Original Article

Diagnosis of Pebrine Disease in Silkworm Using Molecular Methods

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How to cite this article: **Bagheri M, Zahmatkesh A, Moharrami M, Nematollahian S, Torkaman M. Diagnosis of Pebrine Disease in Silkworm Using Molecular Methods. Archives of Razi Institute. 2023;78(4):1185-91.**

DOI: 10.32592/ARI.2023.78.4.1185

Article Info:
Received: 30 October 2022
Accepted: 24 November 2022
Published: 31 August 2023

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ABSTRACT

Since pebrine disease, as the most important and dangerous disease in silkworms, spreads horizontally through the spores and vertically through the eggs, combating the disease and eliminating it completely from livestock production has been associated with numerous problems. This project aimed to identify the molecular cause of pebrine disease in silkworms using a sensitive, specific, and accurate method. To this purpose, a 136 bp fragment was selected based on the *Nosema bombycis* partial SSU rDNA sequence, and a pair of primers was designed. Afterward, using the conventional polymerase chain reaction (PCR) method, the target fragment was amplified and sequenced. After that, to determine the detection sensitivity, using the Real-Time PCR method, 5-fold serial dilutions of *N. bombycis* DNA were prepared, and the last dilution that produced a fluorescent signal was considered the minimum detection limit. All tests were performed in duplicates. Based on the results of the sensitivity test, the standard curve including Ct values and DNA concentration was used for analysis. Moreover, 80 unknown samples examined by light microscope were evaluated using conventional PCR and Real-Time PCR. Both PCR results showed no amplification for the negative control samples. The findings demonstrated that the lowest detection limit for *N. bombycis* was less than 6 pg of DNA, while, this amount was 8 ng for conventional PCR. Out of 80 samples examined, 55, 60, and 62 samples were positive for light microscope, conventional PCR, and Real-Time PCR methods, respectively. The findings suggested that the Real-Time PCR method had a higher ability to detect the causative agent of pebrine disease than the conventional PCR method, and both methods were superior to light microscopy. Therefore, due to the fewer steps and higher accuracy of Real-Time PCR, it can be introduced as a suitable method for diagnosing pebrine disease.

Keywords: Silkworm, Pebrine disease, *Nosema bombycis*, Real-Time PCR method

1. Introduction

Pebrine disease, as the most important and dangerous disease in silkworms, has caused tremendous economic losses in the silk industry. Since pebrine disease spreads horizontally and vertically by transmission from spores, it is too difficult to eradicate it from sericulture and silk industries, and every year it causes the loss of significant amounts of sericulture products (1). The most applied method of diagnosing pebrine disease in Iran is the light microscope method; however, this method lacks acceptable accuracy (2).

In recent years, pebrine disease has been the main problem in the silkworm fields in Iran, and even commercial lines have been affected. During 2016-2019, the spread of pebrine disease on a large scale in an egg production farm in Gilan province practically disrupted the production of commercial hybrid eggs and forced the import of silk products from 2019 until now. This industry is highly important because Iran had become one of the few countries in the world that has a complete silk cocoon production line (including line, ancestors, parents, and commercial hybrids) from 20 years ago. Indeed, this industry has been completely endogenous, and combating this disease is one of the main priorities of the country's breeding industry (3). It has been shown that microsporidian spores can reduce nutrition and metabolism as well as protein production, which would lead to a decrease in larval weight in *Bombyx mori*. In addition, microsporidiosis has a negative effect on all economic traits in *B. mori*, while the higher spore amounts result in worse results (4).

Detection of *Nosema bombycis* in eggs using Real-Time polymerase chain reaction (PCR) with specific primers and probes has shown a high ability to the detection of suspicious samples. Moreover, the cost of preparing Real-Time PCR materials decreases with an increase in the number of samples; therefore, it can be a complementary or alternative method for diagnosing pebrine disease (5). There is a high correlation between the results of conventional PCR and Real-Time PCR. However, Real-Time PCR has fewer steps and is more sensitive and accurate. It offers a more significant advantage over conventional PCR and accelerates analysis while improving the accuracy of diagnosis in suspicious samples (6).

