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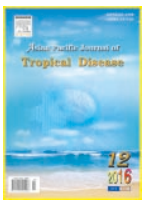

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Development and evaluation of loop-mediated isothermal amplification for rapid detection of *Nosema ceranae* in honeybee

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ABSTRACT

Objective: To develop loop-mediated isothermal amplification (LAMP) to detect *Nosema ceranae* (*N. ceranae*) in honeybee samples.
Methods: LAMP primers were designed recognizing six distinct fragments of 16s rRNA gene and LAMP reaction was determined by optimizing the concentration of reagents, such as forward inner primer and backward inner primer, deoxynucleoside triphosphate and betaine, time and temperature. Ten-fold serial dilutions of DNA were used to determine the detection limit and accuracy using both LAMP and PCR tests.
Results: LAMP required 1.2 μmol/L of forward inner primer and backward inner primer primers, 0.2 μmol/L of forward outer primers and backward outer primer, 2 μmol/L of Mg^{2+}, 0.6 mol/L of betaine, 0.6 μmol/L of deoxynucleoside triphosphate, 4.8 IU of Bst DNA polymerase and 30 ng of DNA. The optimal temperature was 63 °C and after a 40-min incubation time, a clearly ladder-like pattern of LAMP product appeared in the gel electrophoresis. LAMP appeared more sensitive than a standard PCR in detection of *N. ceranae*.
Conclusions: LAMP gave a good results and it could be an alternative diagnostic tool instead of PCR to detect *N. ceranae* infection in honeybee.

1. Introduction

Nosemosis or nosema disease is an important disease which can cause colony collapse disorder (CCD) in honeybee apiaries, which is a phenomenon that occurs when the most of worker bees in a colony disappear. CCD causes significant economic losses for affected beekeepers as it can lead to the death of the entire colony. The number of honeybee apiaries in Thailand is about 1 556 apiaries and about 45.12% (702 apiaries) is in Northern Thailand. Beekeeping industry encounters with many problems, such as bad environment, poor administration, low quality honey and many diseases derived from bacteria, virus, protozoa and fungi.

There are two microsporidian fungi that can cause nosemosis in honeybees. They are *Nosema apis* (*N. apis*) and *Nosema ceranae* (*N. ceranae*). *N. ceranae* was first described in *Apis cerana* or the Asian honeybee in China in 1994 and it was identified as a disease of *Apis mellifera* (*A. mellifera*) or European honeybee in 2004 in Taiwan[1]. *N. ceranae* is the only pathogen that can cause nosemosis among honeybee in Thailand[2]. It has a high infectivity rate in *A. mellifera*, *Apis cerana* and *Apis dorsata*[3]. As this pathogen grows and multiplies in the mid part of the digestive tract of honeybees, the symptoms mostly occur in the digestive system, such as dysentery, extension and swelling of the abdomen of infected bees. These symptoms appear visually when the infection is very severe. That means that when the beekeepers observe the symptoms, it is too late for treatment. Nosemosis can be treated by bicyclohexylammonium fumagillin which is commonly known as fumagilin, and has been the only widely used treatment for nosemosis or nosema disease in *A. mellifera* for about 60 years[4,5]. Fumagilin inhibits the reproduction of *N. ceranae* spores but will not kill the spores.

The action of *N. ceranae* is that it inhibits the methionine aminopeptidase-2 (MetAP2) enzyme[6]. The microsporidian MetAP2 gene is homologous with other eukaryotes with approximately 60% similarity among the eukaryotic organisms[7]. So it is known to be toxic to humans and other vertebrates by interacting with the MetAP2 enzyme, this involves in protein maturation and post translation processes[8]. To decrease the occurrence of this disease in apiaries, beekeepers should be able to diagnose the disease before the infection has progressed to the point that symptoms appear.

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The diagnosis of *Nosema* spp. infection has usually been done by spore detecting though microscopic examination[9]. Whatever, this cannot detect as low level of *Nosema* spp. infection as molecular methods can do. Several methods especially molecular basis methods have been developed to detection and quantification of *N. ceranae* DNA[10-13]. These techniques are rather expensive and time-consuming. Loop-mediated isothermal amplification (LAMP) has been developed for detecting *Nosema* spp. in honeybees. This technique requires non-expensive equipment, is highly sensitive, accurate and timesaving and is not complicated to perform.

