

Effective Silencing of *Dicer* Decreases Spore Load of the Honey Bee Parasite *Nosema ceranae*

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Abstract

Nosema ceranae is an intracellular parasite that infects honey bee mid-gut epithelial cells. Previously, we identified microRNA-like small RNAs and found evidence for expression of the *N. ceranae* gene coding *Dicer*. The *Dicer* protein is critical for small regulatory RNA synthesis and RNA interference. In order to test the effects of *Dicer* on *N. ceranae* reproduction, we designed small interfering RNAs (siRNAs) to suppress *Dicer* gene expression. After inoculating honey bees with *N. ceranae* spores, siRNAs targeting the gene for *Dicer* were fed to individual honey bees for three days post infection at 24 h intervals (siRNA-*Dicer* group). Additional honey bees were fed with non-specific siRNAs (siRNA-scramble group). The third group of honey bees was inoculated with *N. ceranae* spores without any siRNA treatment (infection group). Overall, siRNA-*Dicer* significantly inhibited the spore production (ANOVA, $P < 0.05$). The honey bees fed with siRNA-*Dicer* showed 60% less spores than the honey bees from infection and siRNA-scramble groups (adjusted $P < 0.05$). The expression of *Dicer* was not detected at one day post infection (dpi) in the siRNA-*Dicer* group and the expression level of *Dicer* remained significantly suppressed at two dpi. The results suggest the gene *Dicer* is critical for *N. ceranae* reproduction and provide a new insight for this parasite control.

Keywords: Microsporidian; RNA interference; MicroRNA; Honey bee; *Dicer*

Introduction

Nosema ceranae is an intra-cellular parasite, which infects honey bee mid-gut epithelia cells [1]. After honey bees ingest food contaminated by *N. ceranae*, the spores germinate and extrude polar tubes, which eject the sporoplasm into the host cytoplasm [2]. *N. ceranae* proliferation starts from meronts, leading to daughter sporonts and finally the formation of mature spores [3,4]. The infected cells are filled with spores during infection and the cells may then burst to release the spores. Infected honey bees consumed more sugar water [5] and changed their gene expression [6], which suggest energetic parasitism. Infected honey bees also showed suppressed innate immune responses in some cases [7]. Impacts of *N. ceranae* on the health of colonies in the field differ across studies [8,9] but this parasite is reported to directly cause honey bee colony collapse in Spain [10].

N. ceranae shows high genetic diversity across geographically different locations [11]. The virulence mechanisms of *N. ceranae* remain unclear, with two leading non-exclusive hypothesis for parasite virulence. The first hypothesis suggests that parasites secrete hexokinase, thereby accelerating metabolism of infected cells [12]. The second hypothesis suggests that infection suppresses apoptosis of infected cells, improving parasite reproduction [13]. The latter hypothesis is supported by our previous mRNA-deep sequencing efforts [6]. Also, the honey bees that are known to tolerate *N. ceranae* are reported to overcome apoptosis suppression [14]. Our previous small RNA deep-sequencing efforts identified novel microRNA-like small RNAs from the *N. ceranae* genome along with evidence for expression of the protein-coding gene *Dicer* (Ncer_100079) [6,15]. *Dicer* is a key component of the RNA induced silencing complex, which is critical for regulating developmental and physiological processes and for defending against microbes [16-18]. For many microsporidian species, the gene *Dicer* is lost [19], including *Nosema apis*, sister species of *N. ceranae* [20]. However, *N. ceranae* has maintained a *Dicer* homolog. So far, there is

no functional analysis of *Dicer* in *N. ceranae*. It is also unclear whether *N. ceranae* possesses a functional RNA interference pathway, although prior work has indicated that growth of this parasite is inhibited by RNAi [21]. In order to determine the importance of *N. ceranae* *Dicer* for its reproduction, we designed small interfering RNA (siRNA) to target the parasite gene for *Dicer*. After *N. ceranae* inoculation, honey bees were fed with siRNA at 24 h intervals for three days after infection. Our results showed that *N. ceranae* spore load was significantly reduced after reducing the expression level of parasite gene *Dicer*, indicating a new virulence factor of *N. ceranae*.

