The influence of RNA integrity on the detection of honey bee viruses: molecular assessment of different sample storage methods

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Summary

RNA quality has been considered to be one of the most critical components for the overall success of RNA-based assays. To ensure accuracy of virus diagnosis by the RT-PCR method, it is important to identify an optimal sample storage method that stabilizes RNA and protects RNA from the activities of RNase in intact samples. We conducted studies to evaluate the effects of seven different storage conditions on the integrity of RNA and the influence of RNA integrity on the detection of virus infections in honey bees. RNA was isolated from samples processed under one of six storage conditions: 1) bees stored at 4°C; 2) bees stored at -20°C; 3) bees stored at -80°C; 4) sliced bees immersed in RNAlater at 4°C; 5) crushed bee immersed in RNAlater at 4°C; 6) intact bees immersed in RNAlater at 4°C, or 7) bees immersed in 70% ethanol at room temperature. The results indicated that bee samples stored at -80°C, -20°C, cut in slices and then immersed in RNAlater at 4°C, or crushed into a paste and then immersed in RNAlater at 4°C provided successful RNA stabilization, suggesting any one of these four storage methods is the method of choice for storing bee samples intended for virus analysis. RNA extracted from bee samples stored at 4°C or whole bees immersed in RNAlater at 4°C was partially degraded one week post treatment, suggesting that a temperature of 4°C could not prevent RNA from activities of RNase completely and that the size of tissue is critical for successful stabilization of samples immersed in RNAlater. 70% ethanol caused quick and strong degradation of RNA and therefore bee samples that are stored in 70% ethanol are not the recommended starting material for virus analysis. The information obtained from this study is relevant to other researchers and to apiary inspectors involved in epidemiological surveillance of bee viral infections.

Keywords: Apis mellifera, Agilent 2100 Bioanalyzer, bee viruses, RNA integrity number (RIN), RNA quality, RT-PCR, storage conditions.

Introduction

Bee diseases caused by viruses are significant threats to apiculture. To date, at least 18 viruses have been identified in honey bees, Apis mellifera (Allen & Ball, 1996; Ellis & Munn, 2005). With the exception of filamentous bee virus and Apis iridescent virus, all reported bee viruses are positive-sense single-stranded RNA viruses. In common with other picorna-like viruses, RNA genomes of bee viruses have two functions: firstly, the genomic RNA functions as mRNA and is translated to a single polyprotein that is subsequently cleaved into functional and structural proteins necessary for RNA replication and production of the structural components of the viral particles, virions. Secondly, the genomic RNA can serve as a template for synthesis of a negative-stranded RNA that in turn serves as a template for replication of more positive RNA strands that are packaged into progeny virions. Ribonucleic acid-based methods such as RT-PCR assay have been developed for identification of virus infections by detecting virus specific sequences from infected bees. Completion of genome sequences of bee viruses including acute bee paralysis virus (ABPV) (Govan et al., 2000), black queen cell virus (BQCV) (Leat et al., 2000), deformed wing virus (DfWV) (Lanzi et al., 2006), Kashmir bee virus (KBV) (de Miranda et al., 2004), and
sacbrood virus (SBV) (Ghosh et al., 1999) makes the molecular detection and characterization of these viruses possible. RT-PCR method has become one of the most popular tool for diagnosis of viral diseases in honey bees (Stoltz et al., 1995; Hung et al., 1996; Benjeddou et al., 2001; Evans, 2001; Grabensteiner et al., 2001; Bakonyi et al., 2002; Ribiere et al., 2002; Chen et al., 2004a, 2004b, 2005a, 2005b; Tentcheva et al., 2004a).

