

Detection and quantitation of *Solenopsis invicta* virus in fire ants by real-time PCR

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Abstract

A quantitative real-time PCR (QPCR) method was developed to detect and quantify the amount of *Solenopsis invicta* virus (SINV) infecting individual ants of *S. invicta*. The two-step method utilized a gene-specific oligonucleotide primer targeting the SINV RNA-dependent RNA polymerase (RdRp) for cDNA synthesis. Dithiothreitol used in the cDNA synthesis step was found to significantly decrease the detection sensitivity for SINV RdRp and was therefore omitted. SINV RdRp cDNA was then quantified by QPCR using SYBR Green dye and a standard curve generated from SINV RdRp plasmid clones. A standard curve was successfully constructed from clones of the SINV RdRp region. A strong linear relationship [$r^2 = 0.998$; $y = (-3.63 \pm 0.37)x + (39.19 \pm 1.33)$] between C_T and starting SINV RdRp copy number was observed within a dynamic range of $5-5 \times 10^6$ copies. SINV RdRp copy number was determined with the optimized QPCR method in individual *S. invicta* ants taken from an infected field colony. Worker ants exhibited the highest RdRp copy number (2.1×10^9 copies/worker ant) and pupae exhibited the lowest (4.2×10^2 copies/pupa). Mean RdRp copy number was lowest in early larvae and pupae. Overall, SINV RdRp copy number increased through larval development, sharply declined during pupation, then sharply increased in adults.

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1. Introduction

Solenopsis invicta virus (SINV) is the first virus isolated and characterized from the red imported fire ant, *S. invicta* Buren. The virus has been assigned tentatively to the Dicistroviridae (Mayo, 2002) and named *S. invicta* virus [SINV-1] (Valles et al., 2004). SINV-1 is a positive-strand RNA virus found in all stages of *S. invicta*. It possesses a monopartite, single-strand RNA genome comprised of 8026 nucleotides encoding two, non-overlapping open reading frames (ORFs) (Valles et al., 2004). A second genotype, SINV-1A, was found also to infect *S. invicta* (Valles and Strong, 2005). Comparative sequence analyses with other dicistrovirus genomes revealed that SINV-1 encodes for six copies of the 5', covalently bound, genome-linked protein, *Vpg* (Nakashima and Shibuya, 2006), and a type II internal ribosomal entry site (Jan, 2006)—characteristics hypothesized to facilitate SINV-1 multiplication in host cells. Infected fire ants or colonies

did not exhibit any immediate, discernable symptoms in the field (Valles et al., 2004). However, under stress from laboratory introduction, massive brood death was often observed among infected colonies. These characteristics are consistent with other insect-infecting positive-strand RNA viruses—they frequently persist as inapparent, asymptomatic infections that, under certain conditions, induce replication within the host resulting in observable symptoms and often death (Christian and Scotti, 1998).

Among the best-studied arthropod-infecting, positive-strand RNA viruses are those that infect honey bees. Several reports have postulated and/or provided evidence that colony stress in honey bees, such as environmental factors (Tentcheva et al., 2004), titer (Bowen-Walker et al., 1999; Chen et al., 2005), malnutrition (Chen et al., 2005), and associations with microsporidial or bacterial pathogens (Bailey, 1967; Brødsgaard et al., 2000; Yang and Cox-Foster, 2005) or Varroa mites (Martin, 1998; Shen et al., 2005; Yang and Cox-Foster, 2005) may be responsible for viral activation leading to acute infection. The response of fire ants to SINV appears to fit this hypothesis because nests infected with SINV appeared normal in the field, yet underwent brood die-off when exposed to the

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stress of laboratory conditions (Valles et al., 2004). Thus the fire ant also appears to require some associated stress to induce the acute-lethal phase of SINV infection.

In order to characterize SINV epidemiology in *S. invicta* more thoroughly and determine the factors necessary for viral replication, a real-time quantitative PCR method capable of quantifying SINV genome copy number in individual fire ants was developed and optimized. The two-step method utilizes a gene-specific oligonucleotide primer targeting SINV (genotype-1 and -1A) RNA-dependent RNA polymerase (RdRp) for cDNA synthesis. SINV RdRp cDNA is then quantified by quantitative, real-time PCR (QPCR) using SYBR Green dye and a standard curve generated from SINV RdRp plasmid clones.

2. Methods

2.1. Insects

SINV-1- and -1A-infected *S. invicta* nests were identified in Gainesville, Florida, by one-step, RT-PCR (Invitrogen, Carlsbad, CA) with genotype-specific oligonucleotide primers (Valles and Strong, 2005). SINV-positive nests were excavated and ants were separated from the soil using the floating technique described previously (Jouvenez et al., 1977) and maintained in rearing trays.