Materials and Methods

Ethics statement

Apiaries for bee sample collection were maintained by the USDA-ARS Bee Research Laboratory, Beltsville, Maryland, USA. No specific permits were required for the described studies. The European honey bee (*Apis mellifera*) used in this study is neither an endangered nor protected species.

siRNA design

siRNA sequences were designed using the Invitrogen online oligo

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design tool (<https://rnaidesigner.thermofisher.com/rnaiexpress/>). The selected siRNAs were then aligned to the honey bee and *N. ceranae* genomes to avoid off-target matches with Bioedit [22]. Scrambled siRNA, which do not match any genes, were also designed to assess effects of random siRNAs on the parasite. Two siRNAs were designed for the gene *Dicer*. The two selected siRNAs targeting parasite *Dicer* are 5'-AGAACCCGAAUUUCAUGCUUGUGAA-3' and 5'-UCUGGACUCUACGACUUCUCCUUUA-3'. The selected scrambled siRNA is 5'-GGAACUCUGACAUCGGUUAUCUAA-3'. The siRNAs were ordered from Invitrogen.

Parasite infection and siRNA feeding

N. ceranae spores were isolated from the midguts of heavily infected honey bee workers and purified using a Percoll gradient procedure [23]. Spores were counted using a Fuchs-Rosenthal haemocytometer and *N. ceranae* species status was verified by species-specific PCR [24]. In order to reduce the impacts of genetic variance of host on the parasite reproduction, all the freshly emerged honey bees were collected from the same colony. Eighty newly emerged workers were individually fed with 2 μ l sucrose solution without spores, as uninfected group. An additional 80 newly emerged workers were individually fed with 2 μ l 50% sucrose solution containing 10^5 *N. ceranae* spores without siRNA treatment as an infection group. Eighty newly emerged workers were individually fed with 2 μ l 50% sucrose solution containing 10^5 *N. ceranae* spores and 1.5 μ g siRNA targeting parasite gene *Dicer* as siRNA-Dicer group. Eighty newly emerged workers were individually fed with 2 μ l 50% sucrose solution containing 10^5 *N. ceranae* spores and 1.5 μ g scrambled siRNA as siRNA-scramble group. Forty honey bees were housed in a sterile plastic cup at $34 \pm 1^\circ\text{C}$, 60% relative humidity [25]. In order to include cage effects, each group (N=80) was split into two cups. Sugar water (50%) was provided *ab libitum* as only food source. The honey bees treated with siRNA-Dicer and siRNA-scramble were individually fed for three days post infection at 24 h intervals.

Sample collection and qPCR

Dead honey bees were removed daily. Five living honey bees from each cup were collected from 1 to 6 dpi at 24 h intervals. RNA was extracted from mid-gut tissues of these five honey bees individually using TRIzol and then pooled for replicate qPCR analysis. After 6 dpi, surviving honey bees were collected and individual mid-gut tissue was homogenized to count the spore loads using the Fuchs-Rosenthal haemocytometer. We used qPCR to quantify the expression level of the parasite gene *Dicer* using the described protocols and primers [6]. The honey bee gene *GAPDH*, which is stably expressed during infection, was used as a reference gene to normalize expression levels of the gene *Dicer* [26]. In total, two biological replicates and two technical replicates were conducted respectively.

Statistics analysis

In order to achieve a normal distribution, the spore loads were log transformed (base 2). ANOVA was used to determine the significant level of the siRNA treatments on the *N. ceranae* spore load. Post-hoc corrections (Bonferroni justification for multiple comparisons) were used for pair-wise comparisons. T-tests were used to determine the effect of siRNA treatment on *Dicer* expression levels between the siRNA-Dicer group and infection group for each post infection day (Bonferroni adjusted for multiple comparisons).

