Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

A serological method for detection of *Nosema ceranae*

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Keywords
Apis mellifera, enzyme-linked immunosorbent assay, molecular tool, *Nosema ceranae*.

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2012/1165: received 27 June 2012, revised 9 October 2012 and accepted 10 November 2012

doi:10.1111/jam.12066

Abstract

Aims: We developed a new method for detection of the intracellular parasite, *Nosema ceranae*, one of the most economically devastating pathogens of the honeybee.

Methods and Results: The SWP-32 antibody was used for the development of an enzyme-linked immunosorbent assay (ELISA). We also compared the efficiency of this ELISA to microscopy and quantitative real-time (qRT) PCR, the methods currently in use.

Conclusions: ELISA is comparable in sensitivity with the qRT-PCR, less expensive and faster. When this method is commercialized and made available to bee-keepers, it will allow them to make informed decisions for the application of in-hive chemicals. Hence, bee-keepers may be able to determine when treatments for control of *N. ceranae* are unnecessary and reduce the cost, time and possible side effects of these treatments.

Significance and Impact of the Study: This assay provides the first serological method for detection of *N. ceranae* in bee colonies, which is as sensitive as DNA amplification. It can be easily adopted for both laboratory and field applications.

Introduction

Nosemosis presents special concerns for bee-keepers by threatening to deplete essential pollinators in the United States and around the world (Higes *et al.* 2008). Two species, *Nosema ceranae* and *Nosema apis*, are known to infect honeybees. But only one of them, *N. ceranae*, has been implicated in devastating losses of managed bees. *Nosema ceranae* does not produce clear clinical signs of the disease; so it can go unnoticed for long periods of time. To prevent further dispersal of this intracellular parasite, a simple and robust detection method must be at the forefront of the disease management programme.

Currently, there are two main approaches for detection of *Nosema* in bees: microscopic examinations (Cantwell 1970) and DNA amplification (Fries and Ekbom 1984; Fries 1989; Gatehouse and Malone 1998; Webster *et al.* 2004; Bourgeois *et al.* 2012). Both of these methods require trained personnel and sophisticated equipment and therefore cannot be readily adapted by the bee-keepers.

In this study, we describe a serological approach to *N. ceranae* detection using a highly specific genomic antibody developed against *N. ceranae* SWP-32 spore antigen by Aronstein *et al.* (2011). The advantage of a serological method is that it can be easily adopted for both laboratory and field applications. For field applications, an immunochromatographic assay can be produced in a dipstick format similar to that used for rapid detection of animal and human diseases (Cardinal *et al.* 2006). The laboratory version allows quantification of the environmental spores. With this rapid technique, bee-keepers will be better able to test many individual bee colonies in a short time. By targeting individual colonies instead of entire apiaries, drug applications can be limited to those colonies found to be infected. We also compare the

efficiency of this ELISA to currently used methods, microscopy and quantitative real-time (qRT)-PCR.

Materials and methods

Source of *Nosema*

Nosema ceranae spores were isolated from a single bee collected in a heavily infected commercial apiary (LA, USA) in 2009. Bees infected with a second strain of *N. ceranae* were provided by Dr Lee Solter (University of Illinois, IL, USA) and used as a control for a standard curve analysis for ELISA and qRT-PCR. A second control contained *N. apis* spores from a long-standing culture maintained at Kentucky State University.

Experimental design

Honeybees collected from the Texas apiary were infected in laboratory cages using a range of concentrations of *N. ceranae* (originating from LA, USA) spores at $5{\cdot}0 \times 10^2$, $5{\cdot}0 \times 10^3$ and $5{\cdot}0 \times 10^4$ per bee. Each cage ($12 \times 17 \times 9$ cm) contained 100 newly emerged worker bees provided with water and 50% sugar syrup in gravity feeders placed on top of the cage. *Nosema* spores were fed to 48-h post-emergence bees premixed in a 2-g MegaBee® diet, (Dadant & Sons, Inc. Hamilton, IL, USA). Each treatment group (Nc2, Nc3 and Nc4) was replicated in three cages. Control bees were handled the same way as in the *Nosema* treatment groups except that the protein diet did not contain spores. No *Nosema* spores were detected in newly emerged bees sampled randomly prior to infection. Cages were incubated at 33°C, 50% RH and kept in the dark. Bee samples ($n = 20$) were removed from cages at the end of week 3 and week 4 post-inoculations and stored at −20°C for later analysis.

