Journal of Invertebrate Pathology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

A multiplex PCR assay to diagnose and quantify *Nosema* infections in honey bees (*Apis mellifera*)

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ARTICLE INFO

Article history:
Received 14 April 2010
Accepted 1 June 2010
Available online xxxx

Keywords:
Nosema apis
Nosema ceranae
Identification
PCR
Infection levels
Spore

ABSTRACT

Correct identification of the microsporidia, *Nosema apis* and *Nosema ceranae*, is key to the study and control of Nosema disease of honey bees (*Apis mellifera*). A rapid DNA extraction method combined with multiplex PCR to amplify the 16S rRNA gene with species-specific primers was compared with a previously published assay requiring spore-germination buffer and a DNA extraction kit. When the spore germination-extraction kit method was used, 10 or more bees were required to detect the pathogens, whereas the new extraction method made it possible to detect the pathogens in single bees. Approx. 4–8 times better detection of *N. ceranae* was found with the new method compared to the spore germination-extraction kit method. In addition, the time and cost required to process samples was lower with the proposed method compared to using a kit. Using the new DNA extraction method, a spore quantification procedure was developed using a triplex PCR involving co-amplifying the *N. apis* and *N. ceranae* 16S rRNA gene with the ribosomal protein gene, *RpS5*, from the honey bee. The accuracy of this semi-quantitative PCR was determined by comparing the relative band intensities to the number of spores per bee determined by microscopy for 23 samples, and a high correlation (R^2 = 0.95) was observed. This method of *Nosema* spore quantification revealed that spore numbers as low as 100 spores/bee could be detected by PCR. The new semi-quantitative triplex PCR assay is more sensitive, economical, rapid, simple, and reliable than previously published standard PCR-based methods for detection of *Nosema* and will be useful in laboratories where real-time PCR is not available.

1. Introduction

The diagnosis of Nosema disease has been traditionally done by detecting spores of *Nosema* spp. through microscopic analyses (Shimanuki and Knox, 2000). However, with the recent finding that both *Nosema ceranae* and *Nosema apis* affect western honey bees (*Apis mellifera*), molecular techniques are required that can reliably differentiate between these different species of microsporidia, because the spores of the two *Nosema* species cannot be reliably distinguished by their morphology (Fries et al., 2006). In addition, microscopic analyses are not as sensitive at detecting low levels of *Nosema* infection as molecular methods, such as PCR, can be. Moreover, microscopic examination of *Nosema* spores is costly, laborious and time-consuming.

The first molecular methods used to differentiate between *Nosema* spp. required PCR followed by sequencing (e.g. Higes et al., 2006; Chen et al., 2008) or the use of restriction analysis of PCR products (e.g. Klee et al., 2007). These methods are reliable but require additional steps beyond amplification. A simpler method of detecting *N. apis* and *N. ceranae* was developed by Martin-Hernández et al. (2007). They created a duplex PCR-based method that simultaneously amplified the 16S rRNA regions of *N. apis* or *N. ceranae* using a single reaction with two pairs of primers allowing for parallel detection of both microsporidians. More recently, a real-time duplex PCR assay was developed amplifying the same 16S rRNA regions that also allowed for quantification of *N. apis* and *N. ceranae* in a single reaction (Bourgeois et al., 2010). However, real-time PCR thermocyclers and techniques are not readily available in many laboratories and are more costly than standard PCR thermocyclers and techniques.

Here we describe an improved multiplex-PCR method for the differentiation and quantification of *Nosema* spp. in honey bees in a single reaction. It combines the duplex PCR-based method of Martin-Hernández et al. (2007) with the relative RT-PCR approach of Dean et al. (2002), which includes a house-keeping gene of the host in each reaction for relative quantification based on PCR band intensity. This semi-quantitative method made it possible to estimate *Nosema* infection levels using a standard PCR thermocycler. Also, a different DNA extraction method was included to increase the quantity of DNA obtained from the honey bee samples. We show that the newly improved method has a

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0022-2011/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2010.06.001

number of advantages compared to previously published, standard PCR-based assays and makes it possible to both detect and quantify *Nosema* spores in honey bee samples.

