

Original Article
Microbiology



Rapidly quantitative detection of *Nosema ceranae* in honeybees using ultra-rapid real-time quantitative PCR

A-Tai Truong ^{1,2,3}, Sedat Sevin ⁴, Seonmi Kim ¹, Mi-Sun Yoo ³,
Yun Sang Cho ^{3,*}, Byoungsu Yoon ^{1,*}

¹Department of Life Science, College of Fusion Science, Kyonggi University, Suwon 16227, Korea

²Faculty of Biotechnology, Thai Nguyen University of Sciences, Thai Nguyen 250000, Vietnam

³Parasitic and Honeybee Disease Laboratory, Bacterial Disease Division, Department of Animal & Plant Health Research, Animal and Plant Quarantine Agency, Gimcheon 39660, Korea

⁴Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ankara University, Ankara 06560, Turkey

 OPEN ACCESS

Received: Jun 6, 2020

Revised: Mar 10, 2021

Accepted: Apr 12, 2021

*Corresponding authors:

Byoungsu Yoon

Department of Life Science, College of Fusion Science, Kyonggi University, 154-42 Gwanggyosan-ro, Yeongtong-gu, Suwon 16227, Korea.
E-mail: bsyoon@kyonggi.ac.kr

Yun Sang Cho

Parasitic and Honeybee Disease Laboratory, Bacterial Disease Division, Department of Animal & Plant Health Research, Animal and Plant Quarantine Agency, 177 Hyeoksins 8-ro, Gimcheon 39660, Korea.
E-mail: choys@korea.kr


© 2021 The Korean Society of Veterinary Science

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.


ORCID iDs

A-Tai Truong 

<https://orcid.org/0000-0002-6621-7101>

Sedat Sevin 

<https://orcid.org/0000-0003-0475-9092>

Seonmi Kim 

<https://orcid.org/0000-0003-3784-1949>

ABSTRACT

Background: The microsporidian parasite *Nosema ceranae* is a global problem in honeybee populations and is known to cause winter mortality. A sensitive and rapid tool for stable quantitative detection is necessary to establish further research related to the diagnosis, prevention, and treatment of this pathogen.

Objectives: The present study aimed to develop a quantitative method that incorporates ultra-rapid real-time quantitative polymerase chain reaction (UR-qPCR) for the rapid enumeration of *N. ceranae* in infected bees.

Methods: A procedure for UR-qPCR detection of *N. ceranae* was developed, and the advantages of molecular detection were evaluated in comparison with microscopic enumeration.

Results: UR-qPCR was more sensitive than microscopic enumeration for detecting two copies of *N. ceranae* DNA and 24 spores per bee. Meanwhile, the limit of detection by microscopy was 2.40×10^4 spores/bee, and the stable detection level was $\geq 2.40 \times 10^5$ spores/bee. The results of *N. ceranae* calculations from the infected honeybees and purified spores by UR-qPCR showed that the DNA copy number was approximately 8-fold higher than the spore count. Additionally, honeybees infected with *N. ceranae* with 2.74×10^4 copies of *N. ceranae* DNA were incapable of detection by microscopy. The results of quantitative analysis using UR-qPCR were accomplished within 20 min.

Conclusions: UR-qPCR is expected to be the most rapid molecular method for *Nosema* detection and has been developed for diagnosing nosemosis at low levels of infection.

Keywords: *Nosema ceranae*; microscopic enumeration; ultra-rapid real-time PCR; nosemosis prevention

INTRODUCTION

Nosema ceranae Fries is an intracellular parasite found in honeybees. Although it was first detected in the Asian honeybee *Apis cerana* in 1996 [1], it is now dominant in the European honeybee *Apis mellifera*, and was detected in all members of the colony, including adult bee and brood stages. *Nosema ceranae* is known to contribute to the winter mortality of honeybees

Mi-Sun Yoo <https://orcid.org/0000-0002-3176-579X>Yun Sang Cho <https://orcid.org/0000-0003-1346-7067>Byoungsu Yoon <https://orcid.org/0000-0002-4448-4110>

Funding

This work was supported by the “Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01408002)” Rural Development Administration of the Republic of Korea, as well as by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry, through the Agri-Bio Industry Technology Development Program (318093-03), partially funded by the Animal and Plant Quarantine Agency (Grant No. B-1543081-2019-21-03).

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Yoon B; Data curation: Truong AT, Sevin S, Yoo MS; Formal analysis: Yoon B, Truong AT; Funding acquisition: Yoon B; Investigation: Truong AT, Sevin S; Methodology: Truong AT, Sevin S, Kim SM; Project administration: Yoon B; Writing original draft: Truong AT; Writing review & editing: Kim SM, Sevin S, Yoon B, Cho YS.

worldwide [2-9]. Consequently, the decline of healthy honeybee colonies has a negative influence on honey production as well as on agricultural crops and ecological plants due to the decrease in pollination in flowering plants [10-12]. In Korea, *Nosema ceranae* infection was recorded for the first time in 1996 [13]. The prevalence of nosema disease in apiaries was detected at 56% in 2003 [14], increased to 94% in 2013 [15], and the updated 2019 data from the Honeybee Disease Laboratory, Animal and Plant Quarantine Agency, Republic of Korea, showed that 24% of the surveyed apiaries were infected with *Nosema ceranae* [16].

Microscopy is one of the standard methods for detecting and quantifying *Nosema* [17]. The advantages of this method are that it can be directly observed and infection intensity can be determined by counting spores with the aid of a hemocytometer and a light microscope at 400× magnification [18,19]. However, this method is inadequate for identifying species because different *Nosema* species have similar spore sizes [20]. Furthermore, the identification of all life stages using a conventional light microscope is difficult because of the differences in size and shape among the intracellular life stages of nosema [21]. Therefore, microscopy is not an efficient method for the detection of nosema at a low intensity of infection.

