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Quantitative Real-Time Reverse Transcription-PCR Analysis of Deformed Wing Virus Infection in the Honeybee (*Apis mellifera* L.)

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Deformed wing virus (DWV) can cause wing deformity and premature death in adult honeybees, although like many other bee viruses, DWV generally persists as a latent infection with no apparent symptoms. Using reverse transcription (RT)-PCR and Southern hybridization, we detected DWV in all life stages of honeybees, including adults with and without deformed wings. We also found DWV in the parasitic mite *Varroa destructor*, suggesting that this mite may be involved in the transmission of DWV. However, the detection of the virus in life stages not normally associated with mite parasitism (i.e., eggs and larvae) suggests that there are other modes of transmission. The levels of DWV in different life stages of bees were investigated by using TaqMan real-time quantitative RT-PCR. The amounts of virus varied significantly in these different stages, and the highest levels occurred in pupae and in adult worker bees with deformed wings. The variability in virus titer may reflect the different abilities of bees to resist DWV infection and replication. The epidemiology of DWV is discussed, and factors such as mite infestation, malnutrition, and climate are also considered.

Deformed wing virus (DWV) is a positive-strand RNA virus that was initially isolated from adult honeybees (*Apis mellifera*) from Japan infested with the parasitic mite *Varroa destructor* (5, 6). Under laboratory condition, extracts containing DWV particles were injected into young pupal bees, and the resulting newly emerged adults had deformed wings (5). The disease and mortality caused by DWV have often been reported to be associated with outbreaks of *V. destructor* (11, 14, 23, 24, 25), and Bowen-Walker et al. (13) demonstrated experimentally the role of this mite in transmitting DWV from infected to healthy bees. To date, infection of DWV has been reported in honeybee colonies in Europe, Africa, and Asia (5), and we recently provided evidence of its occurrence in the United States (15).

Several techniques have been employed for detecting bee viruses, including indirect fluorescent-antibody analysis, agarose gel immunodiffusion, an enzyme-linked immunosorbent assay, and reverse transcription-PCR (RT-PCR) (2, 3, 4, 10, 12, 17, 18, 20, 21, 26, 27). While there are techniques for detecting bee viruses, no techniques have been reported for quantification of these viruses. TaqMan real-time RT-PCR is a recently developed technique that allows accurate measurement of virus concentrations and gene expression levels based on fluorescence resonance energy transfer to detect and quantify the amplification product in one step (19). Here, we report the use of TaqMan real-time quantitative RT-PCR to identify DWV in various life stages of honeybees and to determine the relative virus levels in individual adults with and without deformed wings.

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MATERIALS AND METHODS

Sample collection. Two honeybee colonies that were maintained in the USDA-ARS Bee Research Laboratory apiaries in Beltsville, Md., had adult worker bees with deformed wings, and were infested heavily with *V. destructor* were chosen for analysis. Individual frames were removed from each colony and visually inspected for deformed adult bees. In addition, apparently healthy worker bees, pupae, larvae, adult drones, mites, and eggs were collected from each colony. All the samples were stored immediately in a −80°C freezer before they were used for RNA extraction.

RNA extraction. Individual larvae, pupae, adult bees, and mites from each colony were placed in Eppendorf tubes, while 50 eggs collected from the same colony were combined in one tube. All the samples were homogenized in 500 μl of TRIzol reagent (RNA extraction kit; Invitrogen, Carlsbad, Calif.) used according to the manufacturer's instructions for RNA extraction. The resultant RNA pellets were resuspended in diethyl pyrocarbonate-treated water in the presence of RNase inhibitor (Invitrogen).

Primer and probe design. Primer pairs and probes specific for DWV and β-actin were designed based on the previously published nucleotide sequences of DWV (accession no. NC_004830) and *A. mellifera* β-actin (accession no. AB023025) in the GenBank database. The primers and probes used for RT-PCR and TaqMan real-time quantitative RT-PCR were designed by using Primer Expression, version 1.0 (PE Applied Biosystems), and an online program, Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The DWV-specific primers and probe were DWV-sense (5′-ATCAGCGCTTAGTGGAGGAA-3′), DWV-antisense (5′-TCGACAATTTTCGGACATCA-3′), and DWV-probe (6-carboxyfluorescein [FAM]-5′-CGCATGAACAAGTTCGGCGTT-3′-6-carboxytetramethylrhodamine [TAMRA]). A 702-bp DWV-specific product was expected when these primers were used. The β-actin primers and probe were *Apis*-β-actin-probe (FAM-5′-ATGCCAACACTGTCCTTTCTGGAGGTA-3′-TAMRA), *Apis*-β-actin-sense (5′-AGGAATGGAAGCTTGCGGTA-3′), and *Apis*-β-actin-antisense (5′-AATTTTCATGGTGGATGGTGC-3′). A 181-bp fragment was expected when these primers were used. The primers were synthesized by Invitrogen, and the TaqMan probes were purchased from Applied Biosystems.

