane M is DNA size marker; 2, 1.6, 1.2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1kb, respectively. Lane N is no template as a negative control, Lane P is reacted with pBX-Nosema, Lane1 is reacted with Honeybee genomic DNA that was infected by *A. apis* and *A. flavus*. Lane 2 is reacted with Honeybee genomic DNA that was infected by *N. ceranae*. Lane 3 is reacted with artificial Honeybee genomic DNA that was infected by *N. apis*.

ubiquitous exhibiting an extensive range of hosts including honey bees. In many papers previously published, *N. ceranae* has jumped host from *A. cerana* to *A. mellifera* and become distributed almost worldwide (Klee *et al*.,

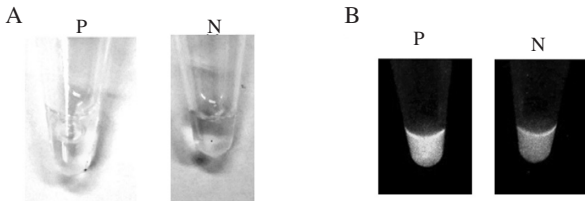


Fig. 9. Gene-Finder™ Nucleic acid fluorescent dye and SybrGreen Ⅰ test of *N. apis*-specific LAMP. Panel A. LAMP product (P) and negative control (N) were stained by Gene-Finder™ Nucleic acid fluorescent dye. Panel B. LAMP product (P) and negative control (N) were stained by SybrGreen Ⅰ. Each product was observed by ultraviolet transilluminator.

2007). A quick and accurate molecular genetic method of detection of microsporidia is important for identification (Klee *et al*., 2006) because species of microsporidia are often difficult to distinguish using morphological criteria (Larsson, 1986; Weiss and Vossbrinck, 1999).

In this study, new LAMP methods for the specific detection of *N. apis* and *N. ceranae* in honeybees were developed and are termed as a *N. apis*-specific LAMP and *N. ceranae*-specific LAMP. In case of *N. apis*-specific LAMP, six primers for *N. apis* specific LAMP (*N. apis* F3, *N. apis* B3, *N. apis* FIP, *N. apis* BIP, *N. apis* Loop F and *N. apis* Loop B) were designed based on *N. apis* large subunit ribosomal RNA gene (GenBank, Accession no.U97150.1). The conditions of the LAMP reaction were optimized by an incubation of the target DNAs; pBX-Nosema apis with the six primers at 54°C for 60 min. Moreover, the diagnostic method using GENECHECKER™, system allowed a direct observation of results by naked eye instead of electrophoresis. The new method has an advantage that can successfully perform amplification of 10^4 molecules target DNA within 30 minutes. And specific *N. apis* primer set is able to achieve specific detection of *N. apis* whereas no amplification to other pathogen like *Ascosphaera apis*, *Aspergillus flavus* and *Nosema ceranae*.

In case of *N. ceranae*-specific LAMP, the *N. ceranae*-specific LAMP method (Lee *et al*., 2010) was modified. Six primers for *N. ceranae*-specific LAMP (Nosema F3, Nosema B3, Nosema FIP, Nosema BIP, Nosema Loop F and Nosema Loop B) were designed based on *Nosema ceranae* small subunit ribosomal RNA gene (GenBank, Accession no.DQ486027). The conditions of the LAMP reaction were optimized by an incubation of the target DNAs; pBX-Nosema with the six primers at 54°C for 60 min. Moreover, the diagnostic method using Genechecker™ system allowed a direct observation of results by naked eye instead of electrophoresis. This method against *Nosema ceranae* can be successfully amplified from 1×10^3 molecules of template DNA. Specific *Nosema ceranae* primer set is able to achieve specific detection of *Nosema ceranae* whereas no amplified to other pathogen like *A. apis, A. flavus* and *N. apis*.

By using Genechecker™ that adopts DC 12V power input and can be connected to a general car power source, these LAMP methods may be expected to be useful tool for rapid, specific detection of *Nosema* diseases in the field and for monitoring of natural infection in *A. mellifera* and *A. cerana*.

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