Veterinary Microbiology 177 (2015) 1–6

Evidence of the synergistic interaction of honey bee pathogens Nosema ceranae and Deformed wing virus

Huo-Qing Zheng [a,b], Hong-Ri Gong [a], Shao-Kang Huang [b,c], Alex Sohr [b], Fu-Liang Hu [a], Yan Ping Chen [b,*]

[a] College of Animal Sciences, Zhejiang University, Hangzhou, Zhejiang, P.R. China
[b] USDA-ARS Bee Research Laboratory, Beltsville, MD, USA
[c] College of Bee Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian, P.R. China

ARTICLE INFO

Article history:
Received 18 September 2014
Received in revised form 31 January 2015
Accepted 2 February 2015

Keywords:
Apis mellifera
Nosema ceranae
Deformed wing

1. Introduction

Due to the importance of honey bees, Apis mellifera, as pollinators of many crops, large-scale losses of honey bee colonies in some countries in recent years have attracted the attention of both the scientific community and the public (Neumann and Carreck, 2010). There is currently insufficient evidence to pinpoint the exact factor as the cause of the complex problems. Nevertheless, several possible contributing factors have been suggested to be responsible for colony losses, either acting solely or in combination (Neumann and Carreck, 2010). Of the factors proposed to be responsible for colony losses, Deformed wing virus (DWV) and Nosema ceranae emerged as two of the key pathogens negatively impacting bee health and are often reported to be implicated in colony declines (Dainat et al., 2012; Higes et al., 2009; Martin et al., 2012).

Among viruses attacking honey bees, DWV is the most common and prevalent infection in honey bee colonies (Allen and Ball, 1996). The association of DWV with the parasitic mite Varroa destructor, was reported to be responsible for the death of millions of honey bee colonies, and has become the most significant threat to apiculture worldwide (Martin et al., 2012). DWV causes overt symptoms of wing deformity resulting in emerging bees that are unable to fly. In the asymptomatic bees, DWV can affect learning behavior, aggressiveness and lifespan. The DWV infection has been suggested as a predictive marker

for honey bee colony losses (Dainat and Neumann, 2013). DWV is now proposed to be the most likely candidate responsible for the majority of colony losses (Schroeder and Martin, 2012).

N. ceranae, an emerging microsporidian parasite that causes the serious disease in honey bees known as nosemosis that is characterized by digestive tract problems and consequently metabolic disorders. For decades, nosemosis of European honey bees was exclusively attributed to a single species of Nosema, N. apis. In 2005, a natural infection of N. ceranae, a species of Nosema which was first found in the Asian honey bee A. cerana, was identified in A. mellifera colonies in Taiwan (Huang et al., 2007). Shortly thereafter, the infection of N. ceranae in A. mellifera was reported worldwide (Chen et al., 2008; Higes et al., 2006; Klee et al., 2007) and the disease caused by N. ceranae in honey bees was found to be far more prevalent than that caused by N. apis (Higes et al., 2009). The infection of N. ceranae has impacts at both the individual honey bee and colony levels and has been associated with honey bee colony losses (Currie et al., 2010; Higes et al., 2008a) although the impact of this parasite on colony health in some other countries still remains controversial (Genersch et al., 2010; Gisder et al., 2010).

A synergistic effect between DWV and N. ceranae is highly plausible, since honey bees have often been reported to harbor two pathogens simultaneously (van Engelsdorp et al., 2010). N. ceranae is a pathogen that causes extensive damage to the midgut epithelial ventricular cells (Fries, 2010). This infection could then create access for other pathogens such as DWV that could be spread by fecal–oral transmission (Chen and Siede, 2007), to pass across the midgut protective barrier and get into the haemolymph. Moreover, it has also been shown that N. ceranae can actively suppress the immune response in honey bees (Antúnez et al., 2009), making N. ceranae-infected colonies more susceptible to viral infections.

The present study examined the effects of N. ceranae and DWV interactions in the co-infected bees under different infective doses of pathogens and nutritional conditions. We provided evidence of the synergistic effect of N. ceranae and DWV in infected bees, however, the synergistic interactions between the two pathogens is dosage- and nutrition- dependent. Our results also showed that the two pathogens did not act synergistically when the titer of DWV reached a plateau.