Sample preparation

Frozen bees were washed briefly in ice-cold ethanol (70%), air dried and the abdomens were cut off using a sterile scalpel to avoid contamination. The abdomen of each bee was homogenized in 200 μl of sterile ddH_2O using disposable tissue homogenizers and placed into a 1·5-ml microcentrifuge tube. Sclerites were removed from the preparations. Each bee sample was divided three ways to conduct different types of analysis as follows: 135 μl was used for DNA extraction, 20 μl for microscopy and 45 μl for ELISA. Bees collected from control cages were processed in the same way. Five biological replicates (a–e) and three technical replicates were used for each analysis. Results are presented as number of spores per bee.

DNA extraction

In each 135-μl sample water was replaced with ice-cold lysis buffer [100 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris–HCl (pH-7·5), 25 mmol l^{-1} EDTA and 0·5% SDS] in 1·5-ml vials. Spores were crushed using manual grinder (Thermo Fisher Scientific, Inc., Rockford, IL, USA) with 45 mg of 0·1-mm Zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA). Samples were then incubated at 70°C for 2 h followed by a standard phenol–chloroform DNA extraction method and ethanol precipitation. DNA pellets were washed with ice-cold 70% ethanol, dried and dissolved in 30 μl ddH_2O each and stored at −20°C for later analysis.

Quantitative real-time PCR (qRT-PCR)

Genomic DNA was amplified using qRT-PCR with SYBR Green I (Molecular Probes Inc., Eugene, OR, USA) on Bio-Rad's CFX96 Real-Time PCR Detection System. We have tested two different sets of *N. ceranae* species-specific primers; all listed on Table 1. The first primer set was designed to amplify a 250-bp fragment of small-subunit (SSU) rRNA of *N. ceranae* (GenBank accession no. DQ486027) as described in the study by Chen *et al.* (2009). The second primer set was designed to amplify a 737-bp fragment of *N. ceranae* spore wall protein *SWP-32* gene that amplifies the same region of DNA used for raising GAT antibody (Aronstein *et al.* 2011). PCR consisted of 1·25 U of GoTaq® Flexi DNA polymerase (Promega Co., Madison, WI, USA) with the colourless 5× GoTaq® Flexi buffer, 0·20 mmol l^{-1} dNTP mix, 2·5 mmol l^{-1} $MgCl_2$, 0·125 μmol l^{-1} of each primer, 0·75 μl of 1000× dilution of SYBR Green I and 1 μl of DNA. After an initial denaturing at 95°C (3 min), reactions ran for 40 cycles at 95°C (30 s), 44°C for *SPW-32* primers or 58°C for (SSU) rRNA primers (1 min). A 72°C (1 min) extension step was used for the *SPW-32* primers. The final extension step was performed at 72°C for 2 min for both primer sets followed by a Melt Curve Analysis step. For each PCR amplification, two negative controls were included: the first one contains no template DNA and the second one contains *N. apis* DNA. All reactions were run in triplicates on the CFX96 Thermocycler (Bio-Rad, Hercules, CA, USA). PCR results were analysed using absolute standard curve method with the CFX Manager Software 2.1 (Bio-Rad). The concentration of a spore suspension for the standard curve analysis was determined using light microscopy (Cantwell 1970).

Table 1 List of primers used for DNA amplification in this study

Primer name	Sequence 5′–3′	An T (°C)	References
16s Ncer F	CGGATAAAAGAGTCCGTTACC-	58°C	Chen *et al.* (2009)
16sNcerR	TGAGCAGGGTTCTAGGGAT	58°C	
SWP32F 5′	ATGGATTTTTATTACCTT	44°C	Aronstein *et al.* (2011)
SWP32R	TTATTTTTCAAAACATCC	44°C	