Results

N. ceranae infection was successful in the infection groups as supported by the spore counting at 6 dpi. Spores were not found

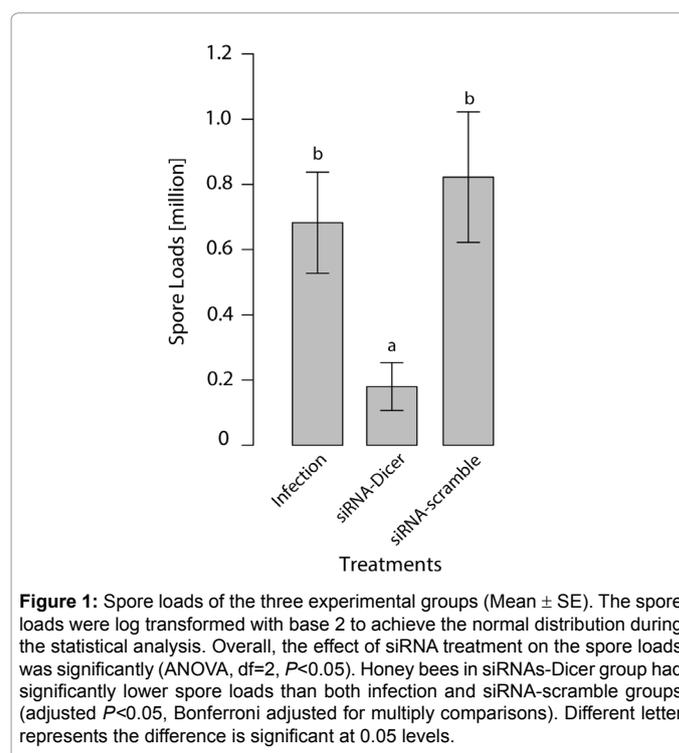


Figure 1: Spore loads of the three experimental groups (Mean \pm SE). The spore loads were log transformed with base 2 to achieve the normal distribution during the statistical analysis. Overall, the effect of siRNA treatment on the spore loads was significantly (ANOVA, $df=2$, $P<0.05$). Honey bees in siRNAs-Dicer group had significantly lower spore loads than both infection and siRNA-scramble groups (adjusted $P<0.05$, Bonferroni adjusted for multiply comparisons). Different letter represents the difference is significant at 0.05 levels.

from the uninfected control group. Overall, the impact of siRNA treatment on spore counts was significant (ANOVA, $df=2$, $P<0.05$) (supplementary table 1). The honey bees in the siRNA-Dicer group showed significantly lower spore loads than honey bees in both siRNA-scramble and infection groups (adjusted $P<0.05$) (Figure 1). On average, in siRNA-Dicer group, *N. ceranae* only produced 30% of the spores isolated from honey bees in infection group. We further quantified the mRNA abundance of *Dicer* between honey bees in infection and siRNA-Dicer groups. The results showed that the expression level of parasite *Dicer* was indeed significantly reduced at 2 dpi (adjusted $P<0.05$) for honey bees in the siRNA-Dicer group when compared with infection group. The expression level of *Dicer* was even below the detection level in the honey bees of the siRNA-Dicer group at 1 dpi and *Dicer* expression was significantly higher at 3 dpi (adjusted $P<0.05$) in honey bees from the siRNA-Dicer group compared with the infection group. From 4 to 6 dpi, the expression levels of *Dicer* were not significantly different between the siRNA-Dicer and infection group (Figure 2).

Discussion

The purpose of this study is to identify the general impacts of parasite *Dicer* on its reproduction. We selected honey bee host from one colony to reduce the impacts of host genetic variance, even though the diversity has been high within the colony for the polyandry honey bee queen.

In our study, suppressing the expression level of *N. ceranae Dicer* for first two days post infection significantly reduced the spore production. Using siRNA to suppress the expression level of *Dicer* is challenging, because *Dicer* itself is a critical component of the RNA-induced silencing complex (RISC) [27,28]. Suppressed expression levels of *Dicer* might reduce the RNA interference efficiency, in some cases disabling this pathway, as shown for *C. elegans* [29]. In our study,