2.2. Development of constructs used as quantitative standards

Plasmid clones of the SINV target region were generated for optimizing QPCR as well as serving as quantitative standards. A specific SINV nucleotide sequence corresponding to the RdRp region of the genome (accession number AY634314, Fig. 1) was reverse transcribed and amplified from total RNA by one-step, RT-PCR with oligonucleotide primers p517 (SINV-1 genome position 3332–3364, 5'-CAATAGGCACCAACGTAT-ATAGTAGAGATTGGA) and p519 (SINV-1 genome position 3559–3585, 5'-GGAATGGGTCATCATATAGAAGAATTG). The 254 bp amplicon was gel-purified, ligated into the pCR4 TOPO vector, and transformed into Top 10 competent cells (Invitrogen). Clones from both virus genotypes (SINV-1 and

-1A) taken from several locations across the United States were generated ([clone #: origin: genotype] 1: Oklahoma: SINV-1; 6: California: SINV-1; 7: Texas: SINV-1A; 8: Florida: SINV-1A; 9: South Carolina: SINV-1A; 12: Florida: SINV-1A). The plasmids were purified with the Quiaquick plasmid extraction kit (Quiagen, Valencia, CA) and further purified by phenol–chloroform extraction at pH 8 (Sambrook and Russell, 2001). DNA concentration was measured spectrophotometrically and the number of SINV RdRp copies per microliter was quantified according to the formula ($[\text{plasmid } (\mu\text{g}/\mu\text{l}) \times 10^{-6} \times \text{Avogadro's number} \times 2] / [\text{molecular weight of the plasmid} + \text{insert} (\text{g/mol})]$). Standard curves were constructed by 10-fold serial dilution of purified plasmid containing the SINV RdRp sequence as target in diethylpyrocyanate-treated, double-distilled water. The standard curve range was from 5 to 5×10^6 copies. Dilutions of the quantitative standards were prepared fresh for each experiment from an aliquot of a plasmid stock solution stored at -20°C . The plasmid inserts were sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida).

2.3. RNA extraction

Total RNA was isolated from individual fire ant workers, early (1st and 2nd instar) and late (3rd and 4th instar) larvae, and male and female alates. Larval stages were identified by the method of Petralia and Vinson (1979). Individual fire ants were placed in a 1.5 ml microcentrifuge tube and homogenized with a plastic pestle in 0.5 ml of Trizol reagent (Invitrogen). Chloroform (0.2 ml) was added to the homogenate which was vortexed briefly and centrifuged at 20,817 g for 5 min at room temperature. The supernatant was transferred to a clean 1.5 ml microcentrifuge tube and 0.5 ml of isopropanol was added. To facilitate RNA precipitation, 1 μl of mussel glycogen (20 mg/ml; Sigma, MO) was added, the tube was inverted several times and then centrifuged at 20,817 g for 5 min at room temperature. The RNA pellet was rinsed once with 70% ethanol, dried, and suspended in 20 μl of DEPC-treated water. The RNA concentration was measured spectrophotometrically. Viral genome copy number was calculated from the total amount of RNA extracted from individual ants and expressed as copy number per ant.

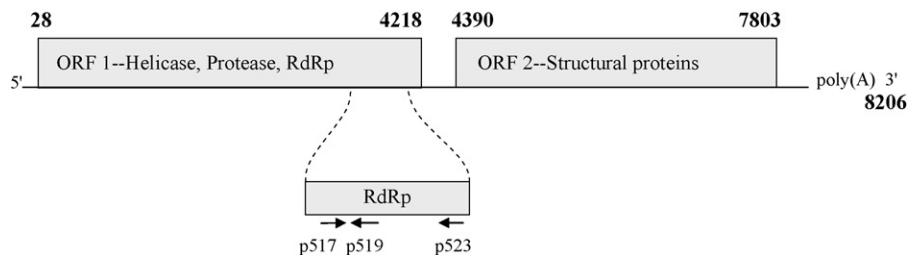


Fig. 1. Diagram illustrating the relative location of the RdRp region amplified within the SINV-1 genome (accession number AY634314). ORFs 1 and 2 on the SINV-1 genome encode putative helicase, protease, and RdRp and structural proteins, respectively. The expanded area represents a portion of the RdRp used in QPCR. Oligonucleotide primers and their respective orientation used in cDNA synthesis and subsequent QPCR are shown. Primer p523 was used for cDNA synthesis and corresponds to genome sequence 3925–3955. QPCR was accomplished with primers p517 and p519, which correspond to genome sequences 3332–3364 and 3559–3585, respectively. ORF positions are denoted by bold numbers above.