Polymerase chain reaction (PCR) detection has been commonly used as a reliable method for diagnosing nosema infection (nosemosis) [22]. PCR detection methods for nosema have been developed for *N. ceranae* and *N. apis* identification based on variations in the size of conventional PCR amplicons [5]. A method to estimate spore counts by semi-quantitative triplex PCR assay was later developed [23], and quantitative real-time PCR was developed for accurate and sensitive detection of *N. apis* and *N. ceranae* simultaneously, using multiple primers in one reaction [17,24,25]. Regrettably, all of these molecular-based methods suffer from the drawback of relying on time-intensive PCR. Recently, a new molecular-based method, known as loop-mediated isothermal amplification (LAMP), was developed for diagnosing nosemosis and was demonstrated to be less time-intensive than PCR detection [26]. However, this method can only be used as an alternative method for conventional PCR because of the requirement of an electrophoresis step after polymerization to verify the specificity of detection [27]. Since quantitative PCR has been used as an efficient tool for detecting and measuring the level of *N. ceranae* infection as well as for related studies, such as the evaluation of virulence of *Nosema* species [25], the influence of *N. ceranae* on its host [28], or interaction between *N. ceranae* and honeybee gut bacteria [29], it is important to develop a sensitive PCR system with stable amplification and less time consumption.

Accordingly, the current study aimed to introduce a molecular detection method that uses a newly developed real-time PCR method, known as ultra-rapid real-time quantitative PCR (UR-qPCR). The rapid heat transfer involved in this system markedly reduces the time required for PCR detection of *N. ceranae*, and it is expected to be an effective tool for rapidly diagnosing low levels of infection and for evaluating the efficacy of drug candidates for mitigating nosemosis in honeybees.

MATERIALS AND METHODS

Sample preparation

The isolation and purification of *N. ceranae* spores were based on the standard method for nosema research [17], with some modifications. Fifty adult bees were ground in 20 mL of

distilled water using a mortar and pestle, followed by the addition of 30 mL of water. The homogenate was filtered through a fine mesh gauze (Two Guys & Tools Ltd., Korea) to remove large bee particles. The solution was vortexed and filtered again using a 70- μ m nylon cell strainer (BD Falcon, USA) to remove large particles that originated from the honeybee alimentary canals. Next, the flow-through solutions were collected in a new 50-mL conical tube and centrifuged at $5,000 \times g$ for 10 min. The supernatant was discarded, and the centrifugation was repeated twice. Finally, the pellets were re-suspended in 5 mL of distilled water and stored at 4°C until further use.

For the evaluation of *N. ceranae* in caged bees, the whole abdomen parts of five bees were ground together with a micropestle in a 1.5 mL tube containing distilled water (0.5 mL). After grinding, the homogenate was vortexed, and only the solution was transferred to a new 1.5 mL tube using a pipette. To minimize the loss of spores, another 0.5 mL of water was added to the remaining substrate. After vortexing, the solution was transferred again to the same new tube. The new tube was centrifuged at $12,000 \times g$ for 1 min using a tabletop centrifuge, and the supernatant was discarded. Next, the pellet was suspended in 1 mL of water and centrifuged again at $12,000 \times g$ for 1 min. After discarding the supernatant, the pellet was resuspended in distilled water to a final volume of 1 mL (corresponding to 0.2 mL solution/bee), and 0.2 mL of the solution was diluted in 0.8 mL and used for microscopic counting. The remaining 0.8 mL spore solution was centrifuged at $12,000 \times g$ for 1 min, and the supernatant was discarded. The resulting pellets were used for DNA extraction and PCR quantification.

DNA extraction

The spore pellet prepared as described in the section on sample preparation was ground in liquid nitrogen using a micropestle from a sample grinding kit (Sigma Aldrich, Korea), and DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. Finally, 100 μ L of DNA solution was acquired from each sample. DNA concentration was measured using a BioPhotometer (Eppendorf, Germany).

Standard DNA and *N. ceranae*-specific primers

A recombinant vector (pCR2.1-Nosema) carrying a 590 bp ribosomal DNA fragment of *N. ceranae* was used as a positive control for *N. ceranae* detection [30]. In addition, *N. ceranae*-specific primers, NO-DC-F3 (5-AGGCAGTTATGGGAAGTAATATTATA-3) and NO-DC-R2 (5-CAGGGTCGTCACATTTTCATCTTTC-3), were designed based on the alignment of the *N. ceranae* rRNA gene sequences. The rRNA gene sequence of *N. ceranae* (GenBank accession No.: JX205151.1), which included a partial 5S sequence, complete sequence small subunit and internal transcribed spacer, and partial large subunit sequence, was used to search for similar sequences in GenBank using the National Center for Biotechnology Information Nucleotide BLAST tool. The rRNA gene sequences of *N. ceranae* and the complete sequences of the small subunit ribosomal RNA gene, internal transcribed spacer, and large subunit ribosomal RNA gene of *N. apis*, a closely related species of *N. ceranae*, were used for alignment using the program Clustal X version 2.0 [31] to design a specific primer for the detection of *N. ceranae* (Fig. 1). Using these two primers, a 216-bp fragment of the small subunit rRNA gene was amplified.

The specificity of *N. ceranae* primers was evaluated by testing cross-amplification of total DNA extracted from honeybee (*Apis mellifera*) and other honeybee pathogens, *Melissococcus plutonius* (ATCC35311), *Varroa destructor*, *Tropilaelaps* spp., and *Paenibacillus larvae* (ATCC9545).

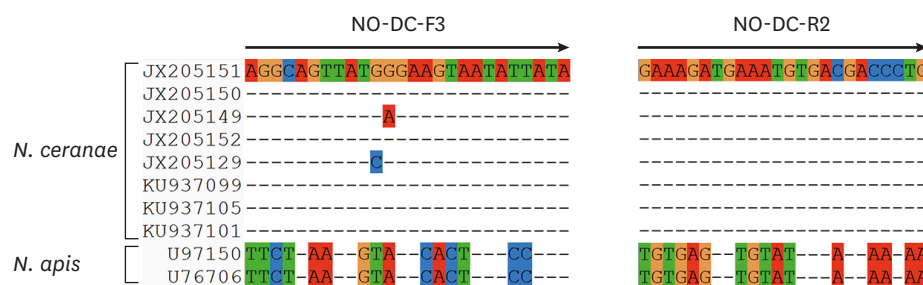


Fig. 1. Alignment of *N. ceranae* and *N. apis* DNA for design of *N. ceranae* specific primers. The specific primer pair NO-DC-F3/NO-DC-R2 for *N. ceranae* detection was designed based on the alignment of ribosomal RNA gene sequences of *N. ceranae* and *N. apis*. NCBI accession No. of the sequences are shown. “---” indicates nucleotide identity with the consensus sequence. NCBI, National Center for Biotechnology Information.

