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Viability and infectivity of fresh and cryopreserved *Nosema ceranae* spores

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ABSTRACT

The microsporidium fungus *Nosema ceranae* is an intracellular parasite that infects the midgut of the honey bee, *Apis mellifera*. A major limitation of research on *N. ceranae* is that the fungus is non-culturable and thus studying it depends on the seasonal availability of *Nosema* spores. Also, spore viability and infectivity can vary considerably, and thus there is a need for reliable methods for determining those traits. This study examined different conditions for *N. ceranae* spore cryopreservation at −70 °C, assessing spore viability and infectivity. Viability was determined by a staining procedure counting total spores numbers with bright field microscopy and un-viable spore numbers with the fluorescent dye, propidium iodide. Spore infectivity was determined with a dilution inoculation assay. Infectivity was dependent on the inoculum dose for the proportion of bees with detectable *Nosema* infections based on the number of spores per bee at 18 days after inoculation; 4000 spores per bee or higher were needed to get approx. 100% of the inoculated bees infected. The median infective dose (ID_{50}) was 149 spores per bee, and the minimum dose capable of causing a detectable infection was 1.28 spores. The proportion of *N. ceranae* infected bees correlated significantly with the number of spores per bee ($r = 0.98$, $P < 0.0001$). *N. ceranae* spores cryopreserved in water or 10% glycerol did not differ in viability compared to fresh spores, but lost infectivity when inoculated into bees. This study shows that while cryopreservation of *N. ceranae* spores can preserve viability, the spores can have reduced infectivity.

1. Introduction

Nosema apis was thought to be the only microsporidium fungus to infect epithelial cells in the midgut of western honey bees (*Apis mellifera*), but in 2005 it was reported both in Taiwan (Huang et al., 2007) and Spain (Higes et al., 2006) that another related microsporidium, *Nosema ceranae*, also infects them. Since then, surveys have shown that *N. ceranae* is the dominant microsporidium infecting honey bees in many countries (Klee et al., 2007; Calderón et al., 2008; Chen et al., 2008; Invernizzi et al., 2009; Fries, 2010; Guzman-Novoa et al., 2011; Emsen et al., 2016). One limitation of studying *Nosema* infections of honey bees is that the pathogens are non-culturable, and thus artificially infecting honey bees with spores requires extracting them from infected bees, which are only seasonably available, typically during spring in temperate countries. Also, extracted spores can vary in their stage of development and thus in their level of infectivity.

A constant supply of viable and infective *N. ceranae* spores requires a reliable technology to preserve spores of the fungus. Deep-freezing (cryopreservation) has been shown to be suitable for many other fungi, maintaining their viability for up to 40 years (Ryan and Smith, 2004). The principle of cryopreservation is to stabilize cells at cryogenic temperatures with a cryoprotectant substance to prevent intracellular formation of ice crystals that can cause significant damage (cryo-injury) to cells. Several studies have examined the effects of freezing temperatures on different species of microsporidia (Amigo et al., 1996; Koudela et al., 1999; Li and Fayer, 2006; Fries and Forsgren, 2009), but the cryopreservation of *N. ceranae* spores has not been investigated.

To assess cryopreservation conditions, a host-free, reliable protocol to determine *N. ceranae* spore viability is required. The majority of viability tests are based on the principle of differential spore staining whereby presumably live cells retain intact membranes, thus excluding any viability dyes, while presumably dead and/or nonviable cells incorporate the dye through a compromised membrane. However, specific organisms and their membrane potentials vary in response to given fluorescent dyes (Mason et al., 1995). For example, Fenoy et al. (2009) used 4′,6-diaminidino-2-phenylindole (DAPI) and Sytox Green to detect viability of *N. ceranae* spores, whereas Amigo et al. (1996) and Leiro et al. (2004) used propidium iodide (PI) to detect viability in microsporidian pathogens of fish and mammals, respectively. In those studies, dye incubation times varied from 10 to 60 min, indicating that the length of exposure to a dye can affect detection.

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Spore viability and spore infectivity are two separate biological qualities. Although viability is important, the ability of the spores to reproduce in the host (i.e., infectivity) is the critical factor that determines a disease. For *N. ceranae*, infectivity is confirmed when newly hatched, uninfected adult honey bees become infected after being force-fed inoculants containing spores (Higes et al., 2007). Experimentally infected bees are kept alive in cages, which is labour intensive and often results in non-uniform infection levels due to variation between individual bees and between variation of groups of bees within and between cages (Malone et al., 2001).

Most fungal pathogen inoculation procedures use far more spores than required to cause infection to insure easily measurable disease development. However, it is also important to know the minimum and median doses for disease development. For artificial *Nosema* infections of honey bees, the minimum and median doses needed to start and develop infections in bees are not well established. Bailey (1972) and Fries (1988) reported that the median infective dose (ID_{50}) for *N. apis* was approximately 100 spores per bee, whereas for *N. ceranae*, Forsgren and Fries (2010) reported a similar dose (85 spores per bee), but Huang et al. (2015) reported a far higher dose (10,000 spores per bee). This large variation may be due to a number of factors including experimental setups, honey bee caste and strain, *N. ceranae* isolates, and environmental effects.

Clearly, honey bee-free methods of keeping a constant supply of viable and infective *N. ceranae* spores are required to facilitate the maintenance of this parasite during periods of low availability as well as to preserve specific *N. ceranae* isolates for future studies. This study examined the response of fresh and cryopreserved *N. ceranae* spores to fluorescent viability dyes and tested the time needed for dye uptake, and also assessed the infectivity of fresh and cryopreserved *N. ceranae* spores. The ultimate aim is improving protocols for testing their viability and long term preservation, as well as for optimal inoculation in live bees for experimental purposes.