In 2014, LAMP was developed to detect *Nosema* spp. in honeybee using 6 primers by Ptaszyńska *et al.*[14]. In this study, a diagnostic method was developed based on the LAMP reaction for the detection of *N. ceranae* and to compare the sensitivity and accuracy of the LAMP and PCR methods. Furthermore, we compare the detection limit of LAMP primers which we have designed and LAMP primers by Ptaszyńska *et al.*[14].

2. Materials and methods

2.1. Collecting samples

The samples of honeybees were collected in Northern Thailand during 2014 and stored at –70 ˚C until use. *Nosema* infection of honeybee samples were detected by microscopic examination and confirmed by PCR. Then, sequencing analysis was performed.

2.2. DNA extraction

Thirty milligrams of homogenized honeybee samples infected with *N. ceranae* was used for DNA extraction using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacture's instruction. DNA concentration and purification was measured by using Beckman Coulter DU 730. Then, the DNA was kept in –70 ˚C before using.

2.3. LAMP

2.3.1. Primer design

LAMP primers were chosen by employing the revealed sequencing of *N. ceranae* (GenBank accession No. DQ486027) and the primer Explorer version 4 software program (http://primerexplorer.jp/elamp4.0.0/index.html) was used. Four LAMP primers were designed, there were forward outer primers (F3), backward outer primer (B3), forward inner primer (FIP) and backward inner primer (BIP). The sequences of the primers and attached position were shown in Table 1.

Table 1

Primers used for the LAMP assay.

Primer	Sequence	Position
F3	5'-CTA CGT TAA AGT GTA GAT AAG ATG T-3'	123–147
B3	5'-AAT ATT ACT TCC CAT AAC TGC C-3'	315–336
FIP	5'-TAC CCG TCA CAG CCT TGT TAA TTT TGT AAG AGT GAG ACC TAT CAG C-3'	153–213
BIP	5'-CGG AGA AGG AGC CTG AGA GAT TTT TCA GAT AAA ATC CAT AGG TCA AG-3'	255–292

2.3.2. Optimization of LAMP reaction

To optimize the LAMP reaction, different reaction temperatures, FIP, BIP and deoxynucleoside triphosphate (dNTP) concentrations, and reaction times were tested.

The LAMP reaction was performed in a 15 μL reaction mixture containing FIP and BIP primer (concentrations varied at 0.8, 1.2 and 1.6 μmol/L), 10 μmol/L F3 and B3 outer primers, dNTPs (concentrations varied at 0.2, 0.4 and 0.6 mmol/L), betaine (concentrations varied at 0.2, 0.6 and 1 mmol/L), 4.8 IU Bst DNA polymerase large fragment (Lucigen), 1 DNA polymerase buffer B [20 mmol/L Tris-HCl, pH 8.8, 10 mmol/L $(NH_4)_2SO_4$, 10 mmol/L KCl, 2 mmol/L $MgSO_4$, 0.1% Triton X-100] and 30 ng of DNA.

The reaction mixture was incubated at a constant temperature (temperatures varied at 56, 58, 60, 63, 65, 67 and 69 ˚C). The incubation time varied at 10, 20, 30, 40, 50 and 60 min and to terminate the reaction the samples were heated to 80 ˚C for 2 min.

2.3.3. Detection and confirmation of LAMP products

2.3.3.1. Using hydroxy naphthol blue (HNB)

LAMP products were visualized by adding HNB to the reaction mixture in the concentration of 100, 120 and 150 μmol/L. The change of colors in the reaction tubes could be seen by the naked eye.

2.3.3.2. Using gel electrophoresis

LAMP products were detected under gel electrophoresis on 1.5% agarose gel and stained with the RedSafeTM (iNtRON Biotechnology Inc., Korea). The ladder-like pattern (many DNA bands in a variation of molecular weights) would be shown in a positive result.