2. Materials and methods

2.1. Samples

Samples of adult honey bees were collected from the entrance of different colonies in Southern Ontario, Canada, and were microscopically examined for presence of *Nosema* spp. spores (Shimanuki and Knox, 2000).

2.2. DNA extraction

Abdomens were excised from honey bees, and DNA was obtained by either: (1) spore germination followed by DNA extraction using the High Pure PCR Template Preparation Kit (Roche Diagnostic, Laval Que., Cat. # 11796828001) as per Martin-Hernández et al. (2007) (i.e., the spore germination-extraction/kit method) or (2) a direct DNA extraction method developed by us based on modifications of the protocol of Hunt (1997) (i.e., the HBRC method).

2.2.1. Germination-extraction kit method

Nosema spores were obtained from the abdomens of 10 bees as per Martin-Hernández et al. (2007). To test the sensitivity of the method, we also used single bees. In order to have relatively clean suspensions of *Nosema* spores required for DNA extraction with the extraction kit, the abdomen(s) of honey bees from each sample were homogenized in a mortar with deionized water (d H_2O) at 1 mL d H_2O per abdomen. The macerated solution was filtered through a plastic screening cloth (77 holes/cm^2 Better Bee Supplies, Cambridge, Ontario, Canada) to remove larger body parts. Then, the solution was filtered with a nylon mesh filter (1000 holes/cm^2; Better Bee Supplies). The suspension was divided into four microfuge tubes (1.25 mL per tube) and then centrifuged at 10,000 rpm for 15 min. The supernatant from each tube was discarded, and the pellets from each of the four tubes were combined into a single tube (final volume of 1 mL). Then, 200 μL of freshly prepared spore-germination buffer (De Graaf et al., 1993) was added and mixed. Preparations were incubated at 37 °C for either 15 min, 2 h, 5 h or 24 h, to study the effect of the time of exposure to the amount of DNA extracted from *Nosema* spores. The DNA was extracted as described in the kit's instructions as per Martin-Hernández et al. (2007). The time and cost of supplies required to extract DNA per sample were calculated.

2.2.2. HBRC method

Abdomens of single honey bees were washed with extraction buffer prepared as per Hunt (1997). The buffer was composed of 0.03 M hexadecyltrimethyl ammonium bromide, 0.05 M tris hydroxymethyl aminomethane, 0.01 M ethylenediamine tetra-acetic acid, and 1.1 M NaCl, in d H_2O. The buffer was titrated to pH 8.0. Each washed bee abdomen was placed in a small mortar with 300 μL extraction buffer and 4 μL Proteinase K solution (20 mg/mL) and then homogenized with a pestle. The total macerated solution was carefully collected avoiding uptake of larger body parts and then transferred to a microfuge tube. The tubes were incubated in a water bath at 60 °C for 3 h. After incubation, the samples were either temporarily stored at −20 °C or immediately subjected to DNA extraction procedures. The homogenized samples were thawed if they had been kept in the freezer. An equal volume (300 μL) of phenol–chloroform mix (1:1) was added to each tube, and the tube contents were mixed gently. The tubes were centrifuged at 13,000 rpm for 15 min, and the supernatant was transferred to a new tube, and the phenol–chloroform extraction was repeated. Then, chloroform (300 μL) was added to the supernatant, mixed gently, and centrifuged at 13,000 rpm for 5 min. The supernatant (300 μL) was transferred to a new tube, and 3 M NaOAc (30 μL) was added followed by 600 μL 95% ethanol. The tubes were mixed gently and placed at −20 °C in a freezer overnight. The samples were then centrifuged at 10,000 rpm for 15 min, and the ethanol was discarded. The pellet was dried and 25 μL of d H_2O was added. The samples were incubated at 65 °C in a water bath for 10 min, and the pellet of each tube was mixed gently. Any un-dissolved material was removed by centrifuging at 10,000 rpm for 10 s. The extracted DNA was stored at −20 °C until utilized. The time and cost of supplies required to extract DNA per sample were calculated.

