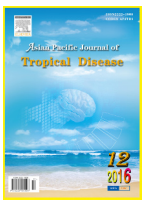
HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd



Parasitological research doi: 10.1016/S2222-1808(16)61163-5

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

Vena Chupia[1], Prapas Patchanee[2], Patcharin Krutmuang[3], Surachai Pikulkaew[2*]

[1]*Graduate school, Chiang Mai University, Chiang Mai, 50200, Thailand*

[2]*Department of Food Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, 50100, Thailand*

[3]*Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand*

ARTICLE INFO

Article history:
Received 17 Jun 2016
Received in revised form 23 Sep, 2nd revised form 30 Sep, 3rd revised form 8 Oct 2016
Accepted 15 Oct 2016
Available online 27 Oct 2016

Keywords:
Nosema ceranae
Rapid detection
Loop-mediated isothermal amplification
Honeybee

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.

*Corresponding author: Surachai Pikulkaew, Department of Food Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, 50100, Thailand.
Tel: 66-53-948-023
Fax: 66-53-948-062
E-mail: surapikulkaew@gmail.com
Foundation Project: Supported by Chiang Mai University Startup fund (Grant No. R12541).
The journal implements double-blind peer review practiced by specially invited international editorial board members.

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