Microscopic enumeration of *Nosema* spores

Microscopic counts of *N. ceranae* spores were conducted according to the standard method described by Human et al. [18]. Briefly, 1 mL of spore solution was prepared for counting. The enumeration was conducted using a Neubauer improved bright-line hemocytometer (Paul Marienfeld GmbH & Co. KG, Germany) and a light microscope at 400× magnification. Two of the 16 (0.025 × 0.025) cm² squares in each of the four corners of the hemocytometer were randomly selected for a total of eight selected squares in each count, and the spores within each randomly selected square were counted. After counting, the hemocytometer was carefully cleaned using distilled water. A new solution of the same sample was added to the hemocytometer for the second enumeration with eight other 8 squares. In total, 16 squares were counted twice for 2 times in each sample. The number of spores per mL was calculated using the following formula: total counted spores/(16 × 0.025 × 0.025 × 0.01 cm³).

Molecular enumeration by quantitative PCR

UR-qPCR was performed using a GENECHECKER PCR machine UF150 (Genesystem Co., Ltd., Korea) and 2× Rapi Master Mix (Genesystem Co., Ltd.). Each 10-μL reaction included 5 μL 2× Rapi Mix, 2 μL primers (10 pmol of each primer), and a maximum of 3 μL with ≤ 10 ng DNA template. SYBR green, which is included in the Rapi Mix, was used as a fluorescent dye for detecting UR-qPCR amplification. The PCR conditions were as follows: 95°C for 30 s, followed by 50 cycles of 95°C for 3 s, 55°C for 3 s, and 72°C for 3 s.

The standard curve for calculation of DNA copy number was established based on amplification using recombinant DNA with 10-fold serial dilution (from 2.05 × 10⁸ to 2.05 × 10⁰ copies per reaction). Linear regression representing the relationship between the log₁₀ initial DNA copy number and the corresponding threshold cycle (Ct) was created from triplicate PCR runs.

The same serially diluted recombinant DNA and specific primers of *N. ceranae* were also used for other real-time PCR systems (The CFX96 Touch Real-time PCR Detection System, BIO-RAD, USA) to compare the sensitivity of *N. ceranae* detection with the UR-qPCR. Twenty-μL reaction mix was prepared consisting of 10 μL 2× Rapi Master Mix (Genesystem Co., Ltd.), 1 μL (10 pmol) of each primer, 1 μL of DNA template, and 7 μL of ddH₂O. PCR conditions were 95°C (3 min), followed by 45 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s.

Limit of nosema detection by microscopic and molecular methods

The spore concentration of the prepared solution was determined to be 2.40×10^7 spores/mL through microscopy. The spore solution was divided into two 1.5-mL Eppendorf tubes (1 mL/tube). The spore solution in the first tube was serially 10-fold diluted using 0.1 mL spore solution plus 0.9 mL of ddH₂O until reaching a concentration of 2.40×10^0 spores/mL and was used for microscopic enumeration. In addition, 10% (0.1 mL) of each dilution (2.40×10^7 to 2.40×10^1 spore/mL), which contained 2.40×10^6 to 2.40×10^0 spores, respectively, was set aside for DNA isolation, in order to generate a spore dilution series. The DNA solution extracted from each spore was 100 μ L. Meanwhile, the spores (2.40×10^7 spores) in the second tube were subjected to DNA extraction to obtain 100 μ L of DNA solution. The DNA solution was then serially 10-fold diluted by adding 10 μ L of DNA solution plus 90 μ L of ddH₂O until reaching a concentration that corresponded to the concentration of DNA obtained from 2.40×10^0 spores, in order to generate a DNA dilution series.

To identify the limit of detection of the two counting methods, UR-qPCR was performed using the DNA solution that originated from the DNA and spore dilutions. Microscopic counting was conducted using a dilution series of the spore solution.

Evaluate the development of *N. ceranae* caged honeybees

The relationship between the two methods was evaluated based on the quantitative results from samples of infected honeybees. *N. ceranae* levels were measured by molecular quantification and microscopic counting from caged bees ($n = 300$), *A. mellifera*, which were divided into three cages (100 bees/cage). The cages were designed according to the instructions of Williams et al. [32]. After starvation for 24 h, the material named Bee happy (Bioforce Co., Ltd., Korea) with 100 \times and 300 \times dilution rates in 50% (w/v) sucrose solution were supplied to the bees in each cage. The bees in the control cage were fed with a 50% sucrose solution. The volume of the feeding solution supplied to each cage was based on an estimated consumption of 50 μ L per bee per day. The severity of *N. ceranae* infection in the bees from each cage was assessed before feeding and every 2 days after feeding until the 8th day. Five bees in each cage were randomly removed for molecular quantification using UR-qPCR and microscopic enumeration at each time point. Caged bees were kept in a dark room at 25°C during the infection period.

RESULTS

Sensitivity and specificity of UR-qPCR for detection of *N. ceranae*

The detection of *N. ceranae* using the primer pair NO-DC-F3/R2 showed a limit of 2.05×10^0 copies of recombinant DNA under PCR conditions of 95°C (30 s) and 50 cycles of 95°C (3 s), 55°C (3 s), and 72°C (3 s) (**Fig. 2A**). The linear regression of \log_{10} values of initial DNA copies and the corresponding Ct values was $y = -3.2283x + 37.796$; $R^2 = 0.9971$, where x and y represent \log_{10} DNA copy number and Ct value, respectively (**Fig. 2B**). The amplification efficiency calculated from the slope of the standard curve was 104.06%. The sensitivity of *N. ceranae* detection using the primer pair NO-DC-F3/R2 was also seen with a limit of 2.05×10^0 copies of recombinant DNA can be detected using other conventional real-time PCR systems (**Fig. 2D**).

The specificity of the *N. ceranae* UR-qPCR system was confirmed by amplification of only *N. ceranae* DNA among the assessed DNA templates of honeybee (*Apis mellifera*), *Melissococcus plutonius*, *Varroa destructor*, *Tropilaelaps* spp., and *Paenibacillus larvae* (**Fig. 2C**).

