Short Communication

External and internal detection of *Nosema ceranae* on honey bees using real-time PCR

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**ABSTRACT**

Numerous methods exist for molecular-based detection of *Nosema ceranae*. Here we determine location of parasite loads, the optimal tissue for pathogen detection, and the likely sources of variability among assays. Bee washes and head/thorax samples revealed substantial *N. ceranae* loads (2.67 × 10^4 ± 1.12 × 10^4 and 1.83 × 10^5 ± 4.14 × 10^4). Midgut samples carried the highest parasite loads (3.42 × 10^6 ± 1.84 × 10^6), followed by the hindgut (5.50 × 10^5 ± 3.24 × 10^5). We recommend using midgut samples for molecular-based detection and quantification of *N. ceranae* because of the low variability among samples.

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The range of the microsporidian *Nosema ceranae* has expanded in recent years to a nearly global scale where it has largely displaced *Nosema apis* as the predominant microsporidian parasite of honey bees (Anderson and Giacon, 1992; Chen et al., 2008, 2009; Fries, 2010). Because of the recency of the host and range expansion of *N. ceranae*, the pathogenicity and long-term effects on colonies is not yet clearly defined (Fries, 2010). A number of molecular-based diagnostic assays have been developed to detect and quantify *N. ceranae* DNA (Higes et al., 2006; Klee et al., 2007; Martin-Hernandez et al., 2007; Chen et al., 2008, 2009; Bourgeois et al., 2010; Hamiduzzaman et al., 2010; Traver and Fell, 2011a,b). However, collectively these studies use varied tissues for DNA extraction and comparisons between studies are difficult. Also, no direct comparisons of tissues for the same bee have been made in an attempt to determine which tissue is optimal. Hence, we sought to determine where *N. ceranae* can be detected on and in honey bees by combining techniques used in the various pathogen assays that have been published.

We measured *N. ceranae* levels in both individual bees (n = 30 from each of 3 colonies) and pooled samples (n = 30 bees from each of 30 colonies) in order to determine levels of individual and colony variation. Four types of samples were taken from each bee: whole bee wash water, head/thorax, midgut, and hindgut. Forceps were washed in 100% ETOH between samples to remove any potential sample to sample contamination.

For individual bee samples, each bee was placed in a separate tube with 250 μL dH2O and agitated in a TissueLyser II (Qiagen, Inc.) at 9 rev/s for 1 min. The bee was removed and de-gastered and the wash water was stored on ice for later processing. The head and thorax were placed into a new tube on ice and cut into approximately 2 mm pieces with dissecting scissors. The midgut and hindgut were dissected and placed separately into two additional tubes on ice. A total of 600 μL dH2O was added to each tissue sample along with 50 μL 1 mm zirconium silicate grinding beads (Next Advance, Inc., Averill Park, NY). Concentrated (3.0%) H2O2 was added in volume appropriate to achieve a final concentration of 0.3% to facilitate spore germination during a 15 min incubation at room temperature. To avoid spore settling and clumping, samples were agitated on an orbital plate shaker 15 min at 250 rpm. In pooled samples, 30 bees were placed in a 50 ml conical tube with 15 mL dH2O (i.e., wash water) and agitated at 175 rpm on an orbital shaker at room temperature. Bees were then removed, de-gastered and dissected as described above. Concentrated (3.0%) H2O2 was added to the wash water in volume appropriate to achieve a final concentration of 0.3%. The solution was then incubated at room temperature with agitation on an orbital shaker at 175 rpm for 15 min. A 100 μL aliquot of wash water was then transferred to tubes with 50 μL beads. Pooled heads and thoraces for each set of bees were homogenized in 30 mL dH2O in a glass homogenizer. Addition of H2O2 and incubation were the same as described above. A 100 μL aliquot of the homogenized solution was transferred to tubes with 50 μL beads. Midguts and hindguts were pooled in groups in sets of 15 in individual tubes. A total of 600 μL dH2O was added to each set of 15 tissue samples as described above. Addition of H2O2 and incubation were also the same as described above.

After incubation in H2O2, all samples (pooled and individual) were homogenized in the TissueLyser II for 4 min at 30 rev/s, as per manufacturer instructions. A 100 μL aliquot of each sample was transferred to a 1 mL deep-well plate for further processing.
Data were converted from copy number to Nosema individuals per bee based on the formula defined in Bourgeois et al. (2010). Data were tested for homogeneity of variance using Levene’s test in PROC ANOVA with SAS 9.2 (SAS Institute, Inc., Cary, NC). Variances were unequal, and were subsequently log-transformed. Differences among individual and pooled samples were evident (P < 0.0001; Fig. 1). There were differences in the concentration of the initial homogenates (i.e., the homogenization step for each sample type; water, head/thorax, midgut, and hindgut), however this was accounted for when sample values were converted from PCR results (number of target sequence copies/PCR reaction) to number of Nosema genomes/bee (hereafter denoted as Nosema/bee). Midgut homogenates were proportionately more (10X) concentrated in pooled samples (30 bees/1.2 mL 0.3% H₂O₂) than individual bees (1 bee/600 µL 0.3% H₂O₂). This could potentially affect the downstream chemistry of the extraction; however the protocol was designed such that large ranges in tissue sizes are equally efficiently extracted. Hindgut homogenates also differed in concentration, but were not as markedly differentiated between the sample groupings. The real-time PCR assay amplifies a wide range of sample concentrations and should have been unaffected.

In all cases, midgut and hindgut infestations were correlated (C1: R² = 0.50, P = 0.005, C2: R² = 0.72, P < 0.0001, C3: R² = 0.64, P = 0.0002, Pooled: R² = 0.73, P < 0.0001), but were also differentiated among the pooled samples (P = 0.001). A relationship between midgut and hindgut samples is logical, as Nosema spores are shed according to Bourgeois et al. (2010) with the following exceptions. Reagent volumes were scaled to the 100 µL starting sample volume (i.e., half volumes from published protocol) and spin times were changed to 20 min @ 4800g to accommodate sample processing in 96-well format.

Detection and quantification were performed on a StepOne™ Real-time PCR System (Applied Biosystems, Carlsbad, CA). Protocols for FAST PCR and reagent specifications followed those described by Bourgeois et al. (2010). Modifications to the protocol were made by elimination of primers and probe reagents for N. apis and addition of distilled water to adjust the reaction volume to 12.5 µL. All other reagents and specifications remained the same. All samples were run in duplicate and were directly quantified by comparison to a standard curve of known levels of N. ceranae DNA copy number. Standards were generated from serial dilutions of a cloned PCR product of the target sequence from N. ceranae (Bourgeois et al., 2010).

Fig. 1. Infestation levels of Nosema ceranae in honey bees processed as individuals (n = 30/colony) and as pooled samples (n = 30 colonies including 30 bees/colony pooled). Pool – pooled samples. C1, C2, and C3 = colony 1, 2, and 3, respectively. Bars represent mean ± SE.
into the lumen of the midgut, enter the hindgut and eventually are dispersed via defecation.

Based on our results, we recommend that the first step in processing samples for molecular-based detection of *N. ceranae* samples be washing in dH₂O to remove spores that may contaminate the external surface of the bee since dissected tissue may come in contact with the bee’s external surface. Also, we recommend that the midgut be used as the tissue of choice. Midgut samples showed the least variance among samples and hence have a greater reliability. Standardization of sample preparation techniques can reduce the variability both within and among studies. This would provide necessary information for a more accurate comprehensive view of *N. ceranae* infestations of honey bee colonies and the relationship to colony losses.

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**References**