RT-PCR amplification and RT-PCR specificity confirmation. The Access RT-PCR system (Promega, Madison, Wis.) was used to perform RT-PCR to examine the DWV infections in the samples of adult bees with wing deformities, apparently healthy worker bees, adult worker drones, pupae, larvae, eggs, and mites of individual honeybee colonies. Amplification was performed in a 25-μl reaction mixture containing 1× avian myeloblastosis virus-*Tfl* reaction buffer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1 μM DWV-sense primer, 1 μM DWV-antisense primer, 2 mM $MgSO_4$, 0.1 U of avian myeloblas-

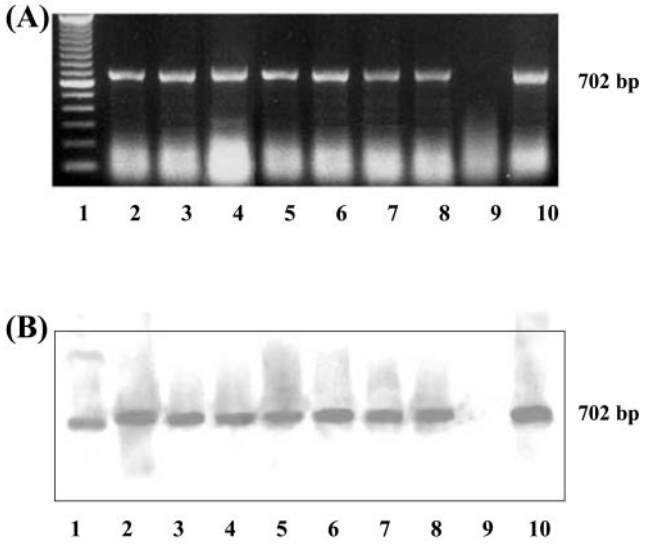

FIG. 1. Determination of DWV infection in a representative bee colony. (A) Gel electrophoresis of RT-PCR-amplified RNA. Total RNA was extracted from samples of apparently healthy adult bees, adult bees with wing deformities, pupae, larvae, eggs, adult drones, and parasitic mites. All samples were subjected to RT-PCR, and a 702-bp band indicating a DWV infection was obtained for all stages of honeybees, as well as the parasitic mites. (B) Southern hybridization analysis. Southern hybridization analysis was used to confirm the specificity of RT-PCR amplification with a DIG-labeled DWV-specific probe. Lane 1, 100-bp DNA ladder; lane 2, RNA extracted from apparently healthy adult bees; lane 3, RNA extracted from adult bees with wing deformities; lane 4, RNA extracted from pupae; lane 5, RNA extracted from larvae; lane 6, RNA extracted from eggs; lane 7, RNA extracted from adult drones; lane 8, RNA extracted from mites; lane 9, negative control (H_2O); lane 10, positive control (previously identified DWV-positive sample).

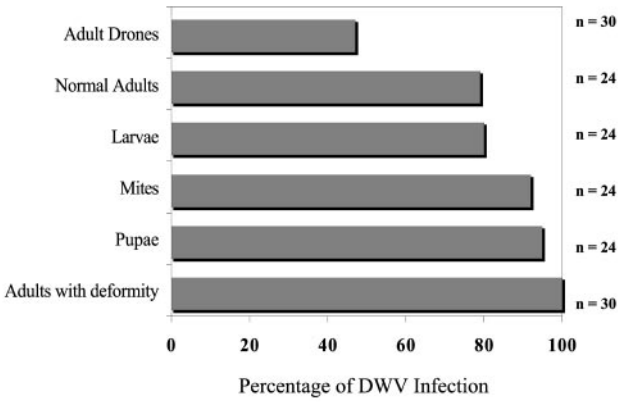

FIG. 2. Percentages of DWV infection in honeybee colonies. The DWV infection rates were different for different stages or castes of bees and *V. destructor*. The percentages of DWV-positive animals were 100% for adult worker bees with deformed wings, 95% for pupae, 92% for parasitic mites, 80% for larvae, 79% for normal-looking adult bees, and 47% for adult drones.

