Further verification on the effects of Hive Alive on Spore viability

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Abstract.

An experiment was carried out to investigate the efficacy of HiveAlive to disrupt the plasma membrane/spore coat of *Nosema ceranae* thereby killing the spores of this microsporidian parasite of honeybees. To determine if spores were dead, the sytox green staining approach using fluorescent microscopy was employed. Earlier investigation showed that after incubation in HiveAlive for a period of three months spores were fluorescing suggesting that the spore coat had been compromised. The majority of spores stored in distilled water for the same length of time were not visible under the fluorescent filter employed suggesting that they were alive. Further testing was needed to confirm these findings. This was performed by comparing spores that had been exposed to HiveAlive for two weeks to control spores that had not been exposed, employing the sytox green nucleic acid stain and fluorescent microscopy. The number of spores that fluoresced was assessed post exposure. There was a clear difference between control and 'treated' spores, where only the 'treated' spores absorbed sytox green suggesting that the spore plasma membrane has been damaged by incubation with HiveAlive.

Introduction:

Fenoy et al., (2009) described a method to determine the viability of *Nosema ceranae* spores utilising DAPI and sytox green stains. The rationale is that all spores would be visible under white light. Unextruded spores would fluoresce with DAPI, which stains nuclear material under the 395- to 415-nm excitation wavelength filter and dead spores would absorb sytox green and fluoresce under the 470- to 490-nm excitation wavelength filter. In this experiment we attempted continue on from the research

performed by Dr McCormack in early 2012 where florescence in HiveAlive treated spores had been observed. The approach used would again repeat the approach of Fenoy et al (2009) and be used to investigate the efficacy of Hive Alive in killing *Nosema* spores by compromising their spore coat or by disrupting their plasma membranes, Rice (2001). Therefore, after treatment with HiveAlive *N. ceranae* and *N. apis* spores should fluoresce under a under the 470- to 490-nm filter after staining with sytox green. Previous attempts to stain *Nosema* spores with DAPI were unsatisfactory and therefore this step was excluded from the following experiment.

Methods:

Materials: For this experiment a concentrated spore suspension of *Nosema apis* was utilised.

Experimental Approach: Two 50µl aliquots of spore suspension (in distilled water) were pipetted into eppendorfs. To one tube 3 µl of HiveAlive was added and the tubes were placed on a shaking thermoblock at 25 ^{o}C for two weeks.

To remove the Hive Alive solution prior to staining, 500 µl of distilled water was added to each tube and mixed gently by pipetting. Spores were pelleted by centrifugation (4,500 rpm for 6 mins), resuspended in the same volume of distilled water and re pelleted. Spores were resuspended in 50 µl of distilled water and stained by adding an equal volume of sytox green solution. Spores were held in the stain at room temperature for 20 min and then washed using 200 µl of distilled water. The final pellet was resuspended in 10 µl of water. This spore suspension was placed on a microscope slide in its entirety and dried, before being viewed on a fluorescence microscope under white light and fluorescence (470- to 490-nm excitation wavelength filter). Photographs were taken under white light (to identify location of spores) and under fluorescence at 400x magnification. Spores (50) were counted from each slide and it was determined whether they were visible under the green filter or not. Spores visible under white and fluorescent light were determined to be dead. Those that were visible under white light but not visible when using the green filter were determined to be alive.

Results:

45/50 spores on the slide that was treated with HiveAlive were visible under the 470- to 490-nm excitation wavelength filter indicating that they were dead (Figure 1A). By comparison, only 4/53 spores on the control slide were visible (Figure 1B).