2.3. Spore counts and dilutions

Spores were purified as described above (Section 2.2.1) and a sub-sample of 10 μL was taken from the pellet to count *Nosema* spores under the microscope with a haemocytometer (Shimanuki and Knox, 2000). Once the number of spores/mL (and per bee) contained in the pellet was known, a series of spore dilutions (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1 and 0.1 spores/mL) were prepared for the experiments. DNA was extracted from each dilution.

2.4. PCR amplifications

DNA samples obtained using the two methods described above were used in the co-amplification of the 16S rRNA gene of *N. apis* or *N. ceranae* (Martin-Hernández et al., 2007) and the honey bee ribosomal protein *RpS5* gene (Thompson et al., 2007) in the same reaction. All PCR reactions were done with a Mastercycler (Eppendorf, Mississauga, Ontario). Each 15 μL reaction contained 1.5 μL 10× PCR buffer (New England BioLabs, Pickering, Ontario), 0.5 μL 10 mM dNTPs (Bio Basic Inc., Markham, Ontario), 1 μL 10 μM for each primer (Laboratory Services, University of Guelph), 0.2 μL 5 U/μL *Taq* polymerase (New England BioLabs, Pickering, Ontario), 2 μL DNA sample (10 ng in 2 μL), and 8.8 μL d H_2O. The thermocycler was programmed to run at 94 °C for 2.5 min, followed by 10 cycles of 15 s at 94 °C, 30 s at 61.8 °C and 45 s at 72 °C, and 20 cycles of 15 s at 94 °C, 30 s at 61.8 °C and 50 s at 72 °C, and a final extension step at 72 °C for 7 min, holding reactions at 4 °C for the rest of the time (modified from Martin-Hernández et al., 2007). DNA obtained from newly emerged, *Nosema*-negative honey bees was used as negative control in the experiments.

Primers for the *Nosema* 16S rRNA gene were MITOC-F (5′ CGGCGACGATGTGATATGAAAATATTAA) and MITOC-R (5′ CCCGGTCATTCTCAAACAAAAAACCG) to yield a 218 bp PCR product specific for *N. ceranae* and APIS-F (5′ GGGGGCATGTCTTTGACGTACTATGTA) and APIS-R (5′ GGGGGGCGTTTAAAATGTGAAACAACTATG) to yield a 321 bp PCR product specific for *N. apis* (Martin-Hernández et al., 2007). Additionally, a honey bee house-keeping gene, the ribosomal protein S5, *RpS5*, was used as a reference (Evans and Wheeler, 2000). Primer pairs RpS5-F (5′ AATTATTTGGTCGCTGGAATTG) and RpS5-R (5′ TAACGTCCAGCAGAATGTGGTA) with 115 bp length product were used in the reactions (Thompson et al., 2007). PCR amplification of *RpS5* was performed for all the samples as a standard control.

2.5. Separation and quantification of PCR amplicons

PCR products were separated by electrophoresis in 1.1% agarose gels and stained with ethidium bromide. Included was a 100 bp DNA ladder (Bio Basic Inc., Markham, Ontario). The intensity of the amplified bands captured in pictures with a digital camera, was measured in pixels using the Scion Image computer software

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(Scion Corporation, Frederick, MD, USA) as per Dean et al. (2002). Then, the ratio of band intensity between the *Nosema* target gene and the honey bee house-keeping gene (ratio of relative expression) was calculated. To determine that the number of amplification cycles was not too great for relative quantification (for calibration purposes), the DNA samples were also amplified with three fewer PCR cycles, and the bands were again quantified by using the Scion Image program.

The ratios of relative expression obtained from 23 samples with different degrees of *Nosema* infection, using spore counts based on microscopy, were plotted against their corresponding infection levels (spores/bee) obtained by microscopic analyses. Data on number of spores per sample obtained by microscopy were regressed on data for ratios of relative expression of the *Nosema* target gene obtained from the same samples. Regression analysis was performed with SPSS v. 16. The experiment was repeated two times.