In recent years, many pathogens, including pathogenic microsporidia, have been detected using the Real-Time qPCR method (7). This method is able to detect pathogens as well as the number of DNA copies of the template by measuring the amount of amplicon using fluorescent light in each cycle (8). In the Real-Time PCR method, all detection process is performed quickly in a closed system. Therefore, it reduces contamination from the environment or other samples (9). Fu, He (9) showed high sensitivity and high reproducibility in the detection of *N. bombycis* in eggs and newly hatched larvae using the qPCR method. Microsporidia are easily detected by light microscopy when the density of spores is high; nevertheless, primary infections with a low number of spores cause errors. Due to this limitation, the study on the prevalence and mode of transmission has been distorted. To overcome these problems, PCR-based techniques have been developed to identify major silkworm pathogens with high specificity and sensitivity.

The present study aimed to diagnose pebrine disease based on molecular techniques (conventional and Real-Time PCR methods) using specific sequences resulted from previous studies (10, 11). Furthermore, the estimation of the detection limit for *N. bombycis* infection was based on the Real-Time PCR method.

2. Materials and Methods

2.1. Data Collection

Infected larvae and mother moths were collected from several farms (Parand, Parnian, Shaft, and Silk Research Center) in the north of Iran, which have the same conditions and management.

2.2. Isolation and Purification of Microsporidia Spores

The pure microsporidia spores were extracted from infected moths as described earlier by Sato,

Kobayashi (12): in brief, 20-30 infected moths were homogenized (crushed together in 25 mL of 0.5% potassium carbonate solution). Afterward, the suspension was filtered twice through absorbent cotton and washed 2-3 times in distilled water. The suspension was centrifuged at 5,000 g for 10 min. Thereafter, the sediments were treated with 5 mL of 2% potassium hydroxide solution. At the next stage, the spore solution was added to a discontinuous sucrose gradient (25%, 50%, 75%, and 100% v/v, each at 5 mL volume in a 50-mL falcon) and centrifuged at 20,000 g for 40 min twice. Finally, pure spores placed between 75% and 100% sucrose levels were obtained, suspended in distilled water, and counted using a hemocytometer under a phase-contrast light microscope.

2.3. DNA Extraction, Polymerase Chain Reaction

DNA was extracted from *N. bombycis* spores using an optimized phenol-chloroform protocol (13). Subsequently, a 136 bp fragment was selected based on the *N. bombycis* SSU rDNA sequence identified in a previous study (10). A pair of primers corresponding to the partial SSU rDNA fragment was designed and synthesized; F: 5'-GTCCCTGTTCTTTGTAC -3' and R: 5'-ATCCTGCTAATGGTTCT -3'. PCR was carried out using 50 ng genomic DNA, 20 pmol of each specific forward and reverse primers, 200 µM dNTP, 2.5 µL 10X *pfu* enzyme buffer with 2 mM Mg^{2+}, and 0.5 U of *Pfu* DNA polymerase (Thermo Fisher Scientific, cat no. EP0501) in a final volume of 25 µL. The condition of PCR was set as an initial denaturing at 94°C for 5 min, followed by 30 cycles of 94°C for 40 secs, annealing temperatures of 49°C for 40 sec, and extension at 72°C for 2 min. The final extension was set at 72°C for 10 min. PCR products were monitored by 1% agarose gel electrophoresis.

2.4. Purification and DNA Sequencing

PCR products were purified using High Pure PCR Product Purification Kit (Roche, Germany) according to the manufacturer's instructions. Two replicates of purified PCR products were sequenced by Microsynth (Switzerland).