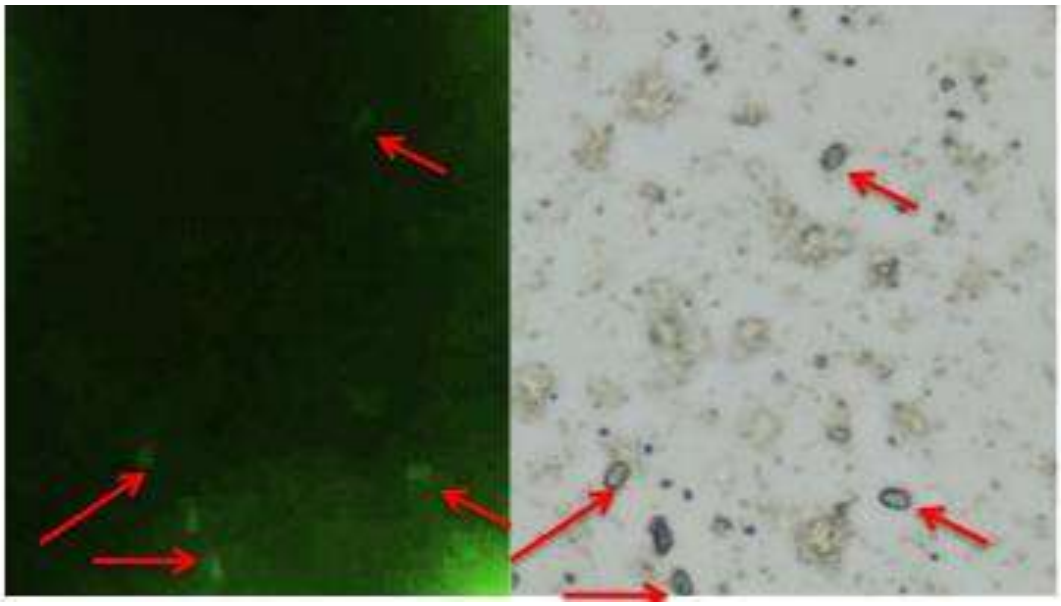

Figure 1 A. Dead Spores from spore suspension held for 14 days in water to which HiveAlive solution was added. The first picture is photographed under the 470- to 490-nm excitation wavelength filter and the second picture is photographed using white light. The slide shows four spores all weakly fluorescing after being stained with sytox green.

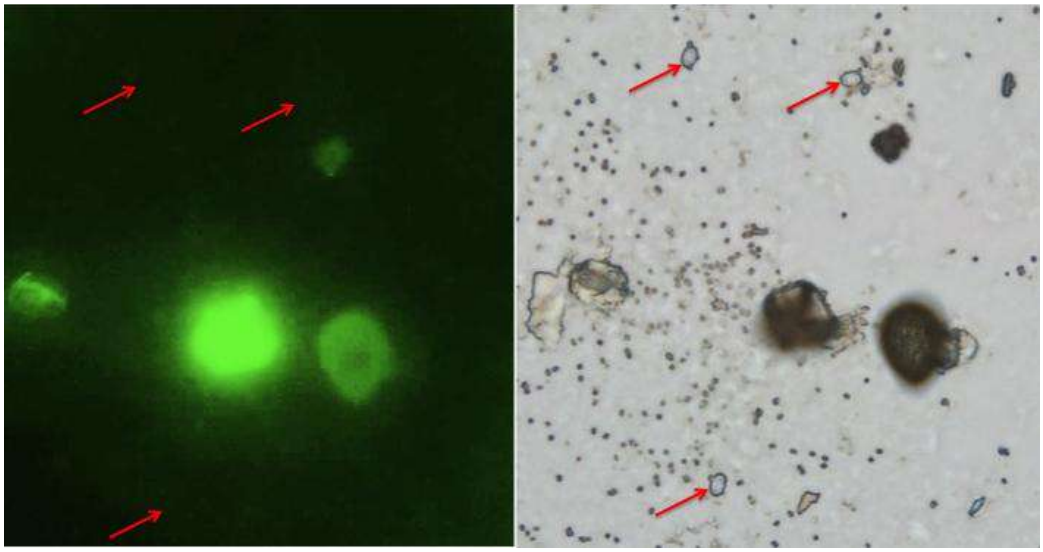

Figure 1 B. Live Spores from spore suspension held for 14 days in water only. The first picture is photographed under the 470- to 490-nm excitation wavelength filter and the second picture is photographed using white light. The slide shows three spores under white light that did not take up the sytox green stain and thus did not fluoresce under the 470- to 490-nm excitation wavelength filter.

Discussion:

This is the second experiment that indicates a clear difference between control and 'treated' spores, where 'treated' spores were stained with the nuclei acid stain sytox green suggesting that the spore plasma membrane has been damaged by incubation with HiveAlive. Results here support those generated in March 2012 although the numbers are smaller. The spore walls of *Nosema ceranae* are similar in structure to *N. apis* and hence the effect of HiveAlive on the *N. ceranae* spore wall would be expected to be comparable with *apis.*

Further research with more incubation times and dilutions would be advantageous.

References

Fenoy S, Rueda C, Higes M, Martín-Herńandez R, del Aguila C. (2009). High-Level Resistance of *Nosema ceranae*, a Parasite of the Honeybee, to Temperature and Desiccation. *Applied and Environmental Microbiology*. 25(1):6886-9.

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