2.3.4. Confirmation of the LAMP product by restriction enzymes

Restriction enzymes were used to confirm that the LAMP test amplified the correct target. The product was digested with *AluI*, *BanI* and *ApaI* at 37 ˚C for 3 h, followed by electrophoresis in 2% agarose gel.

2.4. PCR

PCR reactions were carried out in 20 μL of reaction mixture contained 0.2 μmol/L of each primer, 1.5 mmol/L $MgCl_2$, 1× *Taq* buffer, 0.25 mmol/L deoxynucleotide triphosphates, 0.5 IU *Taq* DNA polymerase and 100 ng of DNA (Fermentas, USA). The PCR condition was as follows: 95 ˚C for 5 min, followed by 35 cycles of 95 ˚C for 30 s, 61.8 ˚C for 30 s and 72 ˚C for 45 s. Then, the final extension step was 72 ˚C for 5 min. The primers used to generate the region in *Nosema* 16S rRNA gene were 218MITOC-F (5'-CGG CGA CGA TGT GAT ATG AAA ATA TTA A-3') and 218MITOC-REV(5'-CCC GGT CAT TCT CAA ACA AAA AAC CG-3') to produce a 218 bp PCR product specific for *N. ceranae* and 321APIS-FOR (5'-GGG GGC ATG TCT TTG ACG TAC TAT GTA-3') and 321APIS-REV (5'-GGG GGG CTT TTA AAA TGT GAA ACA ACT ATG-3') to produce a 321 bp PCR product specific for *N. apis*[15]. The amplified PCR products were detected by electrophoresis on a 1.5% (w/v) agarose gel containing RedSafeTM (0.05 μL/mL) in 1× tris-acetate-ethylene diamine tetraacetic acid buffer (pH 8.0) and 100 bp ladder was included.

2.5. Detection limit and specificity test of LAMP and PCR

The detection limit and specificity of LAMP and PCR methods were carried out under optimal reaction condition. The results were compared with those results of conventional PCR. To study the detection limit, serial 10-fold dilutions of genomic DNA of *N. ceranae* were tested from 30 ng to 0.3 pg. To determine the accuracy, four different pathogens which may infect honeybees and other insects were used. They were *N. apis*, *Metharizium* spp., *Paecilomyces* spp. and *Ascosphaera apis*.

3. Results

3.1. Optimization of LAMP condition

To seek for the optimum concentration of FIP, BIP, dNTP

concentration, betaine concentration, optimal temperature and initiation time of LAMP reaction could be detected the product in 1.5% agarose gel. The number of LAMP reaction products expanded distinctly at 1.2 μmol/L of FIP and BIP (Figure 1A), 0.6 mmol/L of dNTP (Figure 1B) and 0.6 mol/L of betaine concentration (Figure 1C). The optimum temperature was 63 ℃ (Figure 1D). And after 40 min of the incubation time, the LAMP product had clearly appeared in 1.5% agarose gel (Figure 1E). *AluI* restriction enzyme digestion and electrophoresis gave the predicted sizes of about 260 and 200 bp whereas other enzymes including *BanI* and *ApaI* could not digest the LAMP product (Figure 1F).

3.2. Detection of LAMP products with HNB

A sky blue color could be clearly observed with the naked eye in samples with positive result of *N. ceranae* infection while negative and control samples were purple. The optimum concentration of HNB was 120 μmol/L (Figure 2).

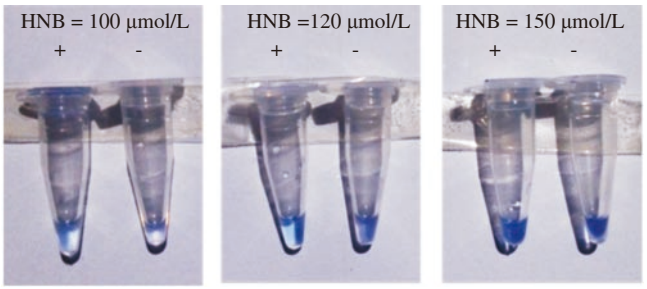

Figure 2. Detection of LAMP products by observing the color of the reaction mixture by naked eye after HNB was added in the concentration of 100, 120 and 150 μmol/L.