tosis virus reverse transcriptase, 0.1 U of *Tfl* DNA polymerase, and 250 to 500 ng of total RNA. Reverse transcription at 48°C for 45 min was followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 2 min and a final extension at 68°C for 10 min. Negative and positive controls were included in each RT-PCR experiment. Five microliters of amplified product was electrophoresed in a 1% agarose gel containing 0.5 μg of ethidium bromide per ml and visualized under UV light.

In order to confirm the specificity of the RT-PCR assay, a DWV amplification band obtained from RNA of an adult bee with a wing deformity was excised from the low-melting-point agarose gel and purified by using the Wizard PCR Prep DNA purification system (Promega). Purified fragments were ligated into a TOPO cloning vector (Invitrogen). Recombinant plasmid DNA was purified by using a Plasmid Mini Prep kit (Bio-Rad, Hercules, Calif.). The nucleotide sequences of the RT-PCR fragments were determined in both the forward and reverse directions, and the sequencing data were analyzed by using BLAST and were compared with the GenBank database at the National Center for Biotechnology Information, National Institutes of Health. Once the sequence of an RT-PCR fragment was verified as DWV specific, recombinant plasmid DNA with an insert of the DWV-specific fragment was digoxigenin (DIG) labeled by using a DIG labeling kit (Roche Applied Science, Indianapolis, Ind.) according to the manufacturer's instructions. RT-PCR amplification products electrophoresed in an agarose gel were transferred from the gel to a nylon membrane (Hybond-N+; Amersham Biosciences, Piscataway, N.J.) for hybridization analysis. The membrane was hybridized with a DIG-labeled DWV-specific probe in DIG Easy hybridization solution for 16 h at 48°C. After hybridization, the membrane was washed twice in low-stringency wash solution (2× SSC–0.1% sodium dodecyl sulfate [1× SSC is 0.15 M NaCl plus 0.015 sodium citrate]) at room temperature for 5 min and twice in high-stringency wash solution (0.1× SSC–0.1% sodium dodecyl sulfate) at 50°C for 15 min to remove the background signals. After the stringency washes, anti-DIG antibody was used to bind to the DIG-labeled DWV probe. The chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-[5′-chloro]tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) was used to visualize antibody binding, and the signal was detected on X-ray film after a 5-min exposure.

TaqMan real-time quantitative RT-PCR. The DWV titers of 10 apparently healthy adult bees, 10 deformed adult bees, 10 pupae, 10 larvae, and 10 adult drones that were determined to be DWV positive by conventional RT-PCR were determined by TaqMan real-time quantitative RT-PCR. Real-time quantitative RT-PCR was carried out by using the one-step Access RT-PCR system (Promega) and was performed with a Stratagene Mx4000 multiplex quantitative PCR system. The TaqMan probe was covalently labeled with a reporter dye (FAM) at the 5′ end and with a quencher dye (TAMRA) at the 3′ end. Fluorescence values were determined, and amplification plots were generated by the Mx4000 System software. The amplification conditions and reaction mixture were the same as those described above for the conventional RT-PCR except that a TaqMan probe at a concentration of 0.2 μM was included in the reaction mixture. A DWV-negative template control, a DWV-positive template control, and a no-template (H_2O) control were included in each reaction run. The amplification results were expressed as the threshold cycle (C_t) value, which represented the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. Finally, 5 μl of amplified product was also electrophoresed in a 1% agarose gel to confirm the correct sizes of the products. In order to normalize the results of DWV quantitation, quantitative RT-PCR was also performed for a housekeeping gene product, *A. mellifera* β-actin, for each sample under the same conditions as those described above for DWV.