Honey bee viruses usually persist as latent infections and cause no apparent signs or disease symptoms in bees. It is difficult to identify bee virus infections based on field observations. The detection of viruses requires the use of sophisticated laboratory equipments and highly technical procedures. Apiary inspectors and beekeepers often need to send their diseased brood and adult bees to the laboratory for analysis of virus infections. Identification of virus infections by the RT-PCR method includes procedures for the isolation of total RNA from putative infected bees, reverse transcription of an RNA molecule into a complementary DNA molecule (cDNA), and PCR amplification of virus specific sequences. Among procedures involving molecular detection of virus infections, collection of live bees and isolation of high-quality RNA are essential for the overall success of RT-PCR analysis. Since honey bee samples freshly collected from the field need to be transported to the laboratory, RNA can be degraded easily by the ubiquitous presence of endogenous RNases during transit if the storage conditions are not ideal. The degradation and fragmentation of RNA may result in a decline of viral RNA concentration and failure to detect viruses in virus-infected bees. Therefore, storage conditions during the period between the collection of the bee samples and the processing of the bee samples are of crucial importance for preserving the integrity of the RNA.

The present study was devised to define the storage conditions that preserve RNA integrity in order to optimize the standard procedures of bee sample handling and storage. To do this, the quantity and stability of RNA extracted from bees stored in different conditions were compared. In addition, the influence of RNA quality and integrity on the detection of virus infections was also evaluated.

Materials and Methods

Collection and storage of bee samples

Honey bees were collected from a bee colony maintained in the USDA-ARS Bee Research Laboratory backyard apiary, Beltsville, MD. Live bees collected from the field were put in a -20°C freezer for five minutes to sedate them before placing them in different storage conditions. Bees were divided into seven groups and treated in one of the following storage conditions: 1) stored at 4°C; 2) stored at -20°C; 3) stored at -80°C; 4) sliced (0.3-0.4 cm in any direction) and immersed in five volumes of RNAlater (25 ml) (Ambion, Inc. Austin, TX) at 4°C; 5) crushed and immersed in five volumes of RNAlater at 4°C; 6) immersed in five volumes of RNAlater at 4°C; and 7) immersed in 70% ethanol at room temperature. Each group consisted of four 50-ml conical tubes containing twenty bees. One tube of bees from each group was subjected to RNA extraction at four time intervals following sample collection: one week, two weeks, three weeks, and four weeks for subsequent evaluation of RNA quantity and quality.

RNA extraction

Total RNA was extracted from bees using an RNA isolation solution, TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In brief, twenty bees were ground together into paste in a pre-chilled mortar. Bees that were stored at 4°C, -20°C and -80°C were directly subjected to grinding. Bees that were immersed in RNAlater or 70% ethanol were dried on a paper towel to remove the excess solution before grinding. Bees that were sliced and immersed in RNAlater or crushed into a paste and immersed in RNAlater were centrifuged at 1,000 rpm for five minutes at 4°C to separate the bee tissues from RNAlater solution, and supernatants were discarded. 2-ml of body juice was collected by crushing the bee paste with a pre-chilled pestle and pipetting out equal amounts of fluid into four microcentrifuge tubes. After the addition of Trizol reagent and chloroform to homogenize the bee extracts and to remove proteins, lipids and DNA, the upper aqueous phase containing RNA was collected. RNA in the aqueous phase was precipitated by adding isopropanol alcohol and the RNA pellet was washed with 70% ethanol. For samples stored in RNAlater, one additional step was required. Following centrifugation of the solution containing the RNA aqueous phase and isopropanol alcohol, instead of forming a gel-like pellet on the side of the bottom of the tube, two phases were formed due to the high concentration of the salts. The top phase was carefully pipetted out and discarded. RNA in the bottom phase was precipitated by the addition of two volumes of 50% isopropanol alcohol. The RNA pellet was washed with 70% ethanol as with the other samples. The resulting RNA pellet was resuspended in DEPC-treated water with Ribonuclease inhibitor (RNaseOut, Ribonuclease, Invitrogen, Carlsbad, CA) added. RNA in four microcentrifuge tubes of the same treatment group were combined together and stored in a -80°C freezer for subsequent molecular analyses after spectrophotometer analysis.

