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Short communication

Sampling and RNA quality for diagnosis of honey bee viruses using quantitative PCR

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Molecular diagnoses of pathogens via ribonucleic acid (RNA) signatures are used widely in honey bee pathology. Such diagnoses can be compromised by ubiquitous and endogenous RNA-degrading enzymes activated after the death of sampled bees. RNA degradation can be minimized by storage at ultra-cold temperatures or by immersion in high-salt buffers. However, these methods are not always available in the field or are costly, driving a search for alternative methods to store and transport bees for RNA analyses. While the impact of storage conditions on RNA integrity has been evaluated, the tolerance of standard RT-qPCR diagnostic methods of honey bee pathogens for suboptimal collection and storage is unknown. Given the short regions of RNA used for pathogen diagnosis (generally amplified regions of 100–200 nucleotides), it is conceivable that even degraded RNA will provide a template for precise diagnosis. In this study, the impact of the two most convenient sample storage and handling methods (+4 °C and ambient temperature) for identifying honey bee virus infections was evaluated by RT-qPCR. The aim was to streamline the methods needed to collect, transport, and store honey bee samples destined for pathogen diagnosis. The data show that samples held at room temperature for times anticipated for sample transport for up to 5 days are suitable for diagnosis of two of the most common and prevalent honey bee viruses, deformed wing virus (DWV) and black queen cell virus (BQCV). The results will be useful for the standardisation of sampling methods across countries and laboratories.

Reverse transcription quantitative PCR (RT-qPCR) has become a crucial tool for investigating the pathogenesis of viral infection of honey bees since most honey bee viruses reported so far are RNA viruses. This method relies on appropriate internal control genes, as well as on the integrity and quality of the RNA (Vandesompele et al., 2002). RNA, however, can be degraded easily by the ubiquitous presence of endogenous RNases. To reduce degradation prior to genetic examination, the most common method has been to maintain samples from the point of collection at low temperatures, ideally −80 °C, until processing. In the field, samples have been preserved using coolers with dry ice or high-salt buffers such as RNAlater® stabilization solution (Ambion, Austin, Texas, USA) that inhibits the enzymatic activity, or an RNA extraction agent (Blow et al., 2008). Alternatively, it has been shown that portable RT-qPCR system and freeze–dried reagents can be used for field work (Takekawa et al., 2010). However, neither dry ice, nor expensive preservatives are always available to beekeepers and fieldworkers for disease regulatory agencies. While the impact of storage conditions on RNA integrity has been evaluated (Chen et al., 2007), the tolerance of standard RT-qPCR diagnostic methods for honey bee viruses, assuming suboptimal collection and storage, has not yet been determined. Given, the short regions of RNA now being tested for viral diagnosis generally amplified regions of ∼100 bp, it is conceivable that even degraded RNA may provide a template for precise diagnosis. In this study the impact of the most convenient sample storage and handling methods on successful RT-qPCR honey bee virus diagnosis was evaluated. The aim was to streamline, if possible, the methods needed to collect, transport, and store honey bee samples destined for examination.

Live bee workers collected from the brood nest and outer frames of six queenright honey bee *Apis mellifera* colonies in Beltsville, MD, USA in March, 2009 were processed as follows ($N = 30$ bee workers each): (1) control sample: live bees frozen immediately at −80 °C; (2) RT: bees killed at −80 °C and then held for 1, 3, 5 days at room temperature; (3) +4 °C: dead bees held 1, 3, 5 days at +4 °C. The two treatments simulated time needed for transportation from the field to the laboratory by various shipping and storage methods. Total RNA was extracted using a standard TRIzol protocol (Invitrogen, Carlsbad, California, USA) from all individual bees. The RNA yield and purity were assessed using a Nanodrop spectrophotometer