The concentration of DWV in honeybees was analyzed by using the comparative C_t (ΔC_t) method (16). Real-time quantitative RT-PCR results were expressed as means ± standard deviations for C_t values for apparently healthy adult bees, deformed adult bees, pupae, larvae, and drones,. For the comparative C_t method to be valid, eight threefold serial dilutions (1,000, 333, 111, 37, 12, 4, 1.37, and 0.45 ng) of a total RNA sample were used for RT-PCR amplification to confirm that the amplification efficiencies of the DWV and β-actin RT-PCRs were similar. The equations for relative standard curves and relative efficiency plots were calculated by using the Statistix7 statistical software (Analytic Software, Tallahassee, Fla.). The virus concentrations of all samples tested were normalized by subtracting the C_t value of β-actin from the C_t value of DWV. The fold differences in the concentrations for different stages of bees were also calculated.

RESULTS

DWV infection in bee colonies. In the colonies with DWV infections, DWV could be detected in eggs, larvae, pupae, drones, apparently healthy adult worker bees, and adult worker bees with wing deformities, as well as in the parasitic *V. destructor* mites that were collected from the colonies. A

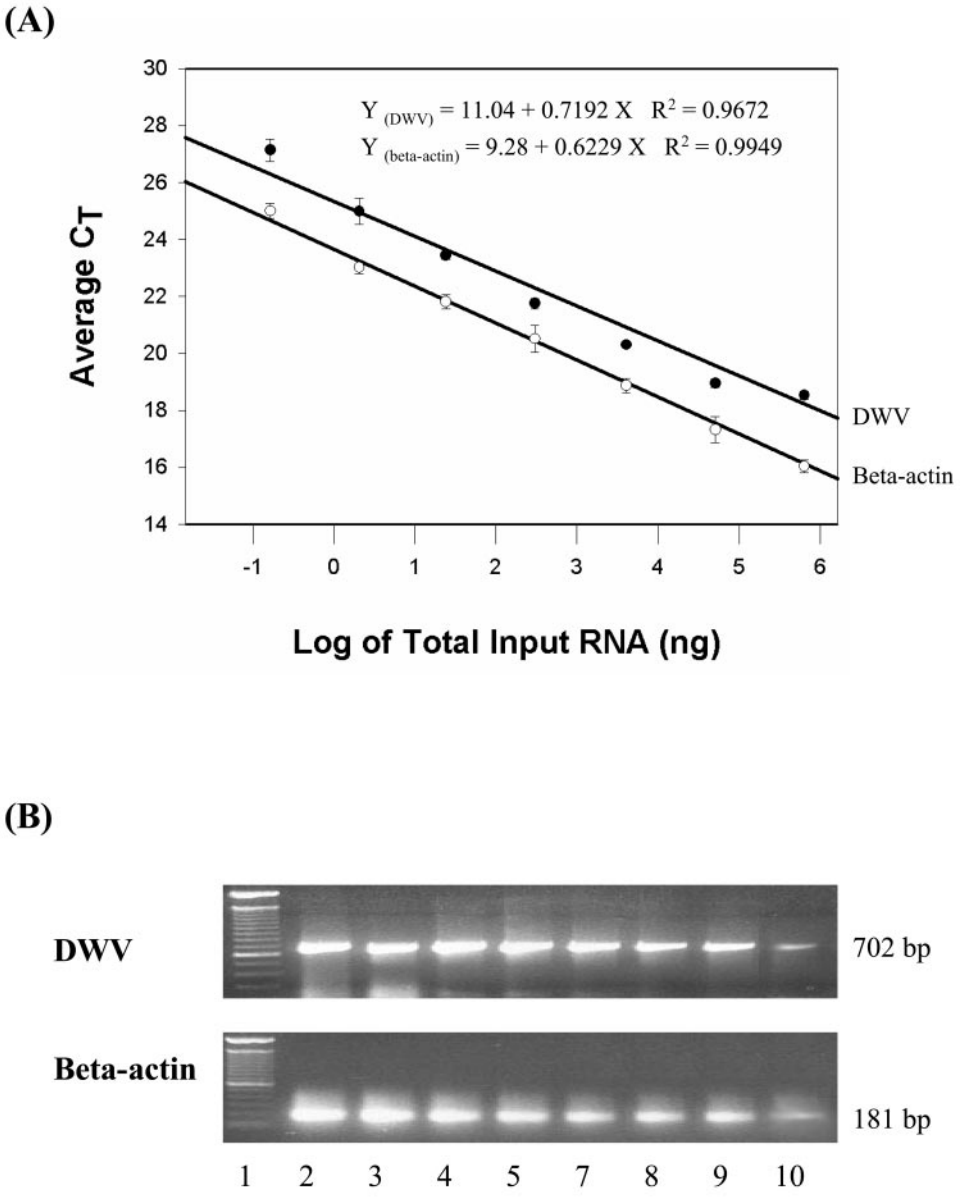

FIG. 3. Dynamic range of detection by TaqMan real-time quantitative RT-PCR. (A) Regression of threshold cycle (C_T) value over the amount of input RNA. To evaluate the sensitivity of the TaqMan real-time RT-PCR assay, amplification of eight threefold dilutions (1,000, 333, 111, 37, 12, 4, 1.37, and 0.45 ng) of a total RNA sample was performed in triplicate, and standard curves for DWV and β-actin were generated individually by plotting C_t values versus the corresponding log input RNA. (B) Amplification products for both DWV and β-actin from eight threefold dilutions of total RNA were electrophoresed in 1% agarose gels. Lane 1, 100-bp DNA ladder; lanes 2 to 10, PCR products for both DWV (702 bp) and β-actin (181 bp) from eight threefold dilutions of total RNA.

702-bp PCR fragment specific to DWV was generated in the samples with DWV infections (Fig. 1A). Southern hybridization analysis indicated that RT-PCR amplification was DWV specific (Fig. 1B). In samples collected from two colonies with apparently DWV-infected worker bees, the percentages of DWV-positive animals were 100% for adult worker bees with wing deformities, 95% for pupae, 92% for parasitic mites, 80% for larvae, 79% for normal-looking adult bees, and 47% for adult drones (Fig. 2).