3. Results

3.1. Comparison of DNA extraction methods

The PCR product specific for *N. ceranae* was obtained when honey bee abdomens were processed with the spore germination-extraction kit method as described by Martin-Hernández et al. (2007). However, *Nosema* DNA extracted from full abdomens was fully visible only when 10 abdomens were used with the kit method (Fig. 1A), but not when single abdomens were used (Fig. 1B). Martin-Hernández et al. (2007) recommended an incubation time of 15 min in the spore-germination buffer of De Graaf et al. (1993) prior to DNA extraction. However, no differences in band intensity were noticeable between samples that were either not exposed to the spore-germination buffer or incubated in it for 15 min, 2 h or 5 h. Furthermore, the germination buffer did not only fail to increase the quantity of the PCR product but actually decreased PCR product intensity after 24 h of incubation, making the PCR band almost imperceptible (Fig. 1A and B). In contrast, direct DNA extraction from honey bee abdomens by the HBRC method resulted in visible PCR products for extracts from both 10 and single abdomens (Fig. 2A and B). Additionally, apparently more DNA was extracted with the HBRC method than with the spore germination-extraction kit method. Based on relative quantification of band intensities, the difference in the amount of PCR product was consistently approx. 4–8 times higher with the HBRC method compared with the germination-extraction kit method (data not shown).

A comparison of the "hands-on" time (excluding the various incubation periods) of the spore germination-extraction kit and HBRC methods showed that the HBRC method took approx. 50% of the time of the spore germination-extraction kit method per sample (15 versus 29 min, respectively). In addition, the cost of supplies needed to extract DNA was 10 times lower with the HBRC method than with the kit method per sample ($ 0.40 versus $ 4.00 CAN, respectively).

3.2. Sensitivity of the HBRC method for Nosema detection

To test the sensitivity of the HBRC method in detecting *Nosema* spores, DNA was extracted from single honey bees infected with *N. ceranae* and a series of 10-fold dilutions resulting in 10^7 to 0.01 spores per PCR reaction. The intensity of the PCR products decreased as the number of spores decreased in the reactions (Fig. 3). Bands were clearly visible with as little as 10 spores per PCR reaction, which is equivalent to 100 spores/bee based on singleplex PCR, which might differ for the multiplexed PCR (Fig. 3, lanes 1–7). Though very faint, a band was also observed with a single spore/reaction (lane 8), while no band was detectable below the dilution level of a single spore/reaction (lanes 9, 10). As expected, no bands were visible for the two negative control samples (lanes 11, 12). In contrast, quantification of *Nosema* spores by using a haemocytometer under a microscope was only possible with 50,000 or more spores/bee.

3.3. Triplex PCR for N. ceranae, N. apis and A. mellifera

A triplex PCR-based method was developed to detect *N. ceranae* and *N. apis*, while amplifying the honey bee *RpS5* gene as a control

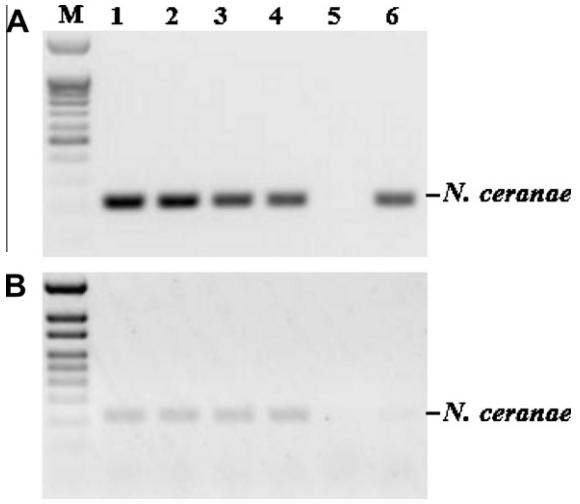

Fig. 1. Detection of the 16S rRNA PCR product specific for *Nosema ceranae* (218 bp). DNA was obtained from spores or abdomens of infected honey bees using the spore germination-kit method. DNA from 10 (A) or single bees (B) used in the reactions. Lanes 1–6 show the amplicon generated after extracting DNA from spores that were not treated with a spore-germination buffer (lane 1), or from spores that were treated with germination buffer for 15 min (lane 2), 2 h (lane 3), 5 h (lane 4), and 24 h (lane 5). DNA was also extracted from total abdomen (s) without germination buffer (lane 6). Lane M (far left) is a 100 bp DNA ladder.