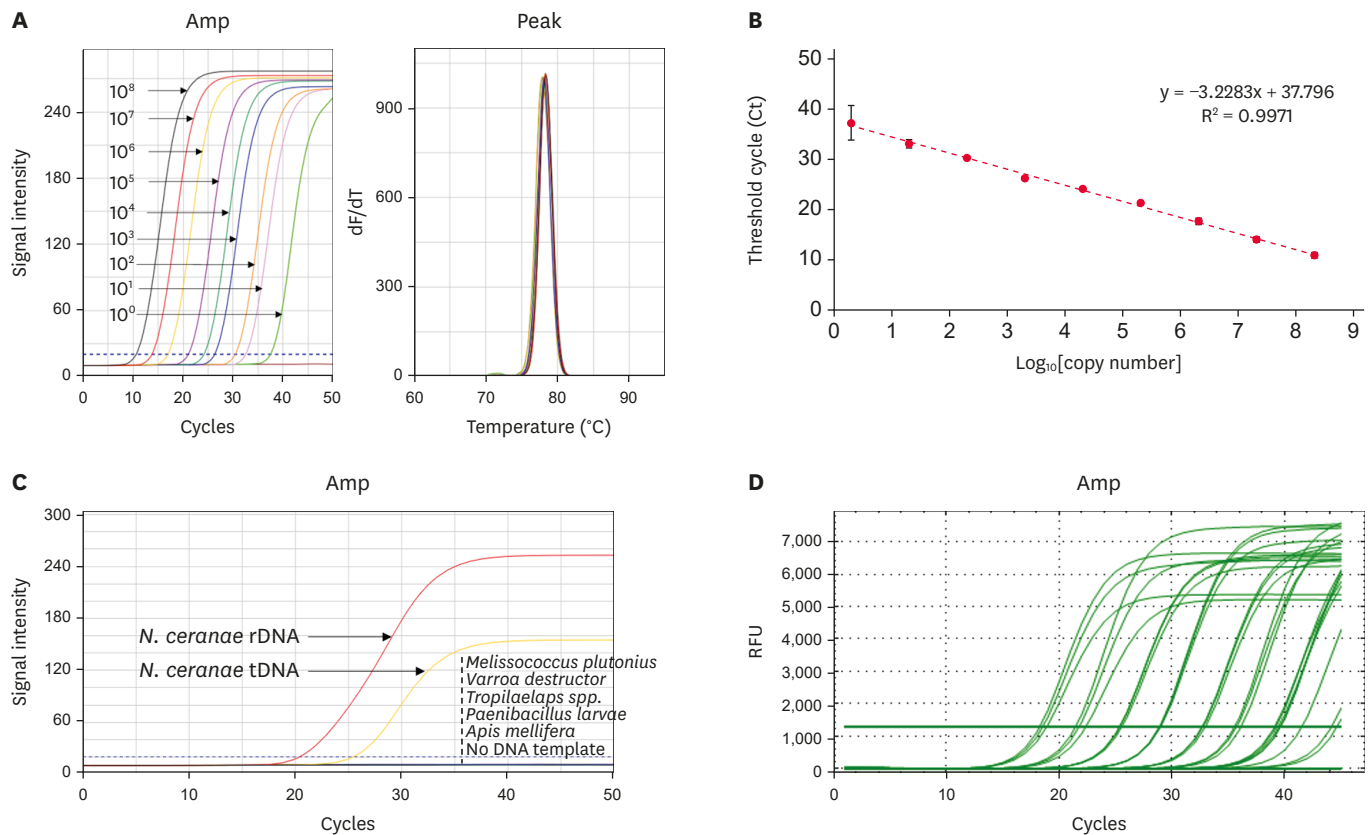


Fig. 2. Sensitivity and specificity of *N. ceranae* detection.

(A) Fluorescent curves represent the amplification of serially diluted recombinant DNA (2.05×10^8 to 2.05×10^0 copies). (B) The linear regression of Ct values and initial DNA copies was established using data from 3 PCR replicates. (C) The specificity of *N. ceranae* primers was seen by the amplification of only *N. ceranae* tDNA and rDNA, and negative amplification was seen on DNA template of honeybee (*Apis mellifera*), *Melissococcus plutonius*, *Varroa destructor*, *Tropilaelaps* spp., *Paenibacillus larvae*, and no DNA template. (D) The sensitivity of detection with a limit of 2.05×10^0 copies of *N. ceranae* recombinant DNA is shown by using the CFX96 Touch Real-time PCR Detection System (BIO-RAD, USA). Ct, threshold cycle; PCR, polymerase chain reaction; tDNA, total DNA; rDNA, recombinant DNA.

Quantification of *N. ceranae* in caged bees

The results showed that the Bee happy solution had possible repressive effects against the development of *N. ceranae* in infected bees. The 100× dilution of Bee happy solution (100× DBS) and 300× dilution of Bee happy solution (300× DBS) induced the increase of *N. ceranae* after 2 days from $3.42 \times 10^6 \pm 6.77 \times 10^5$ to $7.84 \times 10^6 \pm 9.00 \times 10^5$ and $2.00 \times 10^7 \pm 1.97 \times 10^6$ in the 2 feeding, respectively. In the 100× BDS feeding, the efficacy of *N. ceranae* inhibition was seen after 4 days of feeding and the lowest number of spore was seen at the 6th day with $2.26 \times 10^5 \pm 1.70 \times 10^5$ spores/bee before increasing to $5.47 \times 10^6 \pm 8.25 \times 10^5$ spore/bee at the 8th day of feeding period. Meanwhile, the inhibitory effect of 300×BDS diminished after the 4th day, and the spore numbers reached levels comparable to those of the negative control by the end of the feeding period (**Table 1**). The results were confirmed by molecular quantification using UR-qPCR, which showed the same trend of *N. ceranae* development as the microscopic counting results (**Table 2**).