Evaluation of sensitivity and amplification efficiency of TaqMan real-time RT-PCR assay. To evaluate the sensitivity of the TaqMan real-time RT-PCR assay, amplification of eight threefold dilutions of total RNA ranging from 1 μg to 0.45 ng per reaction mixture was performed in triplicate, and standard curves for DWV and β-actin were generated by plotting C_t values against the corresponding log input RNA values. As shown in Fig. 3, for both DWV and β-actin, there were linear relationships between the amount of input RNA and the C_t values within the concentration range from 1 μg to 0.45 ng per reaction mixture [$R^2_{(DWV)} = 0.962$; $R^2_{(\beta\text{-actin})} = 0.9949$; $\rho = 0.05$].

To control for variation in RNA samples, the concentrations

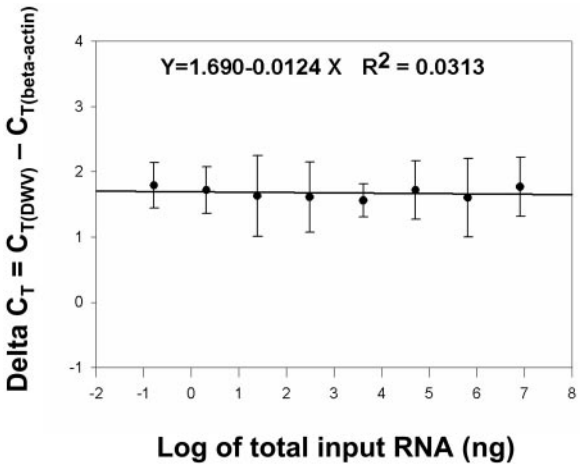

FIG. 4. Validation of amplification efficiency for DWV and β-actin RT-PCR. The difference between the C_t value of DWV and the C_t value of β-actin [$\Delta C_t = C_{t(\mathrm{DWV})} - C_{t(\beta\text{-actin})}$] was plotted versus the log of eight threefold dilutions (1,000, 333, 111, 37, 12, 4, 1.37, and 0.45 ng) of the total sample RNA concentration. The plot of log total RNA input versus ΔC_t has a slope less than 0.1, indicating that the efficiencies of the two amplicons were approximately equal. Therefore, the $\Delta\Delta C_t$ calculation for the relative quantitation of DWV was valid.

of DWV in all samples tested were normalized by the β-actin gene. The difference between the C_t value of DWV and that of β-actin was the ΔC_t. In order to use the ΔC_t method for DWV quantification, the efficiencies of DWV and β-actin amplification in the real-time RT-PCR must be approximately equal. The relative efficiencies of DWV and β-actin amplification were plotted as ΔC_t [$C_{t(\mathrm{DWV})} - C_{t(\beta\text{-actin})}$] versus the log of the corresponding amount of input RNA. As shown in Fig. 4, the efficiency plot for log input RNA versus ΔC_t had a slope of less than 0.1 (the slope was 0.0313), indicating that the efficiencies of DWV and β-actin amplification were approximately equal and that the ΔC_t method was valid for DWV quantification.