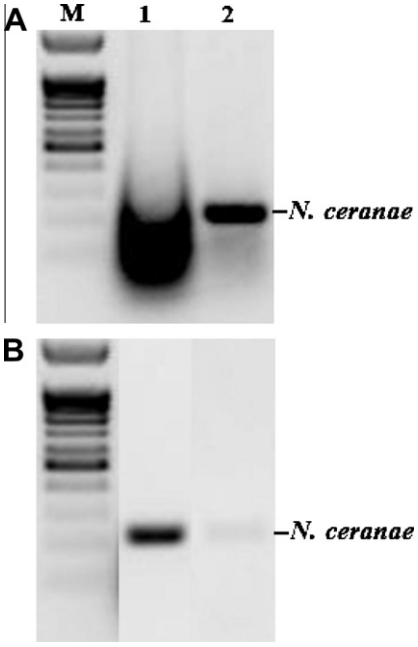

Fig. 2. Detection of the 16S rRNA PCR product specific for *Nosema ceranae* (218 bp). DNA from infected bees (700,000 spores/bee) was extracted with the HBRC method (A) and with a commercial kit (Roche Diagnostic, Laval Que., Cat. # 11796828001) (B). The bands of the amplicon obtained from 10 or single bees are shown in lanes 1 and 2, respectively. Lane M (far left) is a 100 bp DNA ladder.

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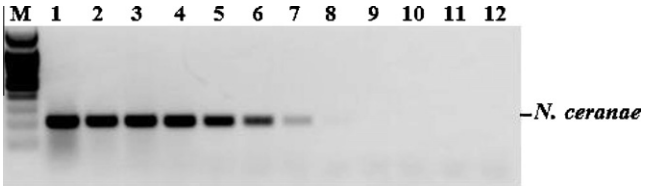

Fig. 3. Detection of the 16S rRNA PCR product specific for *Nosema ceranae* (218 bp) using a series of spore dilutions. DNA was extracted with the HBRC method. Lanes 1–10 show the bands generated with DNA obtained from dilutions ranging from 10^7 to 0.01 spores per reaction. Lane 11 shows results without spores, and lane 12 shows results with purified spores of *N. ceranae* with honey bee material removed prior to DNA extraction. Lane M (far left) is a 100 bp DNA ladder.

at the same time (Fig. 4). By comparison, the duplex PCR detection method of Martin-Hernández et al. (2007) did not include a honey bee control gene. The HBRC method was tested on samples previously diagnosed as *Nosema*-positive by microscopy, and it correctly identified 20 samples infected with *N. ceranae*, 12 samples infected with *N. apis* and 14 samples infected with both microsporidia. None out of 20 samples of newly emerged honey bees from colonies that were *Nosema*-negative gave a positive result for *N. ceranae* or *N. apis* with the HBRC method.

3.4. Quantification of Nosema spores

To estimate the approximate number of spores per bee, a ratio was made of the relative amount of the amplicon specific for *N. ceranae* compared to that of the honey bee *RpS5* gene (Fig. 5). The intensity of the *N. ceranae* amplicon band was lower for samples from honey bees containing fewer spores, while the intensity of the band for *RpS5* did not noticeably change regardless of the *Nosema* infection level (lanes 1–5). A healthy bee (*Nosema*-negative) used as control yielded only the *RpS5* PCR product (lane 6), while only the *Nosema* amplicon was visible when spores were extracted from abdomens homogenized in a mortar and pestle followed by several washes with d H_2O to purify the spores from the ground abdomen (lane 7).

A very high and significant linear relationship was found between the relative amount of *N. ceranae* PCR product and the number of *N. ceranae* spores/bee from samples with different infection levels ($R^2 = 0.95$, $n = 23$; Fig. 6). This linear relationship was observed in samples containing up to 1 million spores/bee, which is considered a level of infection above economic and treatment thresholds (Bailey and Ball, 1991). The strong linear relationship demonstrates that a semi-quantitative PCR based on relative band