Enzyme-linked immunosorbent assay

ELISA analysis was conducted on five bees per cage. Samples consisted of 45-μl tissue homogenate per bee, which ultimately provided three 15-μl replicates per sample. The lowest volume spore preparations tested in this study was 1·66 μl per replicate or 5 μl per sample. Samples were diluted to 50 μl with double distilled autoclaved water and prepared as described previously (Aronstein *et al.* 2011). The Express ELISA Kit for Rabbit Primary Antibodies (GenScript, Piscataway, NJ, USA) was used according to the manufacture's protocol to test the spore suspensions. Samples were bound to EIA Plates (Bio-Rad), and development of the substrate with TMB was carried out at room temperature for 5 min. The stop solution was added and the absorbency measured at 450 nm using the MicroPlate Reader 2.2.1 software (Bio-Rad).

Microscopic analysis

Each 20 μl of macerated tissue was examined microscopically (at 400×; Cantwell 1970). Spores were counted in a Neubauer haemocytometer.

Statistical analysis was conducted using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) with a one-way analysis of variance (ANOVA) and Newman–Keuls multiple comparison test.

Results

Spore concentrations were estimated by three different methods: ELISA, qRT-PCR using two primer sets and microscopy to compare the sensitivity of each test. For the qRT-PCR using *SWP-32* primer set, spore quantification was determined by comparing the threshold C_t values of the experimental samples with standard curves of known spore dilutions. Briefly, known spore concentrations as determined by microscopy were serially diluted and run on the CFX96 in triplicates. Threshold C_t values for each dilution were plotted against the spore concentrations resulting in a linear graph. The line equation for SWP-32 was $y = -3{\cdot}462x + 52{\cdot}026$, $R^2 = 0{\cdot}996$, where $y = C_t$, $-3{\cdot}462$ = the slope (m), x = the number of spores per sample and R^2 = the correlation coefficient, which show how well the standard curve data fit a straight line. Similarly, the relationship between the spore concentration and the rRNA primers for a set of serially diluted samples was determined. The relationship is expressed by the equation $y = -3{\cdot}616x + 32{\cdot}452$ and $R^2 = 0{\cdot}999$. Subsets of standard dilutions were used in all subsequent qRT-PCR of the experimental samples that were labelled as the unknowns in the CFX Manger software. The software calculated the spore concentration of the experimental samples based on C_t values and reported the concentrations as spores/sample. The data were exported to an Excel worksheet, and the concentrations were converted to spores/bee.

For the ELISA test, the relationship to spore concentrations in the experimental samples was determined by generating a standard curve using serial dilutions of a known spore sample in the range of $3{\cdot}0 \times 10^2$–$3{\cdot}0 \times 10^6$ spores per sample. Spore concentrations in the experimental samples were determined by comparing the absorbency readings of the samples to those of a standard curve. Each dilution point was processed individually as described in methods and loaded onto the plates in triplicates. The resulting equation, $\log_{10}(\text{abs}) = 0{\cdot}14[1\log_{10}(\text{conc})] - 2{\cdot}07$ and $R^2 = 0{\cdot}980$, was used to calculate the spore concentration in each bee sample, taking into account the amount of starting material that was used for analysis. As with qRT-PCR runs, all ELISA runs included two negative controls and one positive control.

Statistical analysis of the week 3 results (Fig. 1A) showed a significant match between ELISA measurements and DNA methods used in this study (e.g. ELISA *vs* rDNA, $P < 0{\cdot}05$). To test the pair-wise comparison between the different methods, we performed a one-way ANOVA with the Newman–Keuls multiple comparison post-test that showed significant differences between measurements in several individual bee samples. These differences were detected mostly in samples collected from cages 24c–e and 26a,b ($P < 0{\cdot}001$). However, measurements in most samples using different methods were not significantly different, confirming the one-way ANOVA results, shown above. Data collected 4 weeks post-inoculation (Fig. 1B) showed a significant correlation between the ELISA and the data collected by rDNA qRT-PCR only ($P < 0{\cdot}05$). Measurements using microscopy did not correlate significantly with the DNA and ELISA data ($P > 0{\cdot}05$).