Relationship between microscopic enumeration and molecular quantification

The results of *N. ceranae* quantification from the different caged bees showed that molecular detection was more stable and sensitive than the microscopy approach. The microscopy method negatively detected *N. ceranae* at 2.74×10^4 DNA copies/bee (**Tables 1 and 2**). In the

Table 1. Microscopic count of *N. ceranae* spores per bee during the feeding period

Treatment	<i>N. ceranae</i> spore/bee				
	0 day*	2 days	4 days	6 days	8 days
Control	3.42×10^6 $\pm 6.77 \times 10^5$	4.28×10^6 $\pm 5.94 \times 10^5$	1.54×10^7 $\pm 1.82 \times 10^6$	1.71×10^7 $\pm 1.18 \times 10^6$	1.29×10^7 $\pm 1.29 \times 10^6$
100× DBS	3.42×10^6 $\pm 6.77 \times 10^5$	7.84×10^6 $\pm 9.00 \times 10^5$	3.88×10^6 $\pm 8.30 \times 10^5$	2.26×10^5 $\pm 1.70 \times 10^5$	5.47×10^5 $\pm 8.25 \times 10^5$
300× DBS	3.42×10^6 $\pm 6.77 \times 10^5$	2.00×10^7 $\pm 1.97 \times 10^6$	0	6.80×10^6 $\pm 8.70 \times 10^5$	1.13×10^7 $\pm 1.24 \times 10^6$

DBS, dilution of Bee happy solution.

**N. ceranae* spores were counted before feeding (0 day) and every two days during the feeding period.

Table 2. Molecular quantification of *N. ceranae* DNA per bee during the feeding period

Treatment	<i>N. ceranae</i> DNA copy/bee				
	0 day*	2 days	4 days	6 days	8 days
Control	2.92×10^7 $\pm 9.44 \times 10^6$	2.74×10^7 $\pm 9.22 \times 10^6$	1.13×10^8 $\pm 4.05 \times 10^7$	1.35×10^8 $\pm 3.82 \times 10^7$	1.27×10^8 $\pm 5.59 \times 10^7$
100× DBS	2.92×10^7 $\pm 9.44 \times 10^6$	4.44×10^7 $\pm 1.89 \times 10^7$	2.27×10^7 $\pm 1.03 \times 10^7$	1.42×10^6 $\pm 1.55 \times 10^4$	6.82×10^7 $\pm 2.81 \times 10^5$
300× DBS	2.92×10^7 $\pm 9.44 \times 10^6$	1.85×10^8 $\pm 8.26 \times 10^7$	2.74×10^4 $\pm 1.62 \times 10^3$	5.68×10^7 $\pm 1.79 \times 10^7$	1.11×10^8 $\pm 3.38 \times 10^7$

DBS, dilution of Bee happy solution.

**N. ceranae* DNA was quantified before feeding (0 day) and every two days during the feeding period.

Table 3. Ratio of *N. ceranae* quantitative result by molecular count to microscopic count

Treatment	<i>N. ceranae</i> DNA/spore*					Average
	0 day*	2 days	4 days	6 days	8 days	
Control	8.54	6.40	7.34	7.89	9.84	8.00 ± 1.29
100× DBS	8.54	5.66	5.85	6.28	12.47	7.76 ± 2.87
300× DBS	8.54	9.25	-†	8.35	9.82	8.99 ± 0.68

DBS, dilution of Bee happy solution.

*Ratio of the mean value of DNA copy to spore number from counting every 2 days; †Unidentified result due to the negative detection of microscopic count.

medium (10^6 spores/bee) and high levels (10^7 spores/bee) of *N. ceranae* infection [33], stable detection was achieved by microscopy, and DNA copy numbers were approximately 8-fold higher than spore counts from the same sample (Table 3).

Limit of detection of *N. ceranae*

Microscopic enumeration of the serial spore dilution (from 2.40×10^7 to 2.40 spores/mL) revealed that a minimum of 2.40×10^4 spores/mL are needed for quantitative detection. However, the number of spores at this concentration exhibited the greatest variation from the mean value (Fig. 3). The limit of detection for molecular quantification showed that 3 of 100 μ L DNA solution isolated from 24 *N. ceranae* spores equal to 0.72, spores could be detected in each PCR reaction, and this result was confirmed by the counts of both the DNA dilution series solution and DNA isolated from the spore dilution series (Fig. 4).

DNA copy calculated from each DNA concentration of the DNA dilution series and spore dilution series showed that the quantification result by handling on DNA solution was more stable than on spore solution, as demonstrated by the DNA copy calculated from the DNA dilution series and spore dilution series in comparison with serially diluted spore number. DNA copy number calculated from DNA dilution series at all concentrations was approximately 8-fold higher (7.89 ± 1.24 on average) than spore number, and the relationship between DNA copy and spore number was represented by a linear regression, $y = 0.9656x + 1.0213$; $R^2 = 0.9973$; where x and y are \log_{10} of spore number and the corresponding DNA copy,

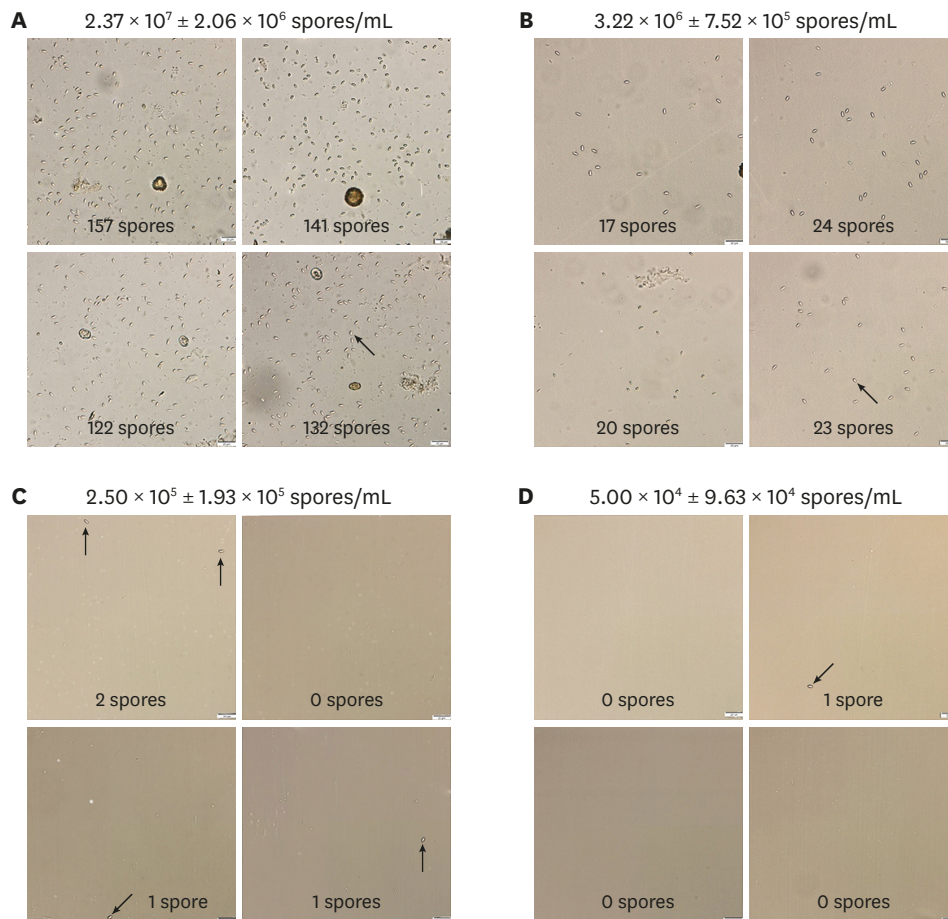


Fig. 3. Microscopic enumeration of purified *N. ceranae* spores. The real concentration of the spore solution was calculated based on the spores counted using each concentration of diluted spore solution, from 2.40×10^7 to 2.40×10^4 , indicated in A to D, respectively. Spores observed for counting in four of the 16 observation fields of a hemocytometer are shown. The white bar in the bottom right corner represents 20 μ m, and the arrows indicate individual spores. The observations were performed at 400 \times magnification under a light microscope.

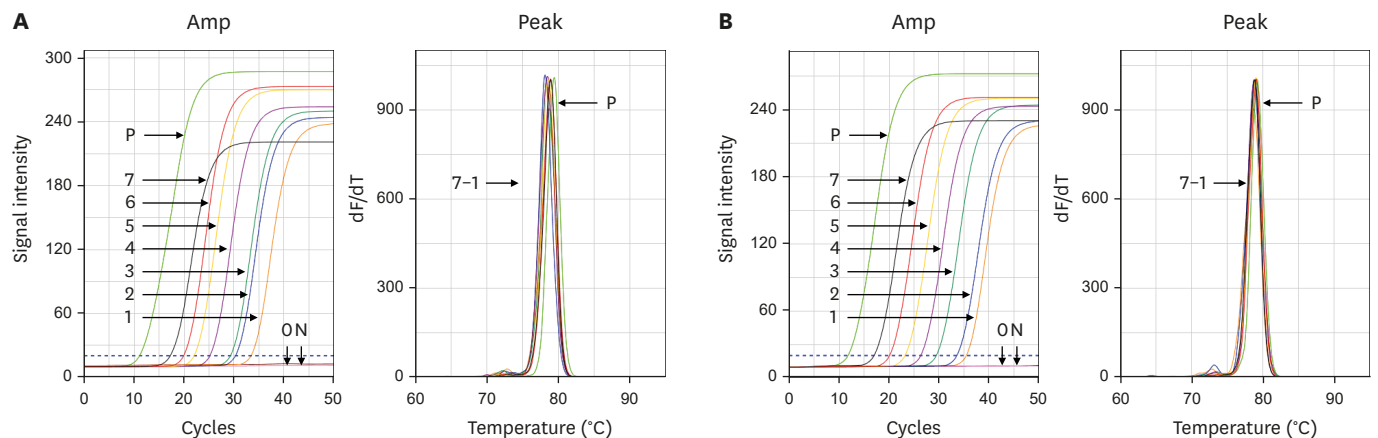


Fig. 4. *N. ceranae* detection in serially diluted DNA solutions. Detection of *N. ceranae* in a spore dilution series and in a DNA dilution series are indicated in panels A and B, respectively. Numbers 0 to 7 indicate the number of spores ranging from 2.40×10^0 to 2.40×10^7 , respectively, which were used for DNA isolation. “N” indicates the negative control without DNA template, and “P” indicates positive control that contained *N. ceranae* recombinant DNA (2.05×10^9 copies).

respectively. Meanwhile, DNA copy calculated from DNA solution originating from spore dilution series at a concentration $\geq 2.40 \times 10^3$ spores/mL was also approximately 8-fold higher than the spore number (8.29 ± 1.52 on average). However, at lower spore concentrations (2.40×10^2 and 2.40×10^1 spores/mL), the dilution of spore solution results in the variation of detected result, which was demonstrated by the increase of DNA copy compared to the expected spore numbers to around 29.42 and 37.97 times, respectively.

DISCUSSION

Quantification of *N. ceranae* by UR-qPCR improved the sensitivity and stability of detection in comparison with microscopic enumeration. The microscopy approach is limited by the ability to observe and count spores, but the visible spores account for only a fraction of the life cycle of the microsporidia that live outside the intestinal cells of the host, and other stages remain invisible [34]. Consequently, low levels of infection can be difficult to detect using microscopic observation. In contrast, molecular quantification relies on PCR performance; the total amount of *Nosema* DNA in the sample was detected and calculated according to a standard curve. Therefore, PCR detection using specific primers has been demonstrated to be useful for the quantitative detection of intracellular parasites [35].

At stable detection levels between 10^5 and 10^7 spores/bee from infected samples and $\geq 2.40 \times 10^3$ spores/mL from the purified spores, the DNA copy numbers were approximately 8-fold higher than the spore counts determined by microscopy. These results were consistent with those of a previous study that considered the *Nosema* genome to contain 10 copies of the targeted ribosomal RNA gene [24].

It has been previously claimed that the limit of detection in one microscopic field is 5×10^4 spores/mL, and the sensitivity is 2.5×10^3 spores/mL when observing 10 fields while counting *Nosema bombycis* spores [36]. However, when one spore was detected in the area of $0.025 \times 0.025 \text{ cm}^2$ and 0.01 cm depth, the limit of detection was 1.6×10^5 spores/mL ($1/0.01 \times 0.025 \times 0.025 \text{ cm}^3$), and when 10 fields were screened, the sensitivity of detection reached 1.6×10^4 spores/mL. The optimal concentrations of purified spore solution for microscopic enumeration were 2.40×10^5 and 2.40×10^6 spores/mL, considering the time and accuracy of quantification. At these concentrations, the number of spores observed in each field was approximately 2 and 20, respectively. At a concentration of 2.40×10^7 spores/mL, more than 100 spores were observed in each area. Therefore, more time was required to count the spores, and it was determined that acquiring images of the observation field and then counting the spores in the image was a more efficient approach. Meanwhile, molecular detection showed stability of detection at 2.40×10^1 spores.

Grinding the spores in liquid nitrogen prior to DNA isolation using DNeasy Blood & Tissue Kit (Qiagen) and followed by UR-qPCR detection showed a sensitivity of *N. ceranae* detection with a limit of 24 spores per bee could be detected when the volume of DNA solution extracted from 24 spores was 100 μL (equal to 0.24 spore/ μL), and 3 of the 100 μL was used for each PCR corresponding to 0.72 spore per PCR reaction can be detected. The results of quantitative PCR compared to spore counting using microscopic methods showed that the number of DNA copies was approximately eight times higher than spore number, multiple copies of the target gene in each spore [24]. Therefore, approximately 5.76 DNA copies (0.72 spore \times 8 copies = 5.76 copies) were used in each PCR reaction. The UR-qPCR and DNA

isolation in this study were much more sensitive than the HBRC DNA isolation method and conventional PCR [23], which had a limit of detection of 100 spores per bee and 10 spores per PCR reaction, and DNA isolation using the DNeasy Plant Mini kit (Qiagen) followed by conventional real-time PCR with a limit of 10 spores per PCR reaction can be detected [37].

The *N. ceranae*-specific UR-qPCR developed in this study showed specificity and was as sensitive as conventional real-time PCR. However, this UR-qPCR system showed the advantages of mobility, simple protocol, and time saving for PCR performance. The time required to complete 50 cycles of UR-qPCR (3 s for each PCR step) was 20 min and 36 s. PCR can be limited by the half-life of *Taq* polymerase (approximately 45 min at 95°C [38]); therefore, the rapidity of UR-qPCR allows for more cycles before degradation of *Taq* polymerase. The UR-qPCR was more rapid than other real-time PCR systems [21,25], and the LAMP methods, which require 30 min to 1 h [26,27], have been developed for the detection of *N. ceranae*.

An appropriate method for *N. ceranae* enumeration using UR-qPCR was evaluated in this study. This method improves upon quantitative detection methods that use real-time PCR. The advantages of this method are the stability and sensitivity of quantification when compared to microscopic enumeration and rapidity when compared to previous molecular-based methods. Additionally, evaluation of the development of *N. ceranae* in honeybees fed with the Bee happy solution showed the repressive potential of this material against *N. ceranae*. These findings provide the basis for further studies that may demonstrate the efficacy and safety of this formulation for the control of nosemosis in honeybee populations.

REFERENCES

1. Fries I, Feng F, Da Silva A, Slemmeda SB, Pieniazek NJ. *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). *Eur J Protistol.* 1996;32(3):356-365.
[CROSSREF](#)
2. Higes M, Martín R, Meana A. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. *J Invertebr Pathol.* 2006;92(2):93-95.
[PUBMED](#) | [CROSSREF](#)
3. Higes M, Martín-Hernández R, Botías C, Bailón EG, González-Porto AV, Barrios L, et al. How natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environ Microbiol.* 2008;10(10):2659-2669.
[PUBMED](#) | [CROSSREF](#)
4. Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, et al. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *J Invertebr Pathol.* 2007;96(1):1-10.
[PUBMED](#) | [CROSSREF](#)
5. Martín-Hernández R, Meana A, Prieto L, Salvador AM, Garrido-Bailón E, Higes M. Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. *Appl Environ Microbiol.* 2007;73(20):6331-6338.
[PUBMED](#) | [CROSSREF](#)
6. Williams GR, Shafer AB, Rogers RE, Shutler D, Stewart DT. First detection of *Nosema ceranae*, a microsporidian parasite of European honey bees (*Apis mellifera*), in Canada and central USA. *J Invertebr Pathol.* 2008;97(2):189-192.
[PUBMED](#) | [CROSSREF](#)
7. Chen Y, Huang ZY. *Nosema ceranae*, a newly identified pathogen of *Apis mellifera* in the USA and Asia. *Apidologie (Celle).* 2010;41(3):364-374.
[CROSSREF](#)
8. Fries I. *Nosema ceranae* in European honey bees (*Apis mellifera*). *J Invertebr Pathol.* 2010;103 Suppl 1:S73-S79.
[PUBMED](#) | [CROSSREF](#)
9. Urbietta-Magro A, Higes M, Meana A, Gómez-Moracho T, Rodríguez-García C, Barrios L, et al. The levels of natural *Nosema* spp. infection in *Apis mellifera* iberiensis brood stages. *Int J Parasitol.* 2019;49(8):657-667.
[PUBMED](#) | [CROSSREF](#)

10. Gallai N, Salles JM, Settele J, Vaissiere BE. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecol Econ*. 2009;68(3):810-821.
[CROSSREF](#)
11. Ollerton J, Winfree R, Tarrant S. How many flowering plants are pollinated by animals? *Oikos*. 2011;120(3):321-326.
[CROSSREF](#)
12. Glenny W, Cavigli I, Daughenbaugh KF, Radford R, Kegley SE, Flenniken ML. Honey bee (*Apis mellifera*) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. *PLoS One*. 2017;12(8):e0182814.
[PUBMED](#) | [CROSSREF](#)
13. Botías C, Anderson DL, Meana A, Garrido-Bailón E, Martín-Hernández R, Higes M. Further evidence of an oriental origin for *Nosema ceranae* (Microsporidia: Nosematidae). *J Invertebr Pathol*. 2012;110(1):108-113.
[PUBMED](#) | [CROSSREF](#)
14. Lee ML, Choi JY, Lee MY, Kim YS. Infection level of honeybee *Nosema* disease in Korea. *Korean J Apic*. 2003;18(2):151-154.
15. Hong IP, Lee MY, Woo SO, Sim HS, Choi YS, Han SM, et al. Prevalence of honeybee *Nosema* disease and black queen cell virus on flowering period of *Robinia pseudoacacia* in 2013. *Korean J Apic*. 2013;28(3):199-203.
16. Annual report of honeybee diseases in Korea [Internet]. Gimcheon: Animal and Plant Quarantine Agency; http://www.qia.go.kr/anp/rchStatus/listwebQiaCom.do?type=79_1hbedp&clear=1. Updated 2020. Accessed 2020 Sep 18.
17. Fries I, Chauzat MP, Chen YP, Doublet V, Genersch E, Gisder S, et al. Standard methods for *Nosema* research. *J Apic Res*. 2013;52(1):1-28.
[CROSSREF](#)
18. Human H, Brodschneider R, Dietemann V, Dively G, Ellis JD, Forsgren E, et al. Miscellaneous standard methods for *Apis mellifera* research. *J Apic Res*. 2013;52(4):1-53.
[CROSSREF](#)
19. Cantwell G. Standard methods for counting *Nosema* spores. *Am Bee J*. 1970;110:222-223.
20. Ptasińska A, Borsuk G, Mullenko W, Demetraki-Paleolog J. Differentiation of *Nosema apis* and *Nosema ceranae* spores under scanning electron microscopy (SEM). *J Apic Res*. 2014;53(5):537-544.
[CROSSREF](#)
21. Chen YP, Evans JD, Murphy C, Gutell R, Zuker M, Gundensen-Rindal D, et al. Morphological, molecular, and phylogenetic characterization of *Nosema ceranae*, a microsporidian parasite isolated from the European honey bee, *Apis mellifera*. *J Eukaryot Microbiol*. 2009;56(2):142-147.
[PUBMED](#) | [CROSSREF](#)
22. Rivière MP, Ribière M, Chauzat MP. Recent molecular biology methods for foulbrood and nosemosis diagnosis. *Rev Sci Tech*. 2013;32(3):885-892.
[PUBMED](#) | [CROSSREF](#)
23. Hamiduzzaman MM, Guzman-Novoa E, Goodwin PH. A multiplex PCR assay to diagnose and quantify *Nosema* infections in honey bees (*Apis mellifera*). *J Invertebr Pathol*. 2010;105(2):151-155.
[PUBMED](#) | [CROSSREF](#)
24. Bourgeois AL, Rinderer TE, Beaman LD, Danka RG. Genetic detection and quantification of *Nosema apis* and *N. ceranae* in the honey bee. *J Invertebr Pathol*. 2010;103(1):53-58.
[PUBMED](#) | [CROSSREF](#)
25. Forsgren E, Fries I. Comparative virulence of *Nosema ceranae* and *Nosema apis* in individual European honey bees. *Vet Parasitol*. 2010;170(3-4):212-217.
[PUBMED](#) | [CROSSREF](#)
26. Ptasińska AA, Borsuk G, Woźniakowski G, Gnat S, Malek W. Loop-mediated isothermal amplification (LAMP) assays for rapid detection and differentiation of *Nosema apis* and *N. ceranae* in honeybees. *FEMS Microbiol Lett*. 2014;357(1):40-48.
[PUBMED](#) | [CROSSREF](#)
27. Chupia V, Patchanee P, Krutmuang P, Pikulkaew S. Development and evaluation of loop-mediated isothermal amplification for rapid detection of *Nosema ceranae* in honeybee. *Asian Pac J Trop Dis*. 2016;6(12):952-956.
[CROSSREF](#)
28. Urbietta-Magro A, Higes M, Meana A, Barrios L, Martín-Hernández R. Age and method of inoculation influence the infection of worker honey bees (*Apis mellifera*) by *Nosema ceranae*. *Insects*. 2019;10(12):417.
[PUBMED](#) | [CROSSREF](#)

29. Rubanov A, Russell KA, Rothman JA, Nieh JC, McFrederick QS. Intensity of *Nosema ceranae* infection is associated with specific honey bee gut bacteria and weakly associated with gut microbiome structure. *Sci Rep*. 2019;9(1):3820.
[PUBMED](#) | [CROSSREF](#)
30. Wang J, Lee D, Ku SJ, Peak MC, Min SH, Lim SJ, et al. Development of a detection method against 11 major pathogens of honey bee using amplification of multiplex PCR and specific DNA-chip. *J Apic*. 2016;31(2):133-146.
[CROSSREF](#)
31. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007;23(21):2947-2948.
[PUBMED](#) | [CROSSREF](#)
32. Williams G, Alaux C, Costa C, Csáki T, Doublet V, Eisenhardt D, et al. Standard methods for maintaining adult *Apis mellifera* in cages under *in vitro* laboratory conditions. *J Apic Res*. 2013;52(1):1-36.
[CROSSREF](#)
33. Yücel B, Goğaroğlu M. The impact of *Nosema apis* Z. infestation of honey bee (*Apis mellifera* L.) colonies after using different treatment methods and their effects on the population levels of workers and honey production on consecutive years. *Pak J Biol Sci*. 2005;8(8):1142-1145.
[CROSSREF](#)
34. Gray FH, Cali A, Briggs JD. Intracellular stages in the life cycle of the microsporidian *Nosema apis*. *J Invertebr Pathol*. 1969;14(3):391-394.
[PUBMED](#) | [CROSSREF](#)
35. Refardt D, Ebert D. Quantitative PCR to detect, discriminate and quantify intracellular parasites in their host: an example from three microsporidians in *Daphnia*. *Parasitology*. 2006;133(Pt 1):11-18.
[PUBMED](#) | [CROSSREF](#)
36. Fu Z, He X, Cai S, Liu H, He X, Li M, et al. Quantitative PCR for detection of *Nosema bombycis* in single silkworm eggs and newly hatched larvae. *J Microbiol Methods*. 2016;120:72-78.
[PUBMED](#) | [CROSSREF](#)
37. Erler S, Lommatzsch S, Lattorff HM. Comparative analysis of detection limits and specificity of molecular diagnostic markers for three pathogens (Microsporidia, *Nosema* spp.) in the key pollinators *Apis mellifera* and *Bombus terrestris*. *Parasitol Res*. 2012;110(4):1403-1410.
[PUBMED](#) | [CROSSREF](#)
38. Lawyer FC, Stoffel S, Saiki RK, Chang SY, Landre PA, Abramson RD, et al. High-level expression, purification, and enzymatic characterization of full-length *Thermus aquaticus* DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. *PCR Methods Appl*. 1993;2(4):275-287.
[PUBMED](#) | [CROSSREF](#)