Quantitative determination of DWV infection in honeybee colonies. After confirmation that the amplification efficiencies for DWV and β-actin were the same, the relative DWV titer could be estimated from the difference in C_t values (ΔC_t): the lower the ΔC_t value, the higher the concentration of virus in the sample. As shown in Table 1, pupae yielded a ΔC_t value of 1.09 ± 0.49, adult bees with wing deformities yielded a ΔC_t value of 1.38 ± 0.18, larvae yielded a ΔC_t value of 3.28 ± 0.17, apparently healthy adult bees yielded a ΔC_t value of 3.35 ± 0.19, and adult drones yielded a ΔC_t value of 4.48 ± 0.21. A representative amplification plot for DWV infection in a bee colony is shown in Fig. 5.

The ΔC_t value for individual samples relative to a calibrator was described as $2^{-}\Delta\Delta C_t$, where $\Delta\Delta C_t = \Delta C_{t(\mathrm{sample})} - \Delta C_{t(\mathrm{calibrator})}$ and where the calibrator represented samples with the lowest virus level. Drones were chosen as a calibrator because they had the lowest ΔC_t value and thus the lowest level of DWV infection. The concentrations of all other samples were analyzed relative to that of drones. As summarized in Table 1, the DWV concentration varied significantly in bees. Compared to drones, the differences in DWV concentrations were 10.5-fold for pupae, 8.57-fold for deformed adult bees, 2.30-fold for larvae, and 2.19-fold for latent infected adult bees.

DISCUSSION

DWV is considered the most widespread bee virus in the United Kingdom and Europe (23, 24), and we recently provided evidence of the presence of this virus in the United States (15). Like many other bee viruses, DWV can persist in bee colonies as latent or inapparent infections. Using molecular techniques, we detected DWV not only in adult bees with deformed wings but also in adults with no apparent symptoms. Our detection of DWV in symptomatic bees is consistent with the findings of Nordstrom et al. (25).

The disease and mortality due to DWV infections have often been reported to be associated with the parasitic mite *V. destructor* (1, 11, 14, 22). Bowen-Walker and colleagues (13) demonstrated that DWV could be transmitted between bees by the feeding activities of these mites. Our detection of DWV in 92% of the mites examined also indicated that *V. destructor* may be involved in virus transmission. However, our detection of DWV in honeybee stages (eggs and young larvae) not normally associated with *V. destructor* suggests that there are other transmission pathways within a bee colony. The detection of virus in or on eggs suggests that there is vertical transmission, although our unwillingness to sacrifice queens to determine their virus status means that this route of transmission has not been determined and requires future study. Another possibility is that young larvae may acquire virus from infected adult bees

TABLE 1. Relative quantification of DWV infection in honeybees by real-time quantitative RT-PCR by using the comparative C_t method

Bees	Avg DWV C_t	Avg β-actin C_t	ΔC_t [$C_{t(\mathrm{DMW})} + C_{t(\beta\text{-actin})}$][a]	$\Delta\Delta C_t$ [$\Delta C_t - \Delta C_{t(\mathrm{calibrator})}$][b]	$2^{-\Delta\Delta C_t}$[c]
Deformed bees	16.40 ± 0.15	15.02 ± 0.10	1.38 ± 0.18	−3.10 ± 0.18	8.57 (7.56–9.71)
Normal bees	18.54 ± 0.17	15.19 ± 0.09	3.35 ± 0.19	−1.13 ± 0.19	2.19 (1.92–2.50)
Pupae	16.20 ± 0.49	15.11 ± 0.07	1.09 ± 0.49	−3.39 ± 0.49	10.48 (7.46–14.72)
Larvae	18.48 ± 0.15	15.20 ± 0.09	3.28 ± 0.17	−1.20 ± 0.17	2.30 (2.04–2.58)
Drones[d]	19.58 ± 0.16	15.10 ± 0.15	4.48 ± 0.21	0.00 ± 0.21	1.0 (0.86–1.16)

[a] The ΔC_t value was determined by subtracting the average C_t value of β-actin from the average C_t values of DWV. The standard deviation(s) of the difference was calculated from the standard deviations of the DWV and β-actin values [$S_{(\mathrm{DWV})}$ and $S_{(\beta\text{-actin})}$, respectively] by using the formula $S = \sqrt{S^2_{(\mathrm{DWV})} + S^2_{(\beta\text{-actin})}}$.