3.3. Detection limits and specificity of LAMP and conventional PCR

For detection limits, the LAMP method clearly detected at 0.3 ng of template DNA concentration, whereas the detection limit of conventional PCR was 3 ng of template DNA concentration (Figure 3).

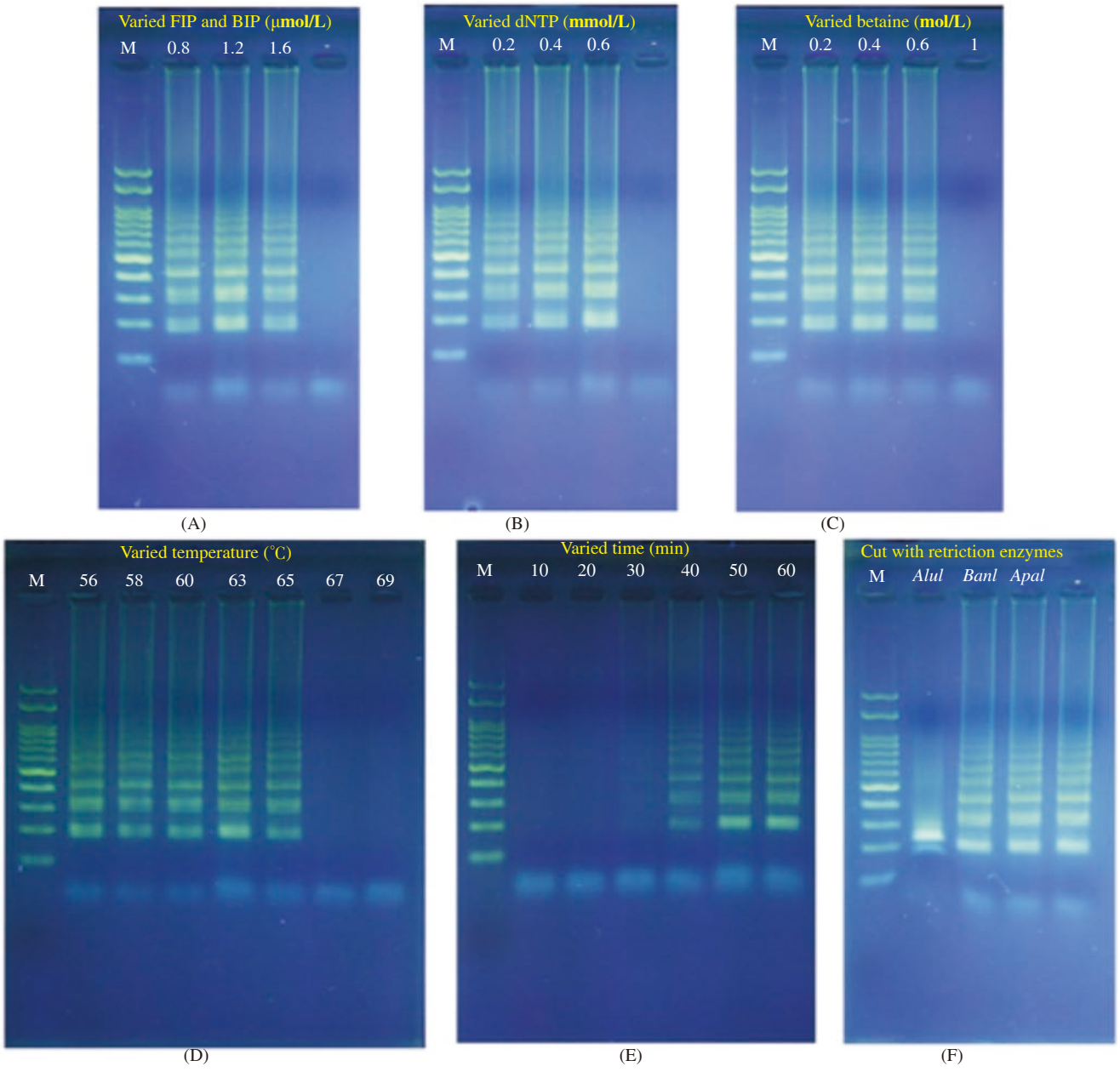

Figure 1. Optimization of LAMP reaction for *N. ceranae*.

