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MICROSPORIDIAN *NOSEMA* SPP. AS A MODEL GASTROINTESTINAL MICROORGANISM OF CARNIOLAN HONEY BEE (*APIS MELLIFERA CARNICA*, POLLMAN, 1879): ASPECTS OF SPORE COUNTING

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ABSTRACT

At high invasion levels, nosemosis can act as an underlying cause of colony collapse disorder, especially in cases of simultaneous invasion of other parasites or/and in combination with virus infections. Rapid quantitative methods for Nosema spp. spore detection are therefore required for various research and diagnostic needs. In this study, the use of haemocytometer (standard method) and Coulter counter is compared. All work was performed with late summer forager bees of Carniolan honey bee subspecies, prepared as isolates and homogenates of the posterior section of the worker bee's intestine. It can be concluded that the Coulter counter provides greater sample dilution flexibility at sufficient repeatability (RSD 0.01–0.1) in the cases of: (i) low concentrations of spores, as well as (ii) samples that required high dilutions ($c \geq 107$ spores ml^{-1}). However, Coulter counter use required additional data processing for obtaining precise concentration estimates at ~ 3 µm mean spore size. Nevertheless, the uncorrected, as-measured values provided concentration ranges which were found to be suitable for general diagnostic purposes.

Key words: bees, Carniolan honey bee, microbiology, microorganisms, cell counting, haemocytometer, Coulter counter

1 INTRODUCTION

Nosema disease, or nosemosis of honey bees is presently known to be caused by two species of microsporidian parasites, *Nosema apis* (Zander, 1909) and *Nosema ceranae* (Fries *et al.*, 1996). Spores of the two *Nosema* species have similar ovoid morphologies. Spores of *N. ceranae* are slightly smaller, reportedly measuring 3.3–5.5 µm length and 2.3–3.0 µm width, whereas *N. apis* spore ranges are 4–6 µm and 2–4 µm, respectively (Fries *et al.*, 1996; Higes *et al.*, 2010). Both species invade the epithelial cells of *ventriculus* (midgut) and have similar life cycles, resulting in release of large number of spores and in severe cases, in consequential death of bees due to destruction of the intestine wall. At high invasion levels, nosemosis can act as an underlying cause of colony collapse disorder, especially in cases of simultaneous invasion of other parasites and/or in combination with virus infections (Bromenshenk *et al.*, 2010).

Due to continuous honey bee colony losses, standing requirements for data collection on parasite pathology, omnipresence within the honey bee population and simplicity of observation, the microsporidian *Nosema* spp. was selected as a model gastrointestinal microorganism for a study on spore counting aspects, which can be extended to cell counting applications.

Routinely, a haemocytometer is used for the quantitative particle analysis of bee gut contents, in particularly for assessment or spores of *Nosema* spp. Simultaneous analyses of other microorganisms residing in the bee gut increase the counting time and typically require preliminary staining procedures. In this study, an insight in reliability of a traditional haemocytometer counting technique for *Nosema* spp. spore assessment is provided and the method is compared to the performance of a portable

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Coulter cell counter for examination of a complex sampling matrix of bee gut homogenate.

2 MATERIAL AND METHODS

All work was performed with the subspecies Carniolan honey bee (*Apis mellifera carnica*, Pollman, 1879). Forager bees of sufficient quantity (approximately 100 bees/hive) were sampled during July and August 2015 from hives located at various field stations operating within the Slovenian national bee monitoring network. Bees were sampled at the hive entrance, in order to avoid underestimation of spore count *via* sampling of recently-hatched bees which are spore-free.

Sample preparation (isolation and homogenisation of the posterior section of the worker bee's intestne) and haemocytometer counting were performed following the standard methods, described by Fries *et al.* (2013) and Human *et al.* (2013), respectively. For all samples analysed, phosphate-buffer saline was used instead of DI water to ensure conductivity of the samples, which was required for particle size determination by means of the Counter principle.