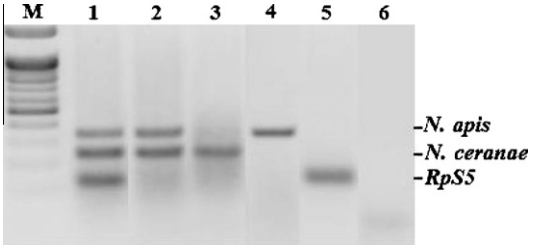

Fig. 4. PCR showing amplicons of different lengths, specific for the detection of the 16S rRNA of *Nosema ceranae*, *N. apis*, and the honey bee house-keeping gene, *RpS5*. Lane 1 shows triplex PCR with sample from honey bee infected with both microsporidians and the *RpS5* gene amplified, lane 2 shows duplex PCR with sample from honey bee infected with both microsporidians but not including the *RpS5* gene, lane 3 is a duplex PCR for a sample infected only with *N. ceranae*, lane 4 is a duplex PCR for a sample infected only with *N. apis*. Lane 5 shows PCR using DNA from healthy bees (*Nosema*-free) with the three pairs of primers, while lane 6 shows a reaction without bee DNA using the three pairs of primers. Lane M (far left) is a 100 bp DNA ladder.

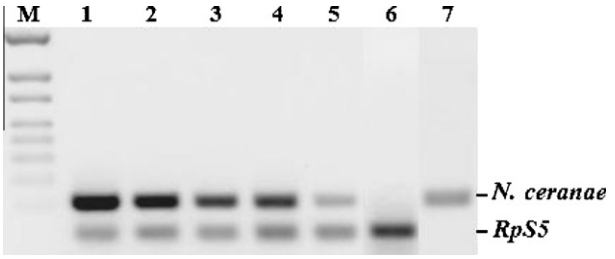

Fig. 5. Co-amplification of the 16S rRNA PCR product specific for *Nosema ceranae* (218 bp) and the PCR product specific for the honey bee house-keeping gene, *RpS5* (115 bp). DNA obtained from single bees with different levels of *Nosema* infection. Infection levels were 700,000 spores/bee (lane 1), 350,000 spores/bee (lane 2), 200,000 spores/bee (lane 3), 100,000 spores/bee (lane 4), and 50,000 spores/bee (lane 5). A healthy bee (*Nosema*-free) was used as control for the reaction in lane 6, and a clean spore dilution (50,000 spores/mL) was also used as control for the reaction in lane 7. Lane M (far left) is a 100 bp DNA ladder.

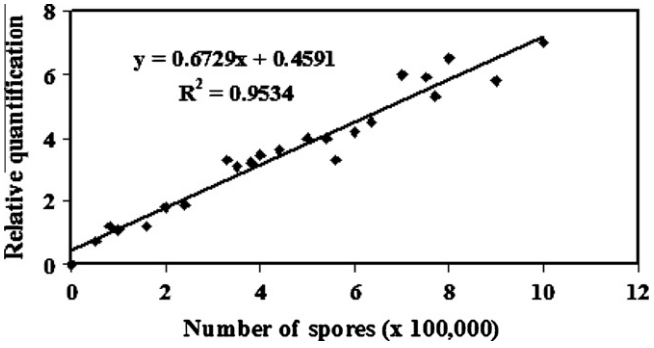

Fig. 6. Relationship between number of *Nosema ceranae* spores determined by microscopy and the quantification of the PCR products of the 16S rRNA specific for *N. ceranae* relative to that of the honey bee gene, *RpS5*. The relative quantification of a PCR product was determined from the ratio of the intensity of the *Nosema*-associated 16S rRNA amplicon to that of the honey bee *RpS5* amplicon in the same reaction, as determined by quantification of the pixels in the gel with the Scion Image program. Twenty-three samples with different levels of infection (0.0–1000,000 spores/bee) were used to generate the linear regression line and equation.

intensities can provide highly accurate spore quantification. The ratio of relative expression can be used to estimate infection levels using the following regression equation: $y = 0.6729x + 0.4591$, where x equals the number of spores per bee from PCR results.

4. Discussion

The new combination of methods described here had several advantages compared to using spore-germination buffer followed by the DNA extraction kit as described by Martin-Hernández et al. (2007). The band of the *Nosema* PCR product obtained with the HBRC method was up to eight times more intense than when using spore-germination buffer followed by a kit DNA extraction, indicating that higher amounts of DNA were obtained. This is supported by the ability of only the HBRC method to detect *Nosema* infections in single bees, even at low levels of infection. Additionally, because abdomen macerates are directly used rather than purified spores and no spore-germination buffer is used, time and money are saved. With the HBRC method, DNA can be extracted in about half the time required with the spore germination-extraction kit method, and the cost of supplies is 10 times lower.