2.5. Real-Time PCR and Standard Curve

After confirming the desired fragment sequence, the Real-Time PCR method was performed to determine the minimum detection limit. To determine the sensitivity, 5-fold serial dilutions of 100 ng to 0.006 ng of *N. bombycis* DNA were prepared, and the last dilution that produced a fluorescent signal was considered the minimum detection limit. All tests were performed in duplicate. The Real-Time PCR reaction was performed by step one ABI (Thermo Fisher), and the reaction was in a volume of 10 mL with 5 mL SYBR Premix Ex Taq (2X), 0.5 mL of each primer, 1 mL template DNA (positive *N. bombycis* spore DNA sample), 0.2 mL ROX Reference Dye (50X) (Takara, Japan) and 2.8 mL dH2O. Additionally, a 10 mL reaction was prepared as a negative control using deionized water instead of the target DNA, and another 10 mL reaction was prepared as a second negative control using the DNA of non-infected larvae. The optimum cycling conditions included an initial denaturation (30 s, 95°C) and 40 cycles (denaturation 5 s, 95°C; annealing 20 s, 55°C, and extension 15 s, 72°C), followed by a melting-curve analysis to confirm the specificity of the PCR amplification. Based on the results of the sensitivity test, the standard curve including Ct values and DNA concentration was drawn. Samples with Ct < 35 were considered positive. Moreover, 80 mother moth samples examined by light microscope were evaluated using both conventional PCR and Real-Time PCR methods. In addition, 30 suspected pooled samples of age 1 and 2 larvae were evaluated through both PCR methods.

3. Results

Results of the isolation and purification of the spores under a light microscope are displayed in

figure 1. The spores were very clear and bluish white and had brown motion under the microscope. Electrophoresis of PCR products on 1% agarose gel is illustrated in figure 2, in which a 136-bp fragment can be observed. After confirming the fragment sequence, Real-Time PCR was performed and the standard curve was drawn (Figure 3). The results showed that the Real-Time PCR method was able to identify samples infected with *N. bombycis* with a detection limit of up to 6 pg DNA, which was much more sensitive than the conventional PCR method (8 ng).

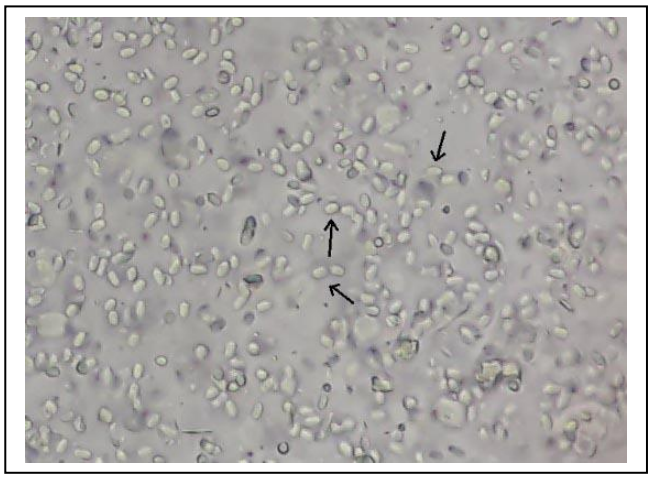

Figure 1. *N. bombycis* spores under 100 X magnification light microscope. Some spores are indicated by arrows

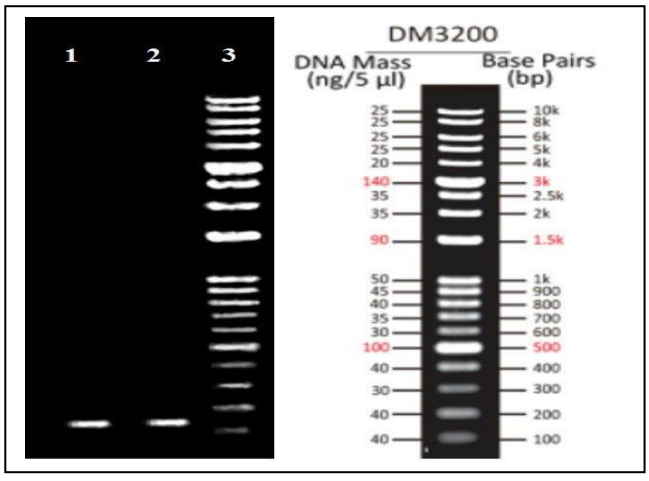

Figure 2. PCR product electrophoresis on 1% agarose gel: lanes 1 and 2: PCR product of the partial SSU-rDNA gene; lane 3: marker