2.4. First-strand cDNA synthesis

cDNA was synthesized from total RNA isolated from infected ants corresponding to the RdRp region (Fig. 1) using SuperScript III Reverse Transcriptase (SsRT; Invitrogen) and a gene-specific primer. In a 0.5 ml, thin-walled PCR tube, 2 μ l of an RdRp-specific primer (1 μ M, p523 [SINV-1 genome position 3925–3955, 5'-CCTCATTGAAGATAAAT-CCTCTCTTGAGAAA], Fig. 1), 1 μ l (10 mM) of a dNTP mix, 9 μ l of H₂O, and 100 ng of total RNA (1 μ l) was heated to 65 °C for 5 min in a PTC 100 thermal cycler (MJ Research, Waltham, MA), followed by incubation on ice for 2 min. Then, 4 μ l of 5 \times first-strand buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl, 15 mM MgCl₂), 2.5 μ l of H₂O, and 0.5 μ l of SsRT (200 U/ μ l) were added. The mixture was pulsed in a centrifuge and incubated at 55 °C for 1 h, followed by inactivation of the RT by heating to 70 °C for 15 min.

Although dithiothreitol (DTT) was a manufacturer-recommended component for cDNA synthesis, it has been reported to increase the background fluorescence in downstream SYBR Green reactions (Lekanne Deprez et al., 2002)—a result confirmed in preliminary studies with SINV. Therefore, an experiment was conducted in which each cDNA synthesis component was omitted and subsequently tested in QPCR to identify any changes in detection sensitivity. The altered cDNA reactions were treated as described above, but did not contain RNA template. Rather, QPCR was conducted for each iteration (complete reaction mixture, complete reaction mixture without oligonucleotide primer p523, without dNTPs, without SsRT, and without DTT) in the presence of 50,000 plasmid copies of the RdRp as template. Critical threshold (C_T) was the response variable measured and three replications were conducted. Data were analyzed by analysis of variance (ANOVA) and Duncan's means separation test in instances where the ANOVA results were significant ($p < 0.05$).

2.5. Quantitative PCR

QPCR assays were performed on an ABI PRISM 7000 Sequence Detection System (SDS) interfaced to the ABI prism 7000 SDS software (Applied Biosystems, Foster City, CA) in a 25 μ l reaction volume. The optimized reaction contained 12.5 μ l of SYBR Green SuperMix (with UDG and ROX, Invitrogen), 0.4 μ l each of oligonucleotide primers, p517 and p519 (10 μ M), 3 mM MgCl₂, 1 μ l of the cDNA synthesis reaction, and 10.7 μ l of H₂O. The thermal conditions were as follows: one cycle of 50 °C for 2 min; 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 56 °C for 15 s; 72 °C for 1 min. Dissociation analysis was conducted after all amplifications to inspect for the formation of primer dimers and extraneous, unintentional amplicons. Samples were also analyzed by agarose gel electrophoresis to verify amplification fidelity. For every QPCR run, non-template control reactions were included as negative controls and quantitative standards which were spiked with 1 μ l of an RT-PCR reaction prepared without RNA template so that comparisons with cDNA synthesized samples would be identical. The RT-PCR blanks

were prepared as described above (first-strand cDNA synthesis).

The first series of experiments were conducted to optimize the quantitative reaction using SINV RdRp plasmid clones as template while manipulating magnesium and oligonucleotide primer concentrations and monitoring reaction efficiency and C_T values. When magnesium concentration was manipulated, the 25 μ l reaction contained 12.5 μ l SYBR Green SuperMix, 0.4 μ l each of oligonucleotide primers, p517 and p519 (10 μ M), and water with magnesium chloride in final concentrations of 3, 4, 5, 7, and 9 mM; 3 mM MgCl₂ was the base concentration of the SYBR Green SuperMix which could not be manipulated. Each treatment condition was evaluated by QPCR with a dilution series of plasmid quantitative standards from 5 to 5 \times 10⁶ copies. The experiment was repeated three times, each with a different plasmid template at different times.

A similar experiment was conducted to optimize the oligonucleotide primer concentrations. The 25 μ l reaction contained 12.5 μ l SYBR Green SuperMix, 0.2 (80 nM), 0.4 (160 nM), 0.8 (320 nM), or 1.25 (500 nM) μ l each of oligonucleotide primers, p517 and p519 (10 μ M), and water. Each treatment condition was evaluated by QPCR with plasmid quantitative standards from 5 to 5 \times 10⁶ copies. The experiment was repeated three times, each with a different plasmid template at different times.

C_T values and reaction efficiencies were analyzed by ANOVA and Duncan's means separation test in instances where the ANOVA results were significant ($p < 0.05$). Reaction efficiency (E) was determined by regressing C_T values against the template copy number (log) and calculated according to the formula [$E = (10^{-1/\text{slope}}) - 1$] (Klein et al., 1999).