It was unexpected that the spore-germination buffer did not increase the amount of *Nosema* DNA extracted and actually inhibited the PCR products after 24 h of incubation with the *Nosema* spores. It is possible that a long incubation time at 37 °C might have degraded the spores along with their DNA. It is known that *Nosema*

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spores are killed by prolonged exposure to temperatures exceeding those of honey bee brood nests (Malone et al., 2001), which are 33–35 °C. It is presumed that the purpose of using the spore-germination buffer of De Graaf et al. (1993) was to improve the extraction of DNA by extracting the DNA from germinated rather than ungerminated *Nosema* spores. However, our results indicate the spore germination does not improve *Nosema* DNA extraction. Other spore-germination buffers could be tested to see their effect on PCR detection of *Nosema*.

The new detection method confirmed the reliability, repeatability and specificity of the primers and PCR conditions developed by Martin-Hernández et al. (2007). However, a more reliable assay is possible by changing from a duplex to a triplex PCR-based method by adding the honey bee *RpS5* gene in the reaction. Three clear and unambiguous bands of specific sizes were repeatedly generated in all cases of mixed infections (one for each *Nosema* spp. and one for the honey bee), which supports the specificity of this method. In healthy bees (not infected with *Nosema*), only the band for *RpS5* was observed, although three pairs of primers were used in the same reaction, indicating the accuracy of this PCR technique. This confirmed that the DNA extraction was successful and there were no problems with the PCR.

The triplex PCR assay was highly sensitive in detecting the presence of very low numbers of *Nosema* spores. Bands were clearly visible even at levels equivalent to only 100 spores/bee, and faint bands were also detectable at levels equivalent to 10 spores/bee based on singleplex PCR. When compared with a microscopic analysis, this is at least 500 times more sensitive in detecting *Nosema* spores, which makes it highly useful for Nosema diagnosis in the early stages of the disease. That detection level is also more sensitive than those reported previously for *Nosema* spp. when standard PCR assays were used (Klee et al., 2007; Martin-Hernández et al., 2007). However, real-time PCR may be even more sensitive as Bourgeois et al. (2010) claimed potential detection levels as low as 1 spore/bee.

The relative expression ratio of the amplicon specific for *N. ceranae* to the honey bee *RpS5* gene was useful in the semi-quantification of a wide range of levels of *N. ceranae*. Band intensity was highly correlated with number of spores per bee ($R^2 = 0.9534$; Fig. 6), which emphasizes the reliability and accuracy of the method. Based on this correlation, a regression line could be used to estimate the approximate number of spores in a sample. As estimates of approximate spore quantifications were possible with a simple PCR thermocylcer rather than with a real-time PCR thermocycler, our method is much less costly, and thus has a greater potential to be adopted by many laboratories where real-time PCR is not available, particularly in developing countries.

In spite of its advantages, this quantification method has limitations because the scale for the ratio of relative expression of the target gene did not allow for quantification estimates of infection levels above 1 million spores per bee unless the sample is diluted and another PCR is carried out. However, because it is widely accepted that the economic and treatment threshold level for *Nosema* infections in honey bee colonies is 1 million spores per bee (Bailey and Ball, 1991), this method would be adequate for detecting infection levels up to that threshold. It is important to note that for those interested in using this method of *N. ceranae* quantification, each laboratory should make its own standard curve from a dilution series to calibrate the method to their thermocyclers and techniques.

The triplex PCR-based method for diagnosing *Nosema* species is specific, relatively simple, reliable, sensitive, rapid and economical for detecting *N. ceranae* and/or *N. apis* and for quantifying *N. ceranae* in honey bees. With the increased concern about honey bee health, including colony collapse disorder, and the concerns that *Nosema* infections are contributing to their decline (Higes et al., 2008; Stankus, 2008), this method provides a new useful alternative for diagnosticians and apiculturists throughout the world.