(A): Effect of primer FIP and BIP concentration; (B): Effect of dNTP concentration on LAMP reaction; (C): Effect of betaine concentration; (D): Effect of temperature; (E): Effect of time for incubation of the LAMP reaction; (F): Result of LAMP product cut by restriction enzymes; Lane M: DNA marker.

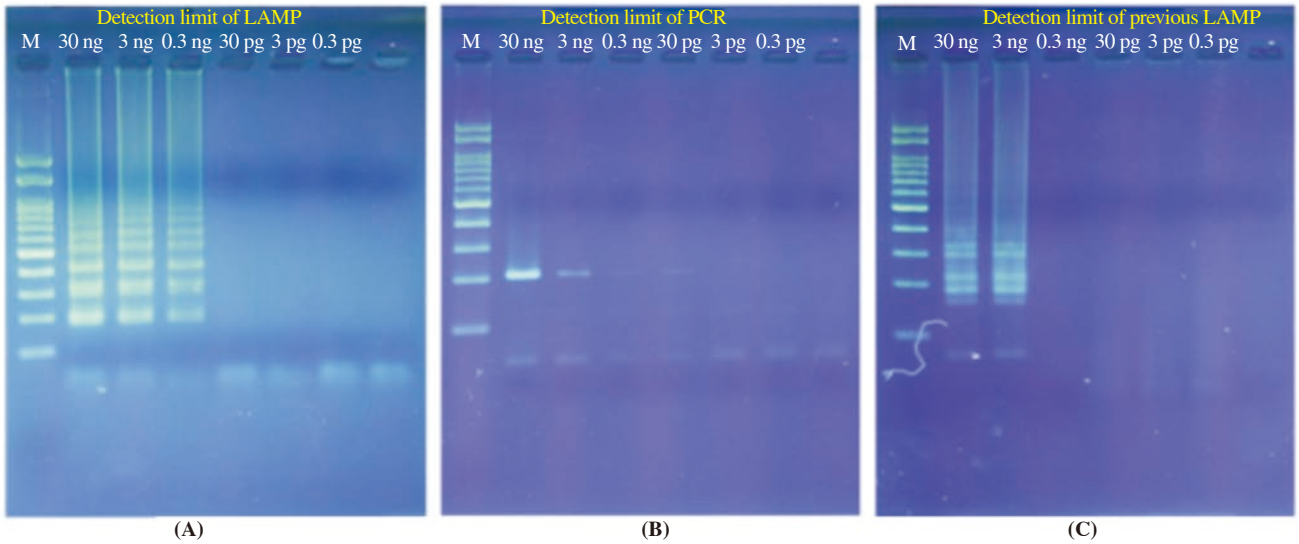

Figure 3. Detection limits of our LAMP (A), PCR (B) and previous LAMP by Ptasynska et al. (C) for *N. ceranae* detection in honeybees.
Lane M: DNA marker; Lane 1: 30 ng; Lane 2: 3 ng; lane 3: 0.3 ng; Lane 4: 30 pg; Lane 5: 3 pg; Lane 6: 0.3 pg; Lane 7: Negative control.

For accuracy test, the LAMP and PCR methods correctly identified *N. ceranae* while no amplification products were detected from any of the other pathogens and negative control. The results were showed via electrophoresis (Figure 4).