3. Results

3.1. Quantitative PCR development and optimization

The magnesium concentration of the SYBR Green SuperMix solution is 3 mM, so magnesium adjustments to improve reaction efficiency and sensitivity could only be increased beyond this level. SINV RdRp amplification reaction efficiency was not altered statistically when the magnesium concentration was increased to up to 9 mM. However, sensitivity of the QPCR reaction significantly decreased as magnesium concentration was increased (Table 1). On average, across the starting template quantities used, the C_T value increased by 2 U (between 3 and 9 mM magnesium concentration), representing a decrease in detection sensitivity equivalent to two orders of magnitude.

Altering the oligonucleotide primer concentrations had a significant effect on both reaction efficiency (Fig. 2) and sensitivity (Table 2). The highest reaction efficiency was observed at oligonucleotide primer concentrations 160, 320 and 500 nM. Generally, across the range of starting template copies employed, reaction sensitivity was highest at 500 nM (Table 2). However, agarose gel electrophoresis of the amplicons revealed noticeable primer dimer formation at 500 nM (data not shown). Therefore, because no significant differences were observed between 160 and 320 nM concentrations, we selected 160 nM as the optimum concentration of each primer in the reaction.

Table 1
Influence of magnesium concentration on C_T value

Template (copies)	C_T at magnesium concentration (mM)				
	3	4	5	7	9
5×10^6	16.1 ± 0.4 A	16.2 ± 0.6 A	16.4 ± 0.5 A	16.8 ± 0.5 A	18.0 ± 0.5 B
5×10^5	19.6 ± 0.5 A	19.9 ± 0.5 A, B	20.0 ± 0.2 A, B	20.6 ± 0.5 B	21.8 ± 0.5 C
5×10^4	23.2 ± 0.3 A	23.2 ± 0.3 A	23.3 ± 0.6 A	24.1 ± 0.4 B	25.3 ± 0.3 C
5×10^3	26.8 ± 0.1 A	27.1 ± 0.4 A	27.0 ± 0.8 A	28.0 ± 0.3 B	28.3 ± 0.3 C
5×10^2	30.8 ± 1.3 A	30.9 ± 1.0 A, B	30.8 ± 0.9 A, B	31.4 ± 1.0 A, B	32.9 ± 1.1 B
5×10^1	34.8 ± 0.9 A	35.6 ± 1.1 A, B	35.0 ± 0.4 A	35.1 ± 1.0 A	36.7 ± 0.2 B
5×10^0	37.1 ± 2.6 A	37.0 ± 1.2 A	36.9 ± 1.8 A	37.8 ± 0.8 A	39.2 ± 0 A

Means within a row with different letters are significantly different by Duncan's means separation test ($p < 0.05$).

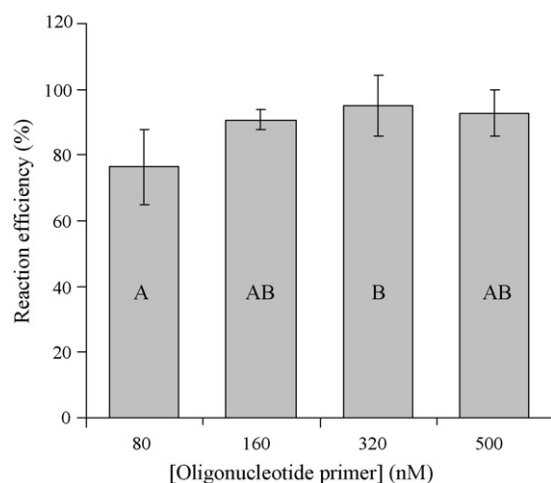


Fig. 2. Influence of oligonucleotide primer concentration on QPCR reaction efficiency of SINRV RdRp. Three independent QPCR runs were carried out using serial dilutions of plasmid clone #6 (5×10^0 , 5×10^1 , 5×10^2 , 5×10^3 , 5×10^4 , 5×10^5 , and 5×10^6). Reaction efficiency means and standard deviations were calculated for each primer concentration and are represented as bars and error bars. Bars with the same letter are not significantly different (Duncan's means separation test).