Discussion

The data analysis revealed that spore counts measured by the ELISA are comparable with the qRT-PCR that is

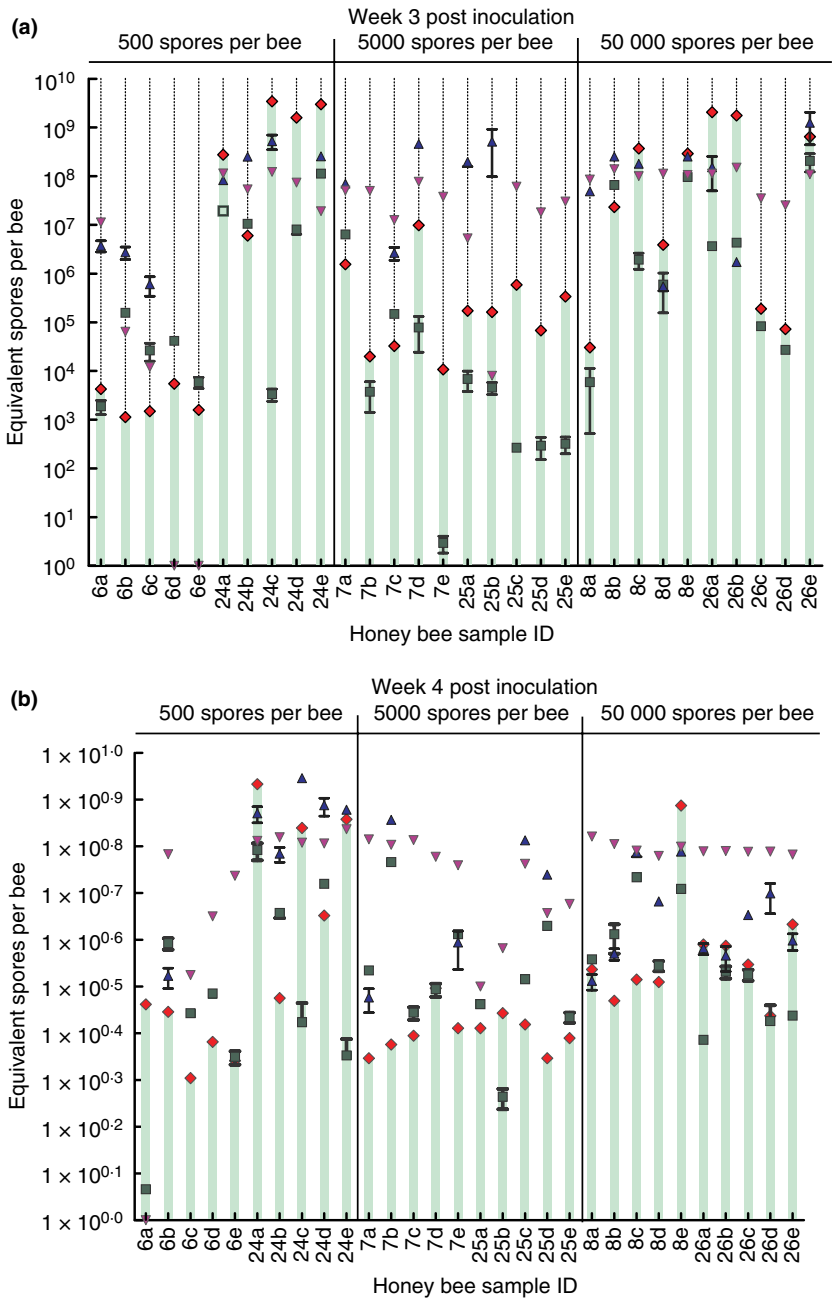

Figure 1 (a) (week 3 post-inoculation) and (b) (week 4 post-inoculation) show the number of *Nosema ceranae* spores in bee samples as determined by four different tests: (i) ELISA indicated by (red diamonds), (ii) qRT-PCR with rDNA primers (green squares), (iii) qRT-PCR with SWP-32 primers (black triangles) and (iv) microscopy (red triangles). Three different treatments ($5{\cdot}0 \times 10^2$, $5{\cdot}0 \times 10^3$ and $5{\cdot}0 \times 10^4$) are shown at the top of the figure. *Y* axis shows number of spores detected in each sample; *X* axis shows cage numbers. Five bees were analysed per each cage (a–e). (■) rDNA; (◆) ELISA; (▲) SWP-32 and (▼) microscopy.

currently the most sensitive method for detection of *Nosema*. In this study, we used two sets of primers to amplify different DNA regions. The results showed that the efficiency of these two reactions differed; the rDNA fragment amplification was much more robust than the *SWP-32* gene fragment (Fig. 1A,B). The magnitude in amplification efficiency can be largely attributed to differences in gene copy numbers and to the size of the amplicons.