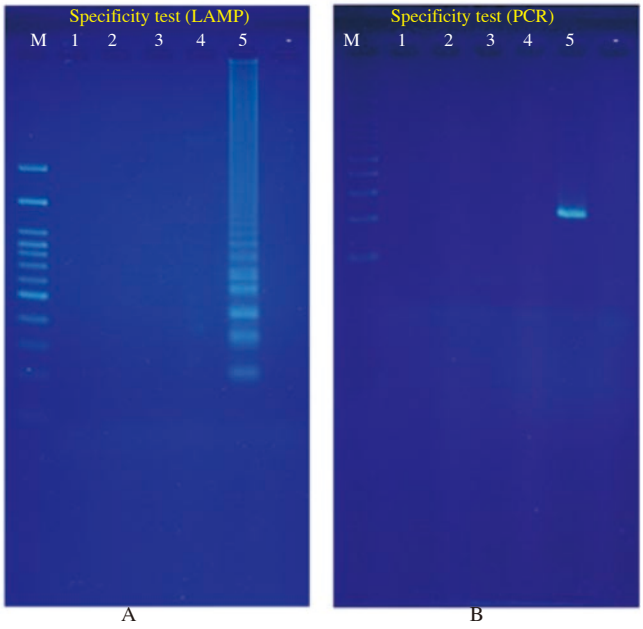

Figure 4. Specificity test of the LAMP (A) and PCR (B).
Lane M: DNA marker; Lane 1: *Ascospheara apis*; Lane 2: *Metarhizium* spp.; Lane 3: *Nosema apis*; Lane 4: *Phacelomyces* spp.; Lane 5: *N. ceranae*; Lane 6: Negative control.

4. Discussion

This pathogen is very significant to commercial beekeeping which is used in the production of honey and other products from honeybees such as bee pollen, propolis and bee venom[16]. Recent tendency indicates that the total amount of western honeybee is decreasing[17,18]. Since 2006, beekeepers across the world have experienced increased hive losses from unknown reasons[19-21], which is called CCD. Nosemosis which occurs from *N. ceranae* may be one of the main diseases causing CCD. The symptoms from *N. ceranae* are not specific but the primary notable symptom is dysentery. The infected bees may be unable to fly due to the disjointed wings. Other symptoms are diarrhea, crawling, and large numbers of dead bees in apiary. So, if the beekeepers can detect this pathogen before the symptom becomes visible, then disease spreading will be prevented in time. Fumagillin is the only antibiotic approved for control of nosemosis in honeybees. But as it may be residues of *N. ceranae* in honey products and because of its toxicity to mammals, disease detection before the symptoms can be seen among the bees is more vital to healthy apiaries than treating with fumagillin.

Many molecular-based methods have been developed for *N. ceranae* detection. But all of them were very expensive, time-consuming and difficult to handle. LAMP is a new molecular-based diagnostic method which is cheap, takes short time to perform (about 1 h) and is not complicated to perform. This method has been developed by Notomi *et al.*[22]. In LAMP reaction, the specific region in DNA sequence is amplified by using the Bst DNA polymerase[22-24]. The LAMP has a high speed under constant temperature condition (60–65 ˚C) and has high accuracy for the specific region.

In 2014, LAMP was develop for *N. ceranae* detection in honeybees using 6 primers by Ptaszyńska *et al.*[14], but our study used 4 primers. On previous result, primer detection limit was 100 fg of target DNA by Ptaszyńska *et al.*[14]. However, when we used the primer set of Ptaszyńska for *N. ceranae,* the detection limit was 3 ng, compared with our primer detection limit was 0.3 ng (Figure 3C). This may be because of the purification of template DNA. In our study, we used the whole abdominal part for DNA extraction whereas in Ptaszynska *et al.*'s, they used the alimentary tract.

In this research, the detection limit of LAMP was lower than PCR. In several studies, a higher level of detection limit was reported for the LAMP as compared to the PCR[25-28]. However, in some experiments, the detection limit result of LAMP was equivalent to PCR[29], this may be because of the characteristic of the primers and the sequence of the specific region in DNA can affect the sensitivity and specificity of molecular technique[30]. In our study, we used hydroxylnapthol blue, a metal indicator. It monitored the change in the Mg^{2+} ion concentration that was the result of LAMP. Because of it, the result can be seen immediately after the LAMP reaction with just the naked eye. UV illumination or gel electrophoresis was neeeded as well.

In conclusion, LAMP method was developed to identify the

infection of *N. ceranae* in honeybees. It has a better sensitivity with a lower limit of detection than conventional PCR. Furthermore, it can be detected with the naked eye immediately after the LAMP reaction by using HNB. This can be a preferable choice for the conventional standard PCR for *N. ceranae* detection in honeybee apiaries.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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