During preliminary experiments, QPCR results were altered by the presence of reagents carried over from the reverse transcription reaction. Systematic removal of each reverse transcription component and testing with a fixed quantity (50,000 copies) of RdRp standard revealed a statistically significant decrease in C_T value when DTT was removed (Fig. 3). Omission of the other components did not significantly alter the C_T value of the reaction. Therefore, cDNA synthesis reactions with SINRV

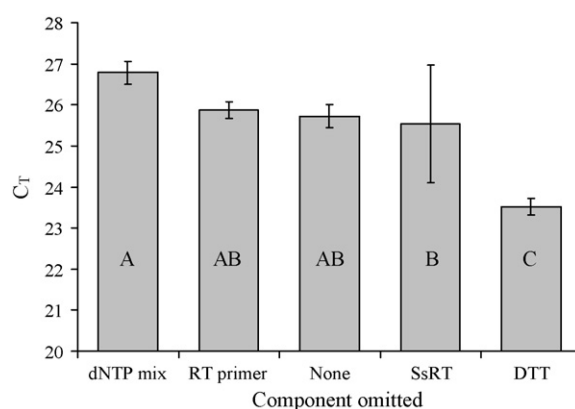


Fig. 3. Influence of RT-PCR reaction components on C_T value. RT-PCR reactions were prepared with missing components (dNTPs, p523, SsRT, DTT, or none [i.e. complete]) following the manufacturer's instructions. The influence of 1 μ l of each of these reactions on QPCR was evaluated in the presence of 5×10^4 copies of RdRp standard plasmid DNA. Three independent QPCR runs were carried out. C_T means and standard deviations were calculated and illustrated as bars and error bars, respectively. Bars with the same letter were not significantly different by Duncan's means separation test ($p < 0.05$).

were conducted without DTT; omission of DTT did not affect the reverse transcription of the SINRV RdRp (data not shown). The presence of DTT decreased dramatically the sensitivity of SINRV detection (Fig. 3). Also note that increased DTT concentration in the QPCR reaction resulted in a corresponding increase in dissociation temperature (Fig. 4). The RdRp amplicon dissociation temperature increased from 76.6 °C in the absence of DTT to 78.5 °C at 1 mM DTT (Fig. 4).

Table 2
Influence of oligonucleotide primer concentration on C_T value

Template (copies)	C_T at primer concentration (nM)			
	80	160	320	500
5×10^6	17.9 ± 1.1 A	16.9 ± 1.7 A	16.4 ± 1.8 A	15.3 ± 0.1 A
5×10^5	21.6 ± 0.6 A	19.7 ± 0.4 B	19.1 ± 0.3 B, C	18.5 ± 0.1 C
5×10^4	25.4 ± 0.7 A	23.6 ± 0.4 B	22.8 ± 0.3 B, C	22.1 ± 0.1 C
5×10^3	29.8 ± 0.2 A	27.2 ± 0.1 B	26.4 ± 0.3 C	25.7 ± 0.1 D
5×10^2	34.0 ± 1.1 A	31.4 ± 1.0 B	30.3 ± 0.4 B, C	29.2 ± 0.1 C
5×10^1	39.0 ± 0.7 A	34.6 ± 0.7 B	33.7 ± 0.3 B, C	32.1 ± 1.8 C
5×10^0	39.0 A	37.3 ± 0.5 A, B	36.5 ± 0.7 B	36.7 ± 0.7 B

Means within a row with different letters are significantly different by Duncan's means separation test ($p < 0.05$).

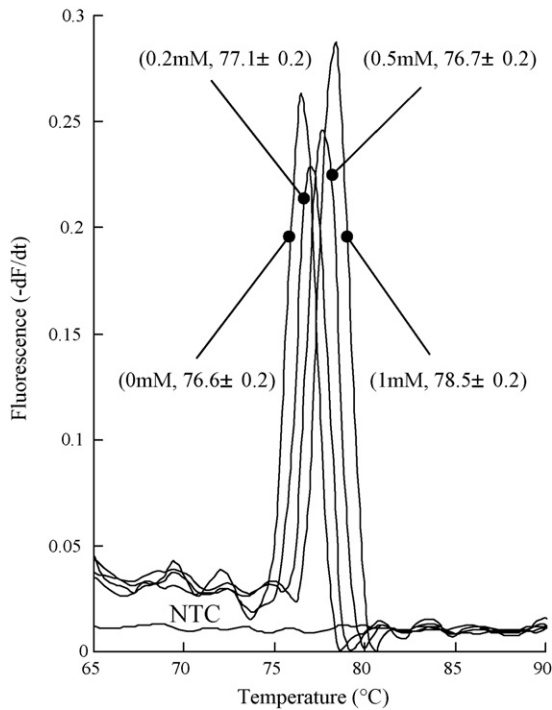


Fig. 4. Dissociation curves of amplicons generated with plasmid #6 as template (5×10^4 copies) in the presence of increasing DTT concentrations. DTT concentration and the mean dissociation temperature (\pm S.D.) are presented for each curve.