Spectrophotometer analysis

The first step after the RNA extraction is to measure the yield and purity of the RNA extracted from the bees that were stored at different conditions and different lengths of time post sample collection. A 1/50 dilution of each RNA sample was used for spectrophotometer analyses. The total RNA yield from each group was determined by measuring the absorption at 260nm, and the purity of RNA was estimated by the absorbance ratio of 260nm / 280nm using a spectrophotometer and 5mm cuvette (Ultrospec 3300 pro, Amersham Biosciences).

Assessment of RNA integrity

RNA samples (200 ng RNA / per sample) along with a RNA 6000 size ladder (Ambion, Austin, TX) were loaded in the RNA 6000 Nano chip (Agilent Technologies, Palo Alto, CA) to generate the Bioanalyzer data. RNA Integrity of individual samples was determined by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The results of RNA quality were displayed on electropherograms and into gel-like images. In addition, the integrity number (RIN) that classifies RNA integrity based on a scale of 1 (completely degraded) to 10 (fully intact) based on entire electrophoretic trace of the RNA was calculated automatically by 2100 expert software (Agilent Technologies) for each RNA sample.
Virus detection by RT-PCR

In order to evaluate the effects of RNA integrity on the detection of bee virus infections, RNA samples were examined for the presence of six bee viruses, ABPV, BQCV, CBPV, DWV, KBV, and SBV by RT-PCR assay at week-1 and week-2. The selection of two time points for examination of viruses was due to the consideration that two weeks was sufficient time to transport samples domestically or internationally. Access RT-PCR system (Promega, Madison, WI) was used for RT-PCR reaction. Primers used in this study were described previously (Chen et al., 2005). The reaction was performed in a total volume of 25 μl with a final concentration of 1X of AMV/Tfl reaction buffer, 0.2 mM of dNTP, 1 μM of sense primer, 1 μM of antisense primer, 2 mM of MgSO4, 0.1 unit of AMV reverse transcriptase, 0.1 unit of Tfl DNA polymerase and 500 ng of total RNA. Amplification was undertaken using the PTC-100 DNA Engine (MJ Research, Waltham, MA) with the following thermal cycling profiles: one cycle at 48°C for 45 min for reverse transcription; one cycle at 95°C for 2 min; 40 cycles at 95°C for 30 sec, 55°C for 1 min, and 68°C for 2 min; one cycle at 68°C for 7 min. Negative (water) and positive controls (previously identified positive sample) were included in each run of the RT-PCR reaction. PCR products were electrophoresed in 1% agarose gel containing 0.5 μg/ml ethidium bromide and visualized under UV light.

Statistical analysis

Statistical analysis was performed using Statistix7 statistical software (Analytic Software). The Fisher’s Least Significant Difference (LSD) Comparison of Means Test was used to test for significant differences of RNA yield and RIN among samples at different storage conditions. The results are expressed as mean ± SD. Differences were considered statistically significant if p value ≤ 0.05.

Results

Effect of storage methods on total RNA yield

The yield of total RNA varied between the groups with different storage treatments at different lengths of time post treatment. The average RNA yield of the four time points was 450 ± 537 ± 152 pg, 653 ± 119 pg, and 223 ± 21 pg from samples stored at -80°C, stored at -20°C, and stored at 4°C, respectively. The RNA was completely degraded at week-3, as shown by the disappearing 18S and 28S peaks (Fig. 1). Compared to week-1, there was a minor drop in RIN at week-2, week-3, and week-4. However, the differences in RIN were not significant among four treatments and among four different time point post treatment (p = 0.05) (Fig. 3). In contrast, the gel-like images and electropherograms indicated that complete degradation was observed in RNA extracted from samples stored in 70% ethanol as shown that 18S and 28S rRNA peaks completely disappeared at week-1 post sample treatment (Fig. 2). Bees stored in 70% ethanol displayed the lowest RIN among all the storage methods and there were no significant differences among RIN obtained at the four time points post sample treatment (p = 0.01) (Fig. 3).