Compared to the qRT-PCR, ELISA is simple to perform. It does not require sophisticated equipment or highly trained personnel. Sample preparation is considerably less labour intensive, whereas DNA extraction is a lengthy process and prone to DNA losses due to insufficient spore lysis (e.g. Fig. 1A, 7e; Fig. 1B, 6a). It is also important to point out that this new ELISA detects only environmental spores of *N. ceranae* (Aronstein *et al.* 2011). Because various developmental stages of *Nosema* are simultaneously found in the host, we expected that qRT-PCR would produce higher counts than those measured by ELISA (Fig. 1A, cages 6, 7 and 25), and the difference would be diminished in advanced stages of the

disease characterized by maturation of the environmental spores.

As indicated above, some spore counts measured by PCR were lower due to partial loss of DNA (Fig. 1A, 25c–e). Crude tissue preparation for ELISA eliminates this problem. In general, microscopy produced relatively lower accuracy results (Fig. 1A, 6d,e), which will highly depend on the technical skills and, in practise, would result in the unnecessary exposure of bees to antibiotics. Taken together, we conclude that ELISA is a very accurate and sensitive approach to detecting *N. ceranae* in bee samples. It is also less time-consuming than other currently used methods and can be adopted for field sampling services by potentially substituting microplate readers with the gradient colour card.

Acknowledgements

We thank Dr Lee Solter (University of Illinois, IL, USA) for providing the control strain of *N. ceranae*. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2009-85118-05718 from the USDA National Institute of Food and Agriculture, awarded in part to K.A.A. and T.C.W.

References

Aronstein, K.A., Saldivar, E. and Webster, T.C. (2011) Evaluation of *Nosema ceranae* spore-specific polyclonal antibodies. *J Apic Res* **50**, 145–151.

Bourgeois, L., Beaman, L., Holloway, B. and Rinderer, T.E. (2012) External and internal detection of *Nosema ceranae* on honey bees using real-time PCR. *J Invertebr Pathol* **109**, 323–325.

Cantwell, G.E. (1970) Standard methods for counting nosema spores. *Am Bee J* **110**, 222–223.

Cardinal, M.V., Reithinger, R. and Gürtler, R.E. (2006) Use of an immunochromatographic dipstick test for rapid detection of *Trypanosoma cruzi* in sera from animal reservoir hosts. *J Clin Microbiol* **44**, 3005–3007.

Chen, Y.P., Evans, J.D., Murphy, C., Gutell, R., Zuker, M., Gundesen-Rindal, D. and Pettis, J.S. (2009) Morphological, molecular, and phylogenetic characterization of *Nosema cerana*, a microsporidian parasite isolated from the European honey bee, *Apis mellifera*. *J Eukaryot Microbiol* **56**, 142–147.

Fries, I. (1989) Observation on the development and transmission of *Nosema apis* Z. in the ventriculus of the honeybee. *J Apic Res* **28**, 107–117.

Fries, I. and Ekbom, G. (1984) *Nosema apis*, sampling techniques and honey yield. *J Apic Res* **23**, 102–105.

Gatehouse, H.S. and Malone, L.A. (1998) The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size. *J Invertebr Pathol* **71**, 97–105.

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E.G., González-Porto, A.V., Barrios, L., Nozal, M.J.D., Bernal, J. L. *et al.* (2008) How natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environ Microbiol* **10**, 2659–2669.

Webster, T.C., Pomper, K.W., Hunt, G., Thacker, E.M. and Jones, S. (2004) *Nosema apis* infection in worker and queen *Apis mellifera*/*Nosema apis* infektion von Arbeiterinnen und Königinnen *Apis mellifera*. *Apidologie* **35**, 49–54.