A portable Coulter counter was used to count samples (Merck, model Scepter 2.0 Cell counter). The basic operational principle of the device consists of pumping a conductive suspension (sample) through a microchannel and determining the particle size of passing solids from measured changes in impedance, which are directly proportional to the volume of the particle traversing the orifice. The particle diameter is then calculated assuming spherical morphologies. The procedure is automated and typical sample handling time ranged from 1 to 3 minutes. For spore counts, sensors with 40 µm aperture were used. Prior use, the apparatus was assessed for reliability with a reference suspension of polystyrene beads (d = 15 µm, $c_p = 2 \times 10^5$ beads ml^{-1}) using 40 and 60 µm sensors (sensor measuring ranges within 3–17 and 6–36 µm, respectively). Reference measurements consistently showed high repeatability of results with both sensor types (relative standard deviation, RSD < 0.06, $\overline{c}_p = 2.3 \times 10^5$ beads ml^{-1}). Cell counts in the desired uniform particle size range were obtained with Scepter Software 2.1 (Merck) cellular analysis platform. In order to extrapolate particle size concentration and distribution below the detection range of the 40 µm sensor, peak fitting (Gaussian amplitude / peak function) was performed with Origin 8.1 (OriginLab Corporation) data analysis software.

In parallel to the novel automated counting method, counting was performed using a Bürker chamber (12 × 12 fields) under Leica DM 2500 light microscope at 400 × magnification. Typical sample handling time for this method ranged from 10 to 20 minutes.

3 RESULTS AND DISCUSSION

Initially, the standardised method was employed for spore counting for an array of samples, which were exhibiting a wide range of *Nosema* spp. levels of infection (from 10^5–10^7 spores ml^{-1}), as shown in Table 1. Preliminary tests showed high variability (P < 0.001,

Table 1: *Spore concentration, average ± standard deviation and haemocytometer counting repeatability (RSD) for seven hives with different Nosema spp. levels of infection*

Replicate N°	Hive I	Hive II	Hive III	Hive IV	Hive V	Hive VI	Hive VII
1	0.45	1.7	3.5	4.3	11.3	20.8	27.8
2	0.5	1.8	3.1	3.8	10.8	20.8	28.4
3	0.7	1.7	3.6	5.7	10.7	19.25	27.2
4	0.6	1.5	3.7	5.4	10.6	19.55	27.9
5	0.65	1.8	3.6	3.9	9.2	20.3	27.1
6	0.6	2.1	3.1	4.1	9.05	20.1	27.3
7	0.6	2.1	2.9	5.1	10.1	19.9	27.1
8	0.45	2.3	4.5	4.0	10.5	19.1	27.5
9	0.55	1.9	3.7	3.7	9.7	20.0	27.1
10	0.7	1.4	3.6	5.2	10.2	20.1	27.3
Average ± SD	0.58 ± 0.09	1.82 ± 0.28	3.49 ± 0.44	4.50 ± 0.75	10.22 ± 0.72	19.99 ± 0.57	27.45 ± 0.44
RSD	0.16	0.15	0.13	0.17	0.07	0.03	0.02

units of concentration: × 10^6 spores ml^{-1}

0.2–0.5 RSD) of spore counts from bees sampled from the same hive for separately prepared and thoroughly homogenized suspensions (n = 20 / 3 suspensions / 4 hives). Therefore in order to increase the level of repeatability, all subsequent analyses were performed with the same primary material *per* hive. Furthermore, ten replicates of the same sample were evaluated by counting either twelve or five fields in the counting chamber and the results were found to be comparable (an average count ratio for 12/5 fields of 0.99/1 was obtained). From there on, five fields were used for counting (1/1; 1/12; 12/1; 12/12; 6/6), at it is mostly done in internal routine counting applications. Using this approach it is evident that at lower concentrations (up to 4.5×10^6 spores ml^{-1}), where 1–20 spores can be counted for each field, the coefficient of variation between replicates was relatively high (Table 1). To increase the repeatability of these results, more fields could have been counted (consequently increasing the time of analysis). At higher concentrations (20–140 spores *per* field), repeatability was high and the coefficient of variation was well below 10 %.