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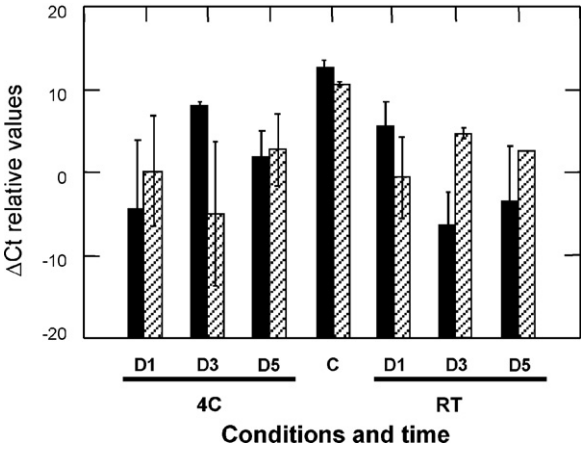

Fig. 1. Results of qPCR for the honey bee viruses DWV and BQCV. ΔCt relative values (*Y*-axis) and standard errors are shown after normalization with beta-actin for the different factors (4 °C and RT) and duration of storage (*X*-axis). Higher ΔCts indicate lower target abundance relative to actin. DWV and BQCV were analyzed from single bees ($N = 6$ for control and $N = 12$ each) (black boxes: DWV; striped boxes: BQCV; RT: room temperature; 4C: +4 °C; C: control; D1 to D5: days).

(Wilmington, Delaware, USA). RNA integrity was assessed across storage regimes with an Agilent Bioanalyser 2100 using an RNA 6000 Nano chip (Agilent technologies, Santa Clara, California, USA) using pooled RNA samples ($N = 6$ for control and $N = 12$ for the other two factors). Total RNA of single bees was then used to generate complementary DNA with Superscript II (Invitrogen) and standard protocols for reactions primed by a random hexamer primer mix. The cDNA was then amplified via quantitative PCR on a Biorad thermocycler CFX 9600 using Express SYBR® GreenER master mix (Invitrogen) and standard conditions (Evans et al., 2006). Reactions were carried out for two common honey bee viruses, deformed wing virus (DWV) and black queen cell virus (BQCV), using published methods (Gauthier et al., 2007; vanEngelsdorp et al., 2009). One primer pair specific to the house keeping gene, β-actin, was used as a reference to standardize RNA levels. Another primer pair of a house keeping gene, RPL8 which spans an intronic sequence was used to ensure no DNA contamination. In each run of qPCR, a positive control and water as a negative control were used. cDNA levels were normalized by subtracting the threshold cycle number of actin from the targets. The data were analyzed with general linear models (GLM), using days as a co-variable and Tukey's honestly-significant-difference post-hoc pairwise test (Systat Software, San Jose, California, USA). The RNA yields with transport duration were analyzed by linear regression least square analyses. The association between RNA yield and actin level was estimated by correlating the data sets using the Pearson correlation coefficient. A Pearson chi-square test was performed to compare the number of successfully amplified actin samples across days. For actin level the results are presented as medians [min; max]. A Kruskal–Wallis test was also applied on BQCV since the assumptions for GLM were not fulfilled.