[b] The $\Delta\Delta C_t$ value was determined by subtracting the ΔC_t value of calibrator from the ΔC_t value of a sample. Drones were chosen as the calibrator as they represented the minimum level of DWV infection as demonstrated by the highest C_t and ΔC_t values. The standard deviation of $\Delta\Delta C_t$ is the same as the standard deviation of ΔC_t.

[c] The $\Delta\Delta C_t$ DWV infection relative to a calibrator (drone) was described as $2^{-\Delta\Delta C_t}$. The range for $2^{-\Delta\Delta C_t}$ is based on $\Delta\Delta C_t + S$ and $\Delta\Delta C_t - S$, where S is the standard deviation of the $\Delta\Delta C_t$ value.

[d] Calibrator.

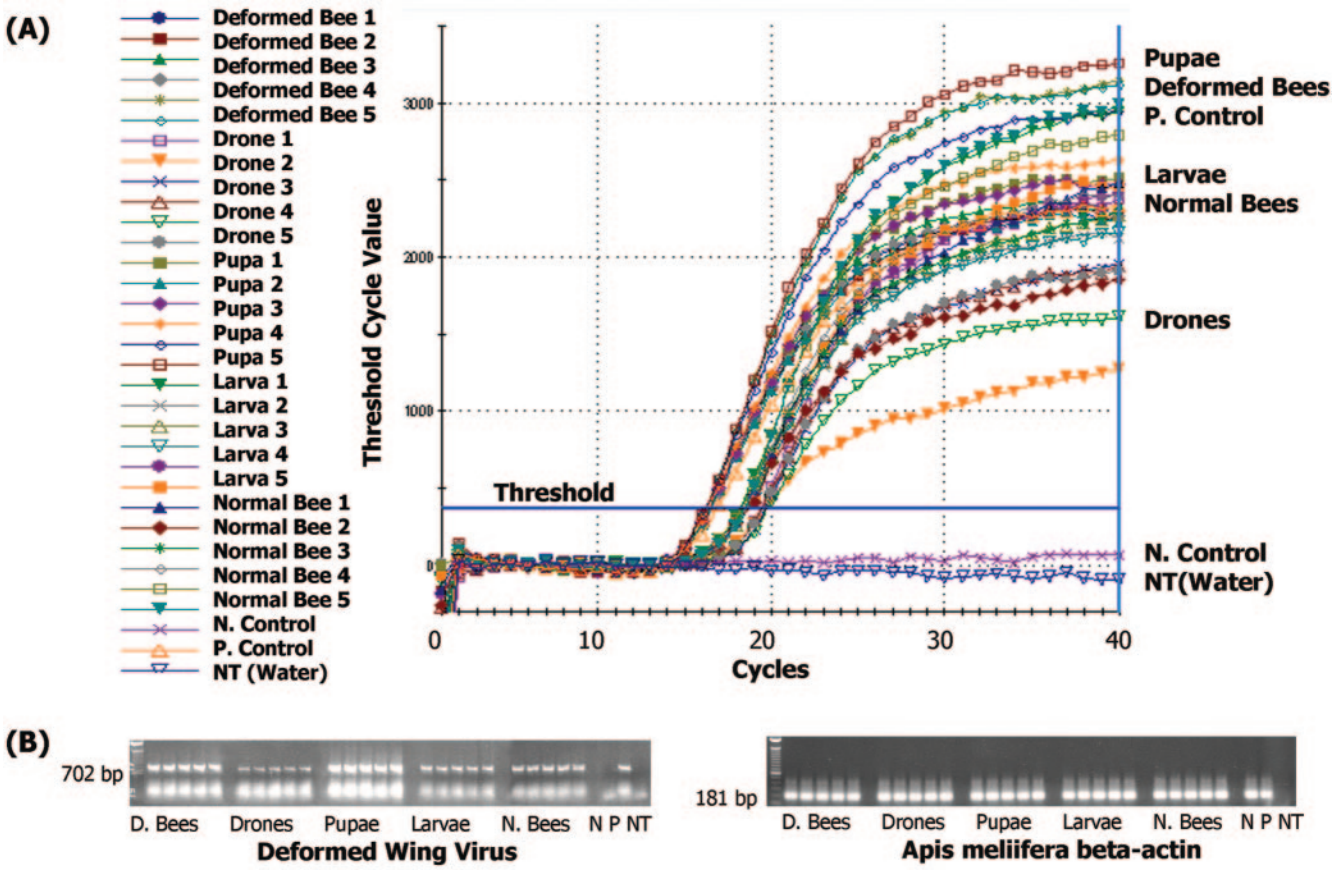

FIG. 5. (A) Representative real-time RT-PCR amplification plot for DWV infection in a bee colony. A PCR fragment specific for DWV was detected in apparently healthy adult bees, adult bees with wing deformities, pupae, larvae, and drones. However, the DWV titers were significantly different for different bee stages. The highest DWV load was detected in pupae, and the lowest viral load was detected in adult drones. The relative concentrations of DWV were in the following order: pupae > deformed adult bees > larvae > apparently healthy bees > adult drones. N. Control, negative control; P. Control, positive control; NT (water), no-template (water) control. (B) PCR products for both DWV and β-actin amplified from total RNA of apparently healthy adult bees (N. Bees), adult bees with wing deformities (D. Bees), pupae, larvae, and drones were electrophoresed individually in 1% agarose gels. A DWV-negative template control (N.), a DWV-positive template control (P.), and a no-template (H_2O) control (NT) were included in each reaction.

through their nursing activities. This possibility also requires additional experimentation.

The amounts of DWV varied significantly in different developmental stages of bees. The highest DWV load was detected in worker pupae, while the lowest viral load was found in adult drones. Pupae had 10-fold more virus than adult drones. The order for the relative concentrations of DWV (from highest to lowest) was pupae > deformed adult bees > larvae > apparently healthy bees > adult drones. The variability in virus titer may reflect the ability of different developmental stages or castes to resist DWV infection and replication. Adult drones appeared to be more resistant to DWV infection and multiplication than