The optimized reaction was comprised of 12.5 μ l of SYBR Green SuperMix, oligonucleotide primers, p517 and p519 (160 nM), MgCl₂ (3 mM), 1 μ l of the reverse transcription reaction (2 mM Tris-HCl, pH 8.3, 3 mM KCl, 0.75 mM MgCl₂, 0.04 μ M p523, 0.04 mM dNTP mix, 1 U SsRT), and 10.7 μ l of H₂O. Quantitative plasmid standards were always spiked with 1 μ l of the reverse transcription reaction (also devoid of DTT). Under these conditions, the primers yielded a 254 bp amplicon from the SINV RdRp target as anticipated. No amplification was observed from uninfected ants. Specificity of the oligonucleotide primers was further demonstrated with agarose gel electrophoresis and dissociation analysis of the amplification reaction by the absence of extraneous amplicons and primer dimers (Fig. 5A). The empirically derived dissociation temperature (e.g. clone #7,

77.8 ± 0.2 °C) of the SINV RdRp amplicon agreed with the theoretical dissociation temperature (77.0 °C).

A representative standard curve constructed from the plasmid clones of the SINV RdRp region is illustrated in Fig. 5B. A strong linear relationship [$r^2 = 0.998$; $y = (-3.63 \pm 0.37)x + (39.19 \pm 1.33)$] between C_T and starting SINV RdRp copy number was observed within a dynamic