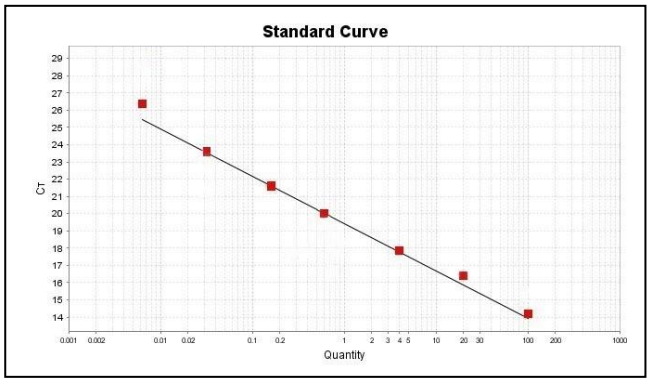

Figure 3. Standard curve using DNA concentration in 5fold dilution

As shown in figure 4, with a decrease in concentration, the signal production took place later and Ct increased. No production was observed for the negative control samples, and the specificity of the reaction was confirmed (Figure 5). R^2 of the standard curve was 0.99, which indicated the precision and accuracy in preparing serial dilutions and the correct pipetting of dilutions into the wells. Out of 80 mother moth samples examined by light microscope, 55 samples were evaluated as positive, and 60 and 62 samples were detected as positive in the conventional and Real-Time PCR methods, respectively. From 30 samples of larvae, all samples were detected as negative by conventional PCR, while, one sample was detected positive by Real-Time PCR.

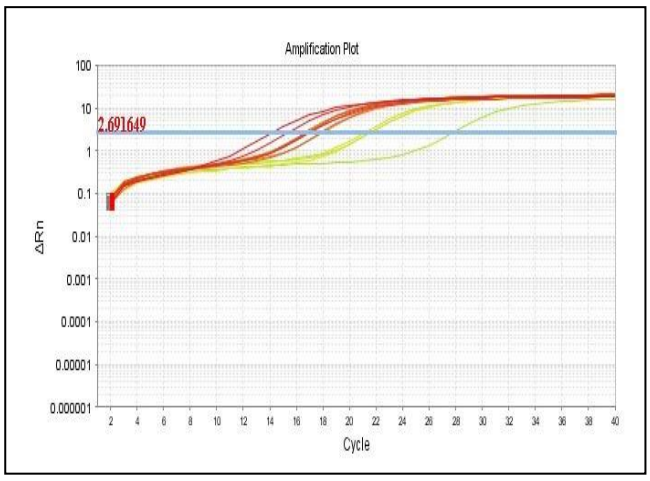

Figure 4. Proliferation curve at 55 degrees of centigrade for the target gene

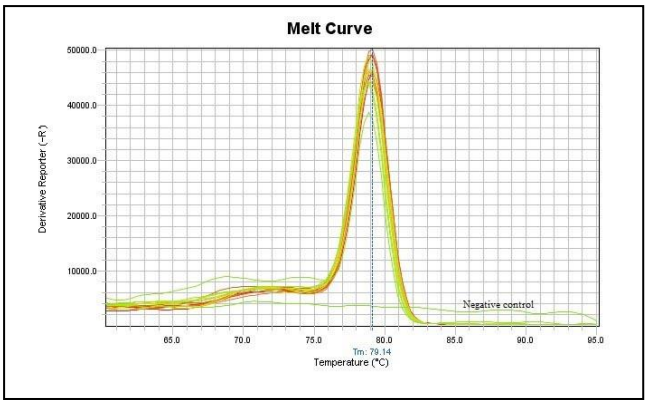

Figure 5. The melting curve of the target gene. As expected, the negative control samples did not show a specific peak

4. Discussion

Sericulture is considered a sustainable economic activity on the farm that benefits the poor rural people in countries like Iran. Due to the demographic structure of the country, the existence of a large population of young job seekers, and also insufficient income from major agricultural occupations, to meet the employment and income needs of the villagers, activities such as silkworm breeding can be helpful. Gilan province, as the main hub of sericulture in Iran, has a background in sericulture, efficient manpower, appropriate climatic conditions, established main research centers, and the management of the sericulture industry. It currently has an important role in producing cocoons, silk threads, and the propagation of mulberry seedlings in the country (2).