The results are shown in Fig. 1. The RPL8 amplification showed no carry-over of DNA. The samples stored at +4 °C showed no significant trend for RNA yield over time ($R = 0.022$; $P = 0.694$), opposite to the one stored at RT which showed a significant negative trend ($R = -0.297$; $P = 0.004$). No correlation was found between the RNA yield and the actin level in the samples at +4 °C ($r_p = -0.044$; $P = 0.820$) and at RT ($r_p = -0.072$; $P = 0.783$). The RNA integrity analyses showed a strong degradation of total RNA. At +4 °C, the RNA was partially degraded by day 1 and day 3 and after day 5 the electropherograms revealed strong degradation of total RNA. The electropherograms showed a low ratio of 28S vs. 18S RNA yield. Similar results were obtained for samples stores at ambient temperatures, but the strong RNA degradation had occurred by day 3. The internal control actin was amplified successfully in all the RT/+4 °C conditions at all time points. When degradation was extreme, no actin could be amplified. At +4 °C, there was no difference between days on the number of samples amplified successfully (Pearson chi-square, $P = 0.100$) whereas there was a strong negative association at RT between successful amplification and days (Pearson chi-square, $P = 0.001$). At day 3 and 5 only 5/12 and 2/12 samples respectively were amplified successfully at RT. Significant differences actin levels were found between temperatures in general ($P < 0.001$) and pairwise between −80 °C and +4 °C ($P < 0.001$); and −80 °C and RT ($P < 0.001$), but not between +4 °C (median [min; max]: 28.5 [24.6; 36.8]) and RT (28.8 [20.2; 32.0]; $P = 0.574$). At −80 °C the highest actin levels were found (i.e. the lower Ct number: 22.7 [21.2; 26.2]). Even after 5 days, no difference could be found between RT and +4 °C ($P = 0.824$) except when compared to the control sample at −80 °C ($P = 0.002$ for RT and $P < 0.001$ for +4 °C). DWV and BQCV in single workers were amplified successfully at every time point under both temperature conditions. The DWV load estimates varied significantly between −80 °C and the other temperatures ($P = 0.008$), but not between RT and +4 °C ($P = 0.735$) and days ($P = 0.875$). Temperature and duration of storage showed interactive effects ($P = 0.001$). Specifically, at day 3 and 5, there was no difference of DWV loads between control and +4 °C ($P = 0.867$ and $P = 0.056$ respectively) whereas this was the case for RT ($P = 0.001$ and $P = 0.045$ respectively). For BQCV, the loads also varied between −80 °C and the other temperatures ($P = 0.001$) but there was no difference between RT and +4 °C ($P = 0.532$). Only one sample was positive after 5 days at RT.

The results show that unpreserved honey bee workers maintained at ambient temperature for several days can be used to diagnose viruses using RT-qPCR. Although significant RNA degradation occurred at +4 °C and RT, both DWV and BQCV could still be detected and even quantified after 5 days, suggesting that amplification could be achieved as long as the fragmentation of RNA did not occur in regions flanked by the specific primers (short fragments from ∼100 bp). Indeed, even the house keeping gene β-actin could be amplified sufficiently after 5 days, at between 4 and 6 cycles later than that observed for control bees, for samples held at +4 °C and room temperature, respectively. Relative quantities of viral loads differ naturally between individual bees, as indicated by the error bar in the figure. Surprisingly, relative viral loads actually increased over time in some cases (Fig. 1). This is interpreted to mean that actin levels had degraded more rapidly than viral loads as measured by this diagnostic method. Since primers for all targets were designed to generate similar product sizes, it appears that viral RNA degrades less quickly than does host actin mRNA. This could reflect muscle tissue degradation or a protective quality of viral capsids on viral RNA. The results indicate that shipment of samples at ambient temperatures is feasible, as are methods such as collection from screened bottom boards with a delay from the time of death until bee collection. Indeed, in another study (Dainat et al., 2009), DWV was detectable in samples stored in RNAlater® which were in transit for days at RT before reaching the laboratory. In the study even DWV minus-stranded RNA, generally found at much lower levels, was detectable. Other studies used bees sent by post for virus surveys (Bakonyi et al., 2002; Tentcheva et al., 2004). In addition, the results show as expected that lower temperature (+4 °C) and shorter period of storage at the same temperature provide better RNA preservation: the samples at RT were degraded completely after >3 days which is the usual transportation time by express delivery services worldwide. Nevertheless, two samples could still be amplified in these degrading conditions after 5 days at RT. The successfully amplified samples showed similar actin levels between +4 °C and RT. In the experiment frozen bees at −80 °C were used as controls, which mimics only post-freezing transport logistics and not initial field sampling. Therefore, it would be of

interest to assay bees which died naturally in future studies. Clearly, it is always preferable, when feasible, to use flash frozen bee samples for diagnosis. It is proposed to utilize standard (honey bee) gene targets as controls and to consistently use one storage and shipping method for any given study. The use of multiple primer pairs in multiple regions is recommended to accommodate for RNA degradation. The results of this study will be useful for future field work and monitoring as well as for the standardisation of sampling methods in honey bee laboratories.

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