For bees stored at 4°C or whole bees immersed in RNA later at 4°C, RNA was partially degraded and fragmentation started to appear at week-1 and further degraded when storage time was prolonged, as shown by the disappearing 18S and 28S peaks and bands in the electropherograms and gel-like images, respectively. The RNA was completely degraded at week-3, as demonstrated by the disappearance of 18S and 28S peaks and bands in the electropherograms and gel-like images (Fig. 2). The RIN of RNA derived from these two treatments showed that RNA started to degrade substantially at week-1 post treatment and reached complete degradation at week 3. Both storage methods yielded RNA of very similar quality and there were no significant differences among these two treatments (p = 0.05) (Fig. 3).

Effects of storage methods on RNA quality

RNA integrity was better preserved when bee samples were stored at -80°C, stored at -20°C, sliced and then immersed in RNA later solution, or crushed and then immersed in RNA later solution. The predominant peaks and bands corresponding to the 18S and 28S rRNA were clearly seen in electropherograms and gel-like images at each time points post sample treatment (Fig. 2).
**Fig. 2a, b** Electropherograms and gel-like images of total RNA. RNA was isolated individually from samples stored at six storage conditions at two points: week-1 (Fig 2a) and week-2 (Fig. 2b) post-treatment. The high quality RNA shows the predominant 28S and 18S rRNA peaks and bands with close to 2 of 28S/18S ratio in electropherogram and gel-like images, such as RNA extracted from samples frozen at −80°C and −20°C, sliced in RNAlater (RNAlater-1), crushed in RNAlater (RNAlater-2). Degradation of the RNA shows a shift in the RNA size distribution toward smaller fragments in electropherogram and gel-like images. RNAs from sample stored at 4°C and whole bees immersed in RNAlater (RNAlater-3) are partially degraded. RNAs from samples stored at 70% ethanol are completely degraded.
RNA integrity in detecting honey bee viruses.

**Effect of sample storage methods on detection of virus infections**

Among six viruses examined, infections of four viruses including BQCV, DWV, KBV, and SBV were identified in bee samples. The expected PCR products for BQCV, DWV, KBV, and SBV were 700 bp, 702 bp, 417 bp, and 824 bp, respectively. As shown in Fig 4, all four viruses were detected in RNA from bees stored at -80°C and at -20°C at week-1 and week-2 post treatment. Three viruses, BQCV, DWV, and SBV, were detected in RNA from bees sliced/crushed in RNAlater at week-1 and week-2. A faint signal of KBV was detected in RNA from bees crushed in RNAlater at week-1. Two viruses, DWV and BQCV, were detected in bees stored at 4°C or whole bees immersed in RNAlater at 4°C. No virus signal could be observed in RNA derived from samples stored at 70% ethanol at any time point.

**Discussion**

Because of the ribonucleic acid nature of the bee virus genomes, RNA may be rapidly degraded by specific or nonspecific endogenous nucleases and the fragmentation of RNA is deleterious for most RNA-based research. Therefore, the immediate stabilization of samples post collection is essential for isolation of high quality RNA and accurate identification of virus infections in bees. Traditionally, the quality of the RNA samples is assessed by running RNA samples on a formaldehyde gel. RNA of good quality will have sharp 28S and 18S rRNA bands and close to 2 of ratio of 28S/18S rRNA band intensity. The common method for the determination of nucleic acid concentration and purity is by measuring the absorption at 260 nm and by calculating the absorbance ratio of 260 nm / 280 nm, respectively, using a UV spectrophotometer. The Agilent 2100 Bioanalyzer (Agilent Technologies) is the state-of-the-art technology to assess both RNA concentration and integrity in one assay. The most powerful feature of the Agilent 2100 Bioanalyzer is its ability to...
provide information about the integrity of an RNA sample, based on a numbering system from 1 to 10. The data of RNA integrity is displayed in the form of electropherograms and mini gel-like images. In this present study, effects of seven storage conditions on preserving the RNA integrity were assessed by running Agilent RNA 6000 Nano assays in an Agilent 2100 bioanalyzer and by RT-PCR assay for detection of bee viruses.