For the purpose of examination of the counting method based on the Coulter principle, we selected a sample exhibiting high *Nosema* spp. level of infection. For each sample, two series of triplicates were measured and respective particle-size distributions were extrapolated below the lower limit of measurement to the full expected diameter range (as shown in Figure 1 for the case of a 100 × diluted sample). Within samples, good repeatability of measurements, as well as a good fit to normal distribution were observed.

In the recent years, several researchers (*e.g.* Csáki *et al.*, 2015; Emsen *et al.*, 2016) and monitoring studies report *N. ceranae* domination in population share, as well as in prevalence. As the need of differentiation amongst the two *Nosema* species has decreased in importance for general diagnostic purposes, spores in this study were regarded as one population with the Coulter counter device. Nevertheless, the counting method does allow for some basic recognition, taking in mind the particle diameters provided by the Coulter counter are represented as sphere diameters, whereas *Nosema* spores exhibit prolate ovoid morphologies. Assuming 50 % *N. apis* and 50 % *N. ceranae* population, prolate ovoids with 3.3–6 µm in length and 2.3–4.0 µm in width would have been observed in the sample, corresponding to an measured average sphere diameter of 3.6 µm. However, as it is evident from Figure 1, the average sphere diameter has been extrapolated to fall below 3 µm (conforming to ovoid shapes with roughly 4 µm in length and 2.5 µm in width), indicating an expected dominance of *N. ceranae* spores and simultaneously confirming the coexistence of *N. apis* spores in the examined samples.

The employed portable Coulter counter is capable of detection of particles, which are larger than 3 µm and at these limitations the device operates within the 5×10^4–1.5×10^6 particle ml^{-1} concentration range. Using the approach presented in Figure 1, it is possible to extrapolate the measured values to obtain total spore counts and dimensions. In comparison to counts obtained with the standard spore counting procedure, the adjusted values were analogous (90.4 % match).

Furthermore, uncorrected, as-measured values ($\overline{c}_{1-3} = 1.22 \times 10^7$ spore ml^{-1}) accounted for approximate-

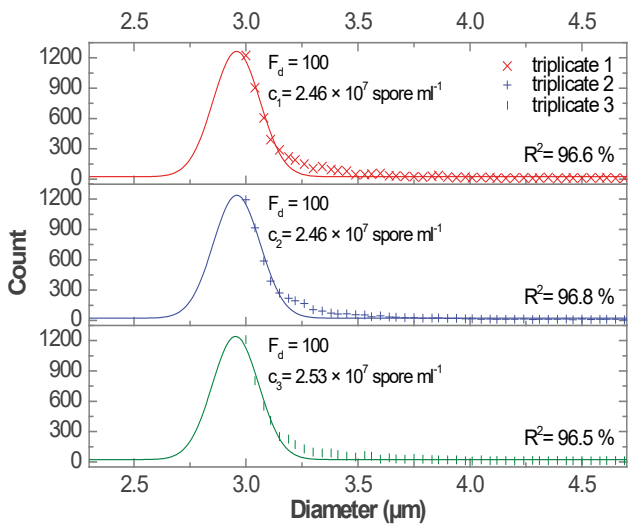

Figure 1: *Particle diameter and count in 100 × diluted sample of bee gut homogenates. Difference between triplicates not statistically significant (P > 0.05).*

ly two fold lower concentrations, yet they remained in the same order of magnitude in comparison to the reference value which was determined by the standard counting method.

As both counting techniques require sample dilution at high spore concentration, a dilution series was prepared to study possible effects. The haemocytometric spore count of the undiluted sample was set as the reference value (Figure 2, denoted as dashed line). For the case of undiluted sample, high repeatability of counts was obtained at > 100 spores for each counting field. However, analyses of larger sets of samples having such a concentration range require a lot of time and focused concentration of the microscope operator. It is therefore not uncommon to count *Nosema* spp. spores in 10 × diluted samples, where reliable counts can be achieved, as Figure 2 shows.