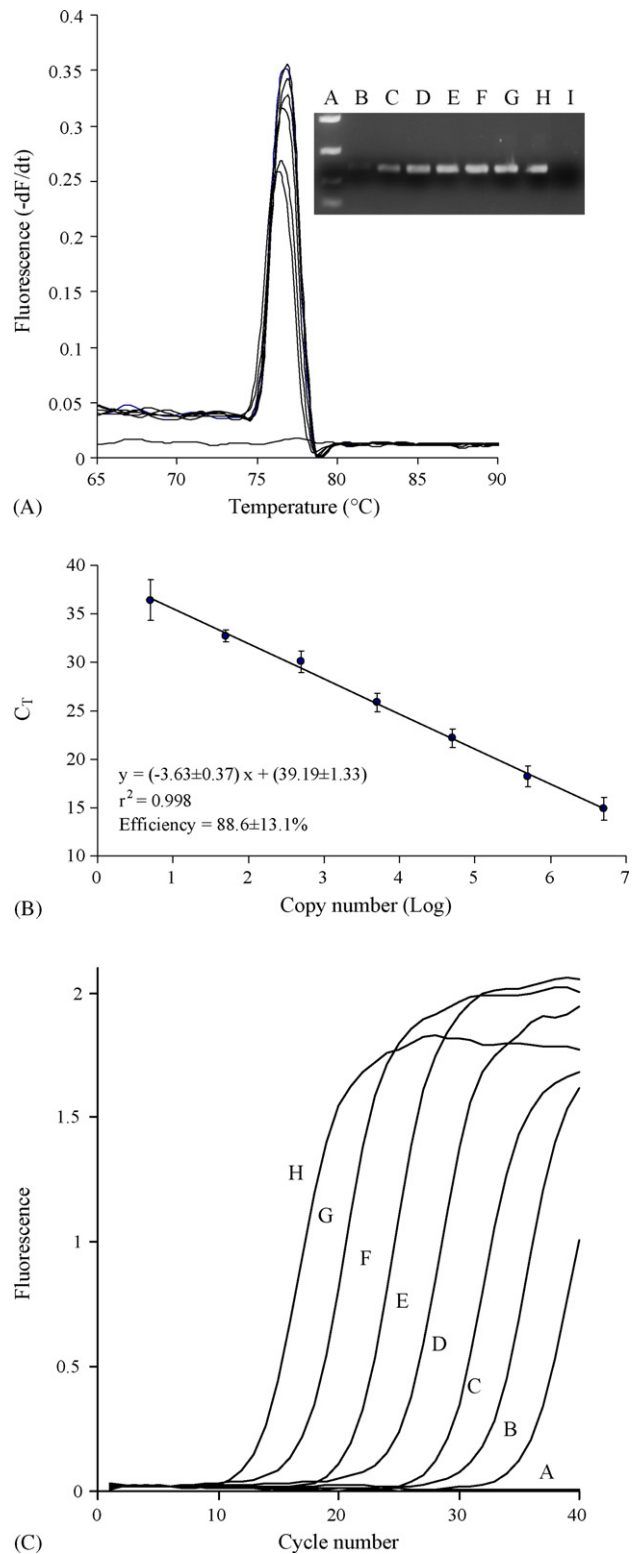


Fig. 5. (A) Representative dissociation profiles of the QPCR amplicons generated from standard RdRp plasmid clones. Amplicons were heated at 0.1 °C/s and the drop in fluorescence is shown as the derivative against temperature. (Inset) The amplicons were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide staining. Lane assignments and initial plasmid copy numbers are as follows: A (molecular weight standards 800, 400, 200, and 100 bp); B (5 copies); C (50); D (500); E (5×10^3); F (5×10^4); G (5×10^5); H (5×10^6); I (non-template control). (B) Linear relationship between C_T and initial template copy number of a series of dilutions of standard plasmids. The number of initial plasmid copies was 5, 50, 500, 5×10^3 , 5×10^4 , 5×10^5 , and 5×10^6 . One microliter of a blank (devoid of RNA and DTT) RT-PCR was added to each standard. Three series of dilutions of each plasmid were run and the mean C_T value for each plasmid copy number was used for establishing the standard curve. Standard deviations are shown as error bars. (C) Amplification profile of a 10-fold dilution series of standard plasmid #6 with initial copy numbers of: A (non-template control); B (5); C (50); D (500); E (5×10^3); F (5×10^4); G (5×10^5); H (5×10^6).

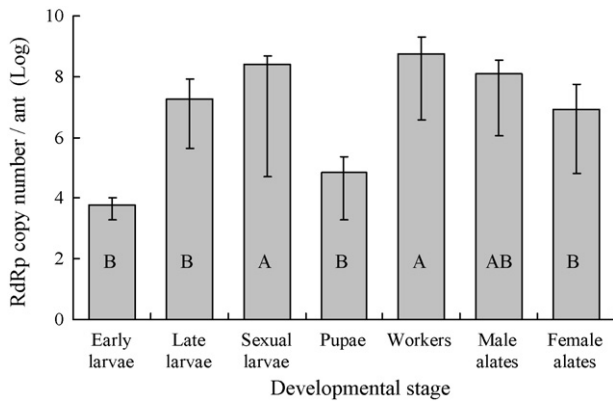


Fig. 6. SINV RdRp copy number determined for individual ants. RdRp copy number was interpolated from a standard curve generated simultaneously. Samples were taken from a field-collected colony and included 10 early larvae, 10 late larvae, three sexual larvae, 13 pupae, 15 workers, three male alates, and 13 female alates. Means are indicated by the bar and error bars represent the highest and lowest value for each developmental stage. Ants with undetectable SINV infections were excluded from data analysis. Bars with the same letter are not significantly different by Duncan's means separation test ($p < 0.05$).

range of $5\text{--}5 \times 10^6$ copies (Figs. 5B and C) representing six orders of magnitude. C_T variability was stable from run to run and with different plasmid preparations; the standard deviation across the dynamic range represented less than 7.9% of the mean value. At the linear threshold level, gaps between adjacent amplification curves were within a range of 3–4. These distances correspond to 8–16 ($2^3\text{--}2^4$) copies of template, a range which is in agreement with the actual 10-fold dilution of the quantitative standards (Fig. 5C). Examination across the entire dynamic range revealed a difference of approximately 22 cycles, which yields an apparent copy number of 2^{22} , or 4.2×10^6 . This empirical value corresponds closely with the actual range. The efficiency (\pm S.D.) of the QPCR reaction was $88.6 \pm 13.1\%$.

3.2. SINV RdRp copies in different developmental stages of *S. invicta*

SINV RdRp copy number was determined with the optimized QPCR method in individual fire ants taken from an infected field colony (Fig. 6). The maximum RdRp copy number was 2.1×10^9 from workers and the lowest value was observed in pupae with 4.2×10^2 copies. Mean RdRp copy number was lowest in early larvae and pupae. However, RdRp copy varied widely even among a single developmental stage; genome copy numbers varied by as much as 1–3 log units. Statistical analysis showed that SINV RdRp copy number increased through larval development, sharply declining during pupation, and sharply increasing in adults.

4. Discussion

A QPCR protocol was developed to estimate the level of SINV infection in individual fire ants by quantifying the number of genome copies. The RdRp region (254 bp amplicon) of SINV was chosen because single-strand RNA viruses have