adult worker bees, as well as brood, as demonstrated by the lowest percentage of infection and lowest titer of virus for the adult drones. To determine what factors are involved in host defense, further investigation focused on the drone immune defense mechanism is needed. Compared to other life stages of bees, pupae seemed to be most susceptible to DWV multiplication, as shown by the high virus titer. DWV could attack all stages of bees, but pupae were probably the most favorable hosts for virus multiplication. Previous studies showed that viruses multiplied readily in pupae, and bee pupae have often been used as hosts for propagating bee viruses (7). The high DWV infection rate and high virus titer in brood together with the fact that a few adult worker bees had wing deformities indicate that brood could tolerate certain levels of DWV without exhibiting deformity and survive to emergence. Bailey and Gibbs (9) reported that live colony bees could contain as much as 10^6 particles of acute bee paralysis virus without paralysis symptoms or mortality occurring. The explanation for this kind of inapparent infection was that the virus was confined to nonvital tissues and replication must have been suppressed, because as few as 10^2 particles of acute bee paralysis virus could cause acute paralysis when they were experimentally injected into the blood (8). The wing deformity in some newly emerged bees might have been caused by a combination of DWV and other factors, such *V. destructor* infestation, malnutrition, and poor climate, that could trigger the development of DWV infection in the brood and result in the early death of brood or newly emerged adult bees with malformed wings. The difference in virus titer between the apparently healthy bees and deformed bees suggests that virus titer

was a key factor in the appearance of wing deformity in the adult worker bees.

In summary, in this paper we provide a quantitative description of DWV infection in honeybee colonies in United States. Quantification of the virus load in living bees provides essential information on virus pathogenesis and epidemiology. Our data demonstrated that real-time quantitative RT-PCR is a specific, sensitive, robust, and reproducible assay with practical applications in the diagnosis of honeybee viral diseases and analysis of virus infection.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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