2. Materials and methods

2.1. N. ceranae spore sources

Samples of forager honey bees were captured with a bee vacuum (Gary and Lorenzen, 1990) when returning from foraging trips at the entrance of randomly selected hives established at the Honey Bee Research Centre of the University of Guelph, Guelph, ON, Canada. Sixty bees per sample were examined by phase contrast microscopy (Olympus BX41; Olympus, Markham, Ontario) for presence of *Nosema* spp. spores at 400× magnification. If positive, infection levels were determined by performing spore counts as per Cantwell (1970). Three colonies that had high *Nosema* spp. spore counts were used as source colonies after confirming that all were infected with *N. ceranae* only as per the species-specific PCR assay of Hamiduzzaman et al. (2010).

2.2. Extraction and purification of N. ceranae spores

N. ceranae spores were extracted and purified from a pool of forager bees from the source colonies. Abdomens were dissected from the bodies of 20–30 bees, the ventriculus removed and placed in an autoclaved mortar, and macerated in 0.1 mL of ddH_2O per individual. A *Nosema* spore purification protocol used by Higes et al. (2007) was modified to increase purity of spore suspensions by filtering and repeated centrifugation. The macerated tissue was strained through a stainless steel funnel screen with 5 mm diameter mesh (U-Lube-It XCel Manufacturing, Windsor, ON, Canada). The homogenate was then filtered through a nylon honey filter with a pore size of 80 holes per inch (Better Bee Supplies, Cambridge, ON, Canada). The filtered macerate was transferred to 2 mL microcentrifuge tubes and centrifuged (Eppendorf, Symphony™, Model 2417R, West Chester, PA, USA) at 800 ×*g* for 15 min. The supernatant was removed and the spore pellets were suspended in 1 mL of ddH_2O and centrifuged two more times. The final pellets were suspended in 2 mL of ddH_2O.

After extraction, 10 μL of the resulting suspension were placed on a haemocytometer. The spores were counted as described above to determine the concentration of spores in the suspension. The spore concentrations were adjusted to either 1×10^4 spores/μL or 1×10^5 spores/μL and kept in sealed 1.5 mL microcentrifuge tubes at 4 °C until needed.

2.3. Infectivity assessments

Newly emerged worker bees were obtained from five colonies of Buckfast bees raised at the Honey Bee Research Centre in Guelph. Capped brood combs were retrieved from these colonies and placed inside emergence cages (5 × 28 × 25.5 cm). The emergence cages were kept at 32 °C and 60% RH in an incubator over night. The following day, the newly emerged bees were shaken into a plastic bin and starved for 2 h prior to treatment. To ensure that the newly emerged bees were not contaminated with *Nosema* spores from the combs, microscopic spore diagnosis was performed using 25 randomly selected bees as described above. The *Nosema*-free status of the bees was later corroborated at the end of the infectivity experiments with an examination for spores in the control bees.

Fifty newly emerged worker bees were individually fed with 10 μL of either 50% sucrose syrup alone (control) or 50% sucrose syrup containing different *N. ceranae* spore concentrations (10,000, 2000, 400, 80, 16, 3.2, 0.64, 0.128, 0.026 spores/μL). Three biological replications for each treatment were conducted.

After feeding, each bee was transferred to a screened hoarding cage (10 × 15 × 15 cm) until 50 bees of the same treatment had been introduced (Williams et al., 2013). Each cage was provided with two gravity feeders, one containing sterile dH_2O and the other 50% sucrose syrup for the bees to feed ad libitum. The cages of all treatments were kept in the dark in an incubator at 32 °C and 60% RH for 18 dpt, and then *N. ceranae* infections were individually determined in all surviving bees as above described. Two samples from each macerate were used and the numbers of *N. ceranae* spores were averaged.

2.4. Optimization of N. ceranae spore viability testing protocols

DAPI and PI dyes were chosen for this study because in preliminary observations they had the lowest autofluorescence of the dyes tested and stained spores with discernible colors. The following procedures were followed to optimize the staining protocol in this study. Initial dye concentrations and dye incubation times were adapted from Fenoy et al. (2009) for DAPI, and from Campbell et al. (1992) for PI with dye incubation times from 0 to 60 min. Stock solutions of DAPI (1 mg/mL in ddH_2O) and PI (1 mg/mL in ddH_2O) were stored in the dark at 4 °C. Three replicates of freshly harvested and purified spore suspensions were incubated simultaneously with PI and DAPI for 10, 20 and 60 min. Each microcentrifuge tube containing 200 μL of spore suspension was incubated with 20 μL of DAPI and 20 μL of PI working solutions in the dark at room temperature. After the dye incubation, spores were rinsed twice by centrifuging at 800 ×*g* for 6 min in 100 μL of ddH_2O, and the final pellet was re-suspended in 100 μL of ddH_2O. Five microlitres of the spore suspension was blotted onto a slide and 5 μL of antifade reagent (ProLong® Gold Antifade, Invitrogen, Burlington, ON, Canada) were added with a pipette tip and mixed until the drop of suspension was spread to 5 mm in diameter. Partial drying was allowed for 5–10 min in the dark and a cover slip was placed over the drop. Slides were cured over-night in the dark, and sealed with clear nail polish the following day.

Slides of stained spores were viewed at 400× magnification on a fluorescence microscope (Leica DM5500B, Leica Microsystems, Wetzlar, Germany). Between 25 and 30 fields containing approximately 25–40 spores per field were photographed under visible light for bright field filters, and then fluorescence light for either DAPI or PI filters based on