2. Materials and methods

2.1. Colony selection

Colonies maintained in the experimental apiary at the USDA-ARS Bee Research Lab, Beltsville, Maryland, USA were monitored and regularly treated for Varroa mite infection, which is positively associated with virus titersin honey bee colonies (Yang and Cox-Foster, 2005). Colonies without any symptoms of Varroa infestation were surveyed for Nosema infection using a routine spore counting method (Shimanuki and Knox, 2000) and PCR analysis to confirm species status (Chen et al., 2009a). The result of PCR analysis showed that only N. ceranae was present in the examined bee colonies in our study. The colonies with infections higher than 5×10^6 spores per bee on average were selected as colony sources for spore purification. Colonies with undetectable Nosema infection were selected for honey bee virus detection. For each colony, thirty adult workers were randomly sampled and pooled for RNA extraction. The presence of seven honey bee viruses, namely Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Chronic bee paralysis virus (CBPV), DWV, Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Sacbrood bee virus (SBV), were checked by RT-PCR using previously reported primers (Ai et al., 2012). Colonies infected with DWV, but not any other viruses, were selected for further analysis. Ten newly emerged bees from each colony were subjected to the quantification of DWV individually by qRT-PCR method To ensure bees used in each experiment had approximately an equal amount of DWV infection from the beginning, only those colonies with variation of relative titer of DWV less than 2.0 among the 10 sampled bees were selected as sources of experimental bees.

2.2. Spore purification and bee preparation

N. ceranae spores were isolated by Percoll centrifugation method (Chen et al., 2009b). After dissecting the intestinal tracts, the midguts were macerated in distilled water using a manual tissue grinder followed by the suspension being filtered through a No. 4 Whatman filter paper. The resulting suspension was cleaned by centrifugation with a Percoll gradient at 3000 g three times and finally resuspended in distilled water. The spore concentration was determined by counting with a hemocytometer chamber and the suspension was prepared for use by mixing with 50% (v/v) sucrose syrup.

Frames of sealed brood obtained from two selected colonies with detectable DWV infection, but undetectable Nosema infection, were kept in an incubator at 34 ± 2 °C to provide newly emerged workers. During a 12-h period, the frames were checked every hour to collect newly emerged workers to decrease the possibility that the bees would consume pollen or honey from the frames, which could affect our experiment by altering the bees' nutrition status and/or, with low possibility, infect the bees with Nosema spores (Higes et al., 2008b). Bees observed being parasitized by Varroa mites, which were encountered very occasionally, were excluded. Worker bees were individually fed using a syringe with 2 μl of 50% sucrose syrup containing a specific amount of spores for inoculation. The bees that did not consume the entire droplet were discarded.

2.3. Experimental setup

In the first experiment, four groups were set up (Table S1). Two of them were inoculated with 1×10^4 spores per bee. The other two served as controls. In the second experiment, eight groups were set up with 2 groups serving as controls (Table S1). The rest were divided into groups of two inoculated with 1×10^3, 1×10^4 or 1×10^5 spores per bee. Each group was composed of three replicates of 30 honey bees in each cage. They were kept

in an incubator at 30 ± 2 °C, 70 ± 5% RH and fed ad libitum with 50% sucrose syrup solution. To compare the effects of different nutrition status, one of the two groups, in either test or control groups, was additionally provided with pollen in a 1.5 ml Eppendorf tube. To avoid viable Nosema spores (Higes et al., 2008b) and/or other pathogens contaminating the pollen, pollen freshly collected at the hive entrances with pollen traps were treated with UV-light. For the treatment, pollen was ground into fine powder, spread into petri-dishes in thin layers and exposed to UV-light for 12 h. During this period, pollen was stirred every 2 h to allow complete exposure.

Dead bees were removed daily and sucrose syrup was changed every two days. Five bees were collected from each cage on days 2, 4, 6, 8, 12 in the first experiment and days 2, 4, 8 in the second experiment for DWV quantification.