Acknowledgments

We thank Paul Kelly and Janine McGowan for collecting and microscopically analyzing samples of honey bees for detecting *Nosema* positive colonies. This study was partially funded by a NSERC discovery grant to EG, by a NSERC strategic grant to P. Kevan (NSERC-CANPOLIN) and by a New Directions grant from the Ontario Ministry of Agriculture, Food and Rural Affairs to EG.

References

Bailey, L., Ball, B.V., 1991. Honey Bee Pathology, second ed. Academic Press, London.

Bourgeois, A.L., Rinderer, T.E., Beaman, L.D., Danka, R.G., 2010. Genetic detection and quantification of *Nosema apis* and *N. Ceranae* in the honey bee. J. Invertebr. Pathol. 103, 53–58.

Chen, Y., Evans, J.D., Smith, I.B., Pettis, J.S., 2008. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J. Invertebr. Pathol. 97, 186–188.

De Graaf, D.C., Masschelein, G., Vandergeynst, F., De Brabander, H.F., Jacobs, F.J., 1993. *In vitro* germination of *Nosema apis* (microspora: nosematidae) spores and its effect on their trehalose/D-glucose ratio. J. Invertebr. Pathol. 62, 220–225.

Dean, J.D., Goodwin, P.H., Hsiang, T., 2002. Comparison of relative RT-PCR and Northern blot analyses to measure expression of ß-1,3-glucanase in *Nicotiana benthamiana* infected with *Colletotrichum destructivum*. Plant Mol. Biol. Report. 20, 347–356.

Evans, J.D., Wheeler, D.E., 2000. Expression profiles during honeybee caste determination. Genome Biol. 2:1, research 0001.1-0001.6.

Fries, I., Martin, R., Meana, A., Garcia-Palencia, P., Higes, M., 2006. Natural infections of *Nosema ceranae* in European honey bees. J. Apic. Res. 45, 230–233.

Higes, M., Martin-Hernandez, R., Meana, A., 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J. Invertebr. Pathol. 92, 81–83.

Higes, M., García, P., Martín-Hernandez, R., Biotas, C., Bailon, E.G., Gonzalez-Porto, A.V., Barrios, L., Del Nozal, M.J., Bernal, J.L., Jimenez, J.J., Palencia, P.G., Meana, A., 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ. Microbiol. 10, 2659–2669.

Hunt, G.J., 1997. Insect DNA extraction protocol. In: Micheli, M.R., Bova, R. (Eds.), Fingerprinting Methods Based on Arbitrarily Primed PCR. Springer-Verlag, Berlin, pp. 21–24.

Klee, J., Besana, A.M., Genersch, E., Gisder, S., Nanetti, A., Tam, D.Q., Chinh, T.X., Puerta, F., Ruz, J.M., Kryger, P., Message, D., Hatjina, F., Korpela, S., Fries, I., Paxton, R.J., 2007. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J. Invertebr. Pathol. 96, 1–10.

Malone, L.A., Gatehouse, H.S., Tregidga, E.L., 2001. Effects of time, temperature, and honey on *Nosema apis* (Microsporidia: Nosematidae), a parasite of the honeybee, *Apis mellifera* (Hymenoptera: Apidae). J. Invertebr. Pathol. 77, 258–268.

Martin-Hernández, R., Meana, A., Prieto, L., Salvador, A.M., Garrido-bailon, E., Higes, M., 2007. Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Appl. Environ. Microbiol. 73, 6331–6338.

Shimanuki, H., Knox, D.A., 2000. Diagnosis of Honey Bee Diseases, US Department of Agriculture, Agriculture Handbook No. AH-690, pp. 61.

Stankus, T., 2008. A review and bibliography of the literature of honey bee colony collapse disorder: a poorly understood epidemic that clearly threatens the successful pollination of billions of dollars of crops in America. J. Agric. Food Inform. 9, 115–143.

Thompson, G.J., Yockey, H., Lim, J., Oldroyd, B.P., 2007. Experimental manipulation of ovary activation and gene expression in honey bee (*Apis mellifera*) queens and workers: testing hypotheses of reproductive regulation. J. Exp. Zool. 307A, 600–610.