At higher dilutions (100 and 1000 ×) beyond the recommended spore concentration of 5–50 spores *per* field (Human *et al.* 2013), the spore numbers became increasingly overestimated (up to 180 %). In the case of the standard method, increasing the dilution did not affect the coefficient of variation (in order of dilution: 13 %, 9 %, and 7 %).

Using the Coulter counter adjusted values at 10 × sample dilution (operating slightly above the recommended concentration) 30 % underestimation of the spore concentration was observed. In contrast to standard counting method, Coulter counter provided relevant values (up to 93 % of the reference value) in subsequent dilutions. In this case, the coefficient of variation was increased from 1 % at 10 × dilution to 10 % at 1000 × dilution.

Similarly to previous observations, uncorrected values corresponded to 40–77 % (2.5–1.3 fold) of the adjusted concentration at 10–1000 × dilution, respectively. This apparent discrepancy can be explained by the fact that as the number of the spores in the suspension decreases due to dilution, the size fraction of the spores which is numerically most represented in the initial (undiluted) population, gains an even greater effect on the overall population estimate. Furthermore, adjusted and unadjusted values exhibited the same coefficients of variation, indicating that the extrapolation has been performed correctly as it preserves the initial characteristics of the measurements within the expanded set of data.

4 CONCLUSIONS

Nosema spp. was selected as a model gastrointestinal microorganism for a study on spore counting aspects, which can be extended to cell counting applications. Two methods, (i) standard haemocytometer counting technique and (ii) the use of a Coulter counter are compared.

It can be concluded, that the Coulter counter provides greater dilution flexibility and exhibited sufficient accompanying repeatability (RSD 0.01–0.1) for the analysis of: (i) spores at low concentrations, or (ii) samples at high dilutions.

Counting spores at low and medium concentrations (6×10^5–4×10^6 spore ml^{-1}) using the standard method resulted in borderline repeatability (RSD up to 0.17), indicating that prolonged sample observation was required, as an increased number of fields would have had of been visually examined.

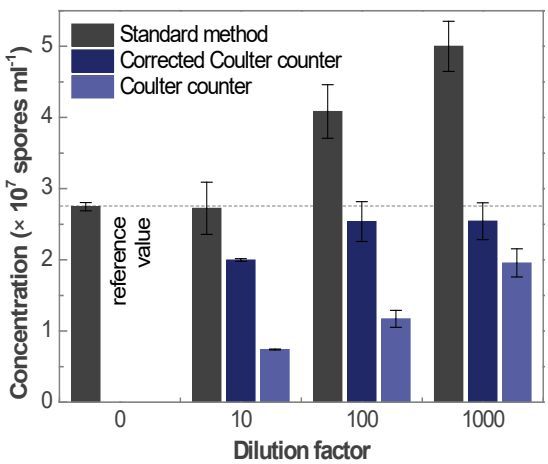

Figure 2: *Spore concentration average at different sample dilutions for each counting method (bars denote standard deviation)*

High sample dilutions may be required, *e.g.* when analysing concentrations $\geq 10^7$ spores ml^{-1}. At higher concentrations (3×10^7 spores ml^{-1}), the repeatability of the traditional technique was high (RSD < 0.05), and the sample handling time was longer as a result of higher spore counts. In the case of unsuitably high dilutions, the measured concentrations were imprecise.

The downside of using Coulter counters is that additional data processing techniques may be required. It is furthermore important to note that the entire spore concentration range, and simultaneous presence of other microorganisms (*e.g.* yeasts with typical d_p = 3–4 µm) were not evaluated in this study and that these effects on *Nosema* spore counting should be considered further. Nevertheless, in comparison to reference value, similar spore concentrations were observed when using adjusted Coulter counter counts. At 100 and 1000 × dilution, adjusted values consistently corresponded to roughly 90 % of the set reference value, showing that this approach can be used for obtaining precise total counts. To allow for time efficient analyses, such devices should allow for the integration of advanced data processing techniques.

Uncorrected values show two to three fold lower concentrations, yet they remain in the same concentration range than the reference value (*e.g.* 10^7 for the presented case) and could therefore be used for routine diagnostic purposes, where the information of the exact concentration is not required.

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