2.4. RNA extraction and qRT-PCR analysis

Total RNA was isolated from individual bees using TRIzol® Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Briefly, each bee was homogenized in 1 ml TRIzol Reagent, shaken vigorously for 30 s and then incubated at room temperature for 3 min. Following precipitation and centrifugation, the resultant RNA pellets were resuspended in 250 μl nuclease-free water. The RNA concentration was measured using a NanoDrop™ spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The titer of DWV was quantified by one-step SYBR Green real-time qRT-PCR. The expression of a housekeeping gene, β-actin, in each sample was also measured for normalization of virus quantification results. The primer pairs for DWV and β-actin were previously reported (Prisco et al., 2011). RT-PCR reactions were carried out in a 20-μl reaction volume, containing 10 μl of 2 × Brilliant® SYBR® Green QRT-PCR Master Mix (Stratagene, La Jolla, CA), 0.4 μM each of forward and reverse primers, and 250 ng of template RNA. The thermal profile for the one-step RT-PCR was as follows: one cycle at 50 °C for 30 min, one cycle at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 30 s. After amplification, a dissociation curve was constructed using 81 complete cycles of incubation where the temperature was increased by 0.5 °C/cycle, beginning at 55 °C and ending at 95 °C to verify presence of a single product. Negative controls (no template) were included in each run of the reaction and yielded no products. Nosema infection of each bee was verified by qRT-PCR using N. ceranae primers (Chen et al., 2009a). qRT-PCR was replicated three times for each sample to address the variability of the analysis process. The relative quantity of DWV and N. ceranae was calculated by subtracting the cycle threshold (Ct) of DWV from Ct of the reference gene (β-actin) (Chen et al., 2005; Chaimanee et al., 2012), averaged between runs and compared between groups.

2.5. Statistical analysis

Data sets were subjected to descriptive analysis and outliers exceeding 1.5 times the length of the box away from either the lower or upper quartiles were excluded. Normality of data sets was tested using the Shapiro-Wilk test. Data sets meeting normal distribution were compared with the parametric student's t-test. Non-normal distribution data sets were analyzed using the lower powered nonparametric Mann-Whitney U test. Data is shown in average ± SE. p-values below 0.05 were considered significant.

3. Results

3.1. DWV proliferation in cage bees

All of the individual bees tested at day 0 had detectable DWV in our study, which enabled us to conduct the study without additionally inoculating virus. Our data showed ready proliferation of DWV in the cage bees. The relative quantity of DWV increased from −16.33 ± 1.03 at day 0 to 7.00 ± 0.21 (calculation of four groups) at day 6 in the first experiment (Fig. 1), which demonstrated an approximate 10 million fold change in the 6 days. Minor increase was observed from day 6 to day 12, showing a plateau period after day 6. Despite DWV titers at day 0, 2, 4 in the second experiment being relatively higher than those at corresponding days in the first experiment, the values at day 8 were similar in both experiments (1st vs 2nd experiment: 7.51 ± 0.17 VS 7.33 ± 0.15), suggesting that a plateau period existed in both trials.

3.2. Effect of N. ceranae infection on DWV

Quantification of N. ceranae revealed that the level of Nosema infection increased after inoculation in our experiments (Fig. S1), which ensured the effect, if any, that Nosema infection in honey bees was established. During the cage experiments, no server mortality occurred as only one to five bees died in each cage. The mortality rate was not significantly different amongst the groups (Kruskal-Wallis H, $p > 0.05$).

The effect of Nosema infection on DWV titer could be observed at day 4 in the first experiment. When bees were restricted from pollen, Nosema infection significantly increased DWV titer ($p < 0.001$). While, when they were supplemented with pollen feeding, there was no significant difference between Nosema-infected bees and non-infected bees on DWV titers ($p = 0.58$). However, a significant difference existed among Nosema-infected bees when they were fed with and without pollen ($p = 0.002$), indicating pollen feeding compensated the effect of Nosema infection, which was also supported by the significant difference observed at day 8 on Nosema-infected bees fed with and without pollen ($p < 0.001$). No significant difference was found between non-infected bees fed with or without pollen at any other time point, including comparisons at days 2, 6, and 12.

3.3. Dosage effect of N. ceranae infection on DWV

With a higher titer of DWV at day 0, bees in the second experiment had relatively higher DWV titers at day 2 and 4 compared with those in the first experiment. The

significant difference between Nosema-infected and non-infected bees occurred as early as two days after inoculation (Fig. 2). When bees were not fed with pollen, significant increases on DWV titer were found when they were inoculated with 1×10^4 and 1×10^5 spores/bee ($p = 0.001$; $p = 0.001$). The increase of DWV titer was not significant when bees were inoculated with 1×10^3 spores/bee ($p = 0.411$). When they were supplemented with pollen, significant increase in DWV titer could only be found when bees were inoculated with 1×10^5 spores/bee ($p = 0.036$). Bees inoculated with $1 \times$p

masked. This highlights the importance of

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