a relatively high mutation rate (Domingo and Holland, 1997; Holland et al., 1982) and this region of the genome is typically more conserved than regions encoding for structural proteins (Koonin and Dolja, 1993). Thus, the probability of detecting more genotypes of SINV would be higher by utilizing the RdRp region. The protocol was established by optimizing the concentrations of oligonucleotide primers, magnesium, and RT-PCR reagents carried over into QPCR. A series of external plasmid DNA standards for interpolation and subsequent estimation of SINV genome copy was employed. QPCR represents a significant advancement over traditional end-point infection or plaque assays to determine virus infection; titer determinations can be evaluated in hours instead of days obviating the need for lengthy, complex bioassays. QPCR has been employed successfully to quantify the level of viral infection in cell culture and various biological samples (Bhattacharya et al., 2004; Chico et al., 2006; Hoffmann et al., 2005; Keyaerts et al., 2006; Laverick et al., 2004; Olmos et al., 2005; Revilla-Fernandez et al., 2005). The most common fluorescence-monitoring systems employed for QPCR are fluorophore release from quenching by the 5' exonuclease activity of DNA polymerase (e.g. TaqMan probes) and fluorophore intensification by binding double-stranded DNA (e.g. SYBR Green). Although each reporting method has distinct advantages, the use of the DNA binding fluorophore SYBR Green was chosen for its simplicity, ability to discern different genotypes based on dissociation curves, and a greater level of certainty that the fluorescence was from the intended amplicon (Ririe et al., 1997). The QPCR method presented is highly sensitive, capable of detecting as few as five copies of the SINV genome. A linear, dynamic range of detection was observed from 5 to 5×10^6 copies. Although C_T values for a given SINV RdRp plasmid copy number were fairly consistent from run to run (standard deviation of 7.9%), plasmid standards ($5\text{--}5 \times 10^6$ copies) were always included for direct, immediate comparison because it has been reported that basing quantitative measurements in QPCR assays on a single standard curve can lead to significant errors (Wolffs et al., 2004).

Although by definition the SINV QPCR assay is considered an absolute quantitative assay, genome copy number values actually determined are relative to a series of standards (Peirson et al., 2003). Thus, assignment of an absolute genome copy or titer value is not considered possible (Niesters, 2001). A number of factors can influence the viral infection value obtained by QPCR, including incomplete nucleic acid purification and recovery, sub-optimal reverse transcription or PCR efficiencies, variation in the standards, pipetting/mathematical errors when extrapolating to the organismal level, polymerase inhibition, RNA degradation, and sample variation (Bustin, 2000; Lekanne Deprez et al., 2002; Niesters, 2001; Peirson et al., 2003; Suslov and Steindler, 2005). Despite these shortcomings, QPCR provides a simple and quick assay to quantify viral infection rate that is as effective as traditional technologies (Niesters, 2001); this point is especially relevant for SINV because virus assays are performed in cell culture which is currently unavailable for *S. invicta*, nor is a surrogate host cell available for SINV.

RNA and DNA standards have been used in QPCR with cogent arguments presented for the use of each. Although the

use of internal RNA standards can aid in compensating for RNA degradation and poor recovery during preparation, external DNA standards are generally more available, easier to produce, and provide a fixed known value for comparison (Nieters, 2001). The additional precision afforded by use of RNA standards is unjustified. The QPCR method described is intended to provide a strong comparative estimate of genome copy number in individual fire ants and use of external DNA standards eliminates standard RNA degradation as a potential source of error. In other words, DNA standards remain reliable for comparison. Furthermore, even under the most favorable conditions, viral RNA yield would not be expected to achieve 100% recovery, limiting the ability to provide an absolute value for SINV genome copy.

DTT used in the cDNA synthesis step significantly decreased the detection sensitivity for SINV RdRp. The addition of DTT (regardless of concentration) decreased the C_T value for a given SINV RdRp copy number by 2U. A similar impairment of QPCR sensitivity by DTT carried over from cDNA synthesis was reported by Lekanne Deprez et al. (2002). They concluded that DTT interfered with SYBR Green dye-binding and fluorescence yield to such an extent as to preclude acquisition of reliable C_T values. This conclusion is supported by the observation that increasing DTT concentrations resulted in corresponding increases in the dissociation temperature of the RdRp amplicon (Fig. 4). Omission of DTT from the reverse transcription reaction did not influence cDNA synthesis (data not shown) and was crucial for reliable QPCR results (Lekanne Deprez et al., 2002).

Up to 2.1×10^9 SINV genome copies were found in an individual worker ant. Reports on RNA virus infection levels in individual insects are fairly sparse (especially those concerned with single-strand RNA viruses). However, viral particle values range from several thousand (Olmos et al., 2005) to several million (Fabre et al., 2003) in individual aphids. Cherry et al. (1997) reported as many 3.3×10^9 occlusion bodies of a nucleopolyhedrovirus from *Spodoptera exempta*, so the level of SINV infection is not without precedent. The high SINV genome copy most likely indicates that the virus is replicating and each infected cell contains multiple copies of nascent RNA. *S. invicta* pupae and early larvae (1st and 2nd instars) contained the fewest genome copies. This result is in direct contrast to honey bees in which deformed wing virus levels were found highest in pupae (Chen et al., 2005).

SINV-infected ants and colonies are often asymptomatic, a characteristic exemplified by many arthropod-infecting positive-strand RNA viruses. In cases where infection is not symptomatic, epidemiological and transmission studies are extremely limited. Therefore, the QPCR method presented can facilitate completion of these studies and provide additional information about this new virus.

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