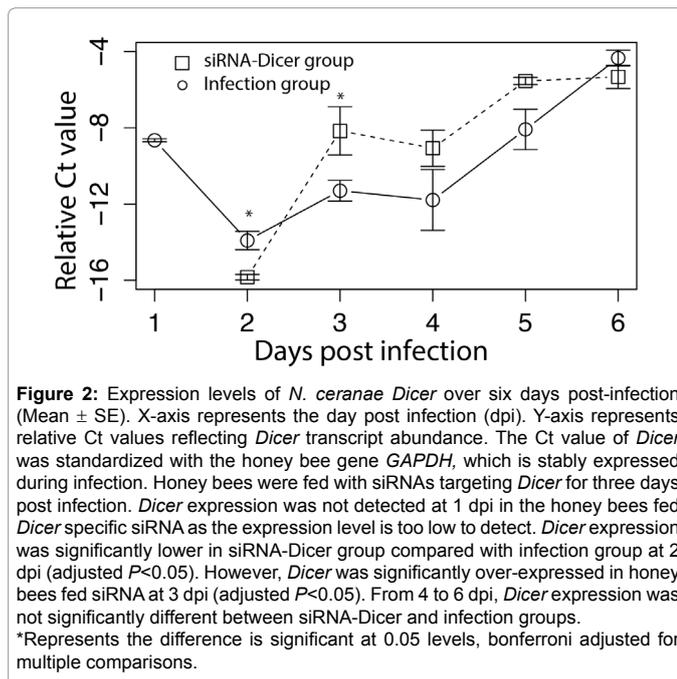


Figure 2: Expression levels of *N. ceranae Dicer* over six days post-infection (Mean \pm SE). X-axis represents the day post infection (dpi). Y-axis represents relative Ct values reflecting *Dicer* transcript abundance. The Ct value of *Dicer* was standardized with the honey bee gene *GAPDH*, which is stably expressed during infection. Honey bees were fed with siRNAs targeting *Dicer* for three days post infection. *Dicer* expression was not detected at 1 dpi in the honey bees fed *Dicer* specific siRNA as the expression level is too low to detect. *Dicer* expression was significantly lower in siRNA-Dicer group compared with infection group at 2 dpi (adjusted $P < 0.05$). However, *Dicer* was significantly over-expressed in honey bees fed siRNA at 3 dpi (adjusted $P < 0.05$). From 4 to 6 dpi, *Dicer* expression was not significantly different between siRNA-Dicer and infection groups. *Represents the difference is significant at 0.05 levels, bonferroni adjusted for multiple comparisons.

Dicer expression was indeed suppressed after siRNA treatment for the first two days post infection. It is necessary to point out that *N. ceranae* is an intra-cellular parasite and the honey bee RNA interference pathway could also be activated to suppress *N. ceranae* gene expression, as suggested from previous study [21]. It is unknown whether honey bees use the siRNA to target the parasite gene *Dicer* or the parasite used the siRNA to silence the expression level of its own *Dicer*. The gene for *Argonaute* is one example for self-silencing. Like *Dicer*, *Argonaute* regulates small RNA syntheses and RNA interference [30]. The gene for *Argonaute* is suppressed with siRNA treatment, leading to enhanced apoptosis of the cell [31]. In our study, the gene for *Dicer* was significantly over expressed 3 dpi in honey bees treated with siRNA-Dicer when compared with the infection group. It is unclear why *Dicer* is over expressed in siRNA-Dicer group at 3 dpi. Two days of *Dicer* gene suppression might impact the parasite RNA interference pathway and also as a biological response to gene suppression, arguably explaining why we observed over expression of the gene for *Dicer* after this time point (Supplementary table 2).

As suggested by our data, *N. ceranae* produced 70% fewer offspring spores when *Dicer* was suppressed for two days. For many microsporidian species, RNAi genes were lost during the evolutionary process and few species have annotated RNAi genes [32]. Still, the functional analyses of the annotated RNAi genes are still missing in Microsporidian species [12,32]. We previously found microRNA-like small RNAs from *N. ceranae*, supporting genomic and expression data involving plausible RNAi pathway members. Those microRNA-like small RNAs were presumably targeting host metabolism genes. Suppressing the expression level of *Dicer* might reduce microRNA-like small RNAs synthesis. Consequently, the metabolism of the host can will be regulated by the infection to support parasite reproduction. Non-exclusively, parasite *Dicer* might be used to regulate its own gene expression for reproduction. For another fungal parasite *Botrytis cinerea*, it could even manipulate the host RNAi system to attack the host immune response [33]. Even though our current efforts cannot explain how the *N. ceranae Dicer* regulates parasite reproduction, or

specific interactions with host genes, this will be an exciting topic to follow up. Nevertheless, our results provide novel insights into the pathogenesis of *N. ceranae* and point to an additional strategy for the control of this important honey bee parasite.

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