Pebrine disease is one of the most important known diseases in silkworms, which has caused a decrease in silk production (1). Therefore, the development of more reliable diagnostic tools to identify the causative agent of pebrine disease has always been one of the most important and necessary strategies to combat this disease (14). Several methods have been proposed and used in practice for the diagnosis, among which, PCR-based methods are more accurate. These methods are able to identify the level of infection in the newly hatched eggs and larvae and have solved the problem of detection in all stages of the life cycle using specific DNA primers (15). The results of this study showed that molecular methods, especially the Real-Time PCR method, can be more accurate leading to a faster diagnosis of *N. bombycis* than the light microscopy technique. Results demonstrated that some samples, which were detected as negative using the light microscope, were detected as positive in the Real-Time PCR method. In the present study, only the samples of the mother moth were examined both microscopically and molecularly, with more positively-detected samples using the molecular method. In addition, the suspected larvae of ages 1 and 2, which were evaluated as negative using the conventional PCR method, except in one case, were also negative in the Real-Time PCR method.

Genetic assays, such as the use of PCR and LAMP methods, have expanded the detection of pebrine disease in eggs (16). Thus, in this study, using a pair of specific primers based on a previous study (10), a detection limit of *N. bombycis* was determined. Findings demonstrated that the detectable amount of infection was less than 6 pg using the Real-Time PCR method, which indicated the high sensitivity of this method;

Detection of microsporidia by PCR using primers designed from SSU-rRNA has been widely used. Roy, Mendoza (17) have used PCR to detect microsporidia with primers designed from conserved regions of the RNA polymerase gene. Microsporidia were detected on day 4, while spores were only visible under the microscope on day 8. Therefore, this method can be useful in the early detection of microsporidia, which is vital in reducing product losses in the silkworm industry. Rafeie, Rezadoust (5) showed that the Real-Time PCR method using a pair of specific primers as well as a probe was able to investigate suspicious samples, and suggested it as a complementary or alternative method for the diagnosis of pebrine disease. Real-Time PCR provides more important advantages over PCR and electrophoresis and increases the speed and accuracy of diagnosis in

suspect samples. Therefore, the Real-Time PCR method is an effective method for identifying pebrine disease (6).

In a qPCR study for the detection of *N. bombycis*, which individually measures silkworm eggs and newly hatched larvae, Real-Time PCR showed high sensitivity and reproducibility assay (9). Fu, He (9) estimated the amount of 0.1 DNA of a spore in one reaction as the limit of detection. From 400 samples produced by infected mother moths, 167 and 195 cases were positive by light microscopy technique and qPCR method, respectively. Therefore, the qPCR method showed higher accuracy and potential for high-throughput screening (9). There is a high correlation between the detection results obtained by the two techniques. However, the qPCR method is very sensitive and accurate, while the standard sensitivity for light microscopy technique in pebrine detection is equivalent to 2.5×10^3 spores/mL. This amount has been reported as about 10 spores for the Real-Time PCR method. In the light microscopy technique, a false negative error may occur, especially when the silkworm spore is low. In the present study, some mother moths, which were evaluated as negative using the light microscopy technique, were evaluated as positive by molecular methods based on PCR. In addition, only healthy mature spores can be identified by light microscopy technique, while vegetative spores of *N. bombycis* cannot be detected even by experienced individuals. In contrast, Real-Time PCR is able to detect a lower amount (18, 19). This method has not only revolutionized the identification and prevention of pebrine disease in the silk industry but it can also be used in ecological studies of *N. bombycis* and silkworm interactions. With this method, more correct planning can be done on vertical and horizontal transfer in silkworm breeding, especially on newly hatched larvae. Furthermore, this diagnostic method is quick and can be used in epidemiological studies.

In conclusion, the present study focused on PCR-based methods according to primers designed based on sequences recently obtained from Iranian *N. bombycis*. The results showed that Real-Time PCR had a higher ability to detect *N. bombycis* than the conventional PCR method, and this method was also superior to the light microscopy technique. Therefore, due to the fewer and more accurate steps of Real-Time PCR, it can be introduced as a suitable method for diagnosing pebrine disease. It was also found that the primer used in the current project had a high efficiency for detecting *N. bombycis*, and therefore, it can be used to design a PCR-based kit.

Acknowledgment

The authors would like to appreciate the staff of the Iran Silkworm Research Center.