In the past, snap freezing of live bee samples in liquid nitrogen or at -80°C have been the most common methods used for sample preservation. Ambion's RNAlater is a storage reagent that stabilizes and protects RNA in tissues at 4°C or even at room temperature. The gel-like images and electropherograms showed that methods of storing bees at -80°C, -20°C, sliced or crushed bees in RNAlater at 4°C, preserved RNA integrity and provided successful RNA stabilization. The spectrophotometer analysis indicated that while RNAlater preserved RNA integrity, it did not improve RNA quantity. Samples frozen at -80°C yielded RNA of the highest quantity among seven treatments, and the yield of RNA descended from samples frozen at -80°C to samples stored at -20°C > whole bees immersed in RNAlater > crushed bees in RNAlater > sliced bees in RNAlater > stored at 4°C > immersed in 70% ethanol. These results suggest that traditional methods of storing samples in liquid nitrogen or with dry ice are the ideal method for shipping bee samples within a time period of one or two weeks and that RNAlater can serve as an alternative for shipping bees intended for virus identification when obtaining liquid nitrogen or dry ice is difficult.

Comparison of three treatments for bees immersed in RNAlater revealed that the size of tissue is critical for reliable stabilization of interior tissues with RNAlater. Storage of whole bees in RNAlater solution does not allow the direct contact of RNAlater with internal organs of the bees and therefore RNA degradation occurred inside of the body. RNA extracted from bees crushed in RNAlater displayed slightly higher RIN than that from bee sliced in RNAlater; a method recommended by the manufacturer's protocol, suggesting that crushing bees in RNAlater can be the method of choice when it is impractical for beekeepers or apiary inspectors to cut live bees in very thin pieces in the field.

Methods with bees stored at 4°C or intact bees immersed in RNAlater at 4°C could not completely prevent RNA from the activities of RNase. RNA from bees stored by these two methods started to degrade at week-1 and completely degraded at week-3. Storing bee samples in 70% ethanol at room temperature had detrimental effects on RNA quality and RNA was readily degraded in 70% ethanol as demonstrated by the absence of 18S and 28S peaks and bands in all electropherograms and gel-like images. Storing samples in 70% ethanol is a common preservation method for samples intended for DNA analysis. Sometimes the practice of transporting bee samples in 70% ethanol for virus diagnosis has even been heard in the honey bee research field. Our results indicated, however, that bees that are stored in 70% ethanol are not recommended starting material for virus assay or any other RNA-based molecular analyses.

The RT-PCR results revealed that RNAs extracted from bees stored at -80°C, -20°C, sliced or crushed in RNAlater at 4°C were of high quality for molecular analysis. The poor quality of RNA extracted from samples in 70% ethanol was indicated by negative electrophoretic bands for all examined viruses. The data of RNA integrity is displayed in the form of electropherograms and mini gel-like images. In this present study, effects of seven storage conditions on preserving the RNA integrity were assessed by running Agilent RNA 6000 Nano assays in an Agilent 2100 bioanalyzer and by RT-PCR assay for detection of bee viruses.

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Thawing of frozen samples can cause the release of RNase from cellular compartments and thereby lead to rapid RNA degradation. Our experiment was designed to isolate RNA from an equal volume of bee tissue extracts for each treatment. As a result, grinding of samples was not conducted in the presence of liquid nitrogen to prevent possible enzymatic activities of RNase. The imperfect RIN of RNA even from samples frozen at -80°C in our studies indicated the occurrence of some low levels of degradation. Therefore, when processing frozen samples, it is important to homogenize the samples at an ultra-low temperature and in the presence of an RNase inhibitor to prevent possible activities of endogenous RNases.

In conclusion, it is very important that the storage methods for bee samples intended for RNA analysis need to provide a high degree of protection of RNA in order to ensure accuracy of virus diagnosis by the RT-PCR method. The recommendations made from our studies may be helpful for researchers, apiary inspectors, and beekeepers involved in epidemiological surveillance of bee viral infections.

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Disclaimer

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