Authors' Contribution

Study concept and design: M. B. and Sh. N.
Acquisition of data: M. B. and Sh. N.
Analysis and interpretation of data: M. B. and A. Z.
Drafting of the manuscript: M. B.
Critical revision of the manuscript for important intellectual content: M. B. and A. Z.
Statistical analysis: M. B. and A. Z.
Administrative, technical, and material support: M. B., Sh. N., A. Z., M. M., and M. T.

Ethics

The procedures were approved by the Ethics Committee of the Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization, Karaj, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

This work was supported by Razi Vaccine and Serum Research Institute and Iran Silkworm Research Center [grant number: 3-18-1824-060-970993].

References

1. Bhat I, Buhroo Z, Bhat M. Microsporidiosis in silkworms with particular reference to mulberry silkworm (Bombyx Mori L.). Int J Entomol Res. 2017;2(1):1-9.
2. Abedi Parijani A, Motamed M, Kavusi Kalashmi M, editors. The role of silkworm breeding in job creation. Proceedings of Rural Development; 2015; Mashhad, Iran.
3. Biabani M, Nematollahian S, editors. A review of the prevalence of Pebrine disease in different provinces in 2017 and its prevention and control methods in Iran Proceedings of the first national silk conference of Iran Rasht, Iran: University of Guilan;2017.
4. Khezrian A, Bagheri M, Sourati Zanjani R, Kheirkhah Rahimabad Y, Nematollahian S, Zahmatkesh A. The effects of feed supplements and Nosema bombycis infection on economical traits in various silkworm breeding lines. Entomol Exp Appl. 2022;170(3):277-83.
5. Rafeie F, Rezadoust MH, Abdoli R. New molecular diagnosis for pebrine inspection in silkworm eggs using a real-time PCR probe. Gene Rep. 2018;12:47-9.
6. Sanders JL, Watral V, Clarkson K, Kent ML. Verification of intraovum transmission of a microsporidium of vertebrates: Pseudoloma neurophilia infecting the Zebrafish, Danio rerio. PLoS One. 2013;8(9):e76064.
7. Chaimanee V, Pettis JS, Chen Y, Evans JD, Khongphinitbunjong K, Chantawannakul P. Susceptibility of four different honey bee species to Nosema ceranae. Vet Parasitol. 2013;193(1-3):260-5.
8. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y). 1993;11(9):1026-30.
9. 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;120:72-8.
10. Kiani-Azad K, Bagheri M, Sadeghi M, Nematollahian S, Zahmatkesh A, Moharrami M, et al. Molecular Characterization of a New Nosema bombycis Strain Detected in Iranian Silkworm Farms. Acta Parasitol. 2022;67(3):1364-71.
11. Moharrami M, Bagheri M, Nematollahian S. Detection and Characterization of Nosema bombycis Using TEM and SEM Techniques. Arch Razi Inst. 2022;77(4):1473-80.
12. Sato R, Kobayashi M, Watanabe H, Fujiwara T. Serological discrimination of several kinds of microsporidian spores isolated from the silkworm, Bombyx mori, by an indirect fluorescent antibody technique. J Sericult Sci Jpn. 1981;50(3):180-4.
13. Undeen AH, Cockburn AF. The extraction of DNA from microsporidia spores. J Invertebr Pathol. 1989;54(1):132-3.
14. Bebitha B, Mohanraj P, s M, Mahalingam CA. Silkworm disease diagnosis through molecular approach and their management. Int J Plant Prot. 2016;9:343-52.
15. 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):11-8.
16. Liu J, Cheng W, Yan Y, Wei J, Yang J. Detection of pebrine disease in Bombyx mori eggs with the loop-mediated isothermal amplification (LAMP) method based on EB1 gene. Acta Entomologica Sinica. 2015;58(8):846-55.
17. Roy F, Mendoza L, Hiebert J, McNall RJ, Bankamp B, Connolly S, et al. Rapid Identification of Measles Virus Vaccine Genotype by Real-Time PCR. J Clin Microbiol. 2017;55(3):735-43.
18. 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):53-8.
19. Polley SD, Boadi S, Watson J, Curry A, Chiodini PL. Detection and species identification of microsporidial infections using SYBR Green real-time PCR. J Med Microbiol. 